KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

COLLEGE OF HEALTH SCIENCES

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

SURROGATE REFERENCE STANDARDS IN QUANTITATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: A CASE STUDY OF THE ANALYSIS OF LAMIVUDINE TABLETS AND LAMIVUDINE ORAL SOLUTION

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NICHOLAS AGYEMANG

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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.

HEAD OF DEPARTMENT

.....

PROF. R.K. ADOSRAKU

22-4-13

SUPERVISOR

.....

for MR. SAMUEL ASARE NKANSAH

CANDIDATE

.....

NICHOLAS AGYEMANG

DEDICATION

This work is dedicated to my dear wife, Akua Nyantakyiwaa, my entire family and also to the memory of my late father, Mr. Edward Asuming Agyemang.

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ABSTRACT

A simple and sensitive isocratic reverse-phase HPLC method was developed for quantitative analysis of Lamivudine in Lamivudine tablets and orals solution using surrogate reference standards with UV detection.

Paracetamol, p-Aminophenol and Metronidazole pure samples were used as surrogate reference standards for the analysis of Lamivudine. The chromatographic separation was performed on stainless steel 5 micron C18 Phenomenex ODS 150 x 4.6mm column. The isocratic mobile phase was Methanol: 1 % v/v glacial acetic acid (15:85 v/v) at a flow rate of 1 ml/min. The determinations were performed using UV-Vis detector set at 280 nm. Mean retention times (minutes) of 1.850 ± 0.056, 5.143 ± 0.1081, 1.397 ± 0.039 and 5.012 ± 0.3291 were recorded for Lamivudine, Paracetamol, p-Aminophenol and Metronidazole respectively. A surrogate constant, K, was determined for Lamivudine using each of the surrogate reference standards. The respective Surrogate constant, K values obtained when Paracetamol, p-Aminophenol and Metronidazole were used as surrogate reference standards were 4.6660 ± 0.013, 7.2885 ± 0.023 and 3.2398 ± 0.024.

The result of the t-test and f-test obtained when the methods were used for the analysis of the Lamivudine tablets and Oral solution were comparable to standard methods of analysis stated in the International Pharmacopoeia. The method showed adequate precision, with a relative standard deviation of (RSD) less than 2% in each case.

The HPLC method developed in this study showed good precision and accuracy and high specificity/selectivity with linearity in the working range and therefore very suitable for quantification of Lamivudine Tablets and Oral solution.

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ABBREVIATIONS

API- Active Pharmaceutical Ingredient

BP- British Pharmacopoeia

DAD- Diode Array Detector

DLI- Direct Liquid Introduction

DVB- Divinyl benzene

GC- Gas Chromatography

HIC- Hydrophobic Interaction Chromatography

IP- International Pharmacopoeia

IUPAC- International Union of Pure and Applied Chemistry

LC- Liquid Chromatography

LLC- Liquid-liquid chromatography

LSC- Liquid-solid Chromatography

LOD- Limit of Detection

LOQ- Limit of Quantitation

RP-HPLC- Reversed-phase High Performance Liquid Chromatography

SEC- Size Exclusion Chromatography

CHAPTER ONE

1.0 INTRODUCTION

Before the discovery of chromatography, techniques like gravimetric analysis, photometry, colourimetry (UV, visible detection), titrimetry (acid base detection) etc. were the sole methods available for analysis. Even though the requirements of analysis for research were simple i.e. there was no necessity for analysis of complex molecules and similar molecules (i.e. molecules with same chemical and physical properties). [1]

However, as research advanced, there were requirements to analyse all the molecules in a given sample matrix for better detection of problems (in clinic), impurities and also deficiencies in industry and research.

This was not possible by the application of simple analytical technique such as photometry and titrimetry alone for the simultaneous detection of myriad of constituents that can be present in a sample. This is because chemicals present in a matrix can have greater physical and chemical similarity or different approaches physically, chemically and biologically.

This challenge was analytically resolved to a large extent by the development of a combined analytical technique whose power of detection and estimation depend on physical and chemical properties of sample constituents. Chromatography as it were, has a great disadvantage of time required in its process. Sometimes a single sample preparation could take a couple of days. In order to overcome limitation, further efforts by scientists led to the development of High Performance Liquid Chromatography (HPLC) with further improvements in speed and

efficiency. Thus, the HPLC technique was discovered to analyse either single component or multicomponent sample matrices at a faster rate with better efficiency. [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]

Due to the widespread use of HPLC in routine analysis, it is imperative that good HPLC methods are developed and thoroughly validated.

In order to develop preliminary HPLC conditions in a timely fashion, scientist use artificial mixtures of Active Pharmaceutical Ingredients (API) and related substances at relatively high concentrations (e.g. 1-2% of related substance relative to API) to develop the preliminary HPLC conditions. The concentration ratio between API and the related substances are maintained to ensure that the chromatography represents that of a real sample. Alternatively, a highly stressed sample (e.g., 5% degradation) can also be used at this stage. With the known composition and high levels of degradation products in the sample, one can evaluate the chromatography to determine whether there are adequate separations for all analytes. The high concentrations of related substances are used to ensure that all peaks will be detected.

An aged HPLC column should be used to develop the initial HPLC conditions. It is usually more difficult to achieve the required resolution with an aged column (e.g., column with about 200

injections). This will reflect the worst case scenario likely to be encountered in actual method uses, and help with the long-term method robustness.

In general, it is usually recommended to develop all methods with HPLC columns from the same vendor. The preferred brand of HPLC column should be selected primarily based on the long term stability and reproducibility.

Sample preparation focuses on the selection of the sample solvent (for extraction) and the proper sample preparation procedures. The effect of solvents of different ratios of organic solvents, pH, extraction volume and extraction procedure on accuracy, precision, sensitivity and the changes in the chromatography (e.g., peak shape, resolution) must be investigated. Whenever possible, the mobile phase must be used in the sample preparation. This will ensure that there will not be any compatibility issues between the sample solution and the HPLC conditions. [4]

The next consideration in the development of HPLC methods is the choice of a detector. There is little use in running a separation if the detector cannot detect the presence of the components of interest. UV-Vis detectors are the most widely used as they can detect a broad range of compounds and have some appreciable degree of selectivity for most analytes. Unfortunately, UV–Vis detectors are not universal detectors so it is worthwhile to look at the chemical structure of the analyte to determine whether it has enough for UV-Visible detection.

Three (3) critical components are very essential in obtaining an efficient method development process; these 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 analyst a chance to critically evaluate the method performance in each component and streamline the final method optimization. Most

analysts, however, focus too much on the HPLC chromatographic conditions and neglect the other 2 components (i.e. sample preparation and standardisation). [4]

It is important that reference standards are available to the analyst for the purpose of validation of the results. These reference standards are normally, pure samples of the compound of interest. In the absence of these pure samples or reference standards, secondary standards or surrogate reference standards may be used.

Surrogate standards are standards of known concentration added to environmental samples for quality control purposes. A surrogate standard is unlikely to be found in environmental samples but has similar properties. Surrogates are intended to monitor for recovery differences, problems during the extraction phase of the analysis and for any potential matrix interferences. [5] Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use. All analytical methods that are intended to be used for analyzing clinical samples will need to be validated. Validation of analytical methods is an essential but time - consuming activity for most analytical development laboratories. It is therefore important to understand the requirements of method validation in more detail and the options that are available to allow for optimal utilization of analytical resources in a development laboratory. [6]

There are many reasons for the need to validate analytical procedures. Among them are regulatory requirements, good science, and quality control requirements. Validation seeks to demonstrate the accuracy, sensitivity, specificity, and reproducibility of either a new or modified analytical method. Finally management of the quality control unit would definitely want to

ensure that the analytical methods that the department uses to release its products are properly validated for its intended use so the product will be safe for human use. [7]

1.1 JUSTIFICATION

The menace of HIV/AIDS in our society today cannot be overemphasized. However, with the influx of anti-retroviral agents onto the Ghanaian market, the emergence of poor quality and substandard anti-retrovirals cannot be underestimated. It is therefore necessary to design analytical procedures that have the potential to overcome the limitations likely to be imposed on quality monitoring of these drugs by the lack of chemical reference standards. It is important to safeguard the quality of these agents because development of resistance by patients to these drugs will not be good for the economic and public health interests of the country. Reference standards, however, play major roles in quantitative HPLC analysis of pharmaceutical products. This is so because the identities and purities of such materials have been ascertained to be of the highest standards and can therefore provide substantive baseline for comparison of analytical results. These reference materials, as a result of the processes involved in their manufacture and purification are very expensive. Most times, and especially in developing economies such as Ghana, chemical reference standards are not readily available because of cost of purchase, importation, delivery period, irregular power supply and facilities for storage. However, these reference materials are an important tool in realising a number of aspects of measurement 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. [8]

Generally, the demand for chemical reference standards 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. [9]

This research therefore seeks to develop an alternative analytical procedure that would make it possible to use quantitative HPLC for assays using compounds related to analyte as surrogate reference standard. Paracetamol, p-Aminophenol and Metronidazole are being used as surrogate reference standards in the analysis of Lamivudine, an antiretroviral drug.

The cost of Lamivudine reference standards in July 2011 is given in Table 1.1. Similar studies have been done successfully with the following pharmaceutical agents: Paracetamol, Aspirin, Diclofenac Sodium, Diazepam, Prednisolone, and Indometacin. Extensive work on anti-infectives is yet to be done and this study is one of the steps to contribute to the new direction. The hypothesis underlying these studies has been proved by the work of Asare-Nkansah *et al* (2011). [10]

Table 1.1 Cost of Lamivudine pure reference samples

Catalog No	Product Description	Current Lot	Previous Lot	Unit Price
1356836	Lamivudine (200mg)	H01378	G0F114 (07/11)	$204.00 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 Lamivudine tablets and Lamivudine Oral solution using HPLC and also to validate the developed method.

1.2.1 SPECIFIC OBJECTIVES

The specific objectives of this research are;

- To develop an HPLC assay procedure for Lamivudine tablets and oral solution using surrogate reference compounds.
- To elute the analyte (i.e. Lamivudine) together with each of the three (3) surrogate reference standards.
- To assess the performance of the method developed using analytical performance parameters such as specificity, selectivity, linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ).
- To determine the surrogate constants (K) for each of the surrogate standards with respect to Lamivudine.
- To determine the percentage content of Lamivudine in various brands of Lamivudine tablets and solution using the method developed.
- To compare the results obtained from the method developed with a standard pharmacopoeial method.

1.3 Hypothesis of study

In instrumental analysis, beyond the limit of quantitation and within the limits of linearity, signal intensity is directly proportional to concentration of solute. If the concentration of a given solute is ~~C and~~ corresponding signal intensity is A, mathematically;

$k = A / C$ where k is a constant of proportionality and specific for a particular substance under a given set of experimental conditions.

Therefore, for two solutions of the same compound with concentrations $C_{analyte}$ and $C_{standard}$, with corresponding signal intensities of $A_{analyte}$ and $A_{standard}$ respectively;

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

However, if the two solutions were for different compounds, that can be co-eluted isocratically with the same respective concentrations and signal intensities as above, their respective constants of proportionality will be the different and consequently;

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

If we assume at this instance that;

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

Then we can deduce that; $$\frac{k_{analyte}}{k_{standard}} = \frac{A_{analyte} \times C_{standard}}{C_{analyte} \times A_{standard}}$$

Therefore;

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

If $\frac{k_{analyte}}{k_{standard}} = K$, then

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

K is a constant that is being reported as the surrogate constant. [10]

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

$A_{standard}$ is the peak response 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, then $C_{analyte}$ which can also be referred to as the actual concentration of analyte, can be determined.

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

Hence percentage content can also be determined 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

2.1.1.1 History and Basic principles of HPLC

High performance liquid chromatography (HPLC) is a technique that has arisen from the application to liquid chromatography (LC) theories and instrumentation that were originally developed for gas chromatography (GC).

Classical liquid chromatography has been around for quite a long time. In the original method an adsorbent, for instance alumina or silica, is packed into a column and is eluted with a suitable liquid. A mixture to be separated is introduced at the top of the column and is washed through the column by the eluting liquid. If a component of the mixture (a solute) is adsorbed weakly on to the surface of the solid stationary phase it will travel down the column faster than another solute that is more strongly adsorbed. Thus the separation of the solutes is possible if there are differences in their adsorption by the solid. This method is called adsorption chromatography or liquid solid chromatography (LSC).

In LC there are other sorption mechanisms that can cause separation, depending on whether one chooses to use liquid or a solid as the stationary phase, or what kind of solid one uses. Liquid-liquid chromatography (LLC) uses a liquid stationary phase coated on to a finely divided inert solid support. Separation here is due to differences in the partition coefficient of solutes between the stationary liquid and the liquid mobile phase. In normal LLC the stationary phase is relatively polar and the mobile phase relatively non-polar, whilst the reverse phase LLC uses a non-polar stationary liquid and a polar mobile phase.

The development of the open-column methods, i.e. paper chromatography (in the 1940s) and thin layer chromatography (in the 1950s), greatly improved the speed and resolution of LC. However, there were still serious limitations compared to modern LC methods, in that, the analysis times were long, resolution was poor and quantitative analysis, preparative separations and automation were difficult.

It was known from gas chromatography theory that the efficiency could be improved if the particle size of the stationary phase materials used in the LC could be reduced. High performance liquid chromatography arose gradually in the late 1960s as these high efficiency materials were produced, and as improvements in the instrumentation allowed the full potential of these materials to be realized. As HPLC has developed, the particle size has become progressively smaller. The stationary phases used today are called microparticulate column packings and commonly uniform, porous silica particles, with spherical or irregular shape, and nominal diameter of 10, 5 or 3 μm. [11]

2.1.1.2 Affinities for Mobile and Stationary Phases

All chromatographic separations, including HPLC operate under the same basic principle; every compound interacts with other chemical species in a characteristic manner. Chromatography separates a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation.

2.1.1.3 Modes of Chromatography

2.1.1.3.1 Adsorption Chromatography (Normal Phase Chromatography)

The separation mechanism depends upon differences in polarity between the different feed components. The more polar a molecule, the more strongly it will be adsorbed by a polar stationary phase. Similarly, the more non-polar a molecule, the more strongly it will be adsorbed by non-polar stationary phase.

During a surface adsorption chromatography process, there is competition for stationary phase adsorption sites, between the materials to be separated and the mobile phase. Feed molecules of low polarity spend proportionally more time in the mobile phase than those molecules that are highly polar, which are retained longer. Therefore the components of a mixture are eluted in order of increasing polarity.

Almost any polar solid can be employed as a polar stationary phase. The choice of stationary phase is governed by the polarity of the feed components. If the feed components are adsorbed too strongly, they may be difficult to remove. Weakly polar mixtures should be separated on highly active absorbents, or little or no separation will occur.

The choice of mobile phase is equally important. The polarity of the mobile phase should be chosen to compliment the choice of stationary phase. In general, good separation is achieved by using fairly polar stationary phases and low polarity mobile phases such as hexane. Water should be noted, as a very polar solvent.

The two most common adsorbents used in chromatography are porous alumina and porous silica gel. Of less importance are carbon, magnesium oxide, and various carbonates. Alumina is a polar

adsorbent and is preferred for the separation of components that are weakly or moderately polar, with the more polar components retained more selectively by the adsorbent, and therefore eluted from the column last. In addition, alumina is a basic adsorbent, thus preferentially retaining acidic compounds. Silica gel is less polar than alumina and is an acidic adsorbent, thus preferentially retaining basic compounds. Carbon is a non-polar (apolar) stationary phase with the highest attraction for larger non-polar molecules.

Adsorbent-type sorbents are better suited for the separation of a mixture on the basis of chemical type (e.g. olefins, esters, acids, aldehydes, alcohols) than for separation of individual members of a homologous series. Partition chromatography is often preferred to the latter, wherein an inert solid (often silica gel) is coated with a liquid phase. Hydrophobic interaction chromatography (HIC) is a special form of surface adsorption chromatography. The materials to be separated should be at least partially hydrophobic in nature. Separation is facilitated by differences in the relative strength of interaction between these materials and a matrix substituted with suitably hydrophobic groups. This type of process is extensively used for the preparative-scale separation of proteins. [13]

2.1.1.3.2 Size-Exclusion Chromatography

Exclusion Chromatography is a technique for separating molecules based on their effective size and shape in solution. The technique is often called gel permeation chromatography if used with organic solvents or gel filtration chromatography if used with aqueous solvents.

The stationary phase used in exclusion chromatography is porous particles with a closely controlled pore size. Unlike other chromatographic mode in exclusion chromatography there should be no interaction between the solute and the surface of the stationary phase.

Depending on their size and shape, solute molecules may be able to enter the pores of the stationary phase particles. Molecules comparable in size with the mobile phase molecules will be able to diffuse throughout the entire porous network. Larger molecules may be excluded from the narrower particles of the porous structure but will be able to move freely in the wider passages. The larger the solute molecules, the fewer places in the porous structure it will find that it can get into. Finally, there may be solute molecules that are so large that they are completely excluded from the pores. These excluded molecules can travel only through the relatively wide channels between the stationary phase particles, and so are rapidly eluted from the column. The smaller the molecule, the more easily it will able to penetrate the pore structures of the stationary phase particles, and the longer it will be retained on the column. [11]

2.1.1.3.2 Partition Chromatography

Unique to chromatography is the liquid-supported or liquid-bonded solids, where the mechanism is absorption into the liquid, also referred to as a partition mode of separation or partition chromatography. With mobile liquid phases, there is a tendency for the stationary liquid phase to be stripped or dissolved. Therefore, the stationary liquid phase has to be chemically bonded to the solid bonding support.

In partition chromatography, the stationary liquid phase is coated onto a solid support such as silica gel, cellulose powder, or kieselguhr (hydrated silica). Assuming that there is no adsorption by the solid support, the feed components move through the system at rates determined by their relative solubilities in the stationary and mobile phases. In general, it is not necessary for the stationary and mobile phases to be totally immiscible, but a low degree of mutual solubility is

desirable. Hydrophilic stationary phase liquids are generally used in conjunction with hydrophobic mobile phases (referred to as "normal-phase chromatography"), or vice versa (referred to as a '"reverse- phase chromatography"). Suitable hydrophilic mobile phases include water, aqueous buffers and alcohols. Hydrophobic mobile phases include hydrocarbons in combination with ethers, esters and chlorinated solvents. [12]

2.1.1.3.3 Ion Exchange Chromatography (IEC)

In this process, the stationary phase consists of an insoluble porous resinous material containing fixed charge-carrying groups. Counter-ions of opposite charge are loosely complexed with these groups. Passage of a liquid mobile phase containing ionised or partially ionised molecules of the same charge as the counter-ions through the system, results in the reversible exchange of these ions. The degree of affinity between the stationary phase and feed ions dictates the rate of migration and hence degree of separation between the different solute species.

The most widely used type of stationary phase is a synthetic copolymer of styrene and divinyl benzene (DVB), produced as very small beads in the micrometer range. Careful control over the amount of DVB added dictates the degree of cross-linking and hence the porosity of the resinous structure.

Resins with a low degree of cross-linking have large pores that allow the diffusion of large ions into the resin beads and facilitate rapid ion exchange. Highly cross- linked resins have pores of sizes similar to those of small ions.

The choice of a particular resin will very much be dependent upon a given application. Cation (+) or anion (-) exchange properties can be introduced by chemical modification of the resin. Ion

exchange chromatography has found widespread uses in industrial processes. This technique is used in the separation of transition metals, the removal of trace metals from industrial effluents and in the purification of a wide range of organic compounds and pharmaceuticals. The resin matrix is usually relatively inexpensive when compared with other types of stationary phase. Ion exchange chromatography is probably the most widely used large-scale chromatographic process, but is limited to ionisable, water soluble molecules. [12]

2.1.1.4 Reverse Phase Chromatography

Reversed phase HPLC (RP-HPLC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica which has been surface-modified with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. With such stationary phases, retention time is longer for molecules which are less polar, while polar molecules elute more readily (early in the analysis). An investigator can increase retention times by adding more water to the mobile phase; thereby making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase. Similarly, an investigator can decrease retention time by adding more organic solvent to the eluent. RP-HPLC is so commonly used that it is often incorrectly referred to as "HPLC" without further specification. The pharmaceutical industry regularly employs RP-HPLC to qualify drugs before their release. [13]

Structural properties of the analyte molecule play an important role in its retention characteristics. In general, an analyte with a larger hydrophobic surface area (C-H, C-C, and generally non-polar atomic bonds, such as S-S and others) is retained longer because it is non-interacting with the water structure. On the other hand, analytes with higher polar surface area

(conferred by the presence of polar groups, such as -OH, $-NH_2$, COO^- or $-NH_3^+$ in their structure) are less retained as they are better integrated into water. Such interactions are subject to steric effects in that very large molecules may have only restricted access to the pores of the stationary phase, where the interactions with surface ligands (alkyl chains) take place. Such surface hindrance typically results in less retention. [13]

2.1.2.0 HPLC Instrumentation

HPLC instrumentation includes a **pump**, **injector**, **column**, **detector** and **data system**. The heart of the system is the column where separation occurs. Since the stationary phase is composed of micrometer size porous particles, a high pressure pump is required to move the mobile phase through the column. The chromatographic process begins by injecting the solute onto the top of the column. Separation of the components occurs as the analytes and the mobile phase are pumped through the column. Eventually, each component elutes from the column as a narrow band (or peak) on a recorder.

Detection of the eluting compounds is important, and this can be either selective or universal. To collect, store and analyse the chromatographic data, a computer, integrator, and other data processing equipment are frequently used. [14][15]

2.1.2.1 Mobile Phases

In HPLC, the type and composition of the eluent is one of the variables that influence the separation. Despite the large variety of solvents used in HPLC, there are several common properties. These include purity, **detector compatibility, solubility** of the sample, **low viscosity and chemical inertness.**

For normal phase mode, solvents are mainly non-polar; for reverse phase, eluents are usually a mixture of water with some polar organic solvents such as **acetonitrile** or **methanol**.

Size exclusion HPLC, however, has special requirements. Eluents have to dissolve polymers, but the most important is that size exclusion eluent has to suppress possible interactions of the sample molecule with the surface of the packing material. [15]

2.1.2.2 Mobile Phase Reservoir

These are made of glass or stainless steel equipped with a means of removing dissolved gases (O2, N2), e.g. degassers, and a means of filtering off dust and particulate matter from solvents, e.g. use of a millipore filter under vacuum (ultrafiltration or ultrasonication). [16] This measure will prevent either base-line drift or continuous spikes on the chromatographic trace. As well as wear to the pumping system.

2.1.2.3 Pumping Systems

High–pressure pumps are needed to force solvents through packed stationary phase beds. Smaller bed particles (e.g. 3 μm) require higher pressures. There are many advantages to using smaller particles, but they may not be essential for all separations. The most important advantages are higher resolution, faster analyses and increased sample load capacity. However, only the most demanding separations require these advances in significant amounts. Many separation problems can be resolved with larger particle packings (e.g. 5 μm) that require less pressure.

Flow–rate stability is another important pump feature that distinguishes pumps. Constant–flow systems are generally of two basic types: reciprocating piston and positive displacement (syringe) pumps. The basic advantage of both systems is their ability to repeat elution volume and peak area, regardless of viscosity changes or column blockage, up to the pressure limit of the pump. Although syringe–type pumps have a pressure capability of up to 540 000 kPa (78 000 psi), they have a limited ability to form gradients. Reciprocating piston pumps can maintain a liquid flow for an indefinite length of time, while a syringe pump needs to be refilled after the syringe volume has been displaced. Dual–headed reciprocating piston pumps provide more reproducible and pulse–free delivery of solvent, which reduces detector noise and enables more reliable integration of peak area. Reciprocating pumps now dominate the HPLC market and are even useful for micro-HPLC applications, as they can maintain a constant flow at flow rates in μL/min ranges.

An additional pump feature found on the more elaborate pumps is external electronic control. Although it adds to the expense of the pump, external electronic control is a very desirable feature when automation or electronically controlled gradients are to be run. Alternatively, this becomes unnecessary when using isocratic methods. The degree of flow control also varies with pump expense. More expensive pumps include such state–of–the–art technology as electronic feedback and multiheaded configurations.

Modern pumps have the following parameters:

- Flow–rate range, 0.01 to 10 mL/min.
- Flow–rate stability, not more than 1% (short term).
- For size exclusion chromatography (SEC), flow–rate stability should be <0.2%.
- Maximum pressure, up to 34 500 kPa (5000 psi). [17]

2.1.2.4 Injectors

An injector for an HPLC system should provide injection of the liquid sample within the range of 0.1 to 100 mL of volume with high reproducibility and under high pressure (up to 27 600 kPa). The injector should also minimise disturbances to the flow of the mobile phase and produce minimum band broadening. Sample introduction can be accomplished in various ways. The injection valve has, in most cases, replaced syringe injection. Valve injection offers rapid, reproducible and essentially operator–independent delivery of a wide range of sample volumes. The most common valve is a six–port Rheodyne valve in which the sample fills an external stainless steel loop. A clockwise turn of the valve rotor places the sample–filled loop into the mobile–phase stream, which deposits the sample onto the top of the column. These valves can be operated manually or actuated via computer–automated systems. One minor disadvantage of valve injection is that the sample loop must be changed to obtain various sample volumes. However, this is a simple procedure that requires a few minutes only. In more sophisticated HPLC systems, automatic sampling devices are incorporated. These auto-samplers have a piston–metering syringe–type pump to suck the preset sample volume into a line and transfer it to a sample loop of adequate size in a standard six–port valve. Most auto-samplers are computer controlled and can serve as the master controller for the whole system.

In HPLC, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. The solvent need not be the mobile phase, but frequently it is wise to choose the mobile phase to avoid detector interference, column–component interference, loss in efficiency or all of these. It is always best to remove particles from the sample by filtration or centrifugation, since continuous injections of particulate material eventually cause blockage of

injection devices or columns. Sample sizes may vary widely. The availability of highly sensitive detectors frequently allows the use of small samples that yield the highest column performance. [17]

2.1.2.5 Columns

Typical HPLC columns are 5, 10, 15 and 25 cm in length and are filled with small diameter (3, 5 or 10 μm) particles. The columns may be made of stainless steel, glass–lined stainless steel or polyetheretherketone (PEEK). The internal diameter of the columns is usually 4.0 or 4.6 mm; this is considered the best compromise between sample capacity, mobile phase consumption, speed and resolution. However, if pure substances are to be collected (preparative scale), larger diameter columns may be needed.

