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THE USE OF SURROGATE REFERENCE STANDARDS IN QUANTITATIVE HPLC; A CASE STUDY OF THE ANALYSIS OF AMLODIPINE AND LISINOPRIL TABLETS

BY

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DECLARATION

I, PRINCE YEBOAH, declare that this research work outlined in this thesis was carried out by the author under the supervision of Prof. J.K. Kwakye at the Department of Pharmaceutical Chemistry, KNUST and that all references mentioned herein are duly acknowledged. There has been no previous tender of this work for a degree anywhere else.

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ABSTRACT

A simple and rapid isocratic RP-HPLC method was developed and validated in accordance with ICH guidelines for the quantitation of Lisinopril and Amlodipine using Diclofenac sodium; Metronidazole and Ibuprofen in place of their own chemical reference standards as surrogate reference standards. The appropriate surrogate constants for each surrogate was determined in relation to each analyte and with these constants the assays of the analytes in commercial formulations were deduced.

The HPLC system used for the determinations comprised of a Schimadzu-CTO20A pump, Prominence programmable UV/Vis absorbance detector SPD-20A, LC-Real time analysis integrator and LUMX Iu C18 5µm, 3.9 x 150mm, Å~100 column. The analysis of Amlodipine and Lisinopril using Diclofenac sodium,

Metronidazole and Ibuprofen in each case was achieved using an ODS column (LUMX Iu C18 5µm, 3.9 x 150mm, Å~100) and a mobile phase system comprising 0.1M orthophosphoric acid: methanol in a ratio 15:85 under isocratic elution mode using a 1.2ml/min flow rate and with UV detection at 219nm and 230nm for lisinopril and amlodipine respectively.

The mean retention times obtained for analysis of Lisinopril using the surrogates were 1.2298 ± 0.0395, 3.0987 ± 0.031, 1.6301 ± 0.0026, 3.2828 ± 0.0029 for Lisinopril, Diclofenac sodium, Metronidazole and Ibuprofen respectively. For the assay of Amlodipine using the surrogate constants. Mean retention times of 1.1761 ± 0.0243, 2.8349 ±0.0001, 1.4987 ± 0.0018, 2.9699 ± 0.0051 were obtained respectively for Amlodipine, Diclofenac sodium, Metronidazole and

Ibuprofen. The surrogate constants obtained for the analysis of Lisinopril were 0.2221 ± 0.008, 0.5916 ± 0.2815, 0.3390 ± 0.018 using Diclofenac sodium, Metronidazole and Ibuprofen respectively. For the analysis of Amlodipine, the constants obtained were 0.7460 ± 0.074, 1.2226 ± 0.737, 1.5203 ± 0.971 using Diclofenac sodium, Metronidazole and Ibuprofen respectively.

The assays obtained for commercial samples of Lisinopril tablets using Diclofenac sodium as surrogate were 97.50 ± 0.850, 98.43 ± 0.954, 101.53 ± 0.793 respectively for brands L-AA, L-BB, and L-CC. When Metronidazole was used as surrogate, the assay values calculated were 97.35 ± 0.189, 98.29 ± 0.045, 101.78 ± 0.469 respectively for brands L-AA, L-BB, and L-CC while the assay values; 97.82 ± 0.008, 98.81 ± 0.002, 101.17 ± 0.203 respectively for brands L-AA, L-BB, and L-CC were obtained when Ibuprofen was employed as surrogate.

The assay obtained for commercial samples of Amlodipine tablets using Diclofenac sodium as surrogate were 94.70 ± 0.086, 97.40 ± 0.084, 96.15± 0.112 respectively for brands A-AA, A-BB, and A-CC. When Metronidazole was used as surrogate, the assay values calculated were 95.83 ± 0.214, 98.27 ± 0.151, 96.50 ± 0.440 respectively for brands A-AA, A-BB, and A-CC while the assay values; 95.14 ± 0.521, 97.96 ± 0.703, 96.80 ± 0.295 respectively for brands A-AA, A-BB, and A-CC were obtained when Ibuprofen was employed as surrogate

The methods developed on comparison with official methods employing the statistical tool; T-test showed no significant statistical difference between the method developed and the official methods. This demonstrates the accuracy of the method and its suitability for routine analysis of the analytes.

The methods developed were validated in accordance with the guidelines of the International Conference on Harmonization (ICH). The results of the validation showed the methods developed were accurate, precise, had good linearity of the working concentration range, robust, sensitive, and specific and can be employed for routine analysis

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 BACKGROUND OF STUDY

Analytical methods for assaying drug entities, either as a whole or part of a formulation, are evolving with modifications centered on more complex assays with the simplest, accurate and cost effective methods (Ahuja and Scypinski 2010, Taverniers et al. 2004). Such changes are exemplified in the everchanging methods of analysis in the official compendia.

These analytical methods may involve cumbersome extraction and separation techniques and these may not be readily replicated to achieve the same accuracy in every analytical laboratory (Russell et al. 2016). The requirements and analytical parameters for the assay of these drug entities may also not be available in literature. Moreover, other official analytical procedures for certain drugs may require expensive reagents and solvents.

Based on the above reasons, it is therefore imperative to develop newer analytical methods for new and known chemical entities. In the development of pharmaceuticals, efficient analytical method development and validation are salient fundamentals. The choice of analytical method in assaying is based on sensitivity, accuracy, and ease of use and the experience level of the scientist as well as the collective expertise of the development and validation unit (Moffat et al. 2011). Good Manufacturing Practices (GMP) outlined by the World Health Organization (WHO) require that every non-compendia analytical method (or modified compendia method) must be validated and the validation results documented (WHO 2007). Significant tests should also be done to ensure

that the method developed shows no substantial difference from the standard method (ICH 2005).

Chromatography can be defined predominately as group of techniques for the separation of a mixture of compounds in between two phases, in which one flows past the other. Chromatography was initially employed to separate components of different chemical/physical properties in a multi-components compounds (Snyder et al. 2011). It has been currently employed in quantitative and qualitative assays when linked with specified detector units (Zhao et al. 2013).

In recent years, separation and analysis of compounds employ HPLC as the main technique. High performance liquid chromatography popularity in usage lies in its ability to combine reproducibility, speed and sensitivity (Snyder et al. 2011).

HPLC is an advanced form of column chromatography in which the solvent is propelled under very high pressures leading to much faster separation. Due to the relatively smaller size of the column, it allows the column packing material to be in a minute particle size creating a large surface area for interaction between the mobile and stationary phases. Not only are these methods highly sensitive but also extremely computerized (Beckett and Stenlake 1988). HPLC separation and analysis of samples is accomplished through the dissolution of the sample in a suitable diluent and injecting into a fast moving mobile phase being propelled through a solid separating substance (stationary phase) filled in a column. This kind of interaction leads to a solid-liquid separation which come about as a result of components of a mixture of compounds' ability to adhere to the stationary phase or remain in the solvent (Watson 2012). As the sample

leaves the column, moving through the detector flow cell; the detector measures the concentration of the sample bands. When no band is passing through the detector, the baseline of the detector is recorded in the form of a constant signal. The difference in the mobile phase properties as the analyte band reaches the detector is observed as a changed in detector signal called peak (Kazakevich and Lobrutto 2007, Scott 1998).

The application of HPLC can be grouped into two owing to the relative polarity of the two phases – mobile and stationary phases: Reversed phase HPLC and Normal Phase HPLC (Kazakevich and Lobrutto 2007). In normal phase HPLC, polar compounds in the mixture passing through the column will form a relatively stronger bond with the stationary phase than non-polar compounds. The non-polar compounds consequently move through the column and exit earlier. However, with reversed-phase HPLC, stationary phase is amended in its polarity property with the attachment of long hydrocarbon chains unto the surface increasing non-polarity. Hence, the polar solvent will interact more with the polar molecules in the mixture being assessed via the column. Polar molecules in the mixture of compounds consequently spend a longer time moving in the mobile phase and elutes first (Arakawa et al. 2010, McMaster 2007, Norwood et al. 2007).

HPLC offers rapidity, reproducibility, and sensitivity altogether. HPLC's popularity and wide usage lies in its versatility. It can be used to separate and analyze chemical entities having a range of polarities in a single run. Almost anything that has the ability to be liquefied can be separated on some type of HPLC column. It can be used to analyze thermally labile compounds and even

volatile compounds (McPolin 2009, Moffat et al. 2011). It can also be used to separate compounds of very wide molecular weight differences (Sousa et al. 2016). The quantity of sample to be detected can vary as small as 10^{-12} g (analytical scale) to 10^{-9}g and 10^{-3}g (semi-preparative scale) to grams (preparative scale). HPLC method development plays a pivotal role in pharmaceutical analysis (Snyder et al. 2011).

Any HPLC method employed in analysis must be able to separate the desirable components from others adequately, be repeatable, rugged as well as produce the appropriate results in order to be useful from time to time without problems. Parameters for assay method validation include robustness, reproducibility among others are prerequisites for acceptance of any newly developed method.

1.1.1 HYPOTHESIS OF THE STUDY

The integrated area under a peak in an HPLC chromatogram is directly proportional to the concentration of the sample injected (Dong 2006, Moffat et al. 2011).

$A \propto C$

$A = QC$ …… Eqn 1.1

Rearranging the equation to make Q the subject:

$$Q = \frac{A}{C}$$

The constant Q for the same compound is equal hence,

$$\frac{\mathrm{A(Analyte)}}{\mathrm{C(Analyte)}} = \frac{\mathrm{A(Standard)}}{\mathrm{C(standard)}}$$ …… Eqn 1.2 But for different compounds,

$$\frac{\text{A(Analyte)}}{\text{C(Analyte)}} \neq \frac{\text{A(Surrogate)}}{\text{C(Surrogate)}} \quad \text{..... Eqn 1.3}$$

Introducing a constant k, the equation for different compounds becomes:

$$\frac{A(Analyte)}{C(Analyte)} = k\frac{A(Surrogate)}{C(Surrogate)} \quad \text{..... Eqn 1.4}$$

Rearranging,

$$C(Analyte) = \frac{A(Analyte)\times C(Surrogate)}{A(Surrogate)\times k}$$

Where A (Analyte)= peak area of analyte, A (Surrogate)= peak area of surrogate

C (Analyte)= concentration of analyte, C (Surrogate)= concentration of surrogate Thus:

$$Percentage\ content = \frac{Actual\ concentration}{Nominal\ concentration} \times 100\% \quad \text{..... Eqn 1.5}$$

1.2 PROBLEM STATEMENT

In quantitative HPLC analysis of pharmaceutical active ingredient, it is imperious to use a chemical reference standard of the desired drug analyte. They are issued only for chemical analysis and may not be suitable for any other purposes. These chemical reference standards are in limited supply even if accessible and very expensive for small-scale industries to afford and provide a lax in quality control stringency methods. Hence the need to assure the quality of formulations produced may therefore be negatively affected. Therefore, the design of HPLC methods that offer quicker, relatively easier, and more importantly, cheaper alternatives is still valid.

1.3 RESEARCH OBJECTIVES

1.3.1 General Objective

This project seeks to explore the prospect of using three compounds as surrogate reference standards for the quantitative analysis of Lisinopril and Amlodipine tablets using RP-HPLC.

1.3.2 Specific Objectives

The specific objectives of this research were to:

1. Establish the conditions for an HPLC assay procedure for Amlodipine and Lisinopril tablets using surrogate reference compounds.
2. Elute the analyte together with a surrogate reference standard at different times.
3. Determine the Detection Limit (LOD), Quantitation Limit **(**LOQ) and the retention times of amlodipine and lisinopril as well as their surrogates.
4. Determine a constant K that can successfully be employed for quantitative analysis.
5. Determine percentage content of Amlodipine and Lisinopril in various brands using the method developed.
6. Compare results obtained from the method developed for Amlodipine and Lisinopril tablets with standard methods.
7. Determine the precision, repeatability, accuracy, as well as the reproducibility of the method developed.