Packing of the column tubing with small diameter particles requires high skill and specialised equipment. For this reason, it is generally recommended that all but the most experienced chromatographers purchase pre–packed columns, since it is difficult to match the high performance of professionally packed HPLC columns without a large investment in time and equipment. In general, HPLC columns are fairly durable and one can expect a long service life unless they are used in some manner that is intrinsically destructive, such as with highly acidic or basic eluents, or with continual injections of 'dirty' biological or crude samples. It is wise to inject some test mixture (under fixed conditions) into a column when new and to retain the chromatogram. If questionable results are obtained later the test mixture can be injected again under specified conditions. The two chromatograms are compared to establish whether or not the column is still useful. [15][17]

2.1.2.6 Detectors

Today, optical detectors are used most frequently in HPLC systems. These detectors pass a beam of light through the flowing column effluent as it passes through a low volume (approx. 10μL) flow–cell. The variations in light intensity, caused by ultraviolet (UV) absorption, fluorescence emission or change in refractive index (depending on the type of detector used) from the sample components passing through the cell, are monitored as changes in the output voltage. These voltage changes are recorded on a strip–chart recorder and frequently are fed into an integrator or computer to provide retention time and peak–area data. The most commonly used detector in HPLC is the ultraviolet absorption detector. A variable wavelength detector of this type, capable of monitoring from 190 to 400 nm, will be found suitable for the detection of the majority of samples.

Other detectors commonly used include diode array, refractive index (RI), fluorescence (FL), electrochemical (EC) and mass spectrometry (MS). Infra–red (IR) and nuclear magnetic resonance (NMR) spectrometers may also be used as detectors.

2.1.2.6.1 Photodiode Array Detector

The photodiode array detector (DAD) is an advanced type of UV detector. Depending on the wavelength, a tungsten lamp and a deuterium lamp are used as light sources. The polychromatic light beam is focused on a flow–cell (volume 8 to 13 μL) and subsequently dispersed by a holographic grating or quartz prism. The spectral light then reaches a chip that contains 100 to 1000 light–sensitive diodes arranged side by side. Each diode only registers a well–defined fraction of the information and in this way all wavelengths are measured at the same time. [15] [17]

2.1.2.6.2 Refractive Index Detector

The RI detector is a universal detector, in that changes in RI (either positive or negative) that arise from the presence of a compound in the eluent are recorded. However, it is also the least–sensitive detector (as much as 100 times less sensitive than UV detection). RI detectors may be used for excipients such as sugars in pharmaceuticals. Many factors influence RI and must be controlled during separation, such as temperature, eluent composition and pressure. The chromatography is best facilitated using a thermostatically controlled cabinet and high–quality pump to minimise pressure fluctuations.

2.1.2.6.3 Fluorescence Detector

In FL detectors, the solute is excited with UV radiation and emits radiation at a longer wavelength. Most detectors allow the selection of both excitation and emission wavelengths. There are only a few drugs and natural compounds that have strong natural fluorescence (e.g. ergot alkaloids), however, many drug derivatives are fluorescent compounds. FL detection can offer great selectivity, since excitation and emission wavelengths as well as retention time can be used to identify drugs. It is necessary to choose eluents carefully when using FL detection. The eluent must neither fluoresce nor absorb at the chosen wavelengths. It is also necessary to consider the pH of the system, in that some drugs only show fluorescence in certain ionic forms.

2.1.2.6.4 Electrochemical Detectors

Electrochemical (EC) detectors measure the current that results from the electrolytic oxidation or reduction of analytes at the surface of an electrode. These detectors are quite sensitive (down to 10^{-15} mole) and also quite selective. Two types of detector are available. Eluents for EC

detection must be electrically conductive. This is accomplished by the addition of inert electrolytes. EC detection is most easily used in the oxidative mode, as use in the reductive mode requires the removal of dissolved oxygen from the eluent. [15][17]

2.1.2.6.5 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. [18]

2.1.2.3 Quantitative analysis In HPLC

The quantification methods incorporated in HPLC is derived mostly from GC methods. The basic theory for quantification involves the measurement of peak height or peak area. To determine the concentration of a compound, the peak area or height is plotted against the concentration of the substance. For peaks that are well resolved, both peak height and area are proportional to the concentration. Three different calibration methods, each with its own benefits and limitations, can be utilised in quantitative analysis, external standard, internal standard and the standard addition method.

2.1.3.1 Ultraviolet-Visible Spectroscopy

The technique of ultraviolet-visible spectroscopy is one of the frequently employed in the pharmaceutical analysis. It involves the measurement of the amount of light of ultraviolet (190-380 nm) or visible (380-800 nm) radiation absorbed by a substance in solution. Instruments which measure the ration or a function of the ratio, of the intensity of two beams of light in the ultraviolet-visible region are called Ultraviolet-visible spectrophotometers. [19]

Ultraviolet–visible spectroscopy or ultraviolet-visible spectrophotometry (UV-Vis or UV/Vis) refers to absorption spectroscopy or reflectance 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 or reflectance 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. The UV-Vis Spectrophotometer measures the intensity of light passing through a sample (I), and compares it to the intensity of light before it passes through the sample (I_o). The ratio I / I_o is called the transmittance, and is usually expressed as a percentage (%T). The absorbance, A, is based on the transmittance:

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

The UV-visible spectrophotometer can also be configured to measure reflectance. In this case, the spectrophotometer measures the intensity of light reflected from a sample (I), and compares

it to the intensity of light reflected from a reference material (I_o)(such as a white tile). The ratio I / I_o is called the reflectance, and is usually expressed as a percentage (%R).

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 (tables of molar extinction coefficients), or more accurately, determined from a calibration curve. [20]

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. A 2nd order polynomial relationship between absorption and concentration is sometimes encountered for very large, complex molecules such as organic dyes (Xylenol Orange or Neutral Red, for example).

The Beer-Lambert law has implicit assumptions that must be met experimentally for it to apply. For instance, the chemical makeup and physical environment of the sample can alter its extinction coefficient. The chemical and physical conditions of a test sample therefore must match reference measurements for conclusions to be valid. [20]

2.2.1 Calibration of UV-Visible Spectrophotometer

The calibration of a spectrophotometer is essential for accurate work in and it must be periodically validated. The instruments absorbance scale and the wavelength scale should be cheeked regularly.

For the control of wavelength, either holmium perchlorate solution containing a 5 % w/v of holmium oxide in 1.4 M perchloric acid or a holmium glass filter is used to calibrate the wavelength scales of the instrument. For either method, only slight differences are observed when the positions of the major wavelengths are compared. For the holmium filter, the International Pharmacopoeia gives the following positions as maxima: 241.5 ± 1, 287.5 ± 1, 360.9 ± 1.5 and 536.2 ± 3 nm. For holmium perchlorate solution, the wavelengths of interest occur exactly at 241.15, 278.2, 361.5 and 536.3 nm. [16]

To check the absorbance scale, a solution containing exactly 0.006% w/v of dry dichromate in 0.001M H_2SO_4, using a I cm cell gives the results as shown in the table below.

Table 2.1: Absorbance Control using 0.00606% w/v Potassium dichromate

Wavelength (nm)	Absorbance (A)	*Permitted Tolerance	A(1%, 1cm)
235	0.748	0.740 - 0.756	124.54
257	0.865	0.857 - 0.874	144.02
313	0.292	0.289 - 0.295	48.62
350	0.640	0.634 - 0.646	106.56
*Permitted tolerance for absorptivity is ± 1%.			

2.2.2 Application in Pharmaceutical Analysis

Absorption spectroscopy is one of the most useful and widely used tools available to the analysts for quantitative analysis. It is used not only for finished pharmaceutical products (such as tablets and capsules) but also for raw materials and intermediate products (such as granules), as well as

tests for tablet dissolution and multi-component mixtures. Also, over 80% of drugs in current use contain chromophoric systems which make them suitable for absorption spectrophotometric analysis. [16]

2.3 Volumetric Analysis

Titration is the process by which a substance is determined by measuring the quantity of a reagent required for the quantitative reaction with the substance. In volumetric analysis, we use standard solution, that is, solutions of accurately known concentration and we measure accurately with a burette the volume of the standard solution (titrant) consumed to complete the titration reaction. The amount of the substance being determined is calculated from the volume of the consumed standard solution, its known concentration, and the equivalent weight of the substance.

The basic goal of a titration reaction is to determine the volume of a standard solution that is chemically equivalent to the substance being titrated. [21]

Titrimetry is widely used for quantitative analysis, and is still the most frequently used in the pharmaceutical assays. It is an absolute method of analysis in which the purity of a substance is determined without a reference standard. The procedure is generally simple and inexpensive, involving the use of volumetric flasks, burettes and pipettes. The choice of titration for an analysis depends on the sensitivity required and the presence of interfering substances and alternate method(s) of analysis. The accuracy and reproducibility of a titrimetric method depends on accurate weighing (using an analytical balance) and preparation of solutions, the tolerance of

the burette, pipette and volumetric glassware used and the ability to locate the end-point of a reaction accurately. [16]

Volumetric methods of analysis are classified in various ways, usually according to the type of the chemical reaction that takes place during the titration. In this way, we have (1) neutralisation (acid-base) titrations, (2) oxidation-reduction (redox) titrations, (3) precipitation titration, and (4) complexometric titrations. [21]

2.3.1 Non-aqueous Titration

A non-aqueous titration involves the use of a solvent other than water as the medium for the titration. This is done to enhance the basicity or acidity of the compound being determined and hence improve the determination of the end-point. The end-point is determined by colour indicator, whose colour depends on the hydrogen ion concentration of the medium. The choice of indicator depends therefore on the pH of the reaction at the end-point; e.g. crystal violet in acetic acid is violet in alkaline, blue-green in neutral and yellowish in acidic pH. Non aqueous titration is usually the method of choice for:

1. Reactants or products which might be insoluble in water or might react with water, or
2. Analytes which are too weakly acidic or basic to be titrated in water.

Thus, weakly basic substances like pyrimethamine and metronidazole, as well as weakly acidic substances like phenobarbitone, some sulphonamides and some organic salts (e.g. ephedrine hydrochloride and pethidine hydrochloride) may be determined by non-aqueous titration. [16]

2.3.2 Redox Titration

Redox titrations are based on an oxidation-reduction reaction between an analyte and a titrant. There must be a sufficiently large difference between the oxidising and reducing capabilities of these agents for the reaction to go to completion and give a sharp end-point; i.e. one should be fairly strong oxidising agent and the other fairly strong reducing agent. The most common oxidising agents in such determinations are iodine, potassium iodate or bromate, ceric ammonium sulphate, potassium permanganate and potassium dichromate. Titanuous chloride, amalgamated zinc and iodine ion are used as reducing agents. The end-point is commonly detected with the use of a redox indicator or by potentiometry; however, with coloured reagents such as $KMNO_4$ and iodine, the reagent itself may act as an indicator. [16]

2.4 Infrared Spectroscopy

Infrared (IR) Spectroscopy is the branch of spectroscopy which is concerned with the measurement of absorption of electromagnetic radiation by molecules due to vibrational energy inherent in them. Even at 0° K, the atoms in a molecule keep vibrating about the bonds linking them. The vibrational energy in a molecule is within the infrared range of the electromagnetic spectrum. [19] The infrared portion of the electromagnetic spectrum is usually divided into three regions; the near-, mid- and far- infrared, named for their relation to the visible spectrum. The higher-energy near-IR, approximately 14000–4000 cm^{-1} (0.8–2.5 μm wavelength) can excite overtone or harmonic vibrations. The mid-infrared, approximately 4000–400 cm^{-1} (2.5–25 μm) may be used to study the fundamental vibrations and associated rotational-vibrational structure. The far-infrared, approximately 400–10 cm^{-1} (25–1000 μm), lying adjacent to the microwave region, has low energy and may be used for rotational spectroscopy. The names and

classifications of these sub-regions are conventions, and are only loosely based on the relative molecular or electromagnetic properties. [22]

Infrared Spectroscopy is one of the most powerful techniques available to organic and analytical chemists. It serves both research applications and more routine studies carried out in application and process-control laboratories. Infrared analysis is applicable to both qualitative and quantitative analyses. Infrared, an invisible part of the electromagnetic spectrum between visible light and radio waves, refers to the radiation (the type of light) used by the spectrometer to perform measurements. Fourier Transform is a complex mathematical computation that is performed by a computer. It is named after the French mathematician and physicist, Baron Jean Baptiste Joseph Fourier, who discovered the mathematical computation FTIR spectrophotometer, is an instrument that provides information about the molecules present in a given sample as well as the quantities. [23]

An infrared source emits light that travels through the optical system of the spectrometer to the matter in the sample compartment. The light excites the molecules in the samples and the molecules in turn absorb some of the light while those remaining are transmitted to the detector. The detector measures the amount of transmitted light converting it into an electrical signal. A display called a spectrum then appears on the computer screen. The spectrum is actually a graphic representation of the sample in terms of the amount of light absorbed (or in other words, transmitted) by the sample at various frequencies (the wavenumbers) along the spectrum. The size and position of the peaks in the sample help in the identification of the composition of the sample. [23]

2.5 Nuclear Magnetic Resonance Spectroscopy (NMR)

2.5.1 Background

Nuclear magnetic resonance spectroscopy (NMR) was first developed in 1946 by research groups at Stanford and M.I.T in the USA. The radar technology developed during World War II made many of the electronic aspects of the NMR spectrometer possible. With the newly developed hardware physicists and chemists began to apply the technology to chemistry and physics problems. Over the past 50 years NMR has developed into the premier organic spectroscopy available to chemists to determine the detailed chemical structure of the chemicals they are synthesizing. Another well- known product of NMR technology has been the Magnetic Resonance Imager (MRI), which is utilized extensively in the medical radiology field to obtain image of soft tissues in the human body. In recent years, NMR has moved out of the research laboratory and into the on-line process analyzer market. This has been made possible by the production of stable permanent magnet technologies that allow high-resolution ^{1}H NMR to be obtained in a process environment. [24]

2.5.2 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. [25]

2.5.3 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. If we take 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. [25]

2.6 Thin Layer Chromatography

Thin-layer chromatography (TLC) is a very commonly used technique in synthetic chemistry for identifying compounds, determining their purity and following the progress of a reaction. It also permits the optimization of the solvent system for a given separation problem. In comparison with column chromatography, it only requires small quantities of the compound (~ng) and is much faster as well. [26]

Although the use of thin layers of adsorbent on glass plates was described as early as 1938, many difficulties were encountered and the technique in its present form was developed much later when suitable materials and apparatus became available as a result of the work of Kirchner (1951-8) and Stahl(1956-8). [19] TLC has achieved phenomenal success not only in its

application to analytical problems (μg scale) and preparative work (mg scale) for which 'thicker" layers can be used, but also in investigating conditions for gravity-feed column and high performance liquid chromatography. [19]

2.6.1 Method

Thin-layer chromatography consists of a stationary phase immobilized on a glass or plastic plate, and an organic solvent. The sample, either liquid or dissolved in a volatile solvent, is deposited as a spot on the stationary phase. The constituents of a sample can be identified by simultaneously running standards with the unknown. The bottom edge of the plate is placed in a solvent reservoir, and the solvent moves up the plate by capillary action. When the solvent front reaches the other edge of the stationary phase, the plate is removed from the solvent reservoir. The different components in the mixture move up the plate at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase. [27]

2.6.2 Visualization

The separated spots on the plate can be visualized in a number of ways and some are listed below.

1. Sulfuric acid/heat: This method is destructive to the compound and leaves charred blots behind.
2. Ceric stain: Also destructive and leaves a dark blue blot behind for polar compounds
3. Iodine: This method is semi-destructive; the iodine gets adsorbed onto the spots though not permanently.

4. UV light: This is non-destructive, long wavelength gives green background and dark spots whereas short wavelength shows glowing compounds on dark plate

2.6.3 Analysis

The components, visible as separated spots, are identified by comparing the distances they have traveled with those of the known reference materials.

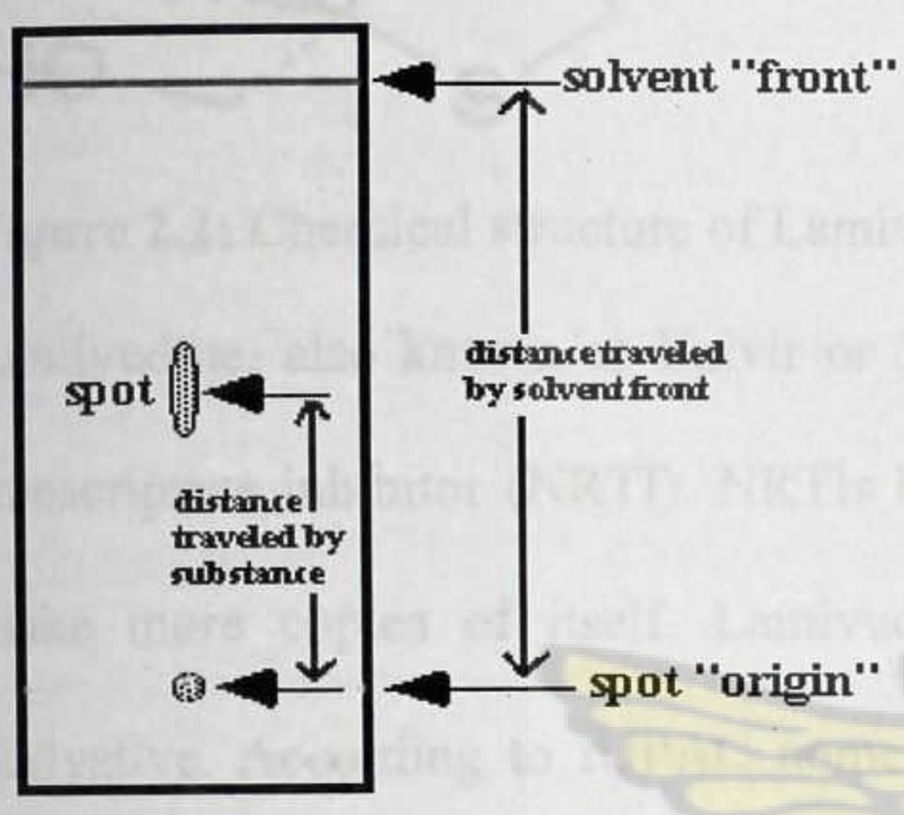

Figure 2.1: TLC plate showing distances travelled by the spot and the solvent after solvent front nearly reached the top of the adsorbent. [28]

The relationship between the distance travelled by the solvent front and the substance is usually expressed as the R_f (retardation factor) value:

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

The R_f depends on solvent system, absorbent (grain size, water content, thickness), amount of material spotted and temperature. Due to the fact that all those variables are difficult to keep constant, a reference compound is usually applied to the plate as well. [26]

2.7 Profile of Pure Analytes

2.7.1 Lamivudine

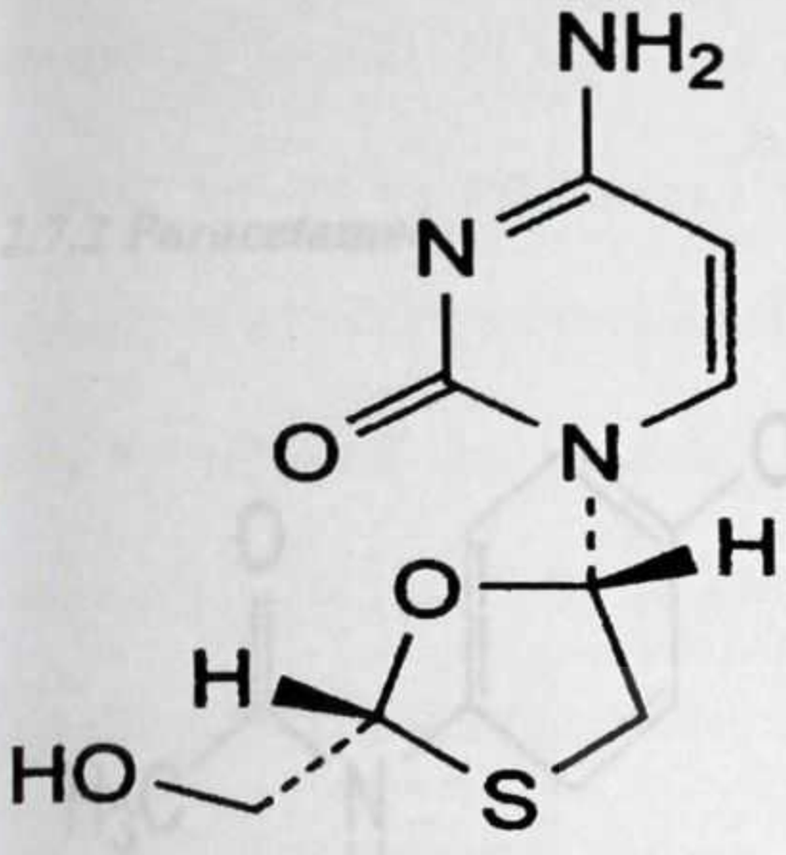

Figure 2.2: Chemical structure of Lamivudine, $C_8H_{11}N_3O_3S$. [29]

Lamivudine, also known as Epivir or 3TC, is a type of medicine called a nucleoside reverse transcriptase inhibitor (NRTI). NRTIs block reverse transcriptase, a protein that HIV needs to make more copies of itself. Lamivudine is a levorotatory pyrimidionone-1, 3-oxathiolane derivative. According to IUPAC nomenclature it is referred to as 4-amino-1-pyrimidin-2-one. Lamivudine is the (-)-enantiomer of a dideoxy analogue of cytidine with a sulfur atom in place of the 3' carbon of the ribose ring of 2-deoxycytidine. Its molecular weight is 229.6 g/mol and its melting point is 160 °C-162 °C.

Lamivudine was approved by the FDA on November 17, 1995, for use in combination with other antiretroviral agents for the treatment of HIV infection in adults and in children more than 3 months old. Although the practice has not received FDA approval, lamivudine may be used to prevent health care workers and others from getting HIV infection after they accidentally come into contact with the virus on the job. Lamivudine alone or in combination with other

antiretrovirals does not cure or prevent HIV infection or AIDS and does not reduce the risk of passing the virus to other people.

2.7.2 Paracetamol

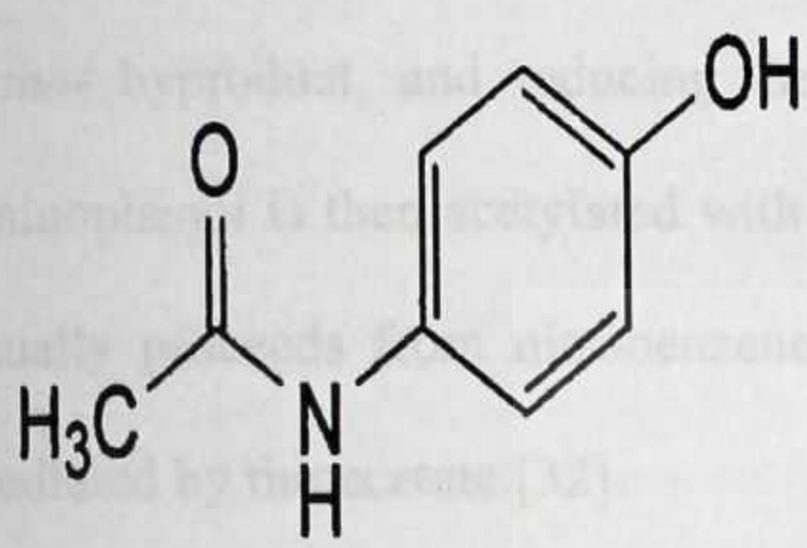

Figure 2.3: Chemical structure of Paracetamol $C_8H_9NO_2$. [29]

Paracetamol is *N*-(4-Hydroxyphenyl) acetamide and in some publications, it is described as 4-hydroxyacetanilide or N-acetyl-p-aminophenol and in the US Pharmacopoeia it is known as acetaminophen. Paracetamol is a white, odourless crystalline powder with a bitter taste, soluble in 70 parts of water (1 in 20 boiling water), 7 parts of alcohol (95%), 13 parts of acetone, 40 parts of glycerol, 9 parts of propylene glycol, 50 parts of chloroform, or 10 parts of methyl alcohol. Paracetamol has a molecular weight of 151.2g/mol and melts at about 168 °C to 172 °C with a specific gravity of 1.263 (BP, 2007).

Paracetamol was first discovered to have both analgesic and antipyretic properties in the late nineteenth century. Prior to this, cinchona bark, which was also used to make the anti-malaria drug quinine, had been used to treat fevers. As cinchona became scarcer, people began to look for cheaper synthetic alternatives. Two of these alternative compounds were acetanilide and

phenacetin, developed in 1886 and 1887 respectively. By this time, Harmon Northrop Morse had already synthesized acetaminophen in 1878 through the reduction of p-nitrophenol with tin in glacial acetic acid. In 1893, the white, odourless crystalline compound with a bitter taste that became known as paracetamol was discovered. Paracetamol may be easily prepared in the laboratory by nitrating phenol with sodium nitrate, separating the desired *p*-nitrophenol from the *ortho*- byproduct, and reducing the nitro group with sodium borohydride. The resultant *p*-aminophenol is then acetylated with acetic anhydride.[30] Industrial preparation of paracetamol usually proceeds from nitrobenzene.[31] A one-step reductive acetamidation reaction can be mediated by thioacetate.[32]

2.7.3 Para aminophenol

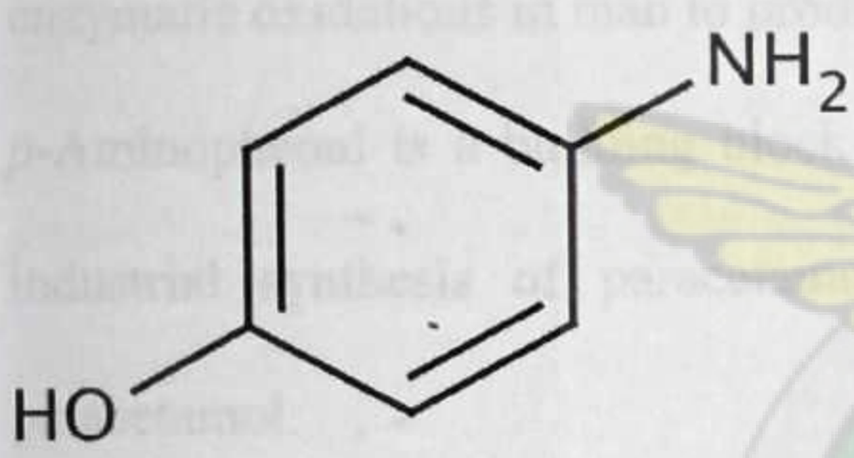

Figure 2.4: Chemical structure of Para aminophenol C_6H_7NO. [29]

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. [32]

Para aminophenol appears as a slightly white or slightly coloured, crystalline powder with melting point of about 186°C, with decomposition. [29] 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: [33]

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

$C_6H_5NHOH \rightarrow HOC_6H_4NH_2$

Para aminophenol reacts with acetic anhydride to give paracetamol. [34]

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. [35]

p-Aminophenol is a building block compound. Prominently, it is the final intermediate in the industrial synthesis of paracetamol. Treating *p*-aminophenol with acetic anhydride gives paracetamol.