1.4 JUSTIFICATION

In separation and analysis of chemical, the commonest technique employed is High performance liquid chromatography (HPLC). This method has gained popularity partly because of the speed, reproducibility, and sensitivity it offers in drug analysis. Also its popularity and wide usage lies in its versatility and has therefore become imperative to find simple HPLC methods for analysis of drugs.

The use of chemical reference standards in quantitative HPLC is very essential. However, they are issued only for chemical analysis and may not be suitable for any other purposes, for example they are not intended for administration to humans or animals. The chemical reference standard may either be used to obtain a calibration curve from which the content can be estimated. Also it may be used to directly obtain the content of drugs after formulation by comparing the area under the curve for the standard and the analyte. Irrespective of such important role chemical reference standards play, they are usually in short supply and even if available, are expensive. Thus the acquisition of these reference standards for use by analyst becomes a problem for relatively smaller pharmaceutical industries and some regulatory agencies. Such difficulties provide a lax in quality control stringency methods. Hence the need to assure the quality of formulations produced may therefore be negatively affected.

Hence, the design of HPLC methods that offer quicker, relatively easier, and more importantly, cheaper alternatives is still valid. One of such alternative methods is the use of surrogate reference standards. Surrogate standards have already provided an alternative for analysis of selected drugs but the universality of such a method is still yet to be ascertained. According to literature, there is

no validated reverse phase high-performance liquid chromatography (RP-HPLC) assay procedure, for amlodipine and lisinopril, which employs the surrogate method. Hence the project sought to design and validate an assay method for two commonly used anti-hypertensives; amlodipine and lisinopril, using the surrogate method.

Table 1.1: Cost of drug reference standards (**www.sigmaaldrich.com**)

Drug	Quantity	Price (Euro)	Source
Lisinopril	300 mg	339.00	Sigma-Aldrich
Amlodipine	350 mg	339.00	Sigma-Aldrich

1.5 LITERATURE REVIEW

1.5.1 Profile of Drug Substances

Amlodipine

Amlodipine is pharmacologically classed as a long-acting 1,4-dihydropyridinecalcium channel blocker. It functions chiefly by stabilizing L-type calcium channels in the vascular smooth muscle (Yeung et al. 1991). By inhibiting calcium influx in smooth myocytes, amlodipine inhibits calcium-dependent smooth muscle contraction and hence prevents vasoconstriction. Another possible mechanism is a pH-dependent inhibition of calcium influx through carbonic anhydrase inhibition (Murdoch and Heel 1991). Amlodipine is 3-Oethyl-5-O-methyl-2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-6-methyl-1,4dihydropyridine-3,5-dicarboxylate with the formula of $C_{20}H_{25}ClN_2O_5$, and a mas of 408.8759 g/mol.

It comes in the form of a white to pale yellow crystalline with an average melting point of 178-179 degrees Celsius. The solubility in water at 25 degrees Celsius is 75.3 mg/ml.

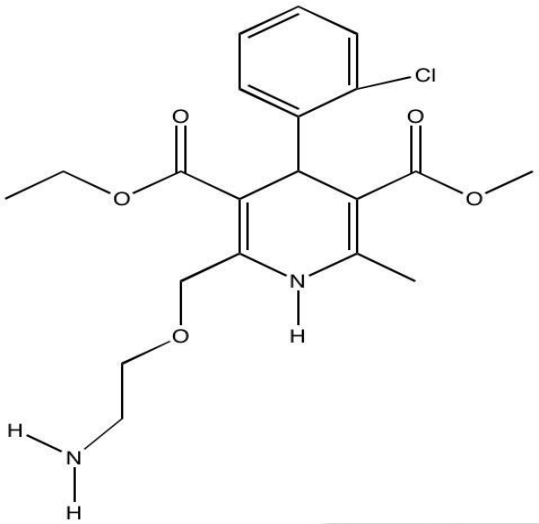

Figure 1- 1: Chemical structure of Amlodipine

Lisinopril

Lisinopril anhydrous possesses an antihypertensive properties and an angiotensin-converting enzyme inhibitor (ACEI) pharmacologically. The inhibition of ACE by lisinopril is achieved through preventing the conversion of angiotensin I to II thereby stopping the vasoconstrictive actions of angiotensin II and subsequently resulting in vasodilation. Lisinopril also reduces angiotensin II-mediated aldosterone secretion by the adrenal cortex. The reduction in aldosterone leads to an increased sodium and water excretion. Lisinopril is (2S)1-[(2S)-6-amino-2-[[(1S)-1carboxy3phenylpropyl]amino]hexanoyl]pyrrolidine2-carboxylic acid with a molecular formula and mass of $C_{21}H_{31}N_3O_5$, 405.4879 g/mol respectively.

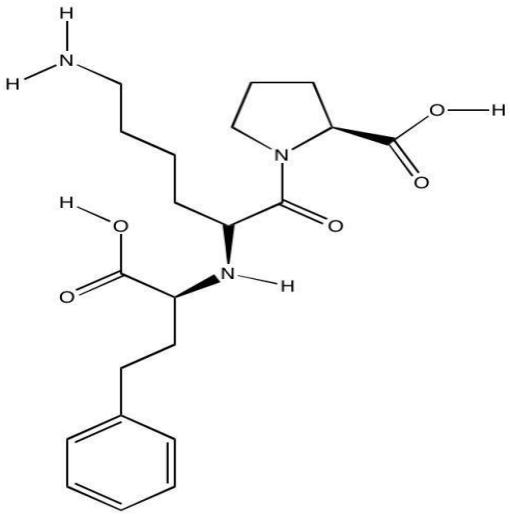

Figure 1- 1: Chemical structure of Lisinopril

Metronidazole

Metronidazole is an Imidazole antibacterial that comes in various preparations such as Metronidazole gel, infusion, suppositories, and tablets. The IUPAC name of Metronidazole is 2-(2-Methyl-5-nitro-1H-imidazol-1-yl) ethanol. It is a white or yellowish, crystalline powder which is slightly soluble in water, acetone, alcohol and methylene chloride. Metronidazole has a melting point of 159 °C to 163°C.

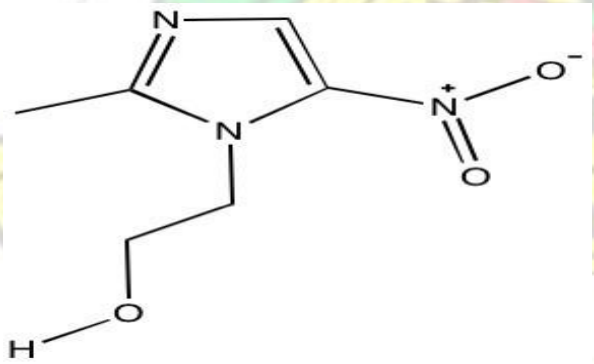

Figure 1- 2: Chemical structure of Metronidazole

Ibuprofen

Ibuprofen is a non-selective cyclo-oxygenase inhibitor, which is utilized as analgesic and anti-inflammatory agent. It is used in preparations such as Ibuprofen Cream, Ibuprofen Gel, Ibuprofen Oral Suspension, and Ibuprofen Tablets (British National Formulary, 2007). Its IUPAC name is (2RS)-2-[4-(2methylpropyl) phenyl] propanoic acid. It presents as a white or off white, crystalline powder or colourless crystals. It is insoluble in water, but freely soluble in acetone and methanol. It has a melting point of 75 °C - 78 °C.

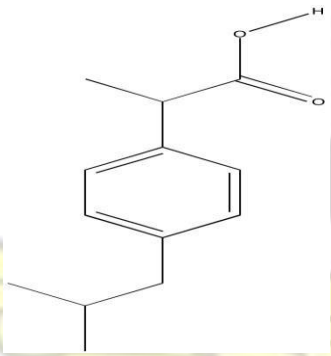

Figure 1- 3: Chemical Structure of Ibuprofen

Diclofenac sodium

It is the sodium salt form of diclofenac, a benzene acetic acid derivative and non-steroidal anti-inflammatory drug (NSAID) with analgesic, antipyretic, and anti-inflammatory properties. Diclofenac is a reversible and competitive inhibitor of cyclooxygenase 1 and 2 isoenzymes, subsequently blocking the downstream production of arachidonic metabolites; that are implicated in the initiation and sustenance of inflammation. This inhibition of prostaglandin E2, one of the downstream products, leads to a blockade of the formation of pain, inflammation and fever. Its IUPAC name is sodium; 2-[2-(2,6-dichloroanilino) phenyl] acetate. It has a molecular formula of $C_{14}H_{10}Cl_2NNaO_2$ and molecular weight of 318.130469 g/mol.

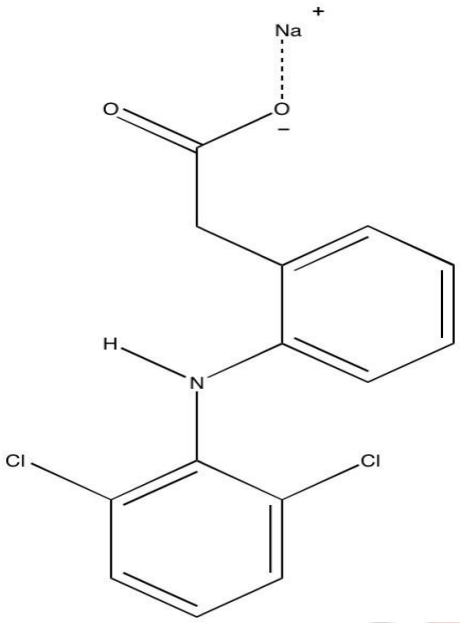

Figure 1- 4: Chemical structure of Diclofenac sodium

1.6 **THEORY OF THE ANALYTICAL TECNIQUE**

1.6.1 Chromatography

Chromatography is basically a physical separation technique, which exploits the differences in distribution on a solid immiscible phase. Its inception is linked to a botanist Michael Twestt who employed chalk columns (stationary phase) and a preferred solvent front (mobile phase) to isolate plant pigments (Ditz, 2005). The principle of all chromatographic techniques is emphasized on the diverse attractions of the compound(s) of interest to the stationary phase and mobile phase. There is repeated occurrence of adsorption and desorption modes of separation as components moves over the stationary phase (Gambhir 2008).

The chromatographic technique is found to be the most extensively used procedure for the separation and purification of mixtures of varying components. In just the past few decades, the original idea of chromatographic separation has seen tremendous changes with the aim of easing operation,

increasing speed of separation and reducing cost. Although the latter has not been achieved with most advanced methods, such advancement has increased the general applicability in separation that was hitherto practically impossible. For example the separation of proteins and even enantiomers of several compounds have been achieved with significant precision and recovery.

Although chromatography is commonly used to isolate new compounds formed during chemical synthesis however in the pharmaceutical industry, during quality control, chromatography is used to monitor the purity of drugs.

The nature of the molecules to be separated and the kind of separation makes one chromatographic technique ideal over the others. There are several types of chromatography based on the physical and chemical nature of the stationary phase, the polarity of the stationary and mobile phases, the principle of separation etc. The different categories of chromatography include: Paper chromatography, thin layer chromatography, column chromatography, gas chromatography, and liquid chromatography.

1.6.2 HPLC

High Performance Liquid Chromatography is a separation procedure that basically involves injection of microliter quantities of a liquid sample into a prepacked column. The components of the sample are forced through the column, using the mobile phase, under high pressure usually delivered by an automated pump. The different molecules of the sample are separated as a result of their differential preference for the packed surface and the mobile phase. The degree of preference for either phase is reflected in the time taken for each separated component to get to the end of the column. Generally, components

with a strong interaction for the mobile phase will elute first. The converse is true for components with a higher degree of attraction for the mobile phase compared to the stationary beads (Kar 2005).

It is highly versatile technique, as it can be used in isolation of a wide range of components, from flavonoids to proteins, provided the right conditions are provided. Compared to liquid chromatography, HPLC is highly sensitive, efficient and relatively easier to operate. As a result, HPLC techniques have been applied in the detection, analysis, quantification and derivation of molecules from mixtures of biological, plant of medical importance by preparative HPLC (Shrivastava and Gupta 2012). Additionally, it is an essential tool in analytical laboratories in pharmaceutical manufacturing industries.