2.7.4 Metronidazole

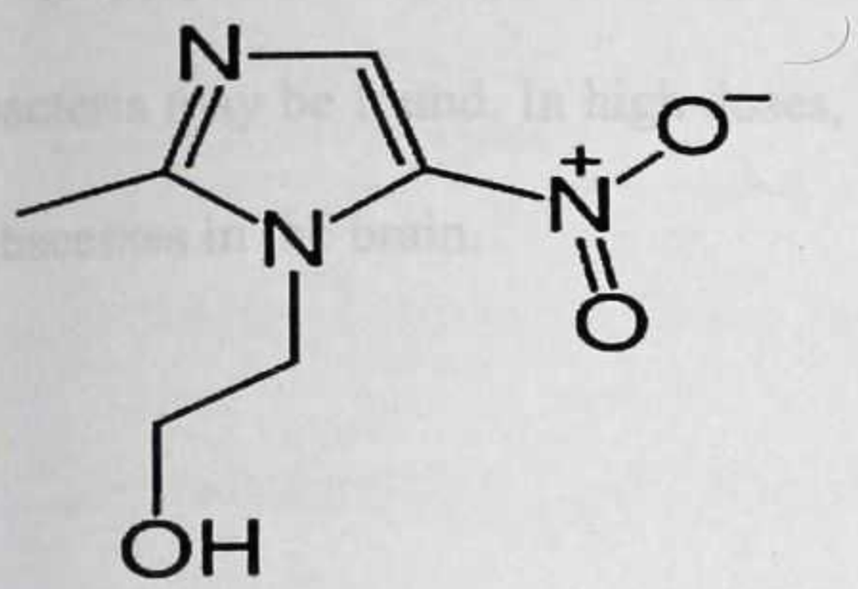

Figure 2.5: Chemical structure of Metronidazole $C_6H_9N_3O_3$ [29]

2-(2-Methyl-5-nitro-1H-imidazol-1-yl) ethanol [29] is the IUPAC name for metronidazole. It 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*. [36]

Metronidazole is white or yellowish, crystalline powder, slightly soluble in water, acetone, in alcohol and in methylene chloride. It darkens on exposure to light but it is stable in air and has a melting point between 159°C and 163°C. [29] 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. [36]

Metronidazole is also used to treat infections with protozoa. These micro-organisms are also anaerobic and include *Trichomonas vaginalis* (which causes trichomonas infection of the vagina) and other protozoa, such as *Entamoeba histolytica* (which causes amoebic dysentry) and *Gardia*

lambila (which causes giardiasis). Metronidazole is also used to prevent infection following surgery, particularly gynaecological surgery and surgery on the gut, where many anaerobic bacteria may be found. In high doses, metronidazole penetrates the brain and can be used to treat abscesses in the brain.

CHAPTER THREE

3.0 EXPERIMENTAL METHODS

3.1 Materials and Reagents

- Cerium (IV) Sulphate (British Drug House-BDH),
- Methanol (HPLC Grade), Hydrochloric Acid, 32 % (BDH),
- Potassium Dichromate, 99.9% (BDH),
- Sulphuric Acid, 98.0% (BDH),
- Glacial Acetic Acid, 99.8% (BDH),
- Sodium Hydroxide pellets, 99.0% (BDH),
- Toluene (BDH), Ethyl Acetate (BDH),
- Perchloric Acid, 70.0% A.C.S (Aldrich).

Table 3.1: Information on Pure samples used

NAME	BATCH NUMBER	DATE OF MANUFACTURE	DATE OF EXPIRY	PERCENTAGE PURITY
Lamivudine	LU1661011	November, 2011	September, 2013	99.86
Metronidazole	09011801	January, 2009	January, 2013	99.90
Paracetamol	11054414	2011	2015	100.2

Source: Metronidazole and Paracetamol are from Amponsah-Efah Pharmaceuticals Limited, Kumasi- Ghana.

Lamivudine pure sample was obtained from Danadams Limited, Accra-Ghana.

4-Aminophenol was obtained from the Department of Pharmaceutical Chemistry, KNUST-Kumasi, Ghana.

Three brands of Lamivudine Tablets and lamivudine Oral Solution manufactured from two different companies were used in this work. Zeffix, the only commercial brand on the market at the time of this project was obtained from a Pharmacy shop (S.K. Osei Pharmacy) in Kejetia, Kumasi. Lamivir Tablet and Lamivudine Oral solution were obtained from The Regional Medical Stores, Kumasi in the Ashanti region of Ghana. Lamdek tablets were also donated by Danadams Limited, Accra-Ghana.

Table 3.2: Profile on Brands of Lamivudine used in the study

DRUG	STRENGTH (mg)	MANUFACTURING COMPANY	BATCH NUMBER	MANUFACTURING DATE	EXPIRY DATE
Zeffix	100	Glaxo Group Ltd. UK.	R504490	-	11/2013
Lamivir	150	Cipla Ltd, India.	KT9345	10/2009	09/2012
Lamdek	150	Danadams Ltd, Ghana	1206157	06/2012	06/2014
Oral Solution	10 / mL	Cipla Ltd, India.	G00136	02/2010	07/2011

Analysis was done in 2011 and 2012

3.2 Instrumentation / Apparatus

A High Performance Liquid Chromatography set up comprising

- Shimadzu Prominence LC-20AT Liquid Chromatograph Pump
- Shimadzu Prominence SIL-20A $_{HT}$ Auto Sampler
- Shimadzu Prominence SPD-20A UV/VIS Detector
- Phenomenex column (15 cm x 4.6 cm) Luna 5 micron C18 100 A
- LCsolution Software and
- Dell Computer Work Station.

Other materials and equipment used include

- Whatman Filter paper 11.0 cm
- Adam PW / 24 Analytical weighing balance
- Cecil CE 2041 2000 Series-UV Spectrophotometer
- Eutech Instrument pH 510/ pHmV/ °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 and
- Sonnicator Bath.

3.3 Identification Tests on Pure Samples

3.3.1 Qualitative Tests (British Pharmacopoeia 2007)

3.3.1.1 Lamivudine Pure Sample

0.0502 g of pure Lamivudine powder was accurately weighed and transferred into a 500 mL volumetric flask. About 400 mL distilled water was added. The mixture was placed in an ultrasonic bath for 5 minutes to dissolve. It was then cooled to room temperature. Distilled water was added to top up to volume. 5 mL of this solution was diluted to 50 mL with 0.1 M sulphuric acid and mixed.

The solution was observed on UV-Visible Spectrophotometer between wavelength range of 210 nm and 300 nm for the presence of absorption maxima at about 280 nm and for specific absorbance between 577 and 637. 50 mL of 0.1 M sulphuric acid was added to 5 mL distilled water and this was used as the blank solution.

3.3.1.2 Paracetamol Pure Sample

1 mL of 1 M hydrochloric acid was added to 0.1 g of Paracetamol to dissolve. The mixture was then heated on a water bath to boil for about 3 minutes. 1mL of distilled water was added and it was cooled in an ice bath. 0.05 mL of 4.9 g/l solution of Potassium Dichromate, which was prepared by dissolving 0.049 g of Potassium dichromate in 10 mL distilled water, was added to the cooled Paracetamol mixture and the mixture observed for colour change.

3.3.1.3 Metronidazole Pure Sample

40.0 mg of pure metronidazole powder was dissolved in 0.1 M hydrochloric acid and diluted to 100 mL with the 0.1 M hydrochloric acid. 5 mL of the solution was diluted to 100 mL with 0.1 M hydrochloric acid. The resulting solution was examined between 230 nm and 350 nm on a UV-Visible Spectrophotometer for wavelengths of absorption maximum and minimum at 277 nm and 240 nm respectively and the specific absorbance.

3.3.1.4 Melting Point Determination

The dry powders of the analyte and each of the surrogate reference standards used in this project were introduced into separate capillary tubes which are sealed at one end. The tubes were filled to about 3 to 5 mm heights by tapping the sealed end of the tubes on a hard surface to enable the powders to pack tightly in the column. Each of them was placed in a digital melting point determination apparatus and their various melting points determined.

3.3.2 Assay of Pure Samples

3.3.2.1 Lamivudine (International Pharmacopoeia 2011)

0.0500 g of pure Lamivudine powder was accurately weighed and transferred into a 500 mL volumetric flask. About 400 mL distilled water was added. The mixture was placed in an ultrasonic bath for 5 minutes to dissolve. It was then cooled to room temperature. Distilled water was added to top up to volume. 5 mL of this solution was diluted to 50 mL with 0.1 M sulphuric acid and mixed.

The absorbance of this solution was measured on UV-Visible Spectrophotometer at a wavelength of maximum absorption at 280 nm. 50 mL of 0.1 M sulphuric acid was added to 5 mL distilled water and this was used as the blank solution.

3.3.2.2 Metronidazole (British Pharmacopoeia 2007)

0.1500 g of pure metronidazole powder 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 Metronidazole.

3.3.2.3 Paracetamol (British Pharmacopoeia 2007)

0.3010 g of pure paracetamol powder was accurately weighed and dissolved in a mixture of distilled 10 mL water and 30 mL dilute sulphuric acid. It was then boiled under a reflux condenser for one hour, cooled and diluted to 100 mL with distilled water. To 20 mL of this solution, 40 mL distilled water was added followed by 40 g ice. 15 mL dilute hydrochloric acid was added. It was titrated with 0.1 M Cerium Sulphate until a greenish-yellow colour was obtained using ferroin solution as the indicator. A blank titration was subsequently carried out.

3.3.3 Thin Layer Chromatography (TLC)

3.3.3.1 Lamivudine Pure Sample and Tablet

100 mg pure lamivudine powder and 100 mg equivalent of lamivudine powdered tablets were separately dissolved in 20 mL of distilled water. 3 mL of distilled water was added to 1 mL each of the lamivudine solutions. The solutions were spotted on a chromatographic plate precoated with 0.25 layer of silica gel, with the aid of a capillary tube. A mobile phase employed consisted of Ethyl Acetate, Methanol and Toluene (11:5:4). A quantity of the mobile phase was poured into a tank to a depth of about 0.5 cm. Equilibration was achieved rapidly by lining the chamber with filter paper which was dipped into the solvent for about 30 minutes after a well fitting lid was placed on it. The plate was placed in an air tight chamber and the solvent was allowed to rise up the plate. The plate was removed and allowed to dry. The spots were examined under the plate under an Ultraviolet light at 254 nm. The Rf values of the samples and pure powder were compared.

3.3.3.2 Metronidazole Pure Sample

200 mg pure metronidazole powder was dissolved in 10 mL of methanol. 1 mL of this solution was pipette and diluted with 3mL of methanol. The solutions were spotted on a chromatographic plate precoated with 0.25 mm layer of silica gel, with the aid of a capillary tube. A mobile phase employed consisted of 15 mL of Ethyl Acetate, 5mL of Methanol and 10 drops of Ammonia solution. A quantity of the mobile phase was poured into a tank to a depth of about 0.5 cm. Equilibration was achieved rapidly by lining the chamber with filter paper which was dipped into the solvent for about 30 minutes after a well fitting lid was placed on it. The plate was placed in the air tight chamber and the solvent was allowed to rise up the plate. The plate was removed and

allowed to dry. The spots were examined under an Ultraviolet light at 254 nm. The Rf value was calculated.

3.4 Standardization of Solutions

3.4.1 Standardization of 0.1M Perchloric Acid

Potassium hydrogen phthalate (0.5007 g) was weighed and transferred into a 250 mL conical flask. 25 mL of glacial acetic acid was added. It was dissolved by warming in a hot water bath. It was then cooled and titrated against 0.1 M Perchloric acid using Oracet blue as the indicator.

3.5 Uniformity of Weight

3.5.1 Uniformity of weight of Lamivudine Tablets [International Pharmacopoeia 2011].

Twenty tablets of each of the three brands were weighed. The tablets were also weighed individually and the average weights, deviations and percentage deviations were determined. This was to ensure that the set of twenty tablets that were randomly selected to define the average weight of a tablet for a brand was uniformly distributed.

3.6 Determination of Percentage Content of Lamivudine in Lamivudine Tablets (150 mg & 100 mg) [International Pharmacopoeia 2011]

Twenty tablets each of the three brands were weighed and powdered. A quantity of the powdered tablets equivalent to 50 mg of lamivudine (0.10074g of lamivir, 0.113925g of Zeffix and 0.12031g of Lamdek) was weighed and dissolved completely in about 400 mL of water and transferred into a 500 mL volumetric flask. The mixture was sonicated for about 5 minutes. It was diluted to the 500 mL mark with water. The solution was filtered through a 0.45 micron filter and the first 20 mL of the filtrate was discarded. 5 mL of the solution was diluted to 50 mL

with 0.1 M sulphuric acid. The absorbance of the solution was measured in a 1-cm layer at the wavelength of 280 nm on a UV-Visible Spectrophotometer against a solvent cell containing the blank. The blank was prepared by diluting 5 mL of water to 50 mL with 0.1 M sulphuric acid in a 50 mL volumetric flask.

3.7 Determination of Percentage Content of Lamivudine in Lamivudine Oral Solution (10 mg/mL) [International Pharmacopoeia 2011]

A quantity of the oral solution equivalent to 20 mg Lamivudine (2 ml of Lamivudine Oral Solution) accurately pipetted, was diluted to 50 mL with water. 1 mL of 0.1 M sulphuric acid was added and extracted with two 30 mL portions of diethyl ether. The combined ether extracts were washed with 20 mL of water. Residual ether was removed by using a current of nitrogen. Sufficient water was added to make 200 mL. 5 mL of this solution was diluted to 50 mL with 0.1 M sulphuric acid. The absorbance of the resulting solution was measured in a 1-cm layer at the wavelength of 280 nm on a UV-Visible Spectrophotometer against a solvent cell containing the blank. The blank was prepared by diluting 5 mL of water to 50 mL with 0.1 M sulphuric acid in a 50 mL volumetric flask.

3.8 HPLC METHOD DEVELOPMENT

3.8.1 Preparation of Mobile Phase

A mobile phase of 1% acetic acid and methanol in the ratio of 85:15 was settled upon after several other ratios were investigated (50:50, 60:40, 70:30). This is because although it involves less amount of the organic phase which is normally expensive, it also gave equally good resolution for the drug substance and all of the surrogate reference standards.

3.8.2 Selection of Wavelength of Maximum Absorption

0.1000g of each sample was weighed and dissolved with methanol into separate 100 mL volumetric flasks. 10 mL each of the resulting solutions were diluted to 100 mL in separate 100 mL volumetric flasks. Another 10 mL each was diluted to 100 mL in separate 100 mL volumetric flasks to obtain solutions of concentrations of 0.01 % w/v. The various solutions were scanned within a wavelength range of 200 nm to 400 nm on a Shimadzu UV- 1800 spectrophotometer. The drug of interest, Lamivudine, was found to have maximum absorbance at wavelength of 280 nm. A concentration of 0.0001% w/v of the analyte and each of the surrogate reference standards were prepared by serial dilution with the mobile phase and injected separately under the chromatographic condition stated below and each of them gave significant peaks. The wavelength of 280 nm was therefore adopted for the study.

3.8.3 Chromatographic Conditions

The following was the condition employed

- ✓ Column: Luna 5µ C18 (2), 150 x 4.6 mm 5 micron; Pore size of 100 A; manufactured by Phenomenex, U.S.A.
- ✓ Chromatograph settings:
- ✓ Flow Rate: 1.0 mL/min
- ✓ Detector: UV-Visible detector, 280 nm;

  Sensitivity: 0.5 AUF
- ✓ Solvent System: 1% Acetic Acid: Methanol in the ratio 85:15.
- ✓ Injection Volume: 20 µL

3.8.4 Stability Studies on Pure sample in solution

Known concentrations of pure lamivudine powder and the surrogates were prepared. The sample solutions were injected at an interval of 30 minutes for six hours and at an interval of two hours for the next six hours. Their peak areas were recorded and a graph of peak area against time was plotted to determine the stability of the drug in the solution within the time stated.

3.9 ANALYTICAL PERFORMANCE PARAMETERS

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

A stock solution of 0.005% w/v of both the drug sample and all the surrogate reference standards were prepared and serially diluted to different concentrations. 0.0006, 0.0005, 0.0004, 0.0002, 0.0001%w/v were the concentrations used for the lamivudine pure powder. 0.0015, 0.00105, 0.00075, 0.00045 and 0.00015%w/v were the concentrations used in Paracetamol. 0.003, 0.0025, 0.002, 0.001, 0.0005 % w/v were those for p-Aminophenol and 0.002, 0.0015, 0.001, 0.0005, 0.0003 % w/v for Metronidazole. Twenty micro litres (20 µL) of the resultant solutions were injected and peak responses recorded. The limit of detection (LOD) and the Limit of Quantitation (LOQ) were determined using the following formulae;

$LOD = 3.3\sigma / S$ and $LOQ = 10\sigma / S$

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.

3.9.2 Linearity

A stock solution of 0.005% w/v of Lamivudine pure powder and all the surrogate reference standards were prepared and further diluted to different concentrations. 0.0006, 0.0005, 0.0004, 0.0002, 0.0001 % w/v were the concentrations used for the lamivudine pure powder. 0.0015, 0.00105, 0.00075, 0.00045 and 0.00015 % w/v were the concentrations used in Paracetamol. 0.003, 0.0025, 0.002, 0.001, 0.0005 % w/v were those for p-Aminophenol and 0.002, 0.0015, 0.001, 0.0005, 0.0003 % w/v for Metronidazole. A twenty micro litres (20 µL) of the different concentrations prepared were injected and the peak responses recorded. A plot of peak response against the various concentrations was carried out.

3.9.3 Reproducibility and Repeatability

Inter-day and Intra –day Variations

The analytes and their respective surrogate reference standards were injected into the chromatograph six times a day for three days. Fresh solvent systems as well as analytes and reference standards were prepared on all of the days. The analyte and the surrogate reference standards were injected repeatedly in each of the analysis to determine the precision between the assays done within the day for each sample.

3.9.4 Accuracy and Precision

The results of the developed method and the standard pharmacopoeia method were subjected to tests to determine their accuracy. The tests used were the relative standard deviation, Standard Error of Mean, the F-Test and the T-Test.

3.10 Determination of Surrogate Constants (K) for the surrogate reference standards.

Four (4) stock solutions of 0.005 % w/v of lamivudine pure powder and the surrogate reference standards were prepared and filtered. Each of these solutions contained 0.00005 mg per ml (0.005 % w/v) of pure lamivudine and the respective surrogate reference standards. Seven different concentrations (0.0006, 0.0005, 0.0004, 0.0003, 0.0002, 0.00015 and 0.0001 % w/v) were prepared from the stock solution.

For Paracetamol, 0.0015, 0.001, 0.00075 and 0.0005 % w/v final solutions were prepared out of the stock. For p-Aminophenol, 0.003, 0.0025, 0.0015 and 0.0005 % w/v final solutions were prepared out of the stock solution. For Metronidazole, 0.002, 0.0015, 0.00066 and 0.0003 % w/v final solutions were prepared from its stock solution. 5 mL each of the lamivudine final solutions and the corresponding concentration of the surrogate reference standard were mixed and 20 µL each of the resulting solutions was injected four times. The corresponding peak responses were recorded.

From the hypothesis of the study, the surrogate constant K relates to the concentration and corresponding peak response of a sample to another. The constant K for any two samples does not depend on the concentration of one since the concentration will be compensated for by the corresponding peak response. The calculated K for each two different concentrations should not change. The constant K for each surrogate reference standard against lamivudine was calculated using the relation for K stated in the hypothesis.

3.11 Analysis of Commercial Samples Using the Surrogate Reference Standards

3.11.1 Lamivudine Tablets

Twenty tablets of each of the three brands of Lamivudine tablets were separately powdered. 0.11393g of powdered Zeffix tablets, 0.10074 g of powdered Lamivir tablet and 0.12031 g of Lamdek tablets which is equivalent to 50 mg lamivudine were weighed and separately dissolved in three 100 mL of the mobile phase (1% Acetic Acid: methanol, 85:15) and sonnicated for 10 minutes. 10 mL each of the resulting solutions were separately diluted to 100 mL with the same mobile phase.

Similarly, 5 mL of Lamivudine oral solution was pipetted and dissolved with 100 mL of the mobile phase and sonnicated for about 10 minutes. 10 mL of the resulting solution was diluted to 100 mL with the mobile phase and filtered discarding the first 20 mL of filtrate.

Each of these solutions contained 0.00005 mg per ml (0.005 % w/v) of pure lamivudine. Seven different concentrations (0.0006, 0.0005, 0.0004, 0.0003, 0.0002, 0.00015 and 0.0001 % w/v) were prepared from the stock solution.

0.005 % w/v each of the surrogate reference standards was prepared and filtered, discarding the first 20 mL filtrate. For Paracetamol, 0.0015, 0.001, 0.00075 and 0.0005 % w/v final solutions were prepared out of the stock. For p-Aminophenol, 0.003, 0.0025, 0.0015 and 0.0005 % w/v final solutions were prepared out of the stock solution. For Metronidazole, 0.00200, 0.00150, 0.00066 and 0.00030 % w/v final solutions were prepared from its stock solution. 5 mL each of the lamivudine final solutions and corresponding concentrations of the surrogate reference standard were mixed and 20 µL each of the final solutions were injected four times. The corresponding peak responses were read and recorded.

CHAPTER FOUR

4.0 RESULTS AND CALCULATION

4.1 Identification of Pure Samples

4.1.1 Qualitative Test

4.1.1.1 Results of Colour and UV-Vis Tests

Table 4.1: Results of Identity Tests on Samples

Sample	Result	Inference
Lamivudine	The solution showed an absorption maximum at 280 nm. The specific absorbance at the maximum is between 577 and 637.	Positive (IP)
Paracetamol	No precipitate was formed upon cooling in an ice bath. A violet colour was finally formed which did not change to red.	Positive (BP)
Metronidazole	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)

4.1.1.2 MELTING POINT DETERMINATION

Table 4.2: Results of Melting Point Determination

Sample	Literature Value (^{O}C)	Experimental Value (^{O}C)
Lamivudine	174 – 180	178 - 180
Paracetamol	168 – 172	168 - 170
Metronidazole	159 -163	160 - 163
Para-Aminophenol	About 186	186 - 188

4.1.2 Assay of Pure Sample

4.1.2.1 Lamivudine Pure Samples and the Surrogate Reference Standards

Table 4.3: Results of Average Percentage Purities of Pure Samples

Samples	Mean Percentage Purity (%)	Standard Deviation
Lamivudine	99.86 ± 0.089	0.1999
Paracetamol	98.92 ± 0.089	0.1757
Metronidazole	100.51 ± 0.269	0.5374

4.1.3 THIN LAYER CHROMATOGRAPHY (TLC)

4.1.3.1 TLC for Lamivudine Tablet

Figure 4.1: TLC plate of Lamivudine Tablet (Zeffix) from Glaxo Group Limited

Table 4.4: Results of Rf Values of Lamivudine pure Sample, tablets and the oral solution

SAMPLE	Rf Value for Pure Sample (Reference)	Rf Value for Drug Product
Lamivudine (Zeffix)	0.695	0.695
Lamivudine (Lamdek)	0.697	0.697
Lamivudine (Lamivir)	0.698	0.698
Lamivudine Oral Solution	0.697	0.697

4.2 Calculation of Factors

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

Amount of $C_8O_4H_5K$ weighed = 0.5007 g

Factor of $C_8O_4H_5K$ = Actual weight / Nominal weight

= 0.5007/0.5

= 1.0014

Volume of $HClO_4$= 25.20 mL

Volume of $C_8O_4H_5K$ = 25 mL

Factor ($HClO4$) = [Factor ($C_8O_4H_5K$) × Volume ($C_8O_4H_5K$)] / Volume ($HClO_4$)

= (1.0014 x 25)/ 25.20

= 0.99345

4.3 Uniformity of Weight

Refer to Table A3 and A4 in the appendix

4.4 Determination of Percentage Content of Lamivudine in Lamivudine Tablets using Standard Method. (International Pharmacopoeia 2011)

Product: Lamivudine Tablet (Zeffix).

100 mg of pure Lamivudine is equivalent to 0.2279 g of Lamivudine tablet

Therefore 0.05 g of pure Lamivudine will be equivalent to 0.1139 g of Lamivudine tablets

Test 1

A [1 %, 1 cm] of pure Lamivudine at 280 nm = 607

Expected Absorbance of 0.001 % Lamivudine at 280 nm = 0.607

Absorbance of test sample at 280 nm = 0.582

% Content of sample = [0.582 / 0.607] x 100

= 95.88 %.

Test 2

A [1 %, 1 cm] of pure Lamivudine at 280 nm = 607

Expected Absorbance of 0.001 % Lamivudine at 280 nm = 0.607

Absorbance of test sample at 280 nm = 0.607

% Content of sample = [0.607 / 0.607] x 100

= 100.00 %.