Problems of Drug An8yalysis by HPLC

Rapid analysis with reduced complexity and steps is of great importance in every quality control laboratory. However, there is a considerable amount of steps, which makes acquisition of data tedious. Invariably such assays/analysis tend to produce a greater degree of imprecision.

Stationary phase

It is the immovable packing substance in the column. A variety of matrices exist for the sustenance of the stationary phase. These include silica, polymers, alumina, and zirconia. Different packing materials are used based on the components to be separated or quantified. However, silica columns appear to be the dominant packing material used in most HPLC columns that are mostly classified based on the size of the particles. (Kupeic *et al*. 2005). The basic explanations for being used extensively lies in its high surface area and porosity

as well as ease of preparation. Additionally, its predominance as a stationary phase material may be due to its robustness (does not easily compress under pressure), chemical stability (for diverse compounds and solvents) and the capacity to easily derivatize the functional groups on its surface. The particles size translates into the quality, backpressure and efficiency of the column. Particle sizes usually ranges from 2-5 μm for analytical columns. The smaller the liquid chromatographic packing size, the higher the efficiency of separation due to the large surface area available. In general, the nature of stationary phase influences to a greater extent the capacity factor, selectivity, efficiency and elution (Dong 2006).

A wide range of functional groups, such as phenyl, nitro and amino, have been bonded to silica to resolve compounds with closely related physical and chemical properties. In spite of this, about half of chromatographers develop methods based on C18 sorbents, probably because it is stable over a broad pH range. Even though their performance has improved over the years, they still lag behind silica in terms of efficiency. Zirconia-cladded support materials are stable from pH 1 to 14 and at elevated temperatures. It can be also be derivatized for reversed - phase applications (Ahuja and Scypinski 2010).

Other non-silica-based sorbents have been used in chromatographic separation. The patronage of stationary phases like alumina and carbon have decreased steadily over the years although alumina for example is stable over a broader pH range compared to silica. Cyano bonded modifications of alumina have proved useful in resolving penicillins, cephalosporins and macrolide antibiotics. Carbon based stationary phases have been employed in reproducible separation

of enantiomers. A Cyclodextran modification of carbon makes it useful for separation of chiral compounds (Cazes 2001).

Mobile phase

Similar to Liquid Chromatography, the mobile phase is the solvent phase that is allowed to move the sample to be separated/detected across the stationary phase. The degree of interaction of the mobile phase with the analyte and the stationary phase greatly influences the retention and separation (McMaster 2007). The identification of an ideal mobile phase for separation of an analyte is a tedious and time-consuming process. However, the general properties such as solubility can provide a general idea as to which mobile phase may be suitable. Irrespective of that fact, an ideal mobile phase should have the following properties:

- Have the ability to bring about the desired analyte separation.
- Analytical grade and relatively inexpensive and
- Compatible with the HPLC stationary material, column hardware and the detection system
- Have low viscosity, less flammable and less toxic
- Highly inert

In most HPLC assays, the mobile phase consist of a mixture of solvents rather than a single solvent. In Reverse HPLC for instance, good separation has been achieved using various ratios of acetonitrile-methanol mixtures. Such binary mixtures offer the flexibility of fine-tuning the polarities of the mobile phase by altering the proportion of the individual components in the mixture.

Some chromatographic separation produce ineffective separation and inconsistent retention time even after employing the most suitable mobile phase or solvent mixture. In such instances, additives such as buffers are added to enhance separation. They are most effective within ± 1.5 units of their pKa. Phosphate buffer is the most common buffer employed in reversed HPLC. Trifluoroacetic acid, phosphoric acid and acetic acid are commonly used to lower the pH of mobile phases to suppress ionization of weakly acidic analytes (Kirkland et al. 1995).

Two elution types propel the mobile phase via the column:

a. Isocratic Elution

It is a popular and simpler form of elution. It employs a mobile phase of fixed composition throughout the separation and analysis. It is achieved by either pumping the pre-mixed mobile phase through a single reservoir or by the delivery of a constant ratio of solvents by the binary/quaternary pumps (Kupeic et al. 2005). Isocratic elution is ideal for simple separation and commonly employed in quality control applications. Additionally, the HPLC system is not expose to fast chemical changes allowing relative stability throughout the separation process.

b. Gradient Elution

This is the type where the mobile phase composition is varied over time.. The composition of one component of the solvent mixture is increased while decreasing the proportion of the second component. Such elution is employed in the analysis of complex sample mixtures characterized by poor separation. It offers complete separation of the components with good peak resolution in relatively shorter period. It can be used in the separation of mixtures containing

compounds with varied polarities in shorter time without compromising resolution (Kazakevich and Lobrutto 2007). It is often used in method development for unknown mixtures. The difficulties associated with the use of gradient elution is in its requirement for complex and pricey equipment as well as maintaining constant flow rate even though, it offers faster and more efficient separation with enhanced detection limits (Schellinger and Carr 2006).

Normal phase chromatography

This is a kind of chromatography in which the stationary phase is polar in nature whilst the mobile phase is relatively non-polar. Commonly used stationary phase is usually silica based whilst hexane and heptane are popular mobile phases employed in such separation (Kazakevich and Lobrutto 2007).

Retention of analyte is primarily dependent on its relative polarity. Due to the arrangement and polarities of the phases employed in this technique, polar samples are retained on the polar surface of the column packing relatively longer than less polar materials. Conversely, samples that are mainly non-polar interact less with the stationary phase and hence elute relatively quicker. Generally, samples that are not water-soluble or that may decompose in water are better separated using normal phase chromatography. This technique is also useful for the separation of isomers and compounds that differ in the number or character of functional groups (Cooper 2006).

RP - high performance liquid chromatography

Amongst the two methods, reversed-phase chromatography (RP-HPLC) is the most extensively used in pharmaceutical drug analysis. Separation in RP-HPLC is based on the hydrophobic binding of the solute molecules in the analyte

solution at different extents to the immobilized hydrophobic ligands on the stationary phase. Solvents most frequently used in include methanol and acetonitrile, water and tetrahydrofuran.

Elution takes place by either isocratic conditions in which the composition of the mobile phase remains unchanged throughout the run, or gradient elution in which the components of the mobile phase is continuously varied throughout the run. The solutes are, therefore eluted in order of molecular hydrophobicity; the more non-polar the molecule, the longer it is retained.

RP-HPLC is a powerful technique for the evaluation of pharmaceutical active principles due to a number of factors that include:

1. Resolution under varied chromatographic conditions for chemically related components.
2. Experimental ease of manipulating chromatographic selectivity through modifications in characteristics of mobile phase.
3. Efficiency and elevated recuperations
4. Reproducibility of repetitive separations somehow due to the stability of the sorbent materials within a widespread of mobile phase conditions.
5. It can be used to analyze both neutral and ionic compounds

The attainment of the preliminary separation conditions and optimization of the experimental conditions is perhaps the most significant challenge faced by RPHPLC users. However, this technique can be used for polar, non-polar, ionizable and ionic molecules, making it very versatile for sample analysis.

Separation mechanisms of HPLC

Liquid chromatography is expanded further according to the type of the interactions between molecules of analyte and the stationary phase surface as well as according to the relative polarity of the stationary and mobile phases.

a. Adsorption chromatography

Adsorption chromatography is one of the oldest chromatographic separation techniques. In this kind of separation, the stationary phase of is a solid material on which the sample compounds are adsorbed. However, the mobile phase can either be a liquid (solid-liquid chromatography) or a gas (gas-solid chromatography). Adsorption chromatography is centered on the interaction between the solute molecules and active sites on the stationary phase. This interaction is dependent on the polarity of solutes. The binding of the analyte to the stationary phase is proportional to the contact surface area around the analyte and the adsorbent. Adsorptive forces as hydrophobic, dipole-dipole, ionic interactions are involved in this technique. If the stationary phase is more polar than the mobile phase then high polar compounds in the mixture will tightly adsorbed to the stationary phase whilst as less polar compound will be loosely bound to the stationary phase. Consequently, less tightly bound compounds will be eluted earlier than the tightly bonded ones. Gas chromatography and thin layer chromatography are typical examples of adsorptive chromatography.

b. Partition chromatography

This chromatographic type makes use of the ability of the mixture components to distribute into two different liquid phases to achieve separation. The separation mechanism is as a result of the differences in coefficients of partition

as the mobile phase flows through the column. Here the molecules get preferential separation in between two liquid stationary and mobile phases. Polar molecules get partitioned into polar phase and vice-verse.

c. Ion-exchange chromatography

This is grounded on the principle of charge-charge interactions between an ionic or ionizable sample and the charges immobilized on the stationary phase. It can be sectioned into cation exchange chromatography, a negatively charged resin is used to bind and separate positively charged ions in the analyte; and anion exchange chromatography, in which a positively charged resin is used to bind negative ions. The elution of the bound molecules is attained by the use of a buffer gradient with continual increment in the ionic strength of the eluent solution. The modification of the eluent pH can be done so as to obtain the analyte or the matrix a charge that will favor the elution of an interest molecule from the resin. Ion exchange is one of the most commonly used separation technique for purification of proteins, polypeptides and nucleic acids.

d. Size exclusion chromatography

In this technique, the column packing material is made up of several pores. Molecules of varying degree of molecular sizes are separated as the smaller molecules penetrate deeply into the pores whilst flowing slowly through the column, and the larger ones pass speedily along with the solvent to exit the column owing to their inability to infiltrate the pores. Subsequently, larger-sized compounds elute from the column relatively quicker compared to smaller molecules. Based on the principle, samples with different sizes are effectively separated due to their differences in elution times.

Size exclusion chromatography consist of gel filtration which utilizes a waterbased mobile phase and a hydrophilic stationary phase and gel permeation which employs hydrophobic stationary and a non-aqueous mobile phase to evaluate molecular weight distribution of polymers such as polysaccharides, proteins etc.

Instrumentation

a. Column

The column is an essential part of the High Performance Liquid Chromatograph. It holds the stationary phase beads, which provides differential retention of components. It is usually a stainless steel tube filled with the packing material, which is inert, relatively low cost and has the capacity to withstand a lot of pressure. Nevertheless, under certain circumstances, stainless steel has been shown to interact with the sample (chloride salts) and the mobile phase. Titanium has been explored as an alternative to stainless steel due to its inert nature. Columns made of glass and KeI-F is usually preferred when operating pressures are up to 4000 psi.

Typical analytical columns are 50 - 250 mm long and 2.0 - 4.6 mm in diameter. Larger columns exist for preparative work. Shorter columns and smaller internal diameter analytical columns offer higher sensitivity, lower solvent usage and reduced analysis time (Dong 2006). Various advantages can be inherent in column size variation. The use of shorter columns 3-10cm in length packed with 3 or 5-micrometer particle size is one approach of harnessing these advantages. To increase sensitivity along with reducing the volume of the eluent, narrowbore columns are primarily preferred.

A guard column which is of the same nature as the main analytical column can be used to trap impurities or particles from the samples. This is because impurities block adsorption sites, change the selectivity of the column and cause peak splitting in the chromatogram. Buffers need to be thoroughly washed off by conditioning the column when they are used. Highly pure HPLC grade solvents can be used. Columns should be stored in the appropriate solvent after use.

b. Pump

The high-pressure pumping system is an important part of the liquid chromatograph. It delivers the vital high pressure to drive the mobile phase and analytes through the densely packed column. Its performance directly affects the retention time and reproducibility. Most conventional pumping systems provide pressure up to 6000 psi.