Average Percentage content = [95.88 + 100] /2

= 97.94 %

Table 4.5: Results obtained from the analysis of Lamivudine Products

SAMPLE	Batch Number	PERCENTAGE CONTENT (%)					
		1	2	3	4	5	Mean Percentage Content
Zeffix Tablet	R504490	97.94	96.61	98.35	99.1	99.34	98.95 ± 0.34
Lamivir Tablet	KT9345	96.54	96.95	96.87	98.19	97.78	97.27 ± 0.31
Lamdek Tablet	1206157	103.05	102.80	102.97	103.13	101.81	102.75 ± 0.24
Oral Solution	G00136	99.75	99.51	99.67	99.84	99.67	99.69 ± 0.05

4.5 HPLC METHOD DEVELOPMENT

4.5.1 UV SPECTRUM OF SAMPLES

Table 4.6 Wavelength of Maximum Absorption of Samples

SAMPLE	WAVELENGTH RANGE (nm) OF ABSORPTION	WAVELENGTH OF MAXIMUM ABSORBANCE (nm)
Lamivudine	200 – 400	280
Paracetamol	200 – 400	250
Metronidazole	200 – 400	312
p-Aminophenol	200 – 400	300

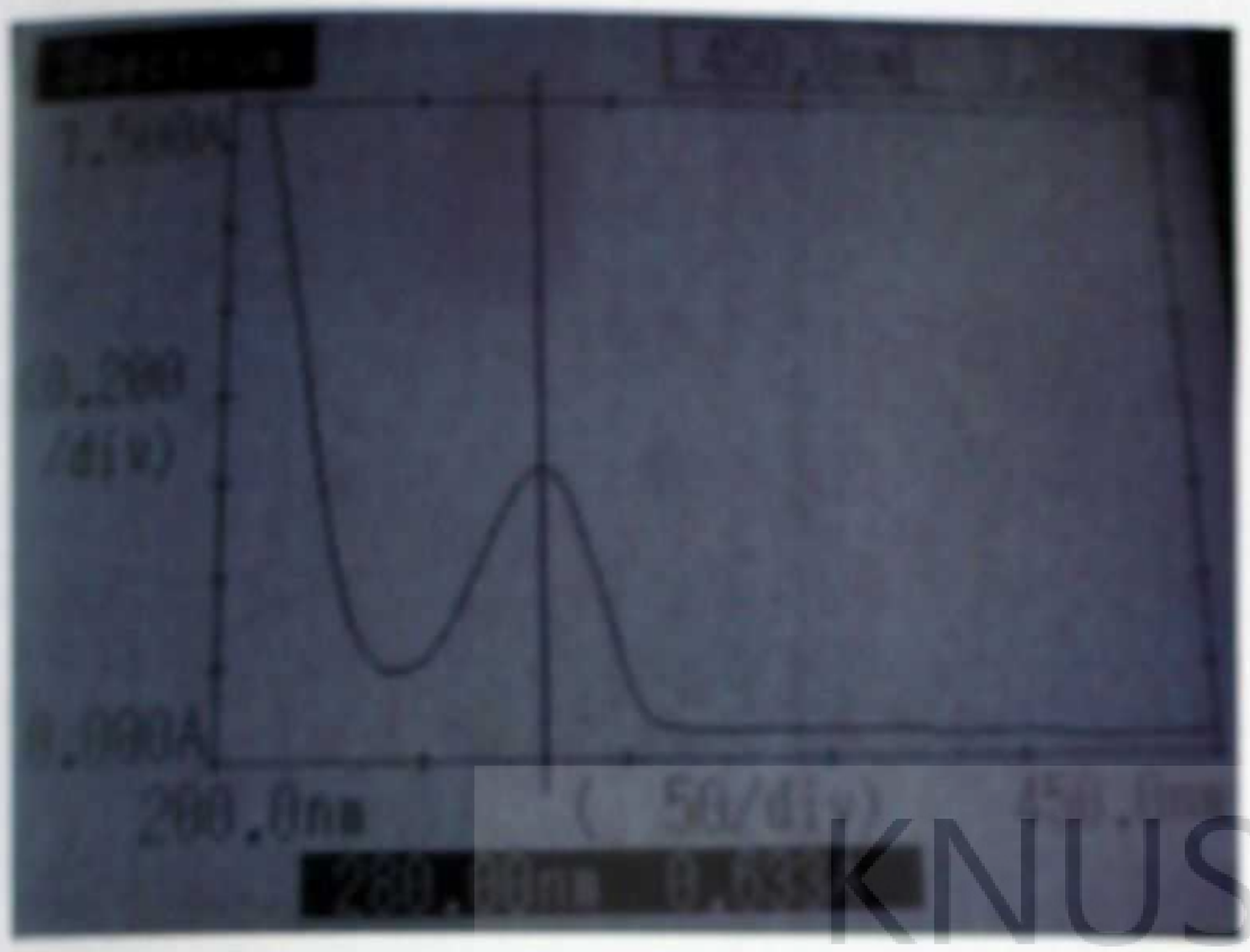

Figure 4.2: UV Spectrum of Pure Lamivudine Powder

Figure 4.3: UV Spectrum of Pure Metronidazole Powder

Figure 4.4 UV Spectrum of Pure p-Aminophenol Powder

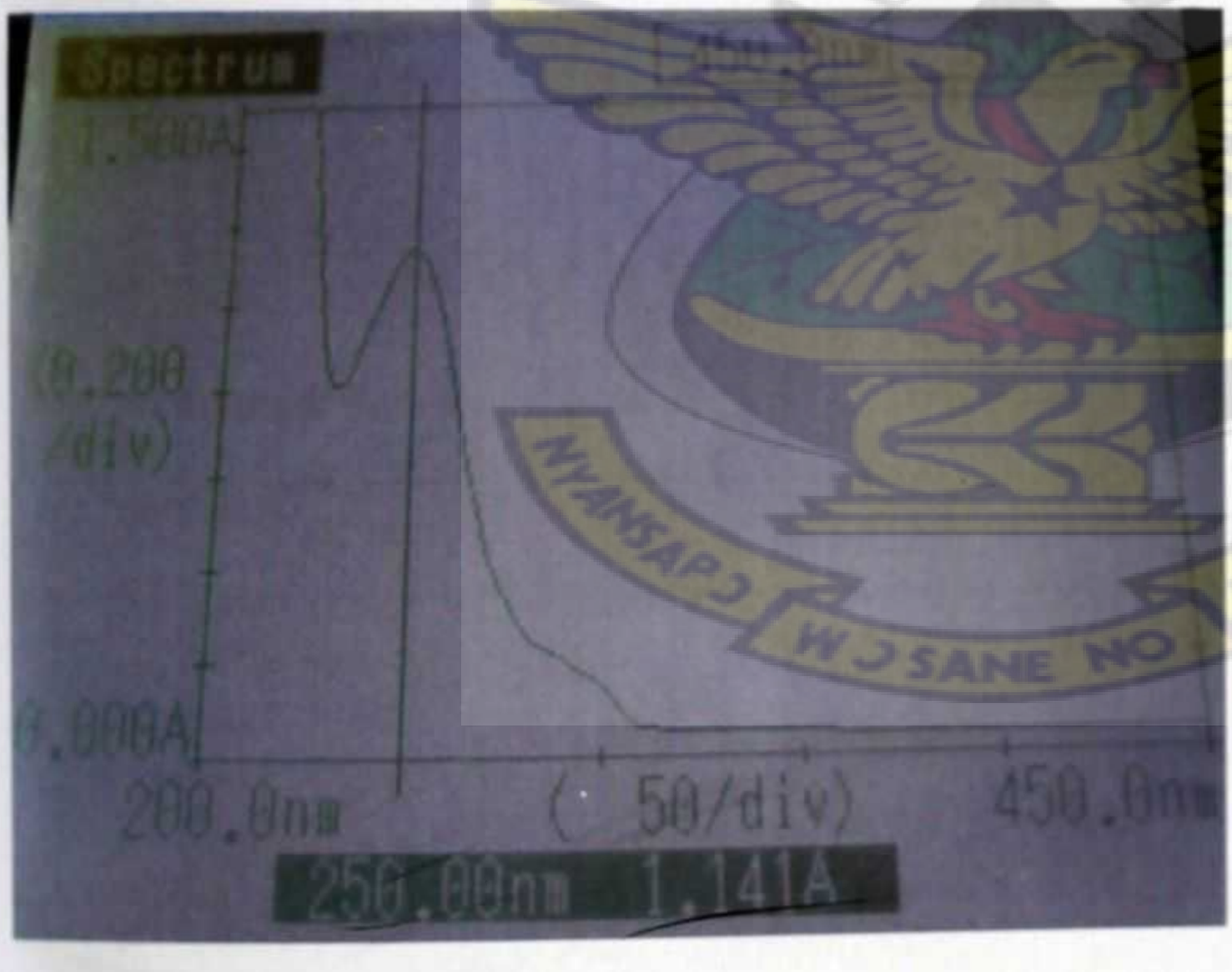

Figure 4.5: UV Spectrum of Pure Paracetamol Powder

4.5.2 Chromatographic conditions for the Analysis of Lamivudine

The HPLC conditions used for the analysis of Lamivudine are as follows

- Mobile phase: 1 % Acetic Acid : Methanol (85:15)
- Stationary phase: ODS C 18 Phenomenex 150 mm x 4.6 mm column
- Detector: UV-Visible Detector
- Wavelength: 280 nm
- Flow rate: 1 mL / min
- Sensitivity: 0.500 AUF
- Injector Volume: 20 µL

Table 4.7 Mean Retention Time for Lamivudine and the Surrogates Standards

SAMPLE	MEAN RETENTION TIME (min)
Lamivudine	1.850 ± 0.056
Paracetamol	5.143 ± 0.1081
P-Aminophenol	1.397 ± 0.0393
Metronidazole	5.012 ± 0.3291

4.5.3 Chromatograms

4.5.3.1 Lamivudine and the surrogate standards

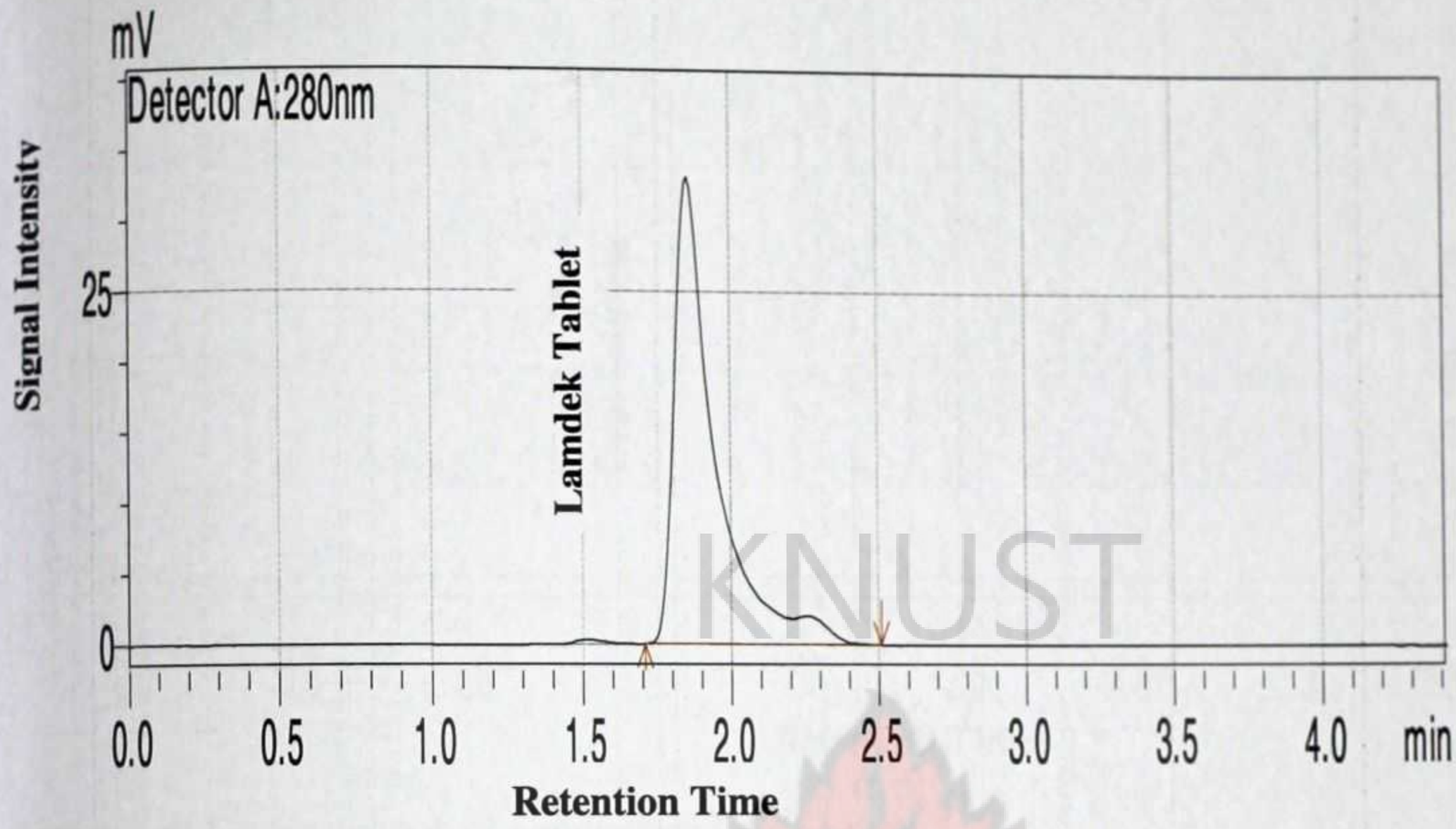

Figure 4.6: Chromatogram of Lamdek tablet

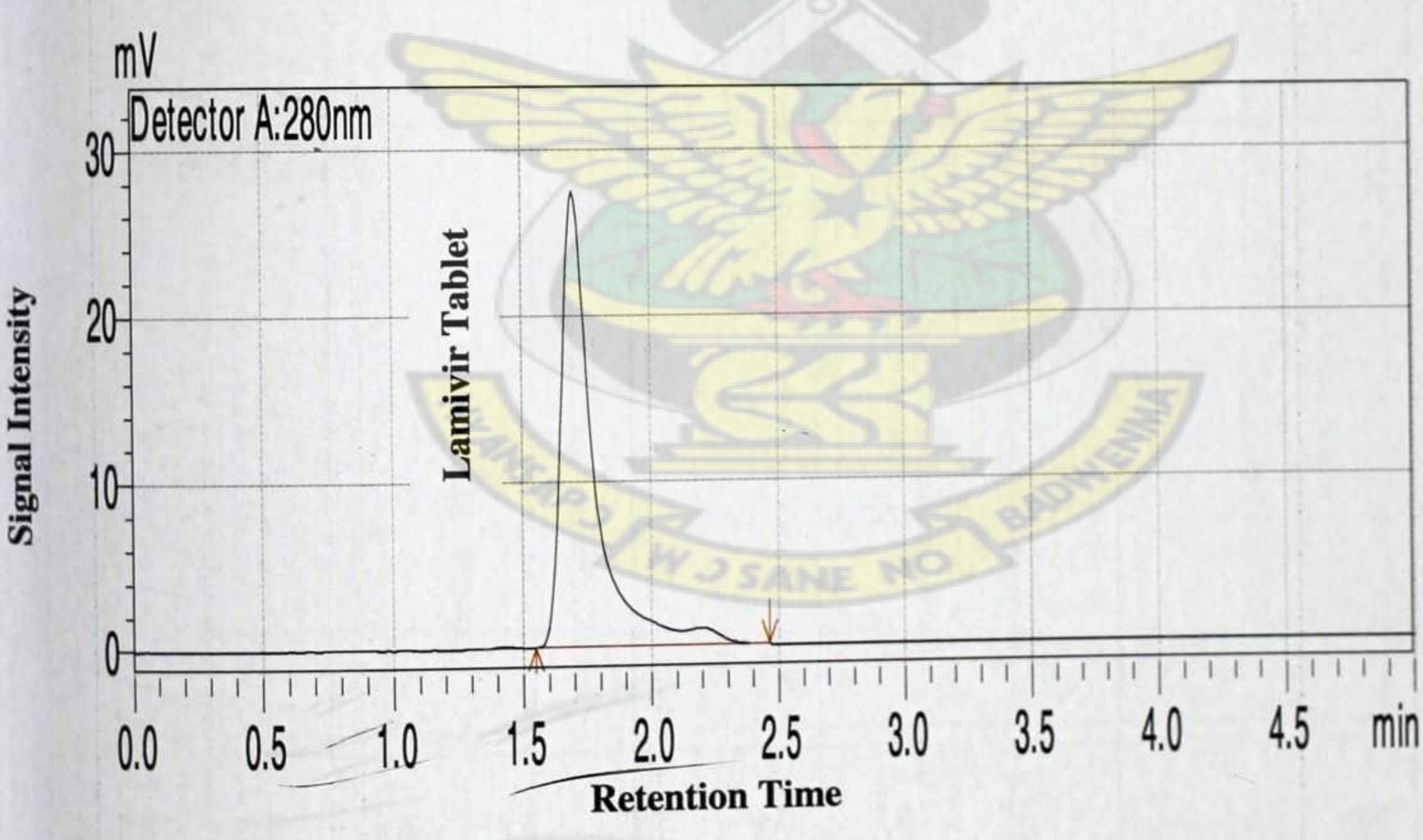

Figure 4.7: Chromatogram of Lamivir tablet

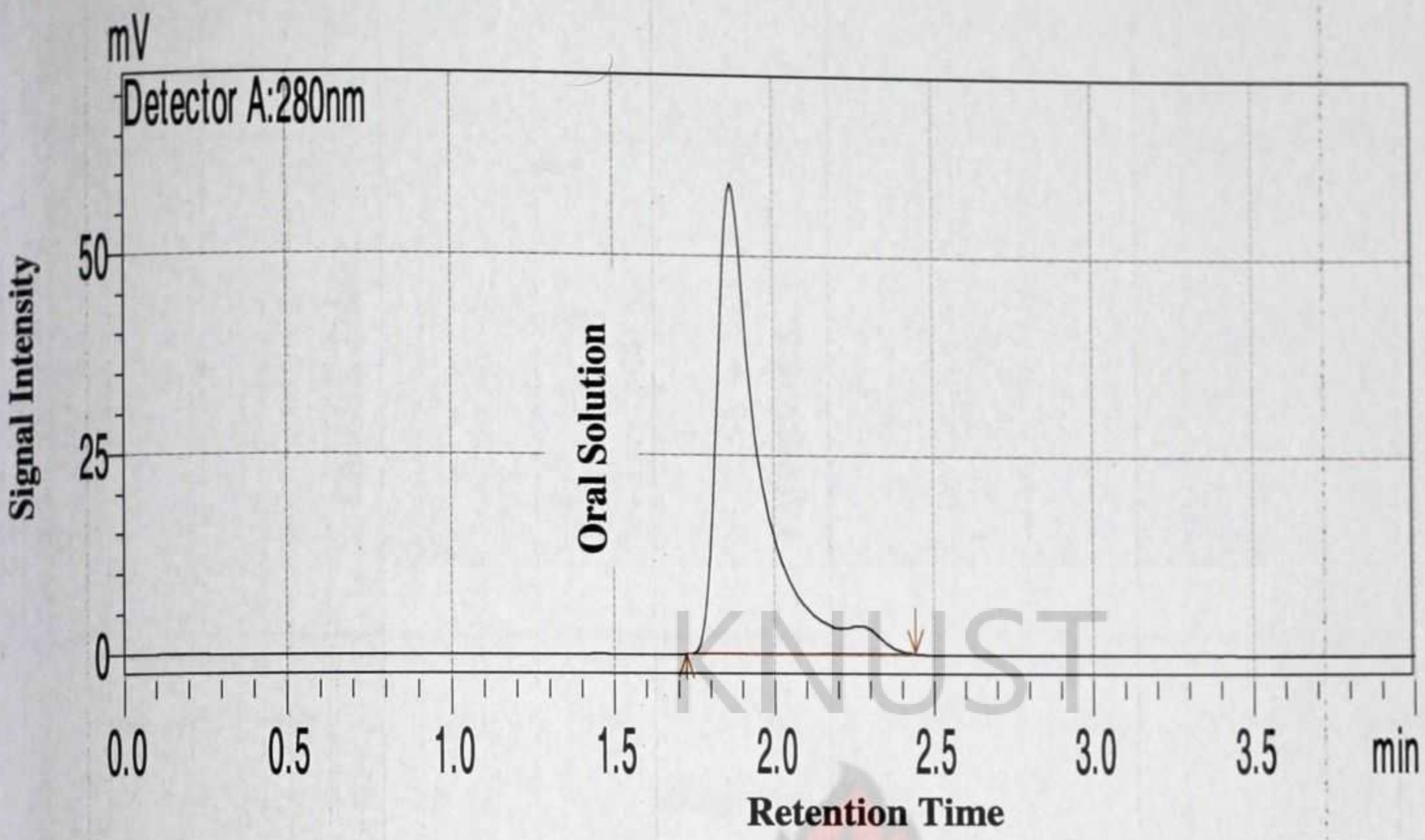

Figure 4.8: Chromatogram of Lamivudine Oral Solution

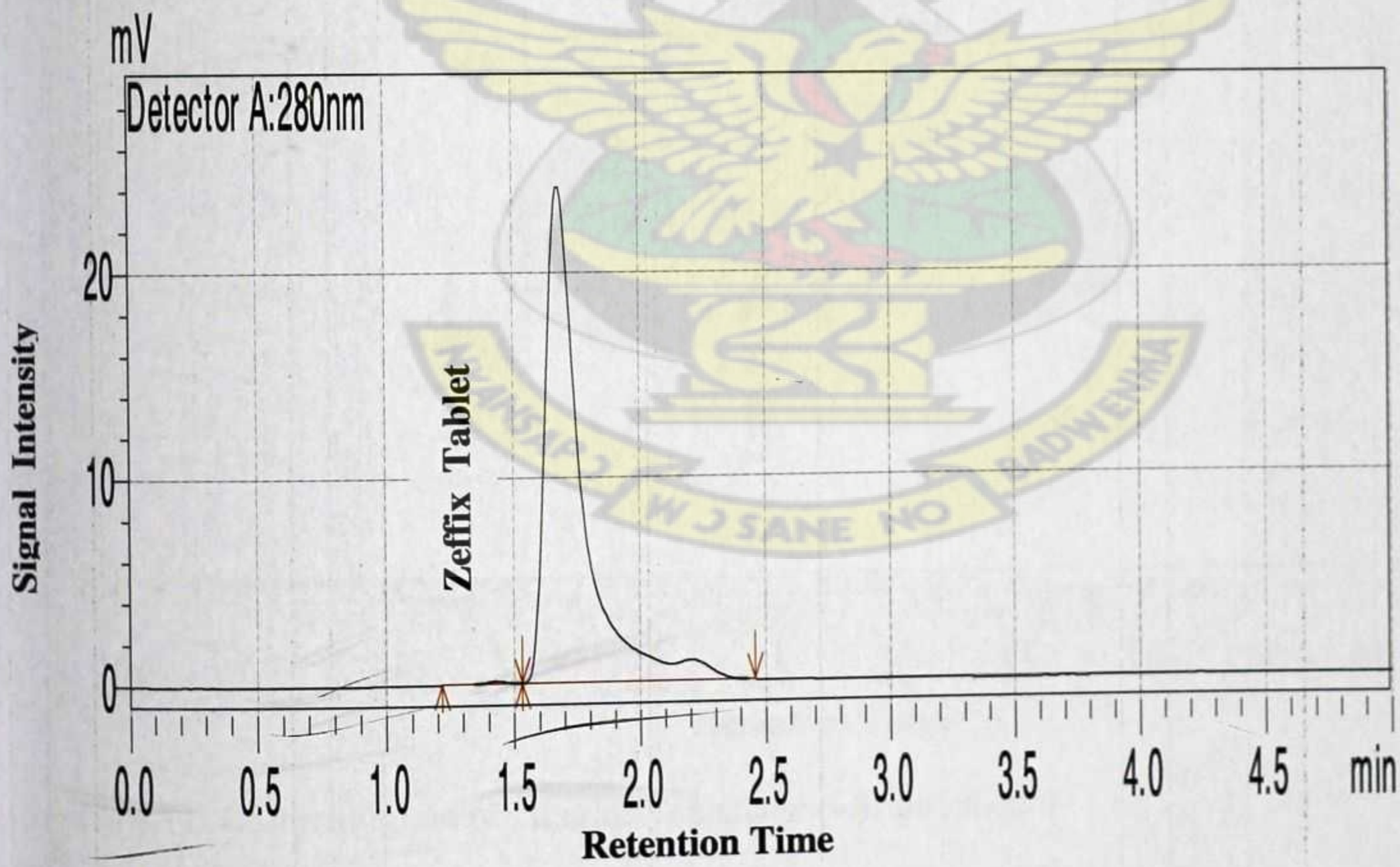

Figure 4.9: Chromatogram of Zeffix Tablet

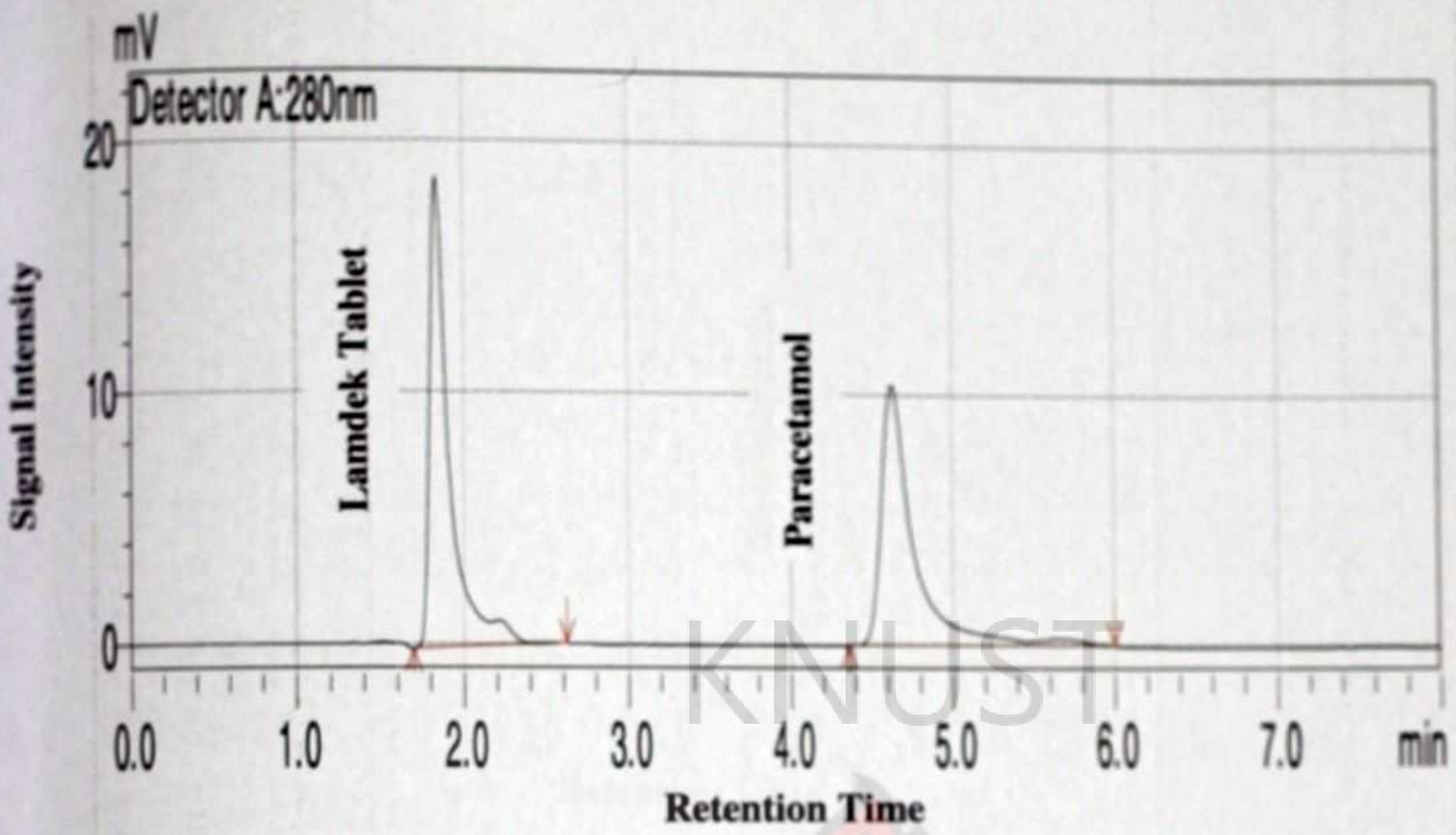

Figure 4.10: Chromatogram of Lamdek Tablet and Paracetamol

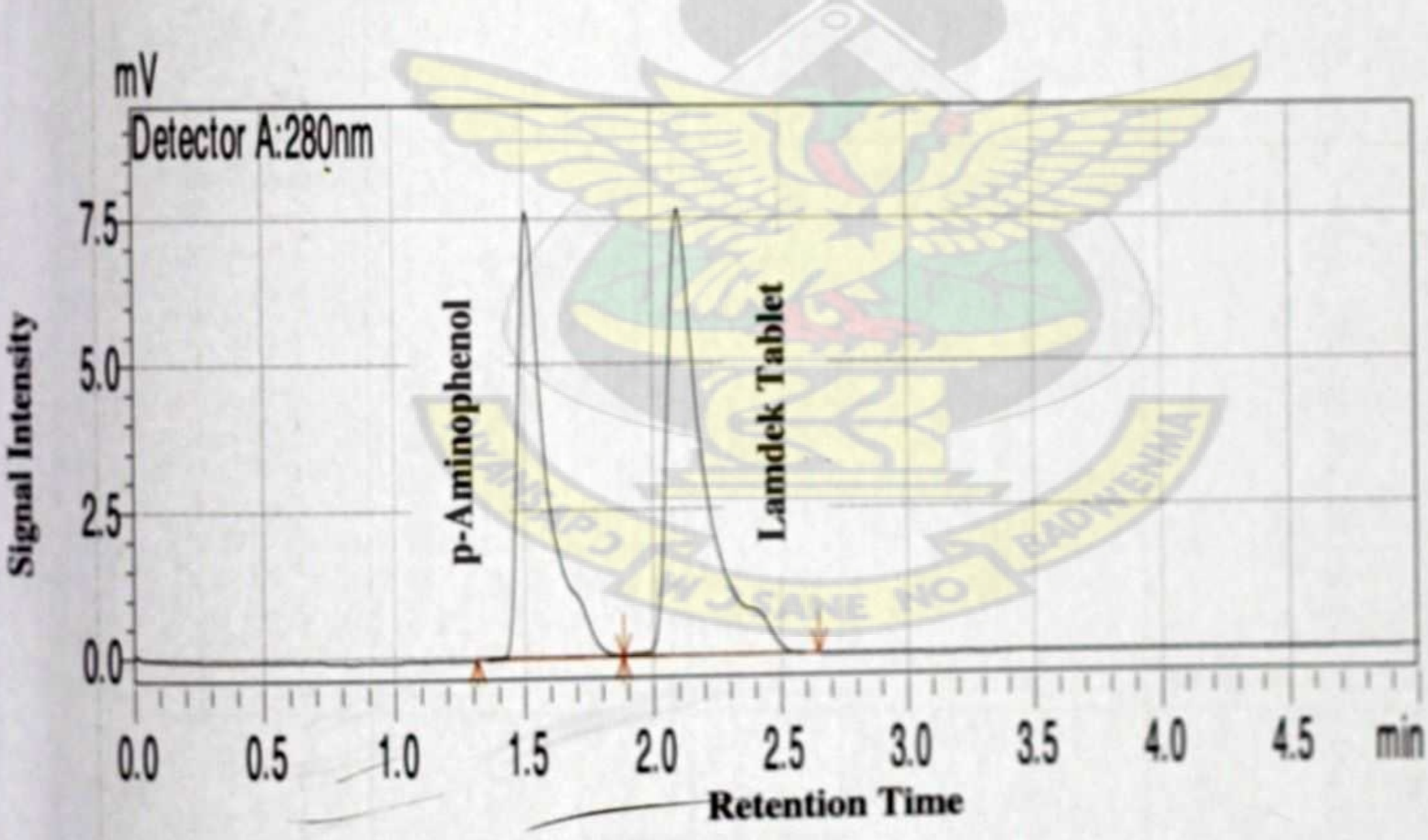

Figure 4.11: Chromatogram of Lamdek Tablet and p-Aminophenol

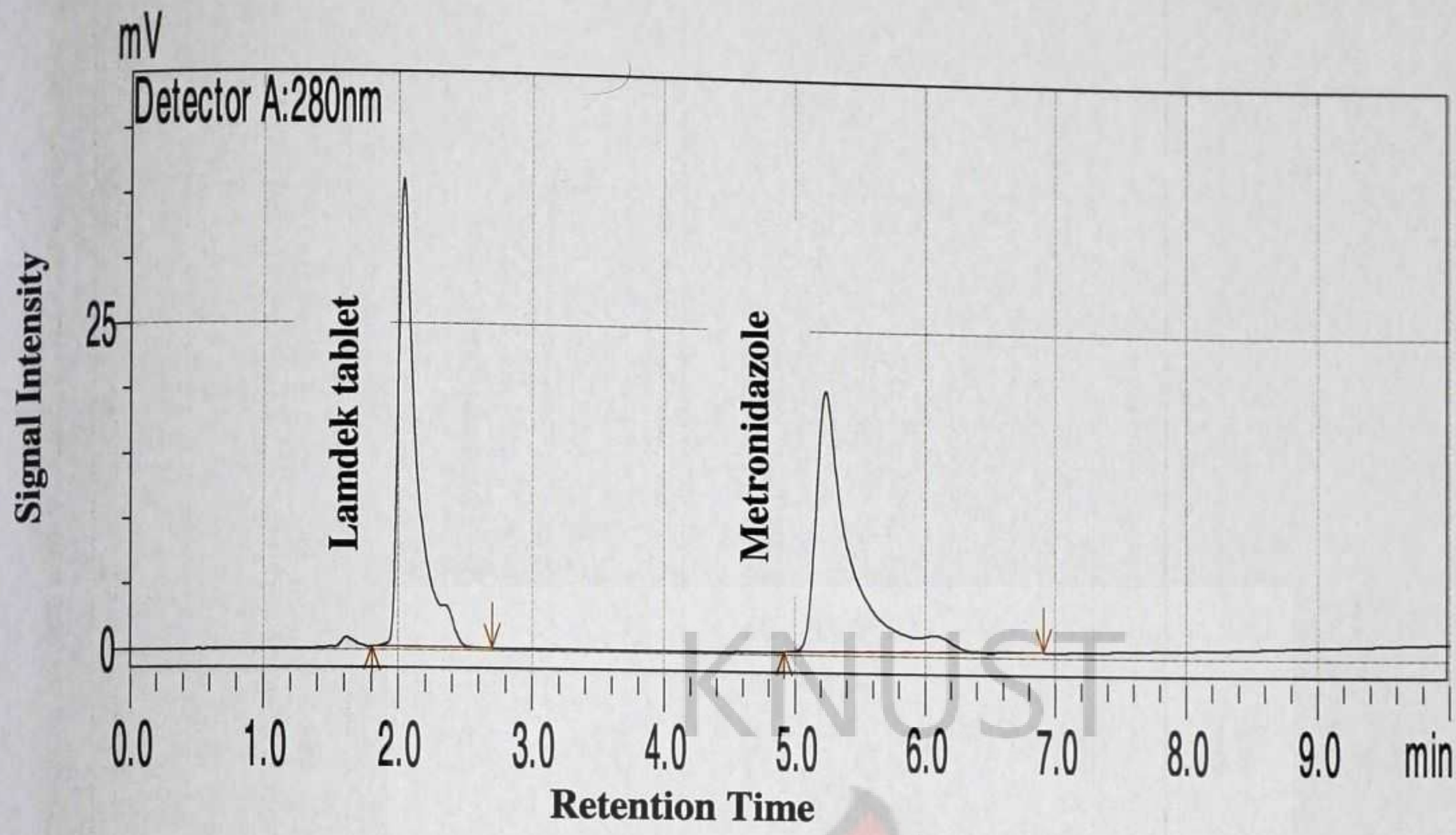

Figure 4.12: Chromatogram of Lamdek Tablet and Metronidazole

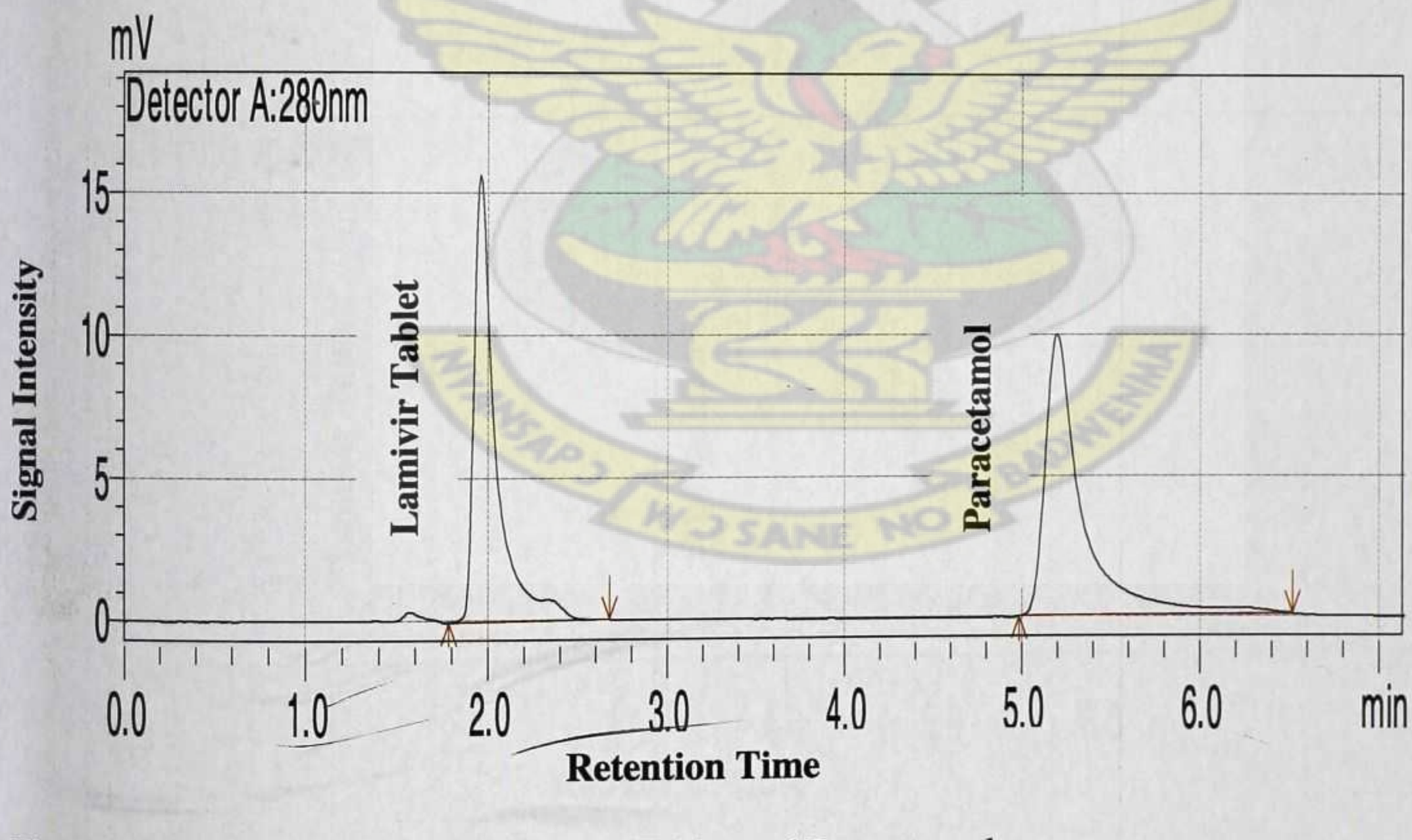

Figure 4.13: Chromatogram of Lamivir Tablet and Paracetamol

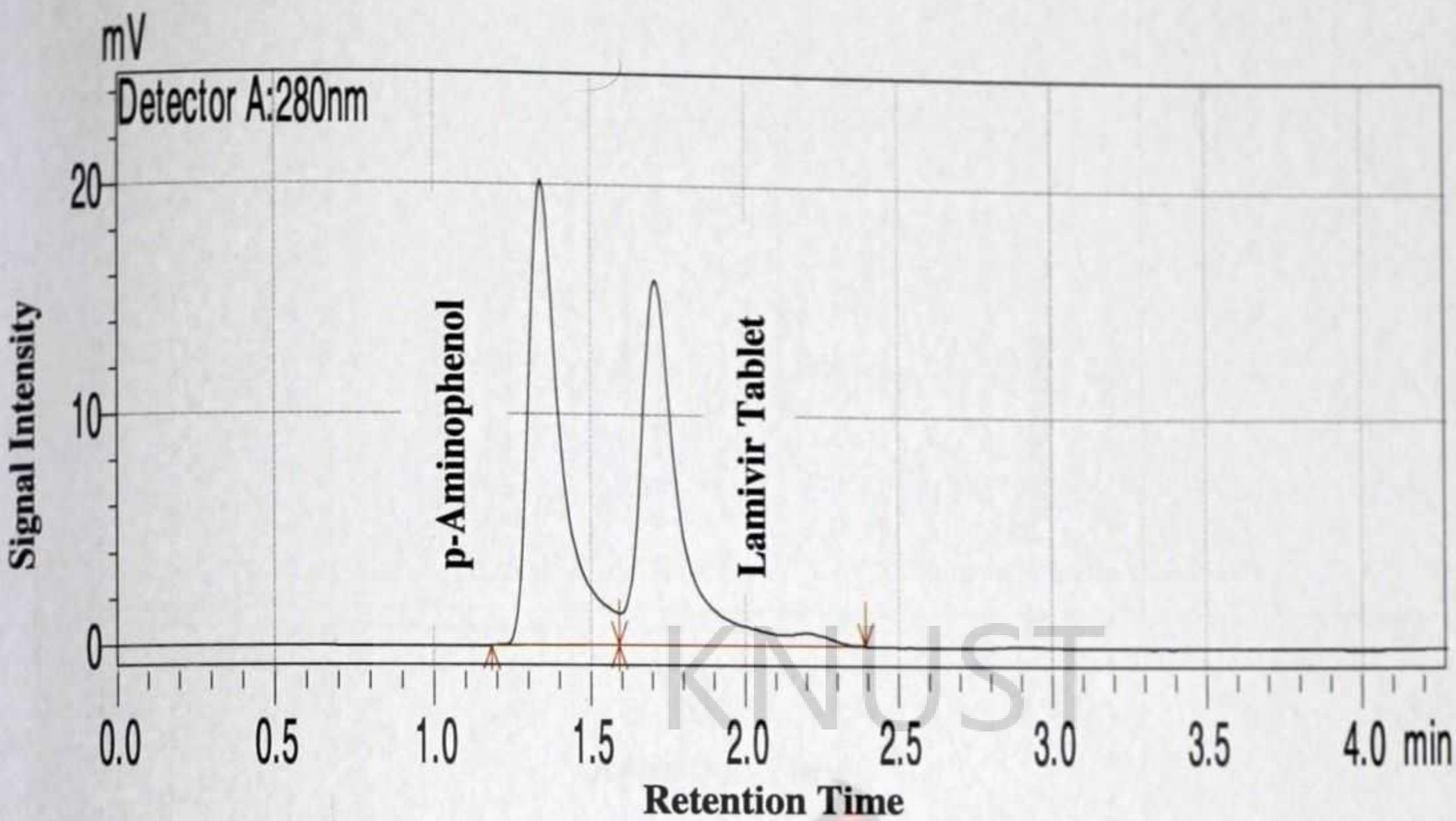

Figure 4.14: Chromatogram of Lamivir Tablet and p-Aminophenol

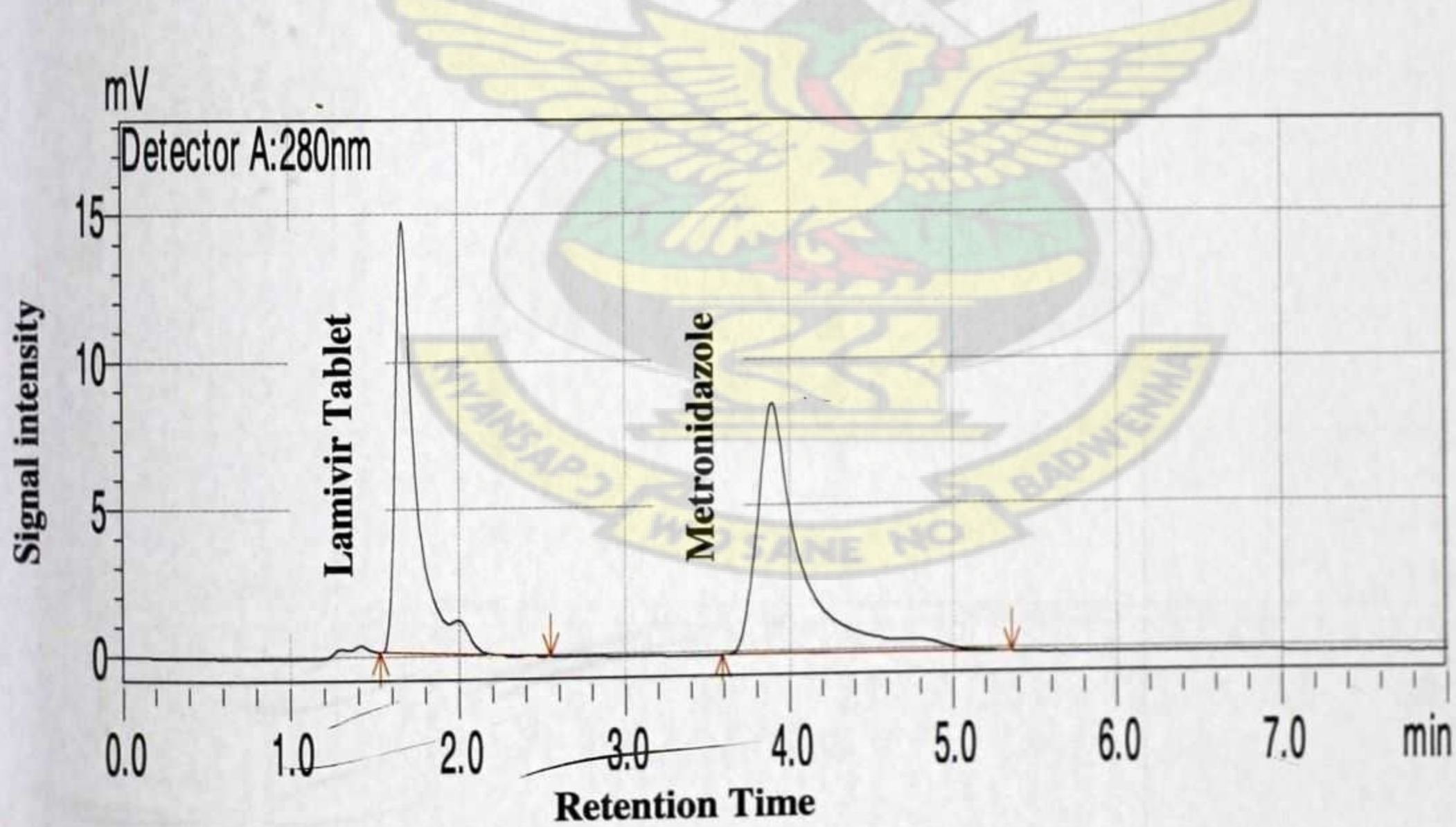

Figure 4.15: Chromatogram of Lamivir Tablet and Metronidazole

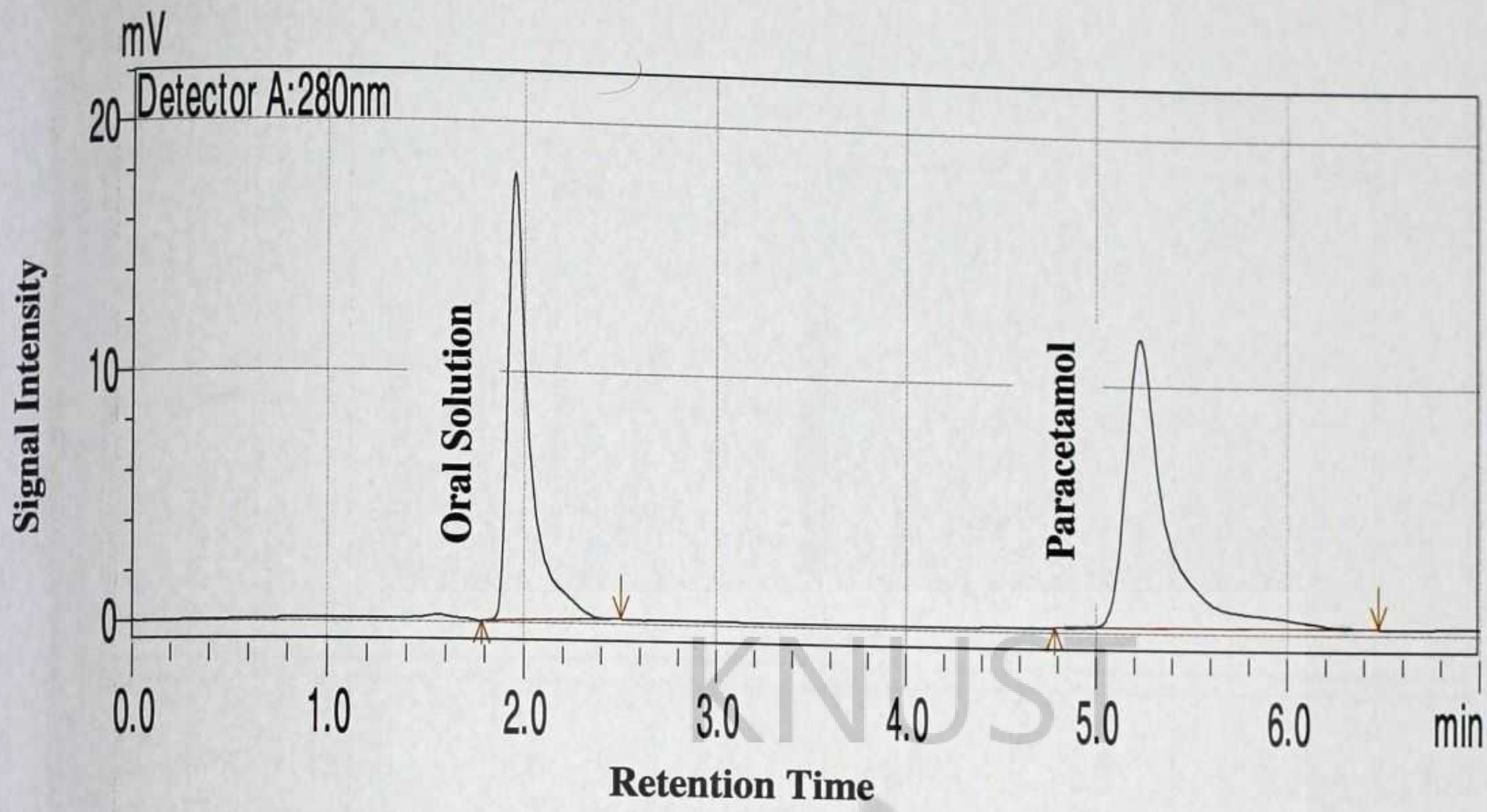

Figure 4.16: Chromatogram of Lamivudine Oral Solution and Paracetamol

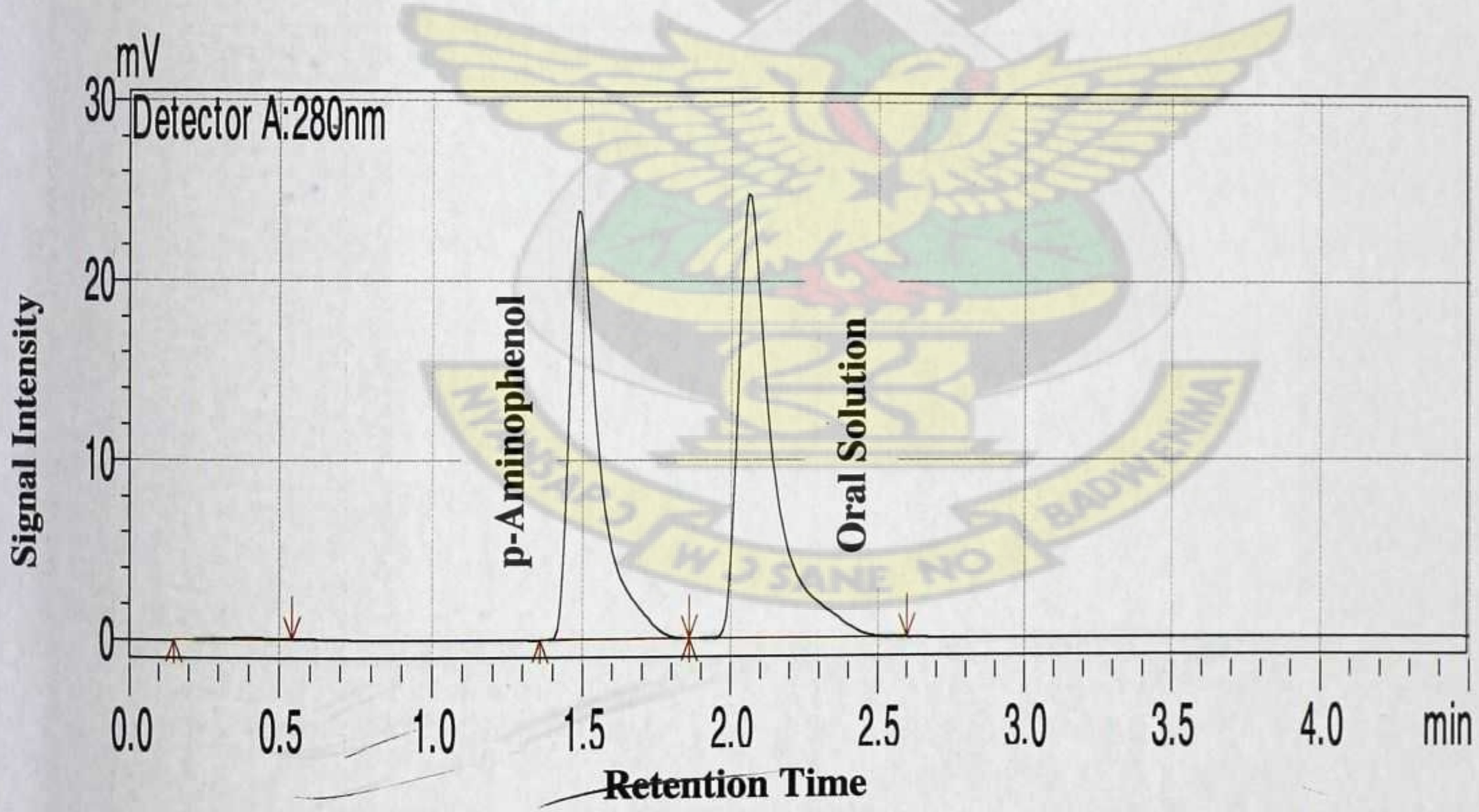

Figure 4.17: Chromatogram of Lamivudine Oral Solution and p-Aminophenol

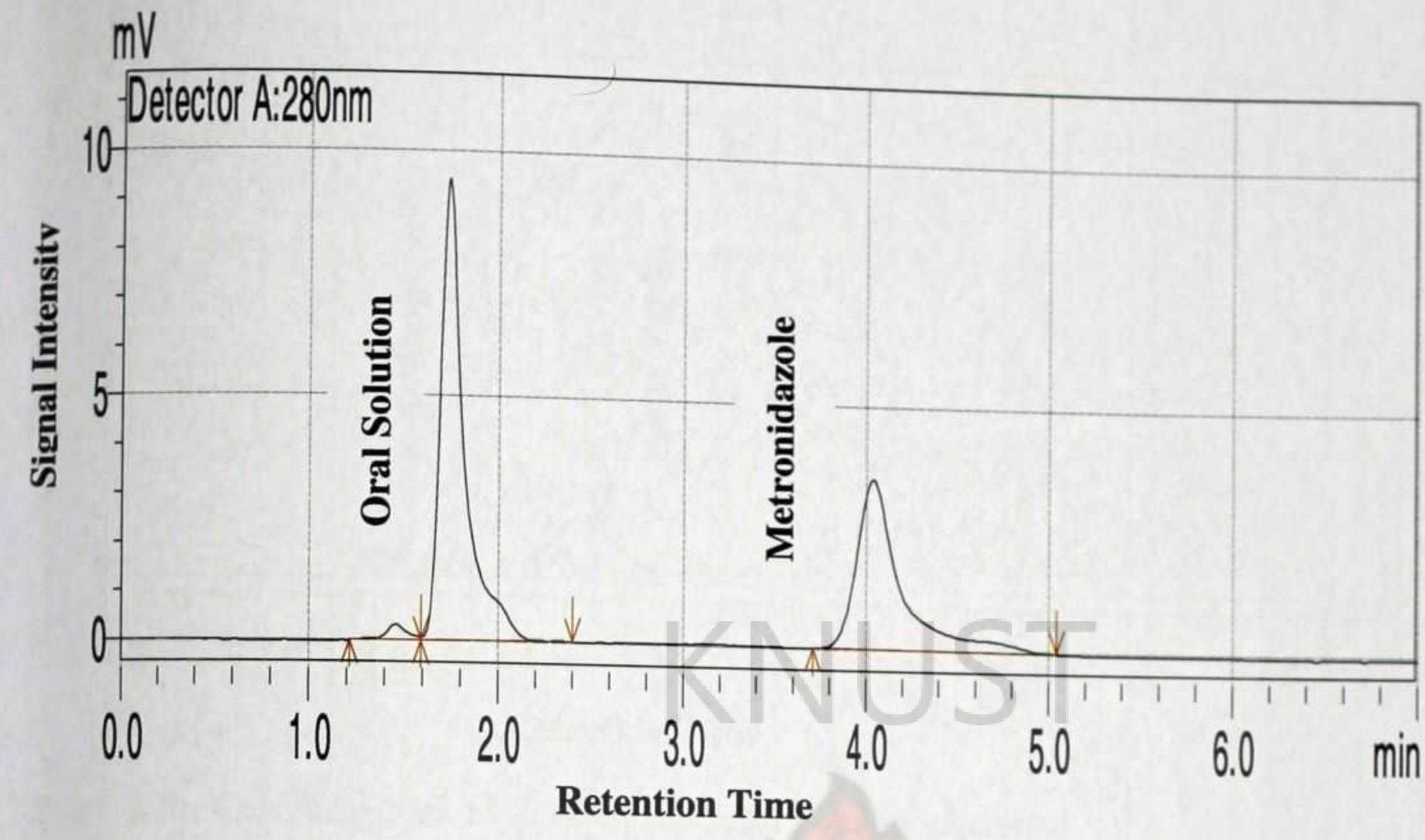