The pumping system must be able to provide constant and reproducible pressure, pulseless output and flow rates ranging from 0.1 to 10 ml/min. (Ayim and Olaniyi 2000, Basett et al. 1989).

c. Injector

Samples are injected into the HPLC through an injection port. The injection port consists of an injection valve and a sample loop. The sample dissolved in an appropriate solvent drawn into a syringe is injected into the loop via the injection valve. The rotation of the valve rotor closes the injection valve and opens the sample loop in order to inject the sample into the stream of the mobile phase. The loop volume ranges from 10µL to over 500µL (Beckett and Stenlake 1988). In more sophisticated HPLC systems, automated sampling devices are

employed where the sample is introduced with the help of auto samplers. This is very useful in multiple analyses (McPolin 2009).

d. Detectors

The detection systems employed by HPLC are designed to respond and measure the extent of change of a particular physical or chemical property of the sample component being eluted. This signal is subsequently processed and recorded as a peak, which gives information about the analyte. Spectrophotometric detectors are by far the most common type of detectors used. Detectors can be broadly classified as;

Ultraviolet/Visible absorption Detector

It is based on the principle of absorption of UV or visible light by the solute according to Beer - Lambert law as it emerges from the column. The UV/Vis absorption detector is the most commonly used detector in pharmaceutical analysis as most pharmaceuticals have chromophores and therefore have UV absorbance. It is highly sensitive, reproducible and easy to operate. It can detect as low as 1 ng of solute (Ayim and Olaniyi 2000). The three types of UV detectors are:

Fixed wavelength detector

A single UV lamp emits light at a specific wavelength. Even though other wavelengths are present the lamp emits light of very high intensity compared with that emitted at the same wavelength by broad-spectrum emission lamps.

Variable wavelength detector

It employs a lamp that emits light over a wide wavelength range. A monochromator may be used to select a particular wavelength for detection purposes.

Diode array detector

The diode array detector is the most powerful UV detector. The xenon or deuterium lamp employed emits light over the UV spectrum range allowing the continuous monitoring of the entire spectrum. It enables the wavelength scan to be taken therefore samples whose maximum wavelengths are unknown can be analyzed (Scott 1998).

Fluorescence Detector

A fluorescence detector monitors the emitted photon or fluorescent light of the solute after excitation by UV radiation. It is not versatile as it is limited to compounds that fluoresce. It is therefore applicable to many biological compounds however derivatisation schemes exist to add fluorophores to nonfluorescing compounds. It is selective and highly sensitive (picoograms to femtograms). It is useful in trace analysis in environmental and forensic analysis **(**Dorsey and Stout, 2002).

Electrochemical Detector

The electrochemical detector measures the current generated with the oxidation and reduction of solutes as it emerges from the column. They include coulometric, polarographic, amperometric and potentiometric detectors. It offers high selectivity and sensitivity in picograms. They are incompatible with

gradient elution. It is conveniently applicable with monoamine assay and quantification.

Nuclear magnetic resonance absorption Detector

Nuclear magnetic resonance absorption detectors have grown rapidly due to improvement in instrumentation in recent years. NMR is used with liquid chromatography reduces analysis time. It allows for the structure elucidation of unknown compounds. This technique however offers very low sensitivity (Norwood et al. 2007).

Mass Spectrometer detector

This type of detector is based on the ionization of compound being separated. This technique offers highly efficient separation, high sensitivity with reduced analysis time (Olaniyi 2000)

1.7 METHOD VALIDATION

It is an analytical procedure in which a series of validation experiments are performed and (statistically) evaluated to objectively demonstrate the method's applicability for the intended purpose. Since the type and extent of validation experiments depend very much on the purpose of the method, various guidance documents specifically addressing analytical method validation in different fields of analysis have been issued by various organizations. It is the steps of confirming the acceptability as well as the efficiency of an analytical method and the proof that the method is dependable and appropriate for it intended purpose. In the pharmaceutical industries validation of analytical methods is established by providing evidence that demonstrates analytically relevant levels of linearity, specificity, accuracy, precision, and robustness amongst others. In

addition to the above, the range, limit of detection as well as limit of quantitation, are further requirement for regulatory submission.

Analytical methods aimed at assessing or assaying a drug entity in drug manufacturing process needs to go through validation process.

1.7.1 Specificity

Specificity describes how well an analytical assay method is able to differentiate the analyte(s) of interest from a mixture of other active components/adjuvants. It is not always possible to demonstrate complete refinement of analytical procedure for the detection of the analyte of interest, however the method developed should ensure there is baseline separation of analyte, in the chromatogram obtained, from all impurities that might be present. This can be achieved by zeroing the detector signal before subsequent analyte traverse the column to reach the detection unit.

The general specificity requirements are that, chromatograms developed from reference standard and the test sample, at the same concentration, should not differ in retention time and normalized peak area by ±10%. In developing an HPLC analytical assay, it is imperative to test for the specificity of the method by deliberately adding specific potential impurities. For analysis of a drug formulation, it is prudent to compare chromatograms for the pure drug sample with one containing traces of all possible synthetic by-products and intermediates, degradation products, and excipients. This makes the assay method useful in both the assay of the pure compound before and after formulation.

1.7.2 Linearity

The purpose of measuring linearity in analytical assays is to ascertain the degree of deviation, or otherwise, of the calibration curve from a straight line. Hence linearity gives a quantitative measure of correlation between the peak area and the concentration of the injected sample. Additionally, linearity proves that the whole analytical system (detector and data acquisition) exhibits direct proportionality over the relevant concentration range for the analyte of interest.

The calibration curve for any analytical method can be assessed for linearity with a minimum of five standard solutions, of known nominal concentrations, ranging from 0.5 to 1.5 times the expected analyte concentration to be used in subsequent assays. To account for analytical variance, this determination is performed in triplicates. The square of the correlation coefficient is a superficial but common measure of linearity.

1.7.3 Accuracy

In general terms, accuracy is a measure the degree of closeness between the accepted true value and the assay experimentally determined value. The accuracy of an analytical method may be inferred from the results of linearity, specificity and precision tests. Accuracy can also be assessed by comparing the results for the method being validated to the results with those of an independent method that has been well characterized. Comparison with a reversed-phase HPLC method, with varied conditions, can be performed using the same detection scheme. Additionally, recovery of the analyte of interest after spiking with a placebo can be used to demonstrate the extent of accuracy of the developed method. In this case, the percentage recovery with the certain acceptance criteria at each specific level is reported. Since standard reference

materials are not readily available and accessible. Usually, triplicate determinations are performed over three concentration levels stretching from 0.5 to 1.5 times the expected sample concentration.

1.7.4 Precision

The measure of how well replicate values agree with each other is referred to as precision. This measure of deviation is usually an indication of random errors in the data set and is usually assessed when the final details of the analytical methods have been finalized. Generally, it can be subdivided into intermediate precision and repeatability.

Repeatability basically analyzes of data from the same experimenter, usually after repeated injection of replicates. Repeatability tests are mandatory for all tests delivering numerical data. Repeatability can further be sub-divided injection and analysis repeatability. Injection repeatability explores the variation that results from multiple injections of a single analyte solution over a short period of time. In analysis repeatability a single experimenter analyzes different concentrations and multiple injections of the same sample on the same day. Another variation, which is the final step in precision assay, is performed on a single sample by different experimenters, with different chromatographic systems, on different days. This provides some knowledge of the extent of inter- and intra-laboratory variability. Adequate precision for analytical methods is assessed using the relative standard deviation or coefficient of variation (*S*rel or %RSD) between the data sets obtained.

1.7.5 Range

All analytical assays assume linearity within certain concentration limits. This concentration limits (upper and lower) over which the linearity, precision and accuracy are acceptable is known as the range.

1.7.6 Limit of Detection and Limit of Quantitation

Limit of detection (LOD) is defined as the minimum concentration of a sample/analyte that can be detected, under the specific experimental conditions, by the detector system coupled to the experimental system. The limit of quantitation (LOQ) however, is the minimum concentration of the analyte in the matrix that can be measured with an acceptable level of accuracy and precision.

Statistically speaking, limit of detection (LOD) is defined for a peak that gives a signal-to-noise ratio of about 3:1, and limit of quantitation (LOQ) is expressed for a peak that provides a signal-to-noise ratio of about 10:1. Some analytical protocols provide guidelines that employ the consecutive injection of five or six samples of these solutions and then calculate %RSD of the multiple peak areas (sample peak).

The acceptable in analytical laboratories limit for %RSD for LOD and LOQ is 120% and 110% respectively

Mathematically, the LOQ and LOD are expressed as follows;

$$LOD = \frac{3\sigma}{S} \quad 3. \qquad \text{……..…. Eqn 1.6}$$

$$LOQ = \frac{10\sigma}{S} \qquad \text{……..…Eqn 1.7}$$

1.7.7 Robustness/Ruggedness

In the development of analytical methods using the HPLC, it is important to ensure reproducibility of results under different analytical environments. Hence, robustness assesses to a large extent the degree of variation of the analytical results of the same sample under the different environmental conditions. However, the components of the solvent mixture are kept constant throughout the assessment of ruggedness of the method. An ideal assay method should be able to withstand slight variations in analytical environment without affecting the assay results significantly. Such changes that can be effected includes buffer composition, temperature and detection wavelength. Significant variations obtained after any single alteration should be stated in the method description as the tolerable limit of the method.

1.7.8 Stability of Solution

Although, generally all analyte samples are freshly prepared before being assayed, it is common in the QC labs of pharmaceutical industries to perform assays at a later time after preparation of the solution. Less stable compounds can degrade and provide false negative results for a sample that might have passed the assay test. Therefore, it is necessary to demonstrate that the sample and reference standard solutions are stable at least during normal duration of an analytical sequence, which ranges from 24 h to 72 h. These assays are performed and compared to a freshly prepared reference standard solution to ascertain the degree of attrition (Garcia et al. 2012).

One of the reasons for a rapidly degrading compound, apart from its inherent instability, may be the solvent system. Substitution or addition of buffers to the solvent system could delay the progress of the most labile compounds.

For drugs it is acceptable to compare the peak area at initial time point and at the defined time *t* (e.g. 24 h). A time course curve and subsequent comparisons with the reference standard peak areas is compared to give an idea of the degree of stability or otherwise of the analyte in the specified solvent system (Watson 2012).

CHAPTER TWO

METHODOLOGY

2.1 MATERIALS

HPLC Grade Methanol (Fisher Scientific UK Limited), Acetic anhydride (BDH), Sodium hydroxide (BDH), Glacial acetic acid (BDH), Sulphuric acid (BDH), Perchloric acid (Qualikems Fine Chem Pvt. Ltd), Phenol red (Fisons Scientific Equipment), Crystal violet (Hembarge Chemical Company), Ethyl acetate (BDH), Anhydrous sodium acetate (BDH), Orthophosphoric acid (BDH), Toluene (BDH), Analar potassium hydrogen phthalate (BDH), sodium 1-hexane sulfonate (SIGMA-Aldrich), acetonitrile (BDH), monobasic potassium phosphate (SIGMA-Aldrich), ammonium acetate(BDH), ethyl

acetate, butan-1-ol, ninhydrin, ethanol (BDH).

Ernest Chemists Limited, Tema - Ghana and Entrance Pharmaceuticals, Spintex - Ghana provided the pure samples of analytes and surrogate reference standards utilized.

2.1.1 INSTRUMENTATION

The equipment utilized in the study include; Shimadzu – CTO – 20A HPLC pump, prominence UV/Vis SPD – 20A detector, LC-Real time analysis integrator, LUMX Iu 150 x 3.9 mm column, Stuart melting point (SMP) 10 apparatus, Sartorius SE623P analytical weighing balance, MAGNA Nylon membrane filters (47mm, 0.45µ), FS 28H Fisher Scientific Sonicator, Eutech Instrument pH meter, PerkinElmer SpectrumTwo Spectrometer – FTIR.

Table 2.1: Profile of pure sample of analytes and surrogate reference standards

Sample	Batch Number	Manufactory Date	Expiry Date
Lisinopril	L/S 5020312	03/2012	02/2017
Amlodipine	OSPZ-019B/022H12	08/2012	07/2016
Ibuprofen	4000/12/0713	08/2012	07/2017
Metronidazole	20131178	11/2013	11/2017
Diclofenac sodium	141222-6	12/2014	12/2018

Table 2.2: Profile of formulations of analytes employed in the study obtained from Retail Pharmacies in Kumasi area.

Sample	Strength	Assigned code	Manufacturing company	Batch number	Expiry date
Amlodipine	10mg	A-AA	Entrance pharmaceutical and research centre	N/15102	10/2017
	10mg	A-BB	Pharmanova Ltd Gh	E013	09/2018
	5mg	A-CC	Sandoz Ltd	ER4632	08/2017
Lisinopril	20mg	L-AA	Accord UK	PR03008	11/2016
	20mg	L-BB	Lupin (Europe) Ltd	G408171	09/2017
	10mg	L-CC	Pharmanova LTD Ghana	E003	08/2018

2.2 METHODS

2.2.1 Identification of Pure Samples of Compounds

Determination of melting points for pure compounds used in study

The melting point of each pure sample was determined as follows:

The loose, dry samples of each analyte were introduced into separate capillary tube sealed at one end. The introduced sample introduced into the lowest part of the capillary tube by tapping the sealed end gently on a hard surface. The process was repeated till a tightly parked mass of a vertical height of

approximately 3 to 5 mm was obtained. The capillary tube was then introduced into the melting point apparatus. The melting point obtained was subsequently recorded. The process was performed in triplicates for each sample.

Determination of infrared spectrum for pure samples of each compound Each sample was analyzed with the FT infrared spectrophotometer to obtain a spectrum for comparison with standard. The spectra for each sample were obtained as follows; a small portion of each sample was taken and run to obtain a spectrum.

Commercial Tablets Identification

Lisinopril Tablets

11 mg of a pure lisinopril powder was dissolved in a solution containing 2 ml of distilled water and 8ml of methanol to obtain a 10 ml, 0.11% w/v solution of lisinopril. Twenty selected lisinopril tablets were crushed in a porcelain mortar with a pestle and a weight of the powder equivalent to 10 mg of lisinopril was weighed and dissolved in a solution containing 2 ml distilled water and 8 ml methanol. About 20 µl portions of the test solution and standard solutions were spotted on a pre-coated TLC plate and allowed to dry. The chromatogram was developed in a saturated chromatank using a mobile phase comprising of ethyl acetate, glacial acetic acid, butan-1-ol and water in equal proportions. The spotted TLC plate was then allowed to stand undisturbed to develop to 15 cm. The plate was subsequently removed, air-dried and sprayed with a 0.2% ninhydrin. The resulting plate was heated in an oven at 105 ^{0}C for 10 minutes and examined in daylight. The Rf values of both the standard and sample were compared. The procedure was repeated for all other brands of lisinopril tablets.

The procedure was repeated for all selected brands of Lisinopril tablets

Amlodipine tablets

A quantity of each powdered brand of tablets was dissolved in Methanol: Water (60:40). It was shaken for 15 minutes on a sonicator and filtered. The filtrate was then analyzed with the FT infrared spectrophotometer to obtain a spectrum for comparison with standard. The spectra for each sample were obtained as follows; a small portion of each sample was taken and run to obtain a spectrum.

2.2.2 Assay of Pure Compounds

Standardizations

Standardization of Approximately 0.1M $HClO_4$ (Perchloric Acid)

20.00ml of glacial acetic acid (25.0ml) was added to 0.500g of analar potassium hydrogen phthalate in a conical flask.

The resulting solution was then warmed, to ensure dissolution of the salt, and allowed to cool. The cooled solution was titrated against 0.1M Perchloric acid using crystal violet as the indicator. The determination was repeated five times and the average titre calculated.

Standardization of Approximately 0.1M Sodium Hydroxide

Standard sulphamic acid solution (25.00 ml) was pipetted and titrated against NaOH (0.1M). Methyl orange was used as an indicator. The determination was performed five times and average titre calculated.

Assay of Ibuprofen

0.450g of the analyte was weighed and introduced into a conical flask containing 50 mL of methanol R. A few drops of phenolphthalein solution R1 was then

added to the flask and titrated with NaOH (0.1 M) until a pink colour was obtained. A blank titration with only 50 ml of methanol R was then performed. 1mL of 0.1M NaOH is equivalent to 20.63mg of $C_{13}H_{18}O_2$.

Assay of Diclofenac sodium

A quantity of 0.250g of diclofenac sodium was weighed accurately and dissolved in anhydrous acetic acid (30ml). The resulting solution was then titrated against 0.1M Perchloric acid VS after the addition of one drop of crystal violet TS until a blue colored end point was obtained.

Assay of Metronidazole

A quantity of 0.150g of metronidazole was weighed accurately and dissolved in anhydrous acetic acid (50ml). The resulting solution was then titrated against 0.1M Perchloric acid VS after the addition of one drop of crustal violet TS until a blue colored end point was obtained.

A unit ml of 0.1M $HClO_4$ (Perchloric Acid) is equivalent to 17.12mg of $C_{17}H_{20}N_2S.HCl$

Assay of Lisinopril

An amount of 0.350g Lisinopril powder was weighed and dissolved in of distilled water (50ml) and titrated with sodium hydroxide (0.1M). The reaction endpoint was determined using methyl orange as an indicator.

1ml of 0.1M NaOH is equivalent to 40.55 mg of $C_{21}H_{31}N_3O_5$.

2.2.3 HPLC Method Development

Amlodipine and Lisinopril like most pharmaceuticals are of intermediate polarity, with significant portions of both polar and non-polar moieties. Hence, reverse phase chromatography employing octadecyl silyl silica (C18) stationary phase was chosen for the work. This permits maximum drugstationary phase interaction owing to the presence of the non-polar moieties in the chosen drugs.

Mobile Phase selection

The two analyte as well as the chosen surrogate standards have significant non-polar in relation to polar moieties. Nevertheless, the presence of carboxylic acid functional groups in the analytes made it imperative to utilize a mobile phase consisting of orthophosphoric acid (0.1M) and methanol in the ratio of 15%: 85% after several trials.

Preparation of mobile phase

The 0.1M orthophosphoric acid was prepared by pipetting approximately 3.1ml of the stock into a 500ml volumetric flask filled with some quantity of distilled water. It was then sonicated for about 5minutes and filled up to the mark. In preparing 1L of the mobile phase, 150ml of the orthophosphoric acid was measured and transferred into 850 ml filled volumetric flask. The mixture was again sonicated and filtered before use.

Detector selection

There are chromophoric groups present in the chemical structures of both the analytes and chosen surrogates standards, which can interact with electromagnetic radiations. In order to attain an appreciable level of interaction, an UV/Vis detector was chosen.

Wavelength selection

The selected wavelengths used in the assay were based on the UV absorption characteristics and previous literature of the analytes. Both drugs however do not have very extensive chromophores hence a low wavelength UV radiation was selected to enable interaction with the available chromophores. Samples of the analytes and surrogates were prepared in a solution of the mobile phase and run in an UV/Visible spectrophotometer between 200 and 300nm. A wavelength of 219nm and 230nm was then selected as the best suited for the analysis of lisinopril and amlodipine respectively.

Flow rate selection

The flow rate influences the retention time and hence operational run time, as well as column backpressure and the resolution between peaks of closely eluting compounds. These factors were considered in selecting the flow rate. A flow rate of 1.2mL/min was selected for both methods after numerous preliminary trials.

2.2.4 Analytical Performance Parameters

Stability Studies on Solutions of Compounds used for the Study

Solutions of the working concentration of each analyte and its surrogates as employed in the methods developed were prepared independently. Each solution was then injected onto the column for analysis at 0, 10, 30, 60, 120, 180, 240, 360, 420, 600, 1200 and 2400 minutes. The corresponding peak areas for each solution at these times were then recorded. A graph of peak area against time for each compound was plotted to analyze the stability of each compound in solution over the specified period.

Linearity

The calibration curves for analysis of lisinopril and amlodipine with surrogates diclofenac, metronidazole and ibuprofen were obtained by preparing stock solutions of each analyte and surrogate. Stock solutions of pure samples of each analyte and surrogate were prepared by weighing 0.20g each and dissolving in a diluent (composed of methanol and 0.1M Orthophosphoric acid; 85:15) to give a solution of approximately 0.2% w/v for each sample. Accurate volumes of each analyte and surrogate were pipetted and diluted serially to obtain six (6) different concentration of for each analyte

(Amlodipine or Lisinopril) and surrogate (Diclofenac sodium, Ibuprofen or Metronidazole) approximately 0.1%. 0.01%, 0.02%, 0.04%, 0.06% and 0.08%. Twenty microliters (20µL) of each solution was then injected unto the column and eluted isocratically with the selected mobile phase. Multiple runs were made for each solution and the average peak areas for analyte and surrogate calculated. The average peak areas were then plotted against their respective concentrations.

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

The limits of detection and quantitation were calculated from the obtained data, in the linearity test, using the formula below:

$$LOD = \frac{3.3\sigma}{S} \quad \text{……..……Eqn 2.1}$$

$$LOQ = \frac{10\sigma}{S} \quad \text{……..……..Eqn 2.2}$$

Where; σ = Standard deviation

Standard deviations were calculated from the calibration curve of the analytes and surrogates employed.

Specificity

Solutions, of analytes and surrogates, with concentrations within the specified working concentration were prepared from their respective stock solutions and injected into the HPLC system for detection and analysis. Similar concentrations were prepared using the selected tablets of the different analytes. The solvent system employed in sample/tablet preparation was also injected for HPLC. The resulting chromatograms obtained for all three injections were then compared.

Accuracy

The standard addition method was employed. Three concentrations of the analyte (lisinopril and amlodipine) were prepared with a standard concentration of a surrogate in each case as follows.

Concentration I (80%)

Stock solutions containing 0.05%w/v of each analyte and surrogate were prepared separately as described above. 2.5mL of 0.05% analyte solution was then pipetted into a 25.0ml volumetric flask with 2.5mL of 0.05% surrogate solution. Another 2.0mL each of the 0.05%w/v analyte and surrogate solution was then added to the volumetric flask, diluted to the mark with the solvent system and the mixed thoroughly. The resulting solution was injected unto the column for analysis with the method developed.

Concentration II (100%)

2.5mL of the 0.05% analyte solution was pipetted into a 25.0ml volumetric flask with 2.5mL of 0.05% surrogate solution. Another 2.5mL each of the 0.05%w/v analyte and surrogate solution was then added to the volumetric flask, diluted

to the mark with the solvent system and the mixed thoroughly. The resulting solution was injected unto the column for analysis with the method developed.

Concentration III (120%)

2.5mL of the 0.05% analyte solution was pipetted into a 25ml volumetric flask with 2.5mL of 0.05% surrogate solution. Another 3.0mL each of the 0.05%w/v analyte and surrogate solution was then added to the volumetric flask, diluted to the mark with the solvent system and the mixed thoroughly. The resulting solution was injected unto the column for analysis with the method developed.

Precision

Intra-day Precision (Repeatability)

Single batches of Lisinopril and Amlodipine tablets were assayed with the developed method at different time points on the same day by the same experimenter. This was achieved by analyzing the approximate concentrations of freshly prepared solutions of samples and running at specified time intervals. Multiple injections were made of each solution containing the analyte tablets and its surrogate to determine the precision between the different assays carried out.

Inter-day Precision (Reproducibility)

Single batches of lisinopril and amlodipine tablets were assayed with the currently developed method at different time points, by, different experimenters, on two different days. Briefly, this was achieved by multiple injections of each solution containing the surrogate and analyte (tablets) and for HPLC analysis.

Ruggedness

The analysis was carried out varying one of the experimental conditions whilst keeping the others constant to scrutinize the ruggedness of the developed method. The parameters varied were the length of column (250cm and 150cm) and different laboratories.