Figure 4.18: Chromatogram of Lamivudine Oral Solution and Metronidazole

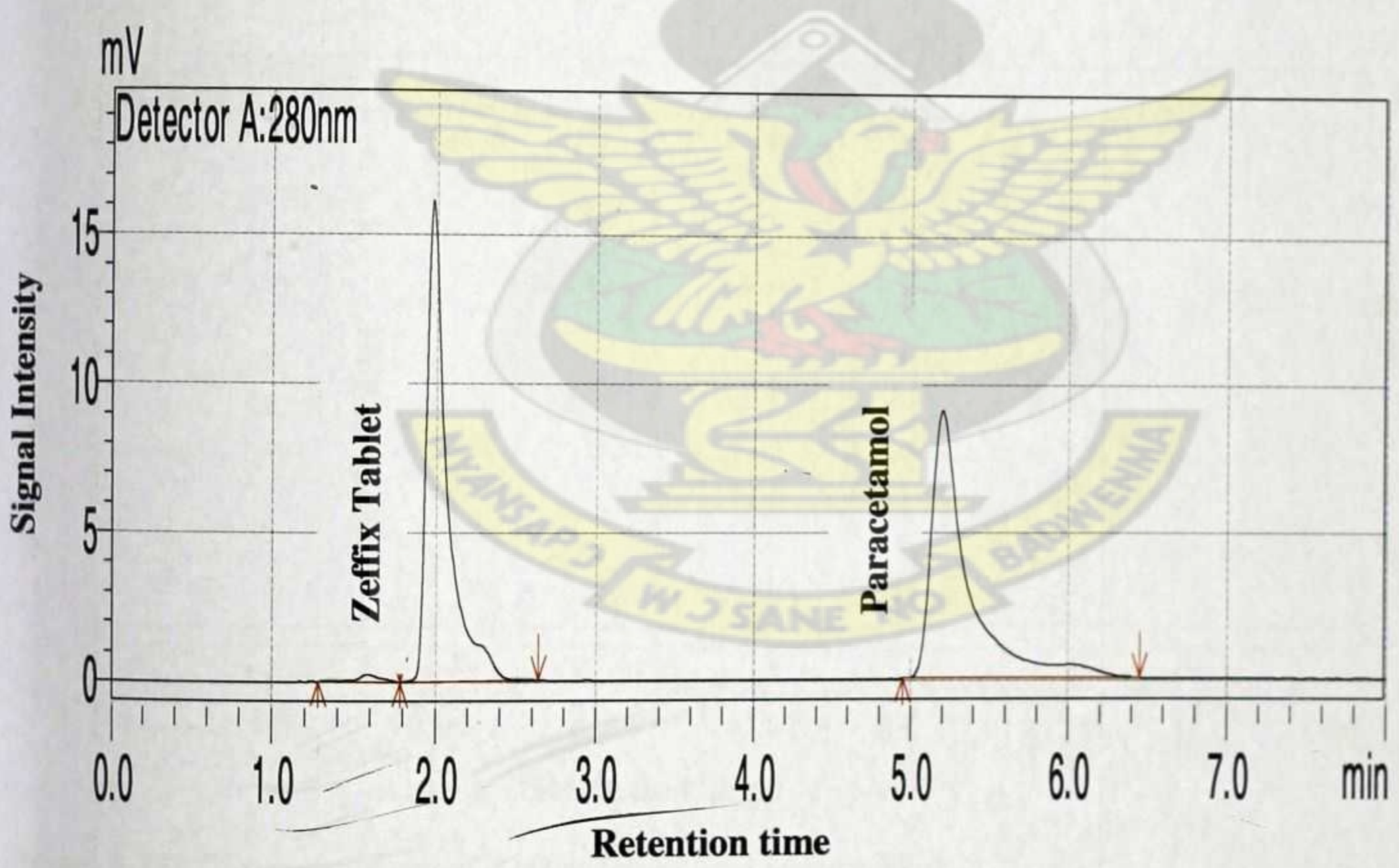

Figure 4.19: Chromatogram of Zeffix Tablet and Paracetamol

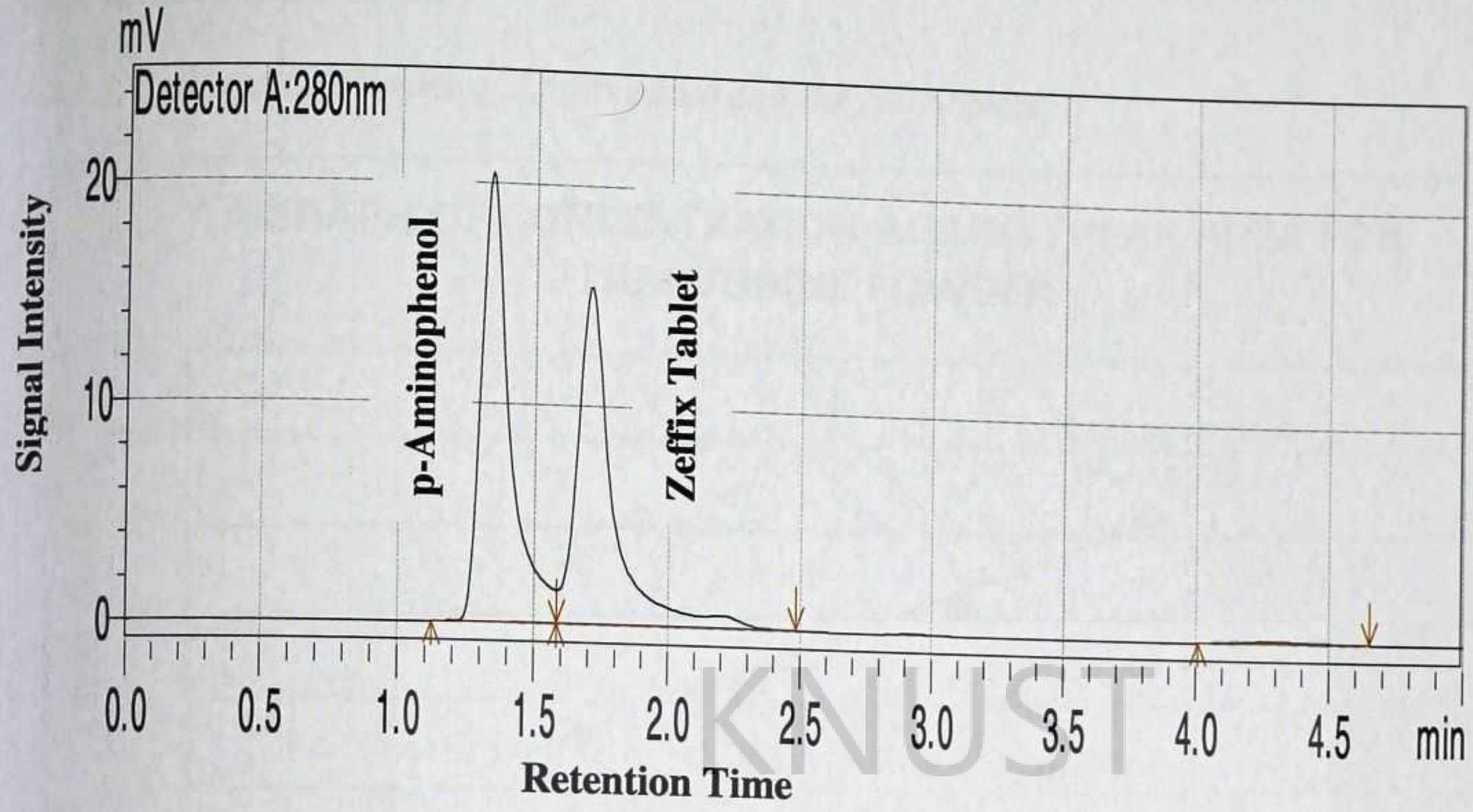

Figure 4.20: Chromatogram of Zeffix Tablet and p-Aminophenol

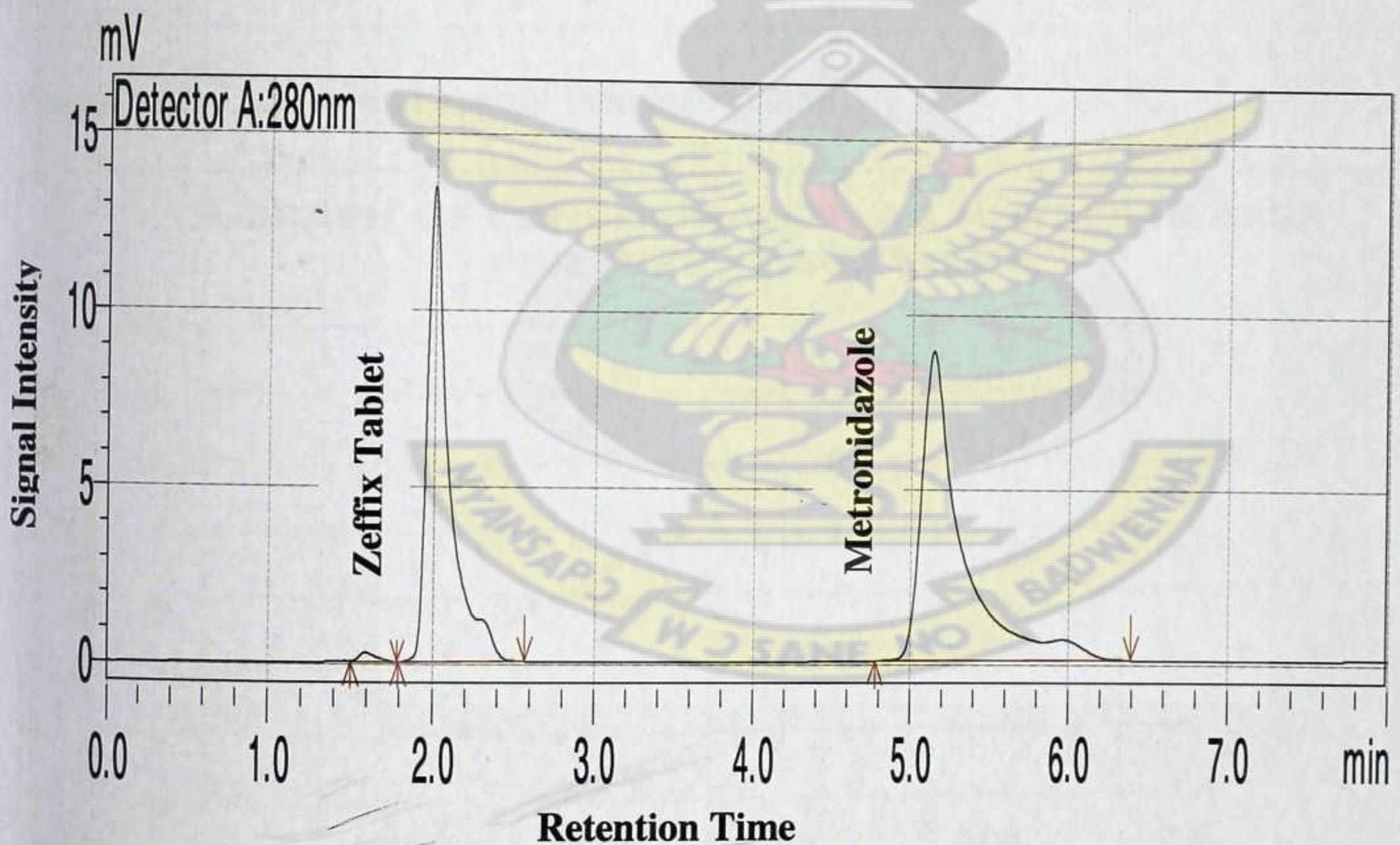

Figure 4.21: Chromatogram of Zeffix Tablet and Metronidazole

4.5.4 Calibration Curves

4.5.4.1 Calibration Curves of Lamivudine and the Surrogates

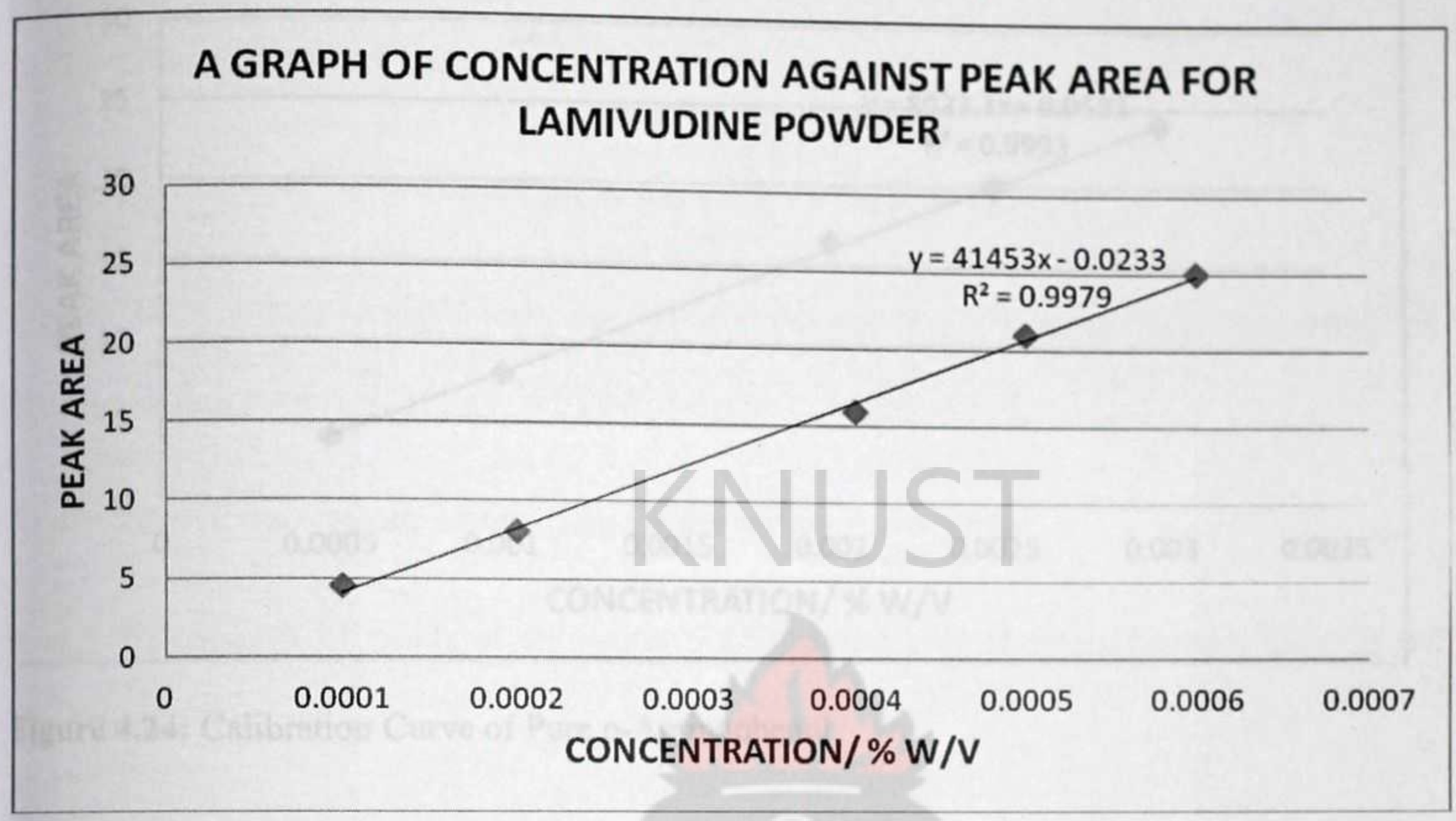

Figure 4.22: Calibration Curve of Pure Lamivudine

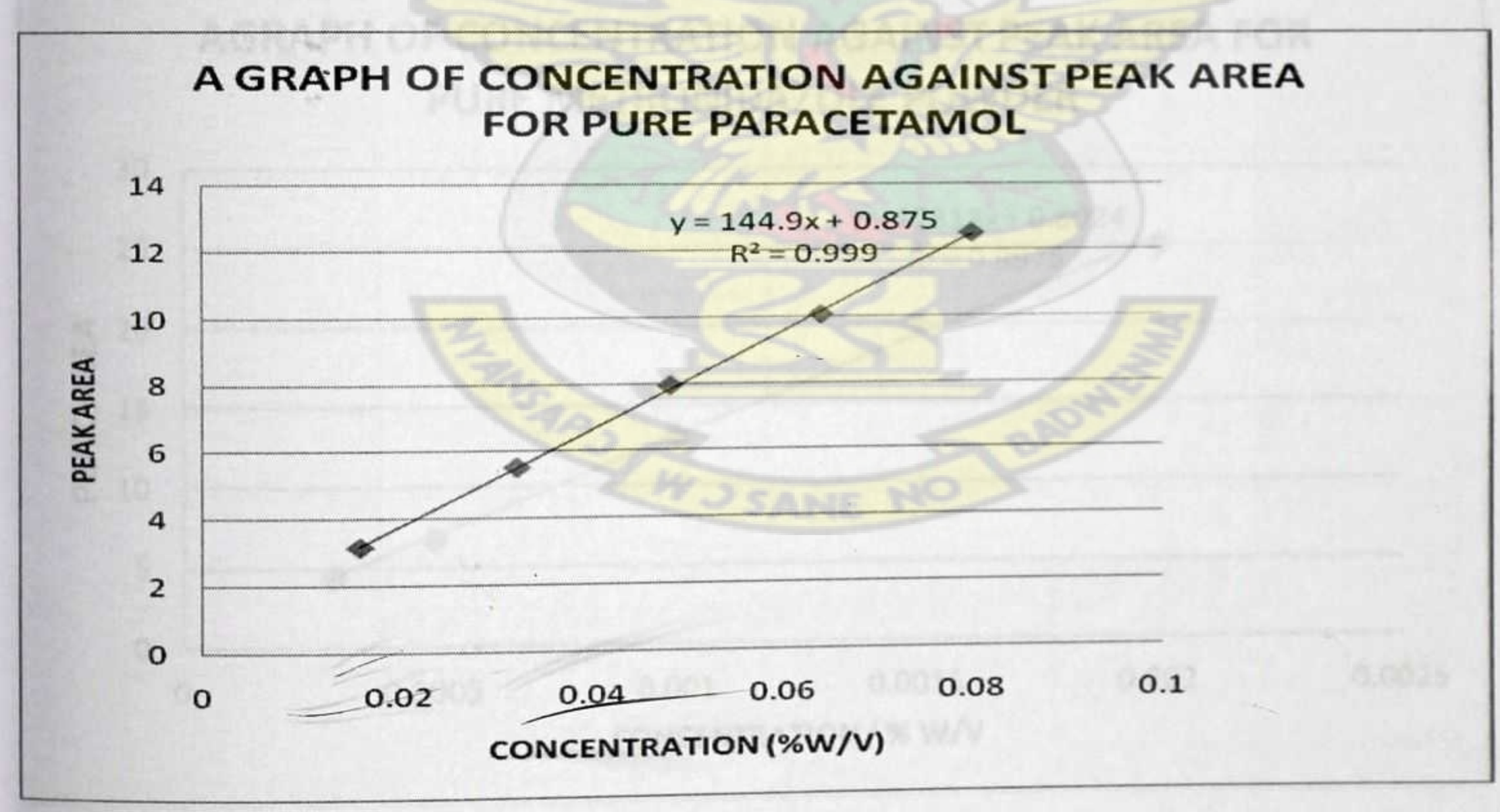

Figure 4.23: Calibration curve of Pure Paracetamol

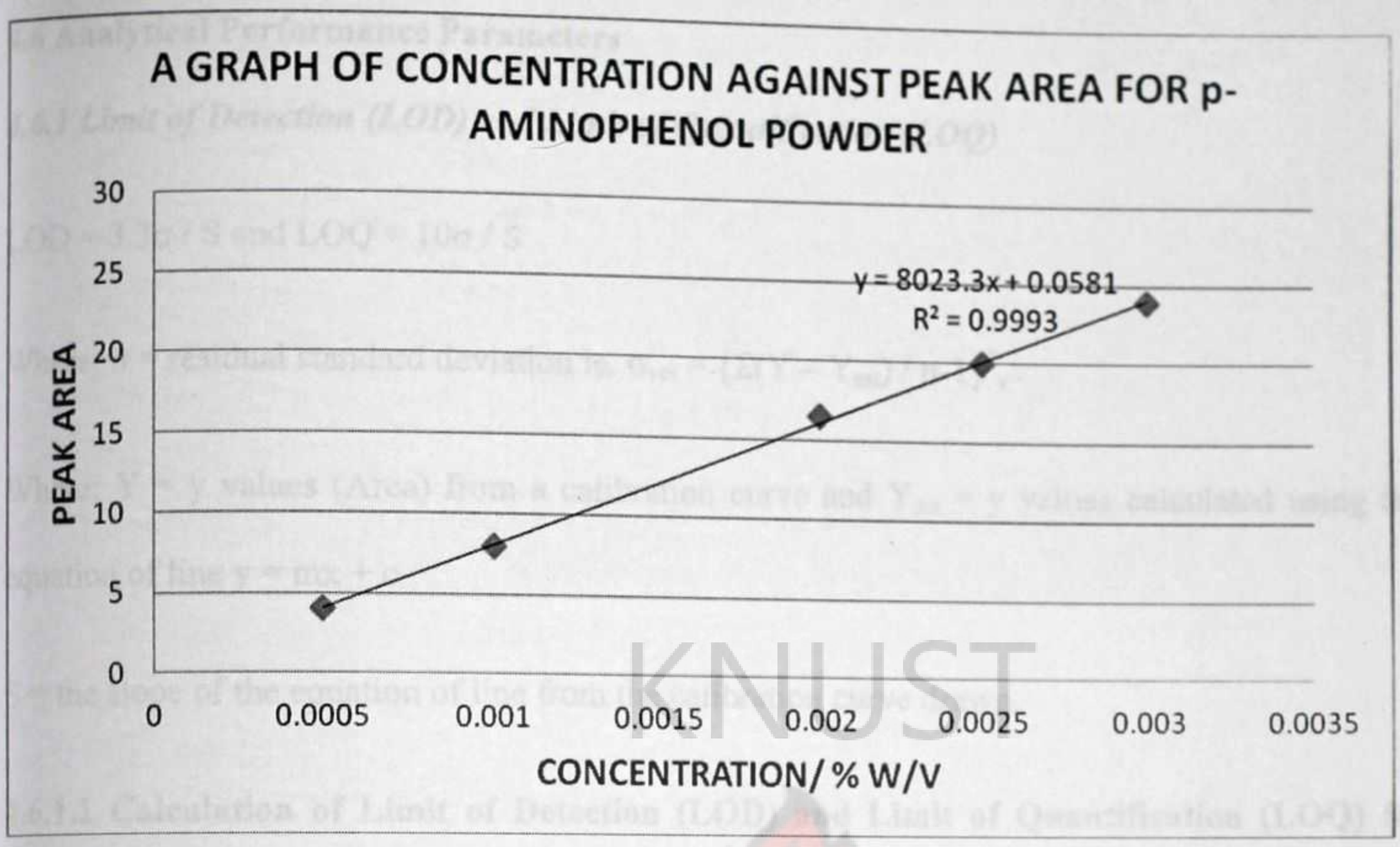

Figure 4.24: Calibration Curve of Pure p-Aminophenol

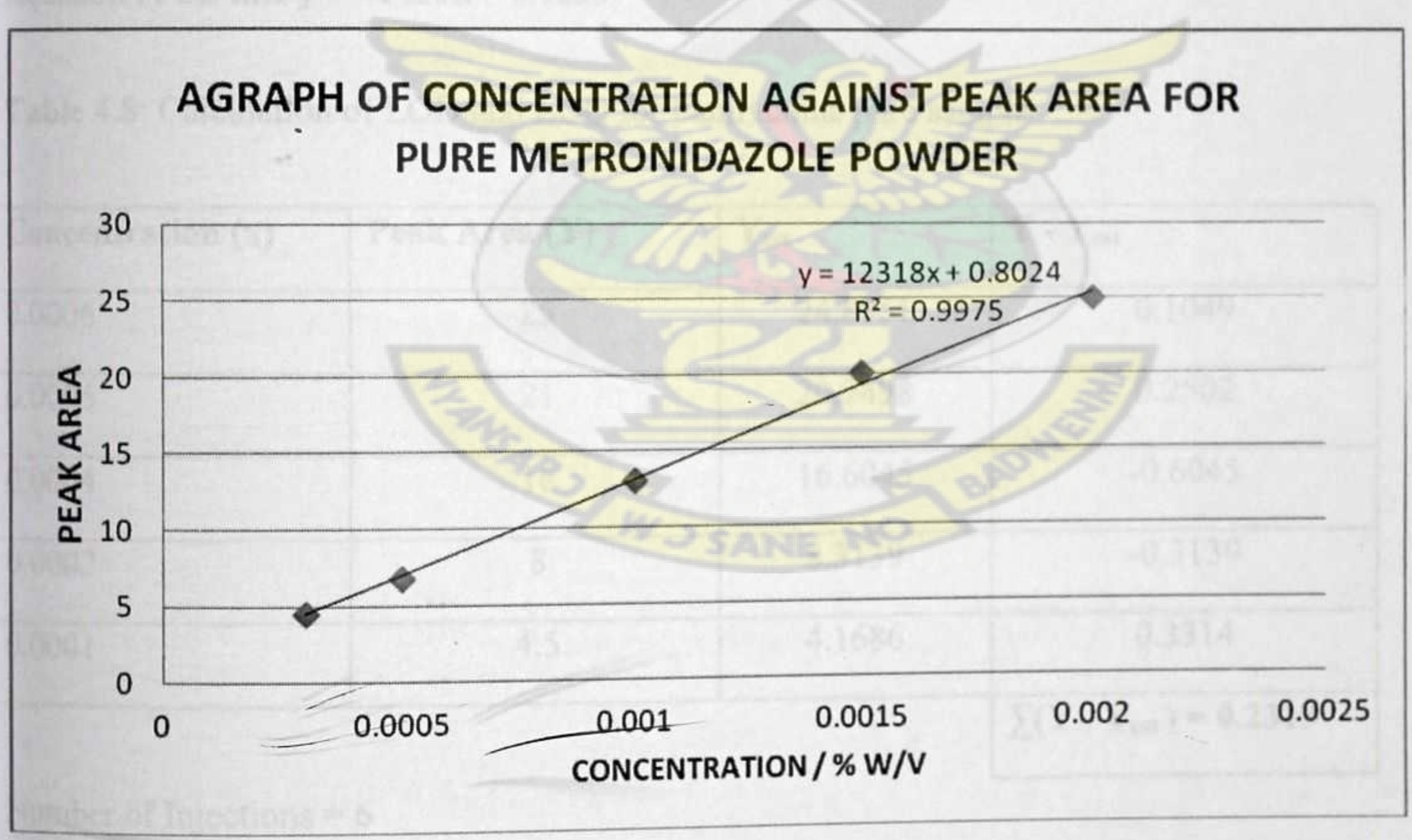

Figure 4.25: Calibration Curve of Pure Metronidazole

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

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 Lamivudine Pure Sample

Equation of the line y = 41453x – 0.0233

Table 4.8: Calculation of LOD and LOQ for Lamivudine pure sample

Concentration (x)	**Peak Area (Y)**	**Y_{est}**	**$Y - Y_{est}$**
0.0006	25	24.8951	0.1049
0.0005	21	20.7498	0.2502
0.0004	16	16.6045	-0.6045
0.0002	8	8.3139	-0.3139
0.0001	4.5	4.1686	0.3314
			$\sum(Y - Y_{est}) = 0.2319$