2.2.5 Determination of Surrogate Constant (K), for Each Analyte Using the Surrogate Reference Standards

Diclofenac sodium, Ibuprofen and Metronidazole were chosen as surrogates for both Amlodipine and Lisinopril. A stock concentration of an approximately 0.1% w/v each of the analytes and surrogates were prepared by weighing 0.1gm of each and dissolving in 100ml of the mobile phase and mixed by sonication. 10ml each of the analyte and surrogates were pipetted into a 25ml volumetric flask and made up to volume with the mobile phase to obtain specific concentrations of both analytes and surrogates.

Approximately, twenty microliters (20µL) of each surrogate-analyte mixture was injected and eluted isocratically with the appropriate mobile phase. The peak areas obtained from the chromatogram were used to calculate the actual concentration of analyte and surrogate in each solution. The peak areas and concentration of the analyte and surrogates were used to calculate the surrogate constant for the surrogate used for analysis of each analyte. The concentrations of analytes and their surrogates were also varied and the process repeated to determine the variability of the surrogate constant at different concentrations.

2.2.6 Tablets Weight Uniformity Test

Twenty (20) tablets of the selected brands of Lisinopril and Amlodipine tablets were weighed accurately individually and cumulatively and subsequently recorded. The deviation and percentage deviation were calculated for each of the individual weights taken.

2.2.7 Assay of Commercial Sample Tablets Using the Surrogate Reference Standards

Amlodipine Tablets

Stock solutions of Diclofenac, Metronidazole and Ibuprofen were prepared by accurately weighing 0.05g of each sample and subsequently dissolving in sufficient diluent (0.1M Orthophosphoric acid: methanol, 15:85). A 100mL solution of each sample was prepared to give an approximate concentration of 0.05% w/v.

Twenty randomly selected tablets of each brand of amlodipine were weighed and powdered. An approximate concentration of 0.05% w/v for each tablet sample was prepared using the same solvent system and method as described in section 2.11. After filtering, 10 ml aliquots of the filtrate was pipetted into a 25mL volumetric flask with 10 mL of each surrogate stock solution. Enough diluent was then added to make the solution up to volume and mixed thoroughly.

Twenty microliters (20 µL) of the resulting mixture each solution (analyte and surrogate) was injected unto the column and isocratically eluted with the mobile phase. The peaks corresponding to amlodipine and each surrogate were then recorded and analyzed to determine the peak area. The content of amlodipine in the tablets were then estimate using the respective surrogate constant (K).

Lisinopril Tablets

Stock solutions of diclofenac, metronidazole and Ibuprofen were prepared by accurately weighing 0.05g each sample and subsequently dissolving in sufficient diluent (0.1M orthophosphoric acid: methanol, 15:85). A 100mL solution of each sample was prepared to give an approximate concentration of 0.05% w/v.

Twenty randomly selected tablets of each brand of amlodipine were weighed and powdered. An approximate concentration of 0.05 % w/v for each tablet sample was prepared using the same solvent system and method as described above. After filtering, 10 ml aliquots of the filtrate was pipetted into a 25mL volumetric flask with 10 mL of each surrogate stock solution. Enough diluent was then added to make the solution up to volume and mixed thoroughly.

Twenty microliters (20 µL) of the resulting mixture of each solution (analyte and surrogate) was injected unto the column and isocratically eluted with the mobile phase. The peaks corresponding to Amlodipine and each surrogate were then recorded and analyzed to determine the peak area. The content of Amlodipine in the tablets were then estimate using the respective surrogate constant (K).

2.2.8 Assay Of Commercial Sample Tablets using the Standards Methods

Amlodipine Tablets

Twenty (20) tablets for each brand of Amlodipine tablets were randomly selected, weighed together and powdered. A weight of the powder equivalent to 50mg of Amlodipine was transferred into a 50ml volumetric flask and an amount of the diluent, ammonium acetate R: methanol R (30: 70 v/v) — mobile phase was added and sonicated for five minutes. It was then filled to volume

with the mobile phase, mixed and filtered. Aliquots of 5.00 ml from the resulting solution were diluted to 100ml with the mobile phase. Twenty microliters of the final solution was then injected at a flow rate of 1.5ml/min
over an ODS column (l=0.15m, Ø=3.9) and detected at 237nm at a temperature of 30°C for fifteen minutes. The reference solution was also run following the same chromatographic conditions (British Pharmacopoeia, 2013).

Lisinopril Tablets

Twenty (20) tablets for each brand of Lisinopril tablets were randomly selected, weighed together and powdered. A weight of the powder equivalent to 20mg (0.2mg/ml) was transferred into a 100ml volumetric flask and an amount of the diluent (water 4: 1 methanol) was added and sonicated for five minutes. The flask was again shaken mechanically for twenty minutes and more diluents added, mixed and filtered. Twenty microliters of the final solution was then injected at a flow rate of 1.5ml/min over an ODS column (l= 0.15m, Ø=3.9) and detected at 215nm. The sample was run at a temperature of 40°C for 15minutes using a mobile phase of acetonitrile and buffer (phosphate solution at pH 2.0 + sodium 1-hexanesulfonate) in a ratio of 1:4. The pure compound was also run following the same chromatographic conditions (United States pharmacopoeia, 29).

CHAPTER THREE

RESULTS AND CALCULATIONS

3.1 IDENTIFICATION OF PURE SAMPLES OF COMPOUNDS

3.1.1 Determination of melting points for pure compounds used in study

Table 3.1: Melting points of test compounds and Surrogate Reference standards employed in the work.

Analyte/ surrogate	Experimental result / ^{o}C	Official value/ ^{o}C
Amlodipine	177 - 178	178 - 179
Lisinopril	146 - 147	146 - 148
Diclofenac Na	280 - 281	280
Metronidazole	160 - 162	159 - 163
Ibuprofen	77 - 78	76 - 78

3.1.2 Determination of infrared spectrum for pure samples of each compound

Table 3.2: Principal peaks' wavenumbers for analytes and surrogates

Sample	Principal Peaks' wavenumber / cm^{-1}
Amlodipine	1262.15, 1192.11; 1089.58
Lisinopril	1609, 1570, 1389.84; 748,739 with a broad peak around 3000 [1]
Diclofenac Sodium	1573.44, 765.49, 1498.07, 1282.1, 1304.34
Ibuprofen	1707.05, 1229.02, 779, 1183.11, 1267.78, 865.66
Metronidazole	1185.07, 1533.83, 1072.93, 1263.83, 743.02, 1157.62

Refer to Appendix A1 for FTIR spectrum

3.1.3 Commercial Tablets Identification

Lisinopril Tablets

- The principal spot of the test sample observed in the TLC chromatogram corresponded in position and colour to that of the standard.
- A retention factor of 0.717 for sample and 0.72 for the standard was produced.

Refer to Appendix A2 for TLC chromatogram

Amlodipine tablets

- Principal peaks at wavenumbers 1262.15, 1192.11; 1089.58cm $^{-1}$ from the IR spectrum was observed

3.1.4 Other Identification and Solubility Tests

Amlodipine

- The compound was freely soluble in methanol, sparingly soluble in absolute alcohol and slightly soluble in water.

Diclofenac sodium

- A blue coloration with the formation of a precipitate was observed and produced the reactions of sodium as per the BP.

Ibuprofen

- Absorption maxima were observed at 272 nm, and 264 nm with a shoulder at 258 nm. The ratio of absorbance at 258nm / 264nm and at 258nm / 272nm were 1.22 and 1.00 respectively.

Metronidazole

- Two peaks were observed at 240nm as the minimum and 277nm as the maxima. The absorbance at 277nm was 0.758.

Lisinopril

- It was soluble in water and insoluble in alcohol.

3.2 ASSAY OF PURE COMPOUNDS

Table 3.3: Percentage purity of various pure samples employed in the research.

Analyte / surrogate	Specified limit (%)	Result (%)
Amlodipine besilate	97.0 – 102.0 (BP)	99.50
Lisinopril	98.0 – 102.0 (USP)	100.60
Diclofenac sodium	99.0 – 101.0 (BP)	99.20
Metronidazole	99.0 – 101.0 (BP)	99.33
Ibuprofen	98.5 – 101.0 (BP)	100.05

Refer to Appendix A3 for sample calculations

3.3 HPLC METHOD DEVELOPMENT

3.3.1 Determination of Wavelength of Maximum Absorption

Table 3.4: Wavelengths of maximum absorption for the test compounds and the Surrogate reference standards in the solvent

Analyte / surrogate	Absorption maxima (λ max)/ nm
Amlodipine	230
Lisinopril	219

Diclofenac Na	230
Metronidazole	235
Ibuprofen	219

Refer to Appendix A1 for UV/VIS spectra of pure samples

3.3.2 Chromatographic Conditions used in The HPLC Method Developed

Amlodipine

1. Stationary phase: LUMX Iu C18 5µm, 3.9 x 150mm, Å~100
2. Mobile phase: 0.1M orthophosphoric acid 15%: methanol 85%
3. Detector: UV/Visible detector
4. Flow rate: 1.2m/min
5. Injection volume: 20µl
6. Detection wavelength: 230nm

Lisinopril

1. Stationary phase: Lumx Iu C18 5µm, 3.9 x 150mm, Å~100
2. Mobile phase: Mobile phase: 0.1M orthophosphoric acid 15%: methanol 85%
3. Detector: UV/Visible detector
4. Flow rate: 1.2m/min
5. Injection volume: 20µl
6. Detection wavelength: 219nm

Sample Chromatograms

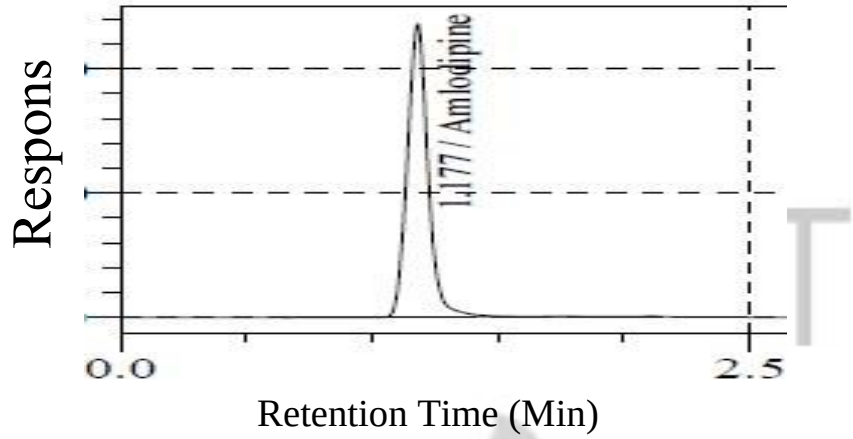

Figure 3- 1: Chromatogram of pure Amlodipine

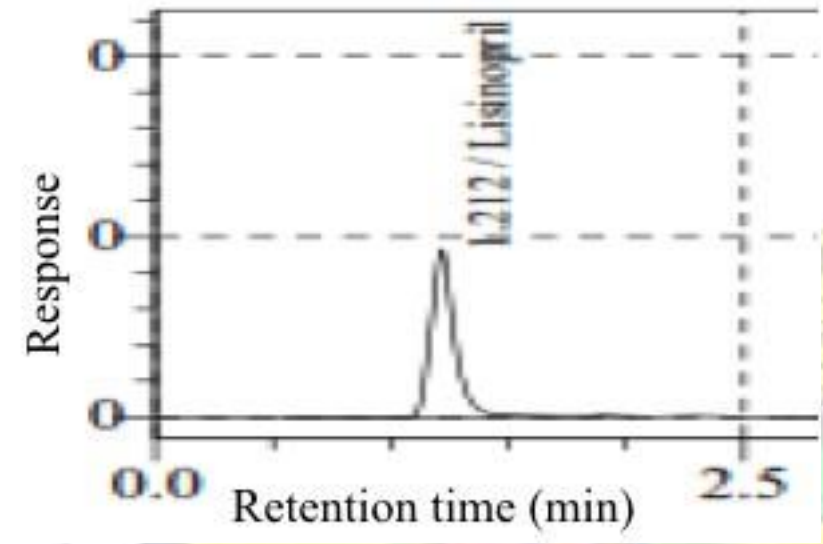

Figure 3- 2: Chromatogram of pure Lisinopril

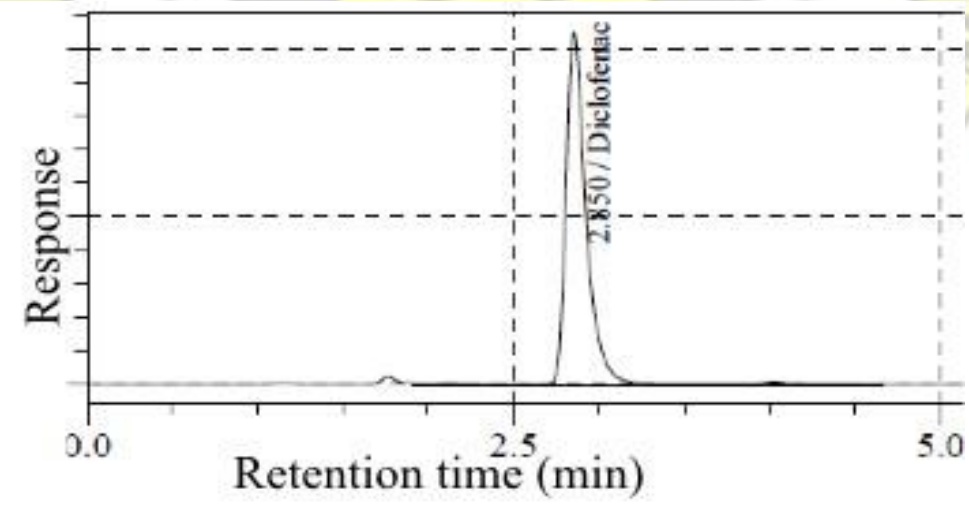

Figure 3- 3: Chromatogram of pure Diclofenac sodium

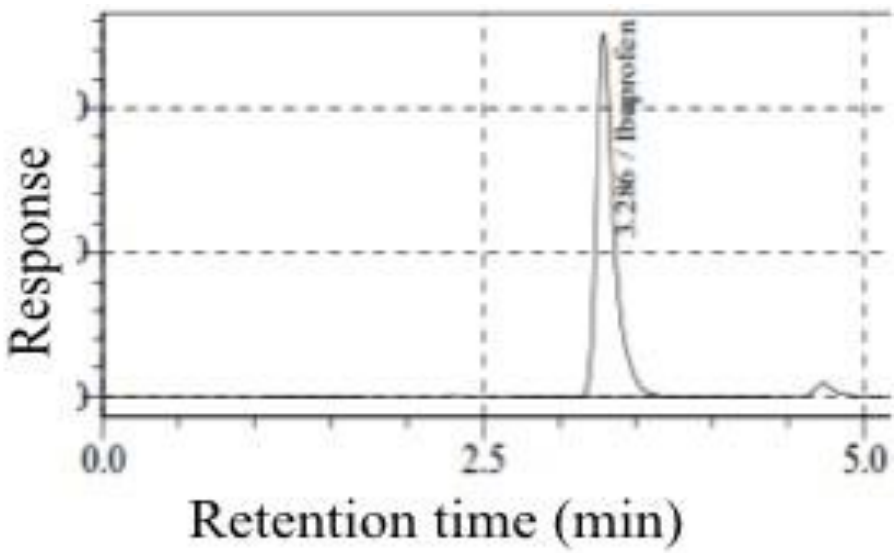

Figure 3- 4: Chromatogram of pure Ibuprofen

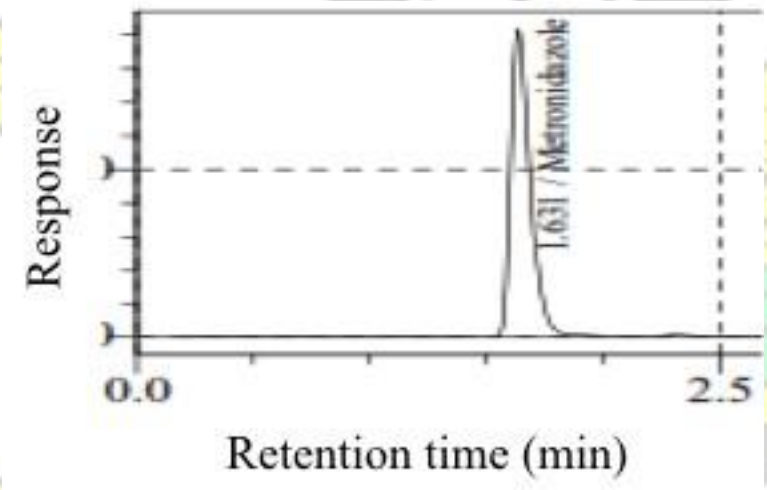

Figure 3- 5: Chromatogram of pure Metronidazole

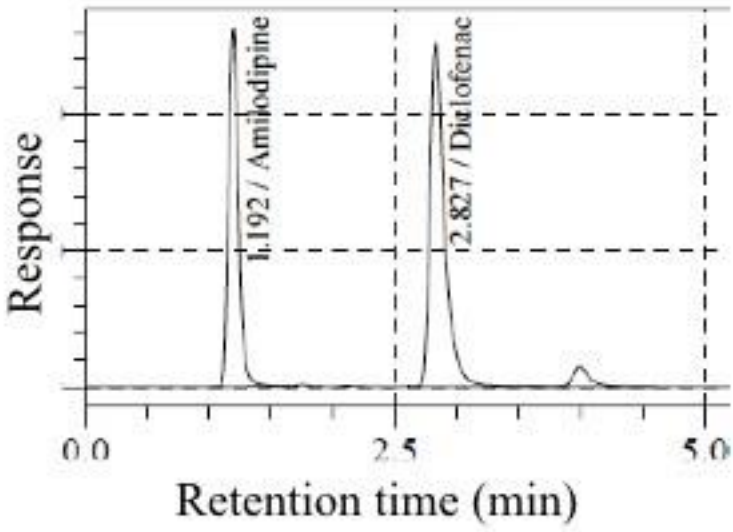

Figure 3- 6: Chromatogram of Amlodipine and Diclofenac

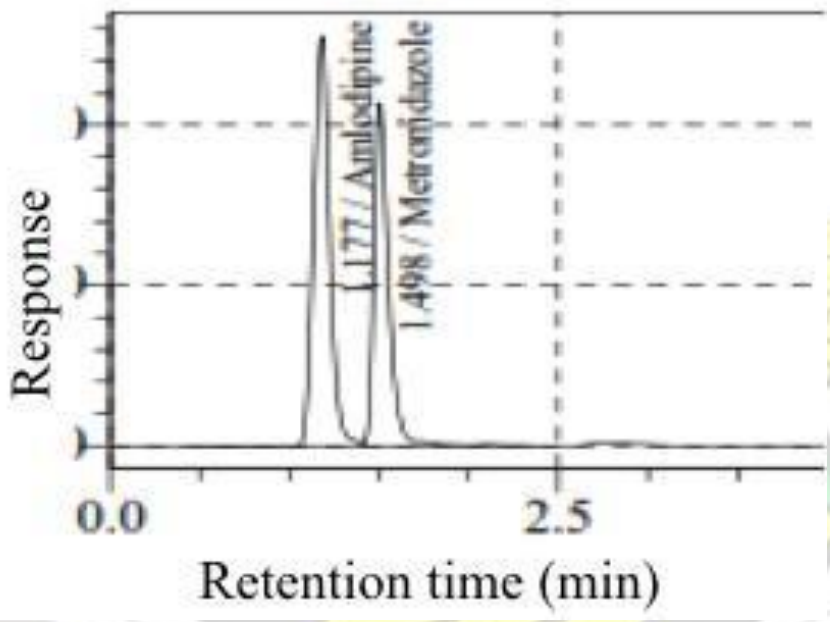

Figure 3- 7: Chromatogram of Amlodipine and Metronidazole

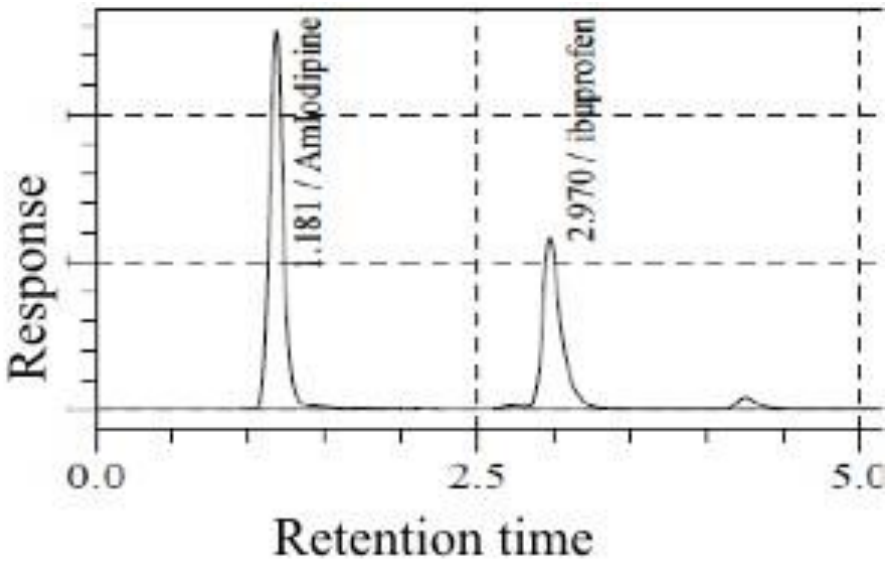

Figure 3- 8: Chromatogram of Amlodipine and Ibuprofen

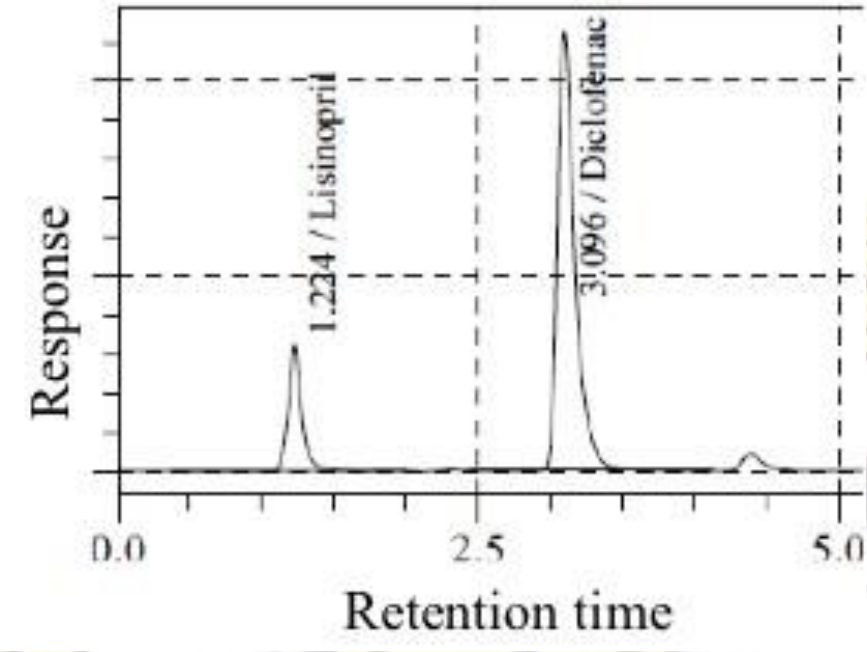

Figure 3- 9: Chromatogram of Lisinopril and Diclofenac

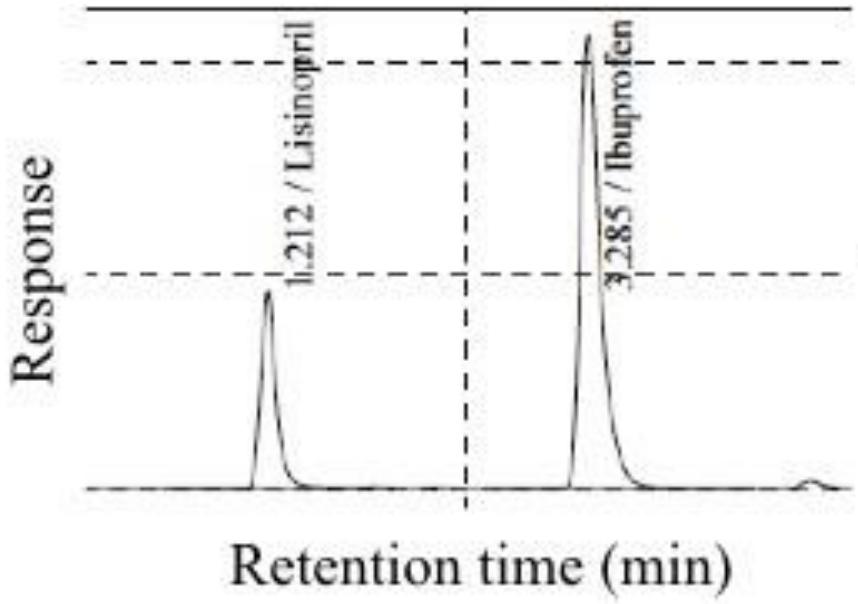

Figure 3- 10: Chromatogram of Lisinopril and Ibuprofen

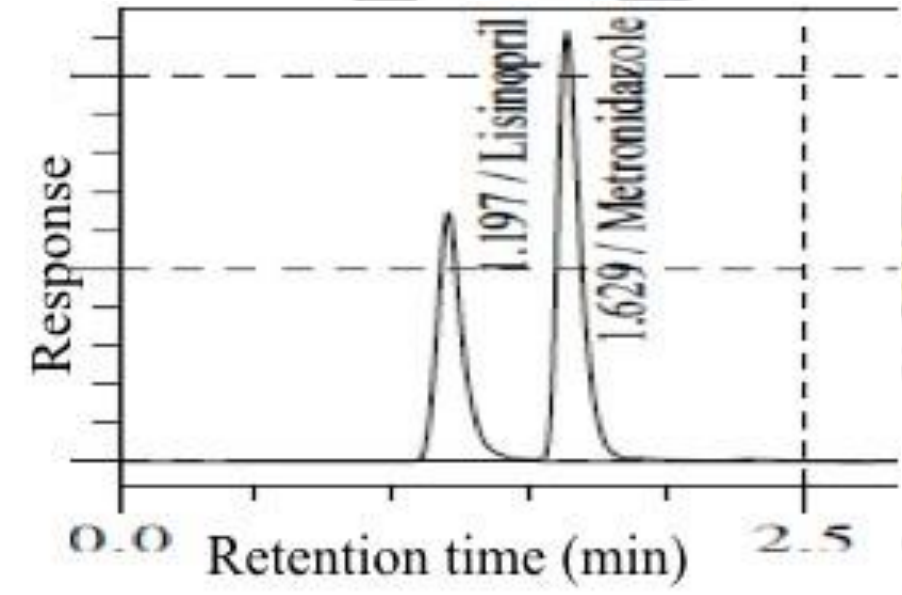

Figure 3- 11: Chromatogram of Lisinopril and Metronidazole

3.3.3 HPLC Retention Times

Table 3.5: Retention times obtained for Amlodipine and surrogates employed in the research (n = 7)

Analyte / surrogate	Average retention time (mean ± SD) / min

Amlodipine	1.1761 ± 0.0243
Diclofenac Na	2.8349 ± 0.0001
Metronidazole	1.4987 ± 0.0018
Ibuprofen	2.9699 ± 0.0051

Table 3.6: Retention times obtained for Lisinopril and surrogates employed in the research (n = 7)

Analyte / surrogate	Average retention time (mean ± SD) / mins
Lisinopril	1.2298 ± 0.0395
Diclofenac Na	3.0987 ± 0.0031
Metronidazole	1.6301 ± 0.0026
Ibuprofen	3.2828 ± 0.0029

3.3.4 Analytical Performance Parameters

Stability Studies on Solutions of Compounds used for the Study

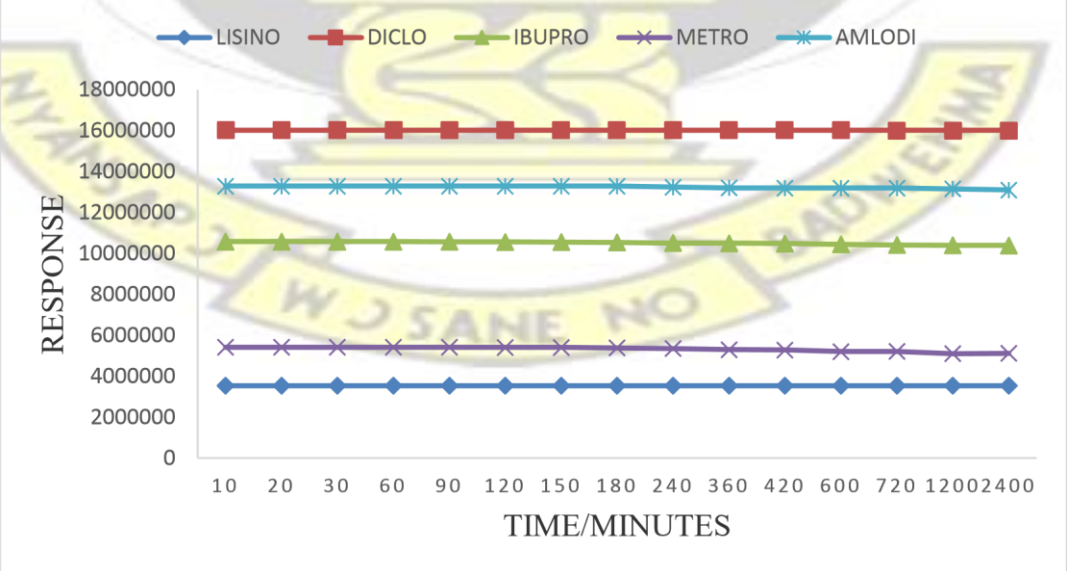

Figure 3- 12: Stability profile of analyte and surrogate reference solutions

Linearity

Table 3.7: Linear equations and coefficient of correlation for analytes and surrogates used in the study with the method developed:

Sample (analyte/surrogate)	Linear equation	R^2
Amlodipine with Ibuprofen as surrogate	Y = 2E+08x + 2E+06	0.9979
Amlodipine with Metronidazole as surrogate	Y= 2E+08x + 617139	0.9986
Amlodipine with Diclofenac as surrogate	Y=2E+08x + 928047	0.9988
Ibuprofen as surrogate for Amlodipine	Y = 1E+08x + 1E+06	0.9963
Metronidazole as surrogate for Amlodipine	Y = 2E+08x + 510406	0.9993
Diclofenac as surrogate for Amlodipine	Y = 3E+08x + 3E+06	0.9978
Lisinopril with Ibuprofen as surrogate	Y = 6E+07x + 294680	0.9987
Lisinopril with Metronidazole as surrogate	Y = 6E+07x + 473500	0.9984
Lisinopril with Diclofenac as surrogate	Y = 6E+07x + 226508	0.9990
Ibuprofen as surrogate for Lisinopril	Y = 2E+08x + 451768	0.9986
Metronidazole as surrogate for Lisinopril	Y = 1E+08x + 599695	0.9994
Diclofenac as surrogate for Lisinopril	Y = 3E+08x + 486132	0.9986

Refer to Appendix A6 for calibration curves