Number of Injections = 6

Degrees of freedom (n-1) = 5

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

$= (0.2319 / 5)^2$

$= (0.04638)^2$

$= 0.0021511044$

$LOD = 3.3\sigma / S$

$= (3.3 \times 0.0021511044) / 41453$

$= 0.000000171$

$LOQ = 10\sigma / S$

$= (10 \times 0.0021511044) / 41453$

$= 0.000000519$

4.6.2 Linearity

Refer to Table A7 to A10 in the appendix

4.6.3 Sensitivity

Refer to Table A11 in the appendix

4.6.4 Precision

Refer to Table A12 to A15 in the appendix

4.7 Results of Stability Studies on Pure Sample in Solution

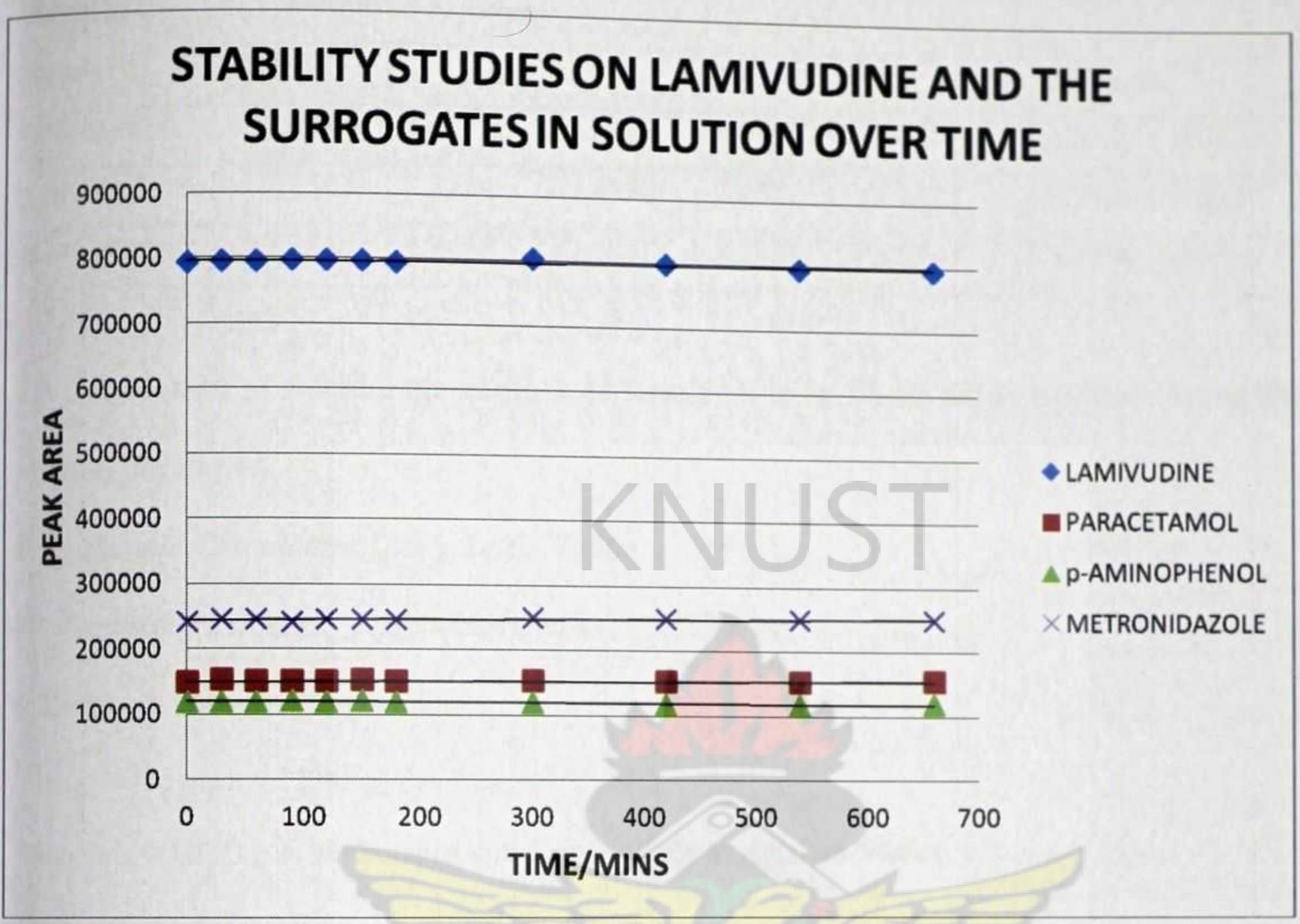

Figure 4.26: Stability studies on Lamivudine and the Surrogates Standards over time

4.8 CALCULATION OF K VALUE

4.8.1 Calculation of the K Value of Lamivudine Using Paracetamol as Surrogate Standard

Concentration of Analyte (Lamivudine), Canalyte = 0.0003 % w/v

Concentration of Standard (Paracetamol), Cstandard = 0.0015 % w/v

Peak Area of Analyte (Lamivudine), Aanalyte = 212896

Peak Area of Standard (Paracetamol), Astandard = 228568

K = [Aanalyte x Cstandard] / [Canalyte x Astandard] = [212896 x 0.0015] / [0.0003 x 228568]

K = 4.6572.

Table 4.9: K values for the various surrogate reference standards in the Analysis of Lamivudine

Surrogate Standard	Calculated K value					
	1	2	3	4	5	Mean K
Paracetamol	4.6945	4.6572	4.6206	4.6774	4.6801	4.6660 ± 0.012
p-Aminophenol	7.2507	7.3284	7.3532	7.2782	7.2321	7.2885 ± 0.023
Metronidazole	3.2534	3.2438	3.1447	3.2758	3.2813	3.2398 ± 0.025

4.9 Calculation of percentage content of Lamivudine in Lamivudine products using the method developed

4.9.1 Sample Calculation Using Zeffix Tablet

Average weight of lamivudine tablet (Zeffix) = 0.22785 g

0.22785 g ≡ 100 mg of Lamivudine

50 mg ≡ (50 mg x 0.22785 g) / 100 mg = 0.11393 g

Therefore, 0.11393 g of zeffix tablet will contain 50 mg of pure Lamivudine.

Concentration of Analyte (Lamivudine), Canalyte = 0.0003 % w/v

Concentration of Standard (Paracetamol), Cstandard = 0.0015 % w/v

Peak Area of Analyte (Lamivudine), Aanalyte = 208690

Peak Area of Standard (Paracetamol), Astandard = 228198

Average K value for Paracetamol = 4.6660

Concentration of analyte = [Area of analyte x Concentration of standard] / [K value x Area of standard]

% Content of Lamivudine in Zeffix Tablet = [208690 x 0.0015] / [4.6660 x 228198]

= [313.035 / 1064771.87]

= 0.000293993

$= [0.000293993 / 0.0003] * 100$

$= 98.00\ \%.$

Table 4.10: Results of Mean Percentage Content of Lamivudine Products Analysed with the Different Surrogate Reference Standards

Surrogate	Mean Percentage Content			
	ZEFFIX	**LAMIVIR**	**LAMDEK**	**ORAL SOLUTION**
Paracetamol	98.35 ± 0.277	97.19 ± 0.330	102.42±0.251	99.48 ±0.090
p-Aminophenol	98.69 ± 0.331	98.35 ± 0.367	102.45±0.225	99.79 ± 0.281
Metronidazole	98.11 ± 0.314	97.05 ± 0.262	101.34±0.222	99.55 ± 0.170

4.10 Comparison of the Method Developed with Standard Method (I.P. 2011) Using t-Test

$t_{exp} = (X_d / S_d) \times \sqrt{N}$ [38]

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 = 0.01) levels for a two-tailed test is 2.78.

4.10.1 Sample Calculation for t_{exp}

The percentage content for Lamivudine in Lamivudine tablets, using the standard method in the IP 2007 and the developed method with Paracetamol as the surrogate reference standard is indicated in the table below:

Table 4.11: Sample calculation for t_{exp}

Percentage content of Lamivudine Tablet (%)		
Standard Method	**New Method**	**Standard Method – New method**
97.94	97.99	-0.05
99.61	97.73	1.88
98.35	98.11	0.24
99.51	98.69	0.82
99.34	99.27	0.07
		$X_d = 0.5920$

$X_d = 0.5920$

$S_d = 0.79364$

$N = 5$

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

$= (0.5920 / 0.79364) * \sqrt{5}$

$= 1.6679$

Table 4.12: t-Test for Zeffix

Standard Method (I.P., 2011) $\%^{w}/_{w}$	**New Method (Paracetamol as surrogate) $\%^{w}/_{w}$**	**New Method (p-Aminophenol as surrogate) $\%^{w}/_{w}$**	**New Method (Metronidazole as surrogate) $\%^{w}/_{w}$**
97.94	97.99	98.29	97.09
99.61	97.73	97.71	97.95
98.35	98.11	99.63	98.05
99.51	98.69	97.69	98.45
99.34	99.27	99.07	99.00
	t = 1.6679	t = 0.5147	t = 3.3513

Table 4.13: t-Test for Lamivir

Standard Method (I.P., 2007) $\%^{w}/_{w}$	**New Method (Paracetamol as surrogate) $\%^{w}/_{w}$**	**New Method (p-Aminophenol as surrogate) $\%^{w}/_{w}$**	**New Method (Metronidazole as surrogate) $\%^{w}/_{w}$**
96.54	96.18	97.9	96.90
96.95	97.88	98.12	96.99
96.87	96.72	98.89	96.99
98.19	97.30	99.46	96.37
97.78	97.86	97.39	97.99
	t = 0.2611	t = 2.7291	t = 0.5396

Table 4.14: t-Test for Lamdek

Standard Method (I.P., 20011) $\%^{w}/_{w}$	New Method (Paracetamol as surrogate) $\%^{w}/_{w}$	New Method (p-Aminophenol as surrogate) $\%^{w}/_{w}$	New Method (Metronidazole as surrogate) $\%^{w}/_{w}$
103.05	102.93	102.93	101.29
102.80	102.93	102.19	101.35
102.97	102.08	102.79	101.35
103.13	101.64	102.63	102.09
101.81	102.53	101.70	100.69
	t = 0.8496	t = 0.5752	t = 9.9425

Table 4.15: t-Test for Lamivudine Oral Solution

Standard Method (I.P., 2007) $\%^{w}/_{w}$	New Method (Paracetamol as surrogate) $\%^{w}/_{w}$	New Method (p-Aminophenol as surrogate) $\%^{w}/_{w}$	New Method (Metronidazole as surrogate) $\%^{w}/_{w}$
99.75	99.18	99.33	99.15
99.51	99.60	99.09	99.91
99.67	99.53	99.91	99.14
99.84	99.4	99.92	99.87
99.67	99.7	100.7	99.69
	t = 0.3870	t = 1.5919	t = 0.7219

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

Table 4.16: RSD and SEM of Lamivudine Products assayed using the I.P. 2011

Name of Product	Mean	Standard Deviation	RSD %	SEM
Zeffix Tablet	98.95	0.7552	0.7632	0.3377
Lamivir Tablet	97.27	0.6895	0.7089	0.3084
Lamdek Tablet	102.87	0.0931	0.0905	0.0042
Oral Solution	99.69	0.1217	0.1221	0.0544

Table 4.17: RSD and SEM of Zeffix Tablet assayed using the new method

Surrogate Reference Standard	Mean	Standard Deviation	RSD %	SEM
Paracetamol	98.36	0.6191	0.6295	0.2769
p-Aminophenol	98.68	0.7395	0.7494	0.3307
Metronidazole	98.11	0.7031	0.7660	0.3144

Table 4.18: RSD and SEM of Lamivir Tablets assayed using the new method

Surrogate Reference Standard	Mean	Standard Deviation	RSD %	SEM
Paracetamol	97.19	0.7379	0.7593	0.3300
p-Aminophenol	98.35	0.8217	0.8355	0.3675
Metronidazole	97.05	0.5864	0.6043	0.2623

Table 4.19: RSD and SEM of Lamdek Tablets assayed using the new method

Surrogate Reference Standard	Mean	Standard Deviation	RSD %	SEM
Paracetamol	102.42	0.5604	0.5472	0.2506
p-Aminophenol	102.45	0.5021	0.4901	0.2246
Metronidazole	101.35	0.4968	0.4902	0.2222

Table 4.20: RSD and SEM of Lamivudine Oral Solution assayed using the new method

Surrogate Reference Standard	Mean	Standard Deviation	RSD %	SEM
Paracetamol	99.48	0.2010	0.2021	0.0899
p-Aminophenol	99.79	0.6283	0.6297	0.2810
Metronidazole	99.55	0.3807	0.3824	0.1702

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.

$F = S_1^2 / S_2^2$ [38]

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

Product: Zeffix Tablet

Mean of Standard method, I.P. 2011 = 97.94

Standard deviation, S of Standard method, I.P. 2011 = 0.7552

Therefore variance, S^2 of Standard method, I.P. 2011 = 0.5704

Table 4.21: F-Test Values for Zeffix Tablet

Surrogate Reference Standard	Mean	Standard Deviation	Variance	F value
Paracetamol	98.36	0.6191	0.3833	1.4881
p-Aminophenol	98.68	0.7395	0.5469	1.0430
Metronidazole	98.11	0.7031	0.4943	1.1540

Product: Lamivir

Mean of Standard method, I.P. 2011 = 97.27

Standard deviation, S of Standard method, I.P. 2011 = 0.6895

Therefore variance, S^2 of Standard method, I.P. 2011 = 0.4754

Table 4.22: F-Test Values for Lamivir Tablet manufactured by Cipla Ltd., India.

Surrogate Reference Standard	Mean	Standard Deviation	Variance	F value
Paracetamol	97.19	0.7379	0.5445	1.1454
p-Aminophenol	98.35	0.8217	0.6752	1.4203
Metronidazole	97.05	0.5864	0.3439	1.3824

Product: Lamdek

Mean of Standard method, I.P. 2011 = 102.75

Standard deviation, S of Standard method, I.P. 2011 = 0.5406

Therefore variance, S^2 of Standard method, I.P. 2011 = 0.2418

Table 4.23: F-Test Values for Lamdek Tablet.

Surrogate Reference Standard	Mean	Standard Deviation	Variance	F value
Paracetamol	102.42	0.5604	0.3141	1.2990
p-Aminophenol	102.45	0.5021	0.2521	1.0426
Metronidazole	101.35	0.4968	0.2468	1.0207

Product: Lamivudine Oral Solution.

Mean of Standard method, IP. 2011 = 99.75

Standard deviation, S of Standard method, IP. 2011 = 0.1221

Therefore variance, S^2 of Standard method, IP. 2011 = 0.015

Table 4.24: F-test Values for Lamivudine Oral Solution.

Surrogate Reference Standard	Mean	Standard Deviation	Variance	F-test
Paracetamol	99.48	0.20104	0.0404	2.6933
p-Aminophenol	99.79	0.6283	0.3948	26.320
Metronidazole	99.55	0.3807	0.1449	9.6600

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Identification test

All the pure samples used in this project were taken through series of tests such as melting point, Thin Layer Chromatography (TLC) and assay to ascertain their identities.

5.1.1.1 Lamivudine

The assay of Lamivudine yielded an average percentage purity of 99.86%. The percentage purity stated in the International Pharmacopoeia ranges from 97.0% to 103.0%. The experimental melting point range was from 178°C to 180°C. The British Pharmacopoeia melting point range for Lamivudine is 174 °C to 180 °C. The substance analysed was Lamivudine.

5.1.1.2 Paracetamol

The assay of Paracetamol yielded an average percentage purity of 98.92%. The percentage purity stated in the British Pharmacopoeia ranges from 98.5% to 101%. The experimental melting point range was from 168 °C to 170 °C. The British Pharmacopoeia melting point range for Paracetamol is 168 °C to 172°C. The substance analysed was Paracetamol.

5.1.1.3 Metronidazole

The assay of Metronidazole yielded an average percentage purity of 100.51%. The percentage purity stated in the British Pharmacopoeia ranges from 99.0% to 101.0%. The experimental melting point range was from 160 °C to 163 °C. The British Pharmacopoeia melting point range for Metronidazole is 159 °C to 163 °C. The substance analysed was Metronidazole.

5.1.1.4 p-Aminophenol

Para-Aminophenol has experimental melting point range of 186 to 188. The experimental melting point range was from 190 °C to 191 °C. The British Pharmacopoeia melting point range for p-Aminophenol is 186. The analyzed sample is therefore p-Aminophenol.

5.1.2 Uniformity of weight

5.1.2.1 Lamivudine

The uniformity of weight test carried out on Zeffix tablets yielded an average tablet weight of 227.85 mg. The average tablet weight of Lamivir tablets was 302.22 mg and that of Lamdek was 360.90 mg. Percentage deviations were calculated for each tablet 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 Tablets (Uncoated tablets and film-coated tablets)

Average Mass of tablet	Percentage Deviation allowed	Number of Tablets
Less than 80 mg	± 10	20
80 mg to 250 mg	± 7.5	20
More than 250 mg	± 5	20

Source: IP 2011

Zeffix had an average weight of 227.85 mg which is less than 250 mg. A look at tables A3 to A5 shows that none of the tablets deviated by more than 7.5 %. Lamivir and Lamdek with average tablet weights of 302.22 mg and 360.90 mg had none of the individual tablets deviating by more than 5 %. It can therefore be inferred that the batch of lamivudine tablets which were taken through the uniformity of weight test are 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 repeatedly at an interval of 30 minutes for six hours and two hour interval for the next 6 hours. A graph of peak area against time was plotted and it showed a linear plot almost parallel to the X axis for all the pure drug samples used suggesting that it is safe to analyze the sample within 12 hours under ambient conditions in the laboratory.

5.1.4 HPLC Method Development

The objective of this work was to develop a cheap, rapid, accurate, precise and reliable HPLC method for the analysis of Lamivudine in Lamivudine tablets and oral solution in the presence of surrogate reference standards, using the most commonly employed C-18 column with UV detector.

Chemical structure and chemical properties are very essential for the chromatographic behaviour of compounds. Very good separation of Lamivudine and its surrogate standards was achieved using an ODS C 18 Phenomenex 150 x 4.6mm column. The best results were obtained using mobile phase composition of Methanol and Acetic acid (15:85). The higher percentage of methanol initially used in mobile phase gave poor resolutions and the samples were eluted very

quickly. However, increasing the percentage composition of the aqueous portion of the mobile phase increased the retention times and also gave very good resolutions. Mean retention times (minutes) of 1.635 ± 0.011, 4.438 ± 0.012, 1.473 ± 0.011 and 4.528 ± 0.007 were recorded for Lamivudine, Paracetamol, p-Aminophenol and Metronidazole respectively.

5.1.5. Analytical Performance Parameters

5.1.5.1 Limit of Detection (LOD)

The limit of detection is the smallest quantity of a substance in a sample (matrix) that can be detected but not necessarily quantitated as an exact value. The Limit of Detection for the analysis of Lamivudine tablets was determined to be 0.000000171, 2.69 x 10^{-10}, 0.00000559, and 0.00000424 % w/v for Lamivudine, Paracetamol, p-Aminophenol and Metronidazole 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. The recorded LODs were vital in the selection of the concentrations used for the analysis to ensure that the samples would be detected at the selected concentrations.

5.1.5.2 Limit of Quantitation (LOQ)

The limit of quantitation is the smallest quantity of a substance in a sample (matrix) that can be quantified as an exact value with acceptable precision and accuracy. The Limit of Quantitation obtained for this work was found to be 0.000000519, 8.16 x 10-^{10}, 0.0000169 and 0.0000128 % w/v for Lamivudine, Paracetamol, p-Aminophenol and Metronidazole respectively. The determinations were 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

The aim of checking the linearity is to derive a direct proportionality between the detector signal and the concentration of a substance in the sample over a certain 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 A7 to A10 in the appendix show an appreciable level of linearity.

5.1.5.4 Specificity/Selectivity

Selectivity is the ability of the technique to detect an analyte that is free from any interference in the presence of other components, such as by-products and degradation products, excipients (matrix) and other impurities.

Such determination can be made by assessing the peak identity and purity.

These parameters were determined by comparing the chromatograms of the Lamivudine tablets with that of the Lamivudine pure sample. The chromatogram of the Lamivudine pure sample and Lamivudine tablets presented peaks with average retention time of 1.850 ± 0.056 minutes.

Additionally, when Lamivudine tablets were injected with each of the surrogates (Paracetamol, p-Aminophenol and Metronidazole), the peaks were well resolved as shown in Figures 4.10, to 4.21 indicating high specificity of the method.

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

5.1.5.5 Precision and Repeatability

Precision expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the

prescribed conditions. It is expressed as the variance, standard deviation or coefficient of variation (relative standard deviation). [37]

The results as shown in tables A13 to A16 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 Surrogate Constant (K)

The peak areas of the analyte and surrogate reference standards and their respective concentrations were used in evaluating the respective K values using the hypothesis for this research. From the results of K values obtained, it was observed that a change in concentration had no significant effect on the K value for each of the surrogate reference standards.

Different K values were obtained for each of the surrogate reference standards used in the analysis. P-Aminophenol was found to have a very high Surrogate constant. A close examination of the chemical structures of all the compounds show that the p-Aminophenol has less of the auxochromes and unsaturated bonds which contributes to a compound's ability to absorb light of a particular wavelength. This makes the other surrogates closer in terms of their structure and the degree of UV absorption to Lamivudine. This may account for the varying surrogate constant for the surrogate reference standards and the high surrogate constant for p-Aminophenol.

5.1.7. Determination of Percentage content using the constant K

Three brands of Lamivudine tablets and a lamivudine Oral solution were analysed 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 A21 to A32 in the appendix. The range of percentage content stated in the International Pharmacopoeia 2011 (IP) for both Lamivudine tablets and Lamivudine oral solution is from 90.0% to 110.0%. From Table 4.10, it could be observed that all the calculated percentage contents were within the range stated in the IP 2011 Monograph for both Lamivudine tablets and Lamivudine oral solution. The percentage contents of the standard method and the developed method were comparable.

5.1.8 Determination of Percentage Content of Lamivudine in Lamivudine Products Using Standard Method in the International Pharmacopoeia (2011)

Lamivudine tablets and Lamivudine oral solution from three (3) different manufacturers were analysed using the standard methods stated in the International Pharmacopoeia (IP) 2011 for both Lamivudine tablets and Lamivudine oral solution. The average percentage contents obtained for the analysis of the Zeffix tablets, Lamivir tablets, Lamdek tablets, and Lamivudine oral solution were 98.95, 97.28, 102.89 and 99.69% respectively. The Lamivudine monograph according to the International Pharmacopoeia, 2011, states that the percentage content for both Lamivudine tablets and Lamivudine oral solution should range between 90.0 and 110%. The percentage content for all the brands analysed were within the range and therefore 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 Lamivudine Products

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 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 knowledge 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) test was employed.

The Null Hypothesis was that the averages of the two methods did not differ significantly at the 95% probability level.

For four (4) degrees of freedom, the critical values of "t" at the 95% (P = 0.01) levels for a two-tailed test is 2.78. [38]

From Table 4.12, the t_{exp} of Zeffix tablets were 1.6679, 0.5147, and 3.3513 for Paracetamol, p-Aminophenol and Metronidazole respectively when they were used as surrogate reference standards. It can be observed that the calculated t-values are smaller than the critical value of 2.78 when Paracetamol and p-Aminophenol were used as surrogate reference standard. 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 Metronidazole was used as surrogate reference standard. Hence, there is a significant difference between the two methods. The null hypothesis is therefore rejected at the 95% probability level.

From the above Table 4.13, the t_{exp} of Lamivir tablets were 0.2611, 2.7291 and 0.5396 for Paracetamol, p-Aminophenol and Metronidazole respectively when used as surrogate reference standard. It can be observed that the calculated t-values are smaller than the critical value of 2.78 when Paracetamol, p-Aminophenol and Metronidazole 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.

From Table 4.14, the t_{exp} of Lamdek tablets 0.8496, 0.5752 and 9.9425 for Paracetamol, p-Aminophenol and Metronidazole respectively when used as surrogate reference standard. It can again be observed that the calculated t-values are smaller than the critical value of 2.78 when Paracetamol and p-Aminophenol were used as surrogate reference standard. Hence, there is no significant difference between the two methods. The null hypothesis is therefore accepted at the 95% probability level. However, the calculated t-value is greater than the critical value of 2.78 when Metronidazole was used as surrogate reference standard. Hence, there is a significant difference between the two methods. The null hypothesis is therefore rejected at the 95% probability level.

From Table 4.15, the t_{exp} of Lamivudine oral solution were 1.5919, 0.3870 and 0.7220 for Paracetamol, p-Aminophenol and Metronidazole respectively when used as surrogate reference standard. It can be observed that the calculated t-values are smaller than the critical value of 2.78 when Paracetamol, p-Aminophenol and Metronidazole 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.

From the above, the t_{exp} values obtained when Paracetamol, p-Aminophenol and Metronidazole 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 (I.P. 2011) and the method developed.

When Metronidazole was used as a surrogate reference standard for the analysis of Zeffix tablets, the t_{exp} values obtained (3.315) was greater than "t" at 95% probability level, hence there was a significant difference between the accuracies of the two methods. Again, when Metronidazole was used as a surrogate reference standard for the analysis of Lamdek tablets, the t_{exp} values obtained (9.9425), was greater than "t" at 95% probability level, hence there was a significant difference between the accuracies of the two methods. The assay results of the Lamivudine tablets and Lamivudine Oral Solution were within the range stated in the International Pharmacopoeia (I.P 2011), as well as those of the standard method.

5.1.11 Relative Precision of the New Method to the Standard Method for the Assay of Lamivudine 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. [38] From Table 4.21, the calculated F-test value of Zeffix tablets using Paracetamol, p-Aminophenol and Metronidazole as the surrogate reference standard against the standard method (I.P. 2011) were 1.4881, 1.0430,

and 1.1540 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.

From Table 4.22, the F-test values obtained for Lamivir tablets using Paracetamol, p-Aminophenol and Metronidazole as the surrogate reference standard against the standard method (I.P. 2011) were 1.1454, 1.4203 and 1.3824 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.

From Table 4.23, the F-test values obtained for Lamdek tablets using Paracetamol, p-Aminophenol and Metronidazole as the surrogate reference standard against the standard method (I.P. 2011) were 1.2990, 1.0426 and 1.0207 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.

From Table 4.24, the calculated F-values are less than the critical value of 9.605 when Paracetamol was used as the surrogate reference standard. There is therefore no significant difference between the two methods for Paracetamol as surrogate and the null hypothesis is accepted at the 95% probability level. However, the calculated F-values when p-Aminophenol and Metronidazole were used as surrogates are greater than the critical value. There is therefore a significant difference between the precision of the two methods. The null hypothesis is however rejected at the 95% probability level.

5.2 Conclusion

The major objective of this project was to find the possibility of using surrogate reference standards to analyse Lamivudine tablets and oral solution. This objective has been successfully achieved and that the method could be used in place of the conventional methods available in the pharmacopoeia. The procedure is simple, fast, accurate, precise, sensitive and reliable and has been fully validated. All the validation procedures confirm that this developed method is suitable for the analysis of Lamivudine tablets and Lamivudine Oral solutions.

The assay results for the standard methods in the International Pharmacopoeia for Lamivudine and the method developed were within the limit stated in the International Pharmacopoeia.

Statistically, there was a significant difference between the accuracies of the standard method and the method developed when Metronidazole was used as surrogate reference standard in the analysis of Zeffix tablets and Lamdek, Ghana. However there was no significant difference between the accuracies of the two methods when Paracetamol, p-Aminophenol and Metronidazole were used as surrogate reference standards in the analysis of both Lamivir tablets and Lamivudine oral solution. There was no significant difference between the precisions of the two methods when Paracetamol, p-Aminophenol and Metronidazole were used as surrogate reference standards for the analysis of Zeffix, Lamivir and Lamdek tablets. There was significant difference in precision when p-Aminophenol and Metronidazole were used as surrogate reference standards in the analysis of Lamivudine Oral Solution. A mobile phase system found suitable for Lamivudine and its surrogates was Methanol: 1% Acetic acid (15:85). The K values obtained when Paracetamol, p-Aminophenol and Metronidazole were used as surrogate reference standards were 4.6660 ± 0.013, 7.289 ± 0.023 and 3.2398 ± 0.025 respectively.

5.3 Recommendations

Similar work must be conducted on several other drugs in order to find surrogate reference standards for their analysis so as to lessen the burden on manufacturing companies, regulatory bodies etc. in developing countries like Ghana as reference standards are 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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7.0 APPENDIX

A.1.0 PREPARATION OF SOLUTIONS

A.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.

A.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.

A.1.0.3 Preparation of 0.1M Cerium (IV) Sulphate

404.3g in 1000ml ≡ 1M Cerium (IV) Sulphate

4.043g in 100ml ≡ 0.1 M Cerium (IV) Sulphates

Cerium (IV) sulphate (4.043g) was weighed and dissolved in distilled water (40ml) topping it up to 100ml in a 100ml volumetric flask. It was then stoppered and labeled.

ASSAY OF PURE SAMPLES

Table A1 Percentage Content of Pure Lamivudine Powder

Replicate determinations	Absorbance of Sample	Percentage Purity (%)
1	0.613	100.98
2	0.610	100.50
3	0.612	100.82
4	0.609	100.33
5	0.613	100.98

Lamivudine Pure Sample

A [1 %, 1 cm] of pure Lamivudine at 280 nm = 607

Expected Absorbance of 0.001 % Lamivudine at 280 nm = 0.607

Absorbance of test sample at 280 nm = 0.613

% Content of sample = [0.605 / 0.607] x 100 = 99.67 %.

Same calculations were done for the samples.

Therefore the average percentage Content was calculated as follows:

[99.67 + 100.12 + 99.84 + 100.00 + 99.67] / 5 = 99.86 %.

ASSAY OF PARACETAMOL

Table A2 Assay of Pure Paracetamol

Burette Reading	1st Sample	2nd Sample	3rd Sample	Blank Titration
Final Reading (mL)	39.90	40.10	40.00	18.7
Initial Reading(mL)	0.00	0.00	0.00	18.0
Titre Value (mL)	39.90	40.10	40.00	0.70

Actual volume that reacted: 39.90 – 0.70 (Blank) = 39.20 mL

Actual weight of paracetamol powder assayed = 0.3003 g

1 mL of 0.1M Cerium Sulphate is equivalent to 7.56 mg of Paracetamol

Therefore 39.20 mL = 0.2964 g

[0.2964/0.3003] x 100 = 98.70%

Sample B and C of pure Paracetamol powder had 99.13 % and 98.93 % respectively.

Average percentage purity = [98.70 + 99.13 + 98.93] / 3 = 98.92 %

Metronidazole Pure Sample

Table A3 Assay of Pure Metronidazole

Sample	A	B	C	Blank
Weight taken	0.1502	0.1501	0.1502	
Endpoint	9.00	9.00	8.90	0.10

Titre value = 9.00 mL – 0.1 mL (Blank) = 8.90 mL

Factor of perchloric Acid = 0.99325

Actual titre = 8.90 x 0.99325

= 8.83993 mL

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

Actual amount of metronidazole = 8.83993 x 0.01712 g

= 0.15133g

Thus the percentage purity (%) = [0.15133/0.15] x 100

= 100.89 %

100.89 % and 99.75 % were the percentage contents obtained for sample B and C respectively.

Average percentage content of Metronidazole pure sample was calculated as follows;

Average percentage content = [100.89 + 100.89+ 99.75] / 3

= 100.51 %

Sample Calculation of Rf Values of Lamivudine

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

Lamivudine tablet (Zeffix)

Rf = 4.3/6.2

= 0.694

Reference

Rf = 4.3/6.2

= 0.694

UNIFORMITY OF WEIGHT

Table A4: Uniformity of Weight (Lamivir Tablet)

Lamivudine Tablet 150 mg (Lamivir Tablet)			
Batch Number: KT 9345			
Weight of 20 tablets: 6.0444 g			
Average Weight: 0.30222 g			
NUMBER	**WEIGTH OF TABLET/g**	**DEVIATION**	**PERCENTAGE DEVIATION**
1	0.3020	0.0002	0.0728
2	0.2993	0.0029	0.9662
3	0.3015	0.0007	0.2382
4	0.3036	0.0014	0.0457
5	0.303	0.0008	0.2581
6	0.3023	0.0001	0.0265
7	0.3073	0.0051	1.6810
8	0.3014	0.0008	0.2713
9	0.2945	0.0077	2.5544
10	0.3017	0.0005	0.1721
11	0.3031	0.0009	0.2912
12	0.2953	0.0069	2.2897
13	0.3077	0.0055	1.8132
14	0.2996	0.0026	0.8669
15	0.3028	0.0006	0.1919
16	0.3041	0.0019	0.6221
17	0.2958	0.0064	2.1243
18	0.3005	0.0017	0.5691
19	0.3089	0.0067	2.2103
20	0.3100	0.0078	2.5743

Table A5: Uniformity of Weight (Zeffix Tablet)

Lamivudine Tablet 100 mg (Zeffix Tablet)			
Batch Number: R 504490			
Weight of 20 tablets: 4.5570 g			
Average Weight: 0.22785 g			
NUMBER	**WEIGTH OF TABLET/g**	**DEVIATION**	**PERCENTAGE DEVIATION**
1	0.2256	-0.0022	0.9853
2	0.2283	0.0005	0.1997
3	0.2295	0.0017	0.7264
4	0.2283	0.0005	0.1997
5	0.2265	-0.0013	0.5903
6	0.2272	-0.0006	0.2831
7	0.2261	-0.0017	0.7659
8	0.2255	-0.0023	1.0292
9	0.2296	0.0018	0.7703
10	0.2284	0.0006	0.2436
11	0.2276	-0.0002	0.1075
12	0.2244	-0.0034	1.5120
13	0.2302	0.0024	1.0336
14	0.2290	0.0012	0.5069
15	0.2275	-0.0003	0.1514
16	0.2278	0.0000	0.0198
17	0.2282	0.0004	0.1558
18	0.2304	0.0026	1.1214
19	0.2291	0.0013	0.5508
20	0.2277	-0.0001	0.0636

Table A6: Uniformity of Weight (Lamdek)

Lamivudine Tablet 150 mg (Lamdek Tablet)			
Batch Number: 1206157			
Weight of 20 tablets: 7.2180 g			
Average Weight: 0.36090 g			
NUMBER	**WEIGTH OF TABLET/g**	**DEVIATION**	**PERCENTAGE DEVIATION**
1	0.3581	0.0029	0.7965
2	0.3610	0.0003	0.0069
3	0.3630	0.0020	0.5610
4	0.3551	0.0059	1.6275
5	0.3651	0.0041	1.1427
6	0.3602	0.0008	0.2147
7	0.3541	0.0069	0.5887
8	0.3631	0.0021	0.5887
9	0.3641	0.0031	0.8657
10	0.3702	0.0092	2.5556
11	0.3541	0.0069	1.9046
12	0.3631	0.0021	0.5887
13	0.3690	0.0080	2.2231
14	0.3720	0.0110	3.0542
15	0.3691	0.0081	2.2508
16	0.3590	0.0020	0.5471
17	0.3600	0.0010	0.2701
18	0.3561	0.0049	1.3505
19	0.3491	0.0119	3.2900
20	0.3540	0.0070	1.9323

TABLE A7: Retention Time for Lamivudine and the Surrogates

SAMPLE	MEAN RETENTION TIME
Lamivudine	1.850 ± 0.056
Paracetamol	5.143 ± 0.1081
P-Aminophenol	1.397 ± 0.0393
Metronidazole	5.012 ± 0.3291

TABLE A8: Limits of Detection and Quantitation for Lamivudine and the Surrogates

SAMPLE	LOD (%W/V)	LOQ (%W/V)
Lamivudine	1.71×10^{-7}	5.19×10^{-7}
Paracetamol	2.69×10^{-10}	8.16×10^{-10}
P-Aminophenol	5.95×10^{-6}	1.695×10^{-5}
Metronidazole	4.24×10^{-6}	1.285×10^{-5}

LINEARITY

LINEARITY FOR LAMIVUDINE

Table A9: Linearity for Lamivudine

RANGE OF CONCENTRATION: 0.0001 – 0.0006 % W/V	
EQUATION OF THE LINE	**CORRELATION COEFFICIENT, R^2**
y = 41453x – 0.0233	0.9979
y = 40756x + 0.0279	0.9992
y = 41116x + 0.2581	0.9990

Table A10: Linearity for Paracetamol

RANGE OF CONCENTRATION: 0.00015 – 0.0015 % W/V	
EQUATION OF THE LINE	**CORRELATION COEFFICIENT, R^2**
y = 13773x + 1.0369	0.9980
y = 13943x + 0.8648	0.9982
y = 14057x + 0.9352	0.9986

Table A11: Linearity for p-Aminophenol

RANGE OF CONCENTRATION: 0.0005 – 0.0030 % W/V	
EQUATION OF THE LINE	**CORRELATION COEFFICIENT, R^2**
y = 8023.3x + 0.0581	0.9993
y = 7976.7x + 0.3419	0.9989
y = 7853.5x + 0.5837	0.9989

Table A12: Linearity for Metronidazole

RANGE OF CONCENTRATION: 0.0003 – 0.0020 % W/V	
EQUATION OF THE LINE	**CORRELATION COEFFICIENT, R^2**
y = 12318x + 0.8024	0.9975
y = 12262x + 0.8026	0.9971
y = 12392x + 0.804	0.9977

SPECIFICITY AND SELECTIVITY

Table A13: Specificity/ Selectivity for Lamivudine

SAMPLE	RETENTION TIME
LAMIVUDINE TABLET	1.850 ± 0.056

PRECISION (REPEATABILITY)

LAMIVUDINE AND ITS SURROGATE

Table A14: Precision for Lamivudine

Relative Standard Deviation of Pure Lamivudine, Number of injections = 6									
Concentration (%w/v)	**Peak Area**						**Mean Peak Area**	**Standard Deviation**	**RSD (%)**
	1	**2**	**3**	**4**	**5**	**6**			
0.0006	805546	805646	806446	805879	805839	805628	805830.7	327.80	0.0407
0.0004	525281	515613	522059	523246	522613	525311	522353.8	3569.37	0.6833
0.0001	148239	145016	141794	145998	146894	146964	145817.5	2245.12	1.5396

Table A15: Precision for Paracetamol

Relative Standard Deviation of Pure Paracetamol, Number of injections = 6									
Concentration (%w/v)	**Peak Area**						**Mean Peak Area**	**Standard Deviation**	**RSD (%)**
	1	**2**	**3**	**4**	**5**	**6**			
0.00150	426767	427047	430929	428032	426965	426117	427642.8	1723.95	0.40313
0.00075	209641	215465	217406	216959	215967	209887	214220.8	3521.28	1.6438
0.00015	67116	67939	65998	65890	65502	65895	66390	934.21	1.4072

Table A16: p-Aminophenol

Relative Standard Deviation of Pure p-Aminophenol, Number of injections = 6									
Concentration (%w/v)	**Peak Area**						**Mean Peak Area**	**Standard Deviation**	**RSD (%)**
	1	**2**	**3**	**4**	**5**	**6**			
0.0030	695183	692286	689390	693215	692586	693422	629680.3	1902.10	0.2746
0.0020	477938	477938	475042	475601	474500	475419	476073	1492.93	0.3136
0.0005	115864	121657	121347	121368	121566	121300	120517	2283.71	1.8949

Table A17: 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**			
0.0020	880226	873184	883747	880198	881200	882335	880148.3	3670.11	0.4170
0.0010	457718	464759	471801	460096	450925	451658	459492.8	7969.06	1.7343
0.0003	158441	154920	154920	156568	155653	154029	155755.2	1565.85	1.0053

DETERMINATION OF THE SURROGATE CONSTANT K FOR LAMIVUDINE

Table A18: Determination Of K Values For Lamivudine Pure Sample Using Paracetamol As The Surrogate. Percentage Purity Of Paracetamol = 98.92 %

Concentration of Paracetamol	Peak Area of Paracetamol	Concentration of Lamivudine	Peak Area of Lamivudine	K Value
0.00150	226996	0.0003	213125	4.6945
0.00100	155234	0.0002	144592	4.6572
0.00075	111368	0.00015	102919	4.6026
0.00050	76899	0.0001	71938	4.6774
0.00150	226950	0.0003	212434	4.6801
Average K value = 4.6660 ± 0.013				

Table A19: Determination Of K Values For Lamivudine Pure Sample Using p-Aminophenol As The Surrogate.

Concentration of p-Aminophenol	Peak Area of p-Aminophenol	Concentration of Lamivudine	Peak Area of Lamivudine	K Value
0.0030	306150	0.0005	369969	7.2507
0.0025	261911	0.0004	303068	7.2321
0.0015	159008	0.0002	155366	7.3282
0.0005	81135	0.0001	55738	7.2782
0.0030	304098	0.0005	372683	7.3532
Average K value = 7.2885 ± 0.023				

Table A20: Determination Of K Values For Lamivudine Pure Sample Using Metronidazole As The Surrogate. Percentage Purity Of Metronidazole = 100.51 %				
Concentration of Metronidazole	**Peak Area of Metronidazole**	**Concentration of Lamivudine**	**Peak Area of Lamivudine**	**K Value**
0.00200	407603	0.0006	384538	3.1447
0.00150	274339	0.0005	299564	3.2758
0.00066	135429	0.0002	133518	3.2534
0.00030	64659	0.0001	69913	3.2438
0.00200	390630	0.00006	384538	3.2813
Average K value = 3.2398 ± 0.024				

DETERMINATION OF PERCENTAGE CONTENT FOR LAMIVUDINE IN LAMIVUDINE TABLETS AND ORAL SOLUTION

Table A21: Percentage Content of Lamivudine in Lamivudine Tablet using Paracetamol as the Surrogate Standard					
Tablet: Zeffix, 100 mg; Average weight = 0.22785 g					
Mean Peak Area of Paracetamol (As)	Concentration of Paracetamol (Cs)	Aa x Cs	K x As	[Aa x Cs] / [k x As]	Percentage Content
228198	0.00150	313.04	1064771.9	0.0002939	97.99
154320	0.00100	140.74	720057.1	0.0001955	97.73
114677	0.00075	78.75	535082.9	0.0001472	98.11
113739	0.00075	78.57	530706.2	0.0001480	98.69
72868	0.00150	33.751	340002.1	0.0000993	99.27

Table A22: Percentage Content of Lamivudine in Lamivudine Tablet using Paracetamol as the Surrogate Standard					
Tablet: Lamivir, 150 mg; Average weight = 0.30222 g					
Mean Peak Area of Paracetamol (As)	Concentration of Paracetamol (Cs)	Aa x Cs	K x As	[Aa x Cs] / [k x As]	Percentage Content
226087	0.00150	304.37	1054921.9	0.00028853	96.18
148289	0.00100	135.45	691916.5	0.00016576	97.88
15730	0.00075	71.57	493336.2	0.00014508	96.72
73810	0.00050	33.51	344397.5	0.00009730	97.30
73762	0.00050	33.68	344173.5	0.00009783	97.86

Table A23: Percentage Content of Lamivudine in Lamivudine Tablet using Paracetamol as the Surrogate Standard					
Tablet: Lamdek, 150 mg; Average weight = 0.30690 g					
Mean Peak Area of Paracetamol (As)	Concentration of Paracetamol (Cs)	Aa x Cs	K x As	[Aa x Cs] / [k x As]	Percentage Content
225976	0.00150	328.71	1064557.2	0.00030878	102.93
149619	0.00100	143.72	698122.3	0.00020308	102.93
149056	0.00100	142.0	695495.3	0.00020416	102.08
113248	0.00075	82.8	543379.0	0.00015246	101.64
76285	0.00050	36.5	355945.8	0.00010253	102.53

Table A24: Percentage Content of Lamivudine in Lamivudine Oral Solution using Paracetamol as the Surrogate Standard					
Tablet: Lamivudine Oral Solution 10 mg/5 mL					
Mean Peak Area of Paracetamol (As)	Concentration of Paracetamol (Cs)	Aa x Cs	K x As	[Aa x Cs] / [k x As]	Percentage Content
215065	0.00150	298.59	1003493.3	0.00029755	99.18
215272	0.00150	300.13	1004459.1	0.00029880	99.60
145757	0.00100	135.38	680102.2	0.00019906	99.53
105652	0.00075	73.72	492972.2	0.00014955	99.70
71953	0.00050	33.39	335732.7	0.00009944	99.44

Table A25 Percentage Content of Lamivudine in Lamivudine Tablet using p-Aminophenol as the Surrogate Standard					
Tablet: Zeffix, 100 mg; Average weight = 0.22785 g					
Mean Peak Area of p-Aminophenol (As)	Concentration of p-Aminophenol (Cs)	Aa x Cs	K x As	[Aa x Cs] / [k x As]	Percentage Content
306051	0.0030	1096.28	2230652.7	0.00049146	98.29
260280	0.0025	749.04	1897050.8	0.00039484	98.71
147107	0.0015	213.64	1072189.4	0.00019926	99.62
56499	0.0005	40.23	411793.0	0.00009794	97.69
56503	0.0005	40.80	411822.1	0.00009907	99.07

Table A26: Percentage Content of Lamivudine in Lamivudine Tablet using p-Aminophenol as the Surrogate Standard					
Tablet: Lamivir, 150 mg; Average weight = 0.30222 g					
Mean Peak Area of p-Aminophenol (As)	Concentration of p-Aminophenol (Cs)	Aa x Cs	K x As	[Aa x Cs] / [k x As]	Percentage Content
306261	0.0030	1092.62	2232183.3	0.00048948	97.90
306794	0.0030	1097.00	2236068.1	0.00049059	98.12
261343	0.0025	753.44	1904798.5	0.00039555	98.89
147267	0.0015	213.51	1073355.5	0.00019892	99.46
56737	0.0005	40.28	413527.6	0.00009739	97.39

Table A27: Percentage Content of Lamivudine in Lamivudine Tablet using p-Aminophenol as the Surrogate Standard					
Tablet: Lamdek, 150 mg; Average weight = 0.30690 g					
Mean Peak Area of p-Aminophenol (As)	Concentration of p-Aminophenol (Cs)	Aa x Cs	K x As	[Aa x Cs] / [k x As]	Percentage Content
300254	0.0030	1126.29	2188401.3	0.00051466	102.93
262210	0.0025	781.85	1912108.8	0.00040877	102.19
260913	0.0025	781.95	1901664.4	0.00041119	102.80
158664	0.0015	237.37	1156422.6	0.00020526	102.63
55314	0.0005	41.0	403156.1	0.00101696	101.70

Table A28: Percentage Content of Lamivudine in Lamivudine Oral Solution using p-Aminophenol as the Surrogate Standard					
Tablet: Lamivudine Oral Solution; 10 mg/5 mL					
Mean Peak Area of p-Aminophenol (As)	Concentration of p-Aminophenol (Cs)	Aa x Cs	K x As	[Aa x Cs] / [k x As]	Percentage Content
305001	0.0030	1104.10	2222999.8	0.00049667	99.33
261892	0.0025	763.31	1925709.2	0.00039638	99.09
146334	0.0015	213.13	1066555.4	0.00019983	99.91
145437	0.0015	211.82	1060017.6	0.00019983	99.92
56428	0.0005	41.42	411275.5	0.00010071	100.70

Table A29: Percentage Content of Lamivudine in Lamivudine Tablet using Metronidazole as the Surrogate Standard					
Tablet: Zeffix, 100 mg; Average weight = 0.22785 g					
Mean Peak Area of Metronidazole (As)	Concentration of Metronidazole (Cs)	Aa x Cs	K x As	[Aa x Cs] / [k x As]	Percentage Content
412062	0.00200	777.72	1334998.5	0.00057855	97.09
301130	0.00150	477.82	975601.0	0.00048977	97.95
132411	0.00066	84.12	428985.2	0.00019609	98.04
132030	0.00066	84.23	427750.8	0.00019691	98.45
62164	0.00030	19.94	201398.9	0.00009899	99.00

Table A30: Percentage Content of Lamivudine in Lamivudine Tablet using Metronidazole as the Surrogate Standard					
Tablet: Lamivir, 150 mg; Average weight = 0.30222 g					
Mean Peak Area of Metronidazole (As)	Concentration of Metronidazole (Cs)	Aa x Cs	K x As	[Aa x Cs] / [k x As]	Percentage Content
415055	0.00200	651.47	1344695.2	0.00048448	96.90
419828	0.00200	659.60	1360158.8	0.00048494	96.99
303764	0.00150	381.82	984134.6	0.00038797	96.99
133630	0.00066	83.45	432934.5	0.00019275	96.37
62136	0.00030	19.73	201308.2	0.00009799	97.99

Table A31: Percentage Content of Lamivudine in Lamivudine Tablet using Metronidazole as the Surrogate Standard					
Tablet: Lamdek, 150 mg; Average weight = 0.30690 g					
Mean Peak Area of Metronidazole (As)	Concentration of Metronidazole (Cs)	Aa x Cs	K x As	[Aa x Cs] / [k x As]	Percentage Content
41360	0.00200	815.89	1342443.5	0.00060776	101.29
301029	0.00150	494.20	975273.8	0.00050673	101.35
301355	0.00150	494.66	976329.9	0.00050665	101.33
133030	0.00066	88.00	430990.6	0.00020417	102.09
64367	0.00030	21.00	208536.2	0.00010069	100.69

Table A32: Percentage Content of Lamivudine in Lamivudine Oral Solution using Metronidazole as the Surrogate Standard					
Tablet: Lamivudine Oral Solution; 10 mg/5 mL					
Mean Peak Area of Metronidazole (As)	Concentration of Metronidazole (Cs)	Aa x Cs	K x As	[Aa x Cs] / [k x As]	Percentage Content
378767	0.00200	730.04	1227129.3	0.00059492	99.15
286090	0.00150	463.01	926874.4	0.00049954	99.91
136818	0.00066	87.89	443263.0	0.00019827	99.14
64396	0.00030	20.84	208630.2	0.00009987	99.87
64489	0.00030	20.83	208931.5	0.00009969	99.69