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

Detection Limit (LOD) and Quantitation Limit (LOQ) of samples employed in the study by method developed.

Table 3.8: LOD and LOQ Results

Sample	**LOD (%w/v)**	**LOQ (%w/v)**
Lisinopril using Diclofenac as surrogate	1.18×10^{-2}	3.57×10^{-2}
Lisinopril using Metronidazole as surrogate	2.77×10^{-2}	8.38×10^{-2}
Lisinopril using Ibuprofen as surrogate	1.53×10^{-2}	4.63×10^{-2}
Metronidazole, used as surrogate for Lisinopril	1.96×10^{-2}	5.94×10^{-2}
Diclofenac, used as surrogate for Lisinopril	5.3110^{-3}	1.61×10^{-2}
Ibuprofen used as surrogate for Lisinopril	7.3810^{-3}	2.24×10^{-2}
Amlodipine, using Diclofenac as surrogate	1.31×10^{-2}	3.98×10^{-2}
Amlodipine, using Metronidazole as surrogate	9.2110^{-3}	$2.79\times1\ 0^{-2}$
Amlodipine, using Ibuprofen as surrogate	3.26×10^{-2}	9.86×10^{-2}
Diclofenac, used as surrogate for Amlodipine	3.50×10^{-2}	1.06×10^{-1}
Metronidazole, used as surrogate for Amlodipine	9.1110^{-3}	2.76×10^{-2}
Ibuprofen used as surrogate for Amlodipine	3.50×10^{-2}	1.06×10^{-1}

Accuracy

Table 3.9: Assay of Lisinopril formulations using Metronidazole as surrogate

LEVEL	% RECOVERY	ACCEPTANCE CRITERIA (ICH)
80% (0.04%w/v)	98.40	98% - 102%
	98.50	
	99.40	
100% (0.05%w/v)	99.20	
	98.70	
	99.04	
120% (0.06%w/v)	101.20	
	100.64	
	101.00	

Table 3.10: Assay of Lisinopril formulations using Diclofenac sodium as surrogate

LEVEL	% RECOVERY	ACCEPTANCE CRITERIA (ICH)
80% (0.04%w/v)	98.03	98% - 102%
	98.50	
	99.01	
100% (0.05%w/v)	98.40	
	100.40	
	100.40	
120% (0.06%w/v)	98.80	
	98.80	
	100.10	

Table 3.11: Assay of Amlodipine formulations using Diclofenac as surrogate

LEVEL	% RECOVERY	ACCEPTANCE CRITERIA (ICH)
	100.22	

80% (0.04%w/v)	99.67	98% - 102%
	102.00	
100% (0.05%w/v)	101.40	
	98.18	
	99.82	
120% (0.06%w/v)	99.44	
	100.34	
	101.60	

Table 3.12: Assay of Lisinopril formulations using Ibuprofen as surrogate

LEVEL	% RECOVERY	ACCEPTANCE CRITERIA (ICH)
80% (0.04%w/v)	100.22	98% - 102%
	100.22	
	100.21	
100% (0.05%w/v)	101.12	
	100.70	
	101.40	
120% (0.06%w/v)	99.92	
	100.02	
	99.94	

Table 3.13: Assay of amlodipine formulations using Ibuprofen as surrogate

LEVEL	% RECOVERY	ACCEPTANCE CRITERIA (ICH)
80% (0.04%w/v)	98.72	
	98.74	
	99.07	
	101.70	

100% (0.05%w/v)	100.45	98% - 102%
	98.94	
120% (0.06%w/v)	102.00	
	101.74	
	101.70	

Table 3.14: Assay of Amlodipine formulations using Metronidazole as surrogate

LEVEL	% RECOVERY	ACCEPTANCE CRITERIA (ICH)
80% (0.04%w/v)	100.36	98% - 102%
	100.08	
	99.71	
100% (0.05%w/v)	99.2.0	
	98.92	
	99.01	
120% (0.04%w/v)	100.40	
	99.43	
	100.00	

Precision

Intra-day precision (Repeatability)

Table 3.15: Assay of Lisinopril with Diclofenac sodium as surrogate

INTRADAY PRECISION	
RUN #	MEAN ASSAY
1	99.50
2	99.41

3	99.43
4	99.50
5	99.53
MEAN	99.474
SD	0.04586938
RSD	0.046111929

Table 3.16: Assay of Lisinopril with Ibuprofen as surrogate

INTRADAY PRECISION	
RUN #	MEAN ASSAY
1	100.50
2	100.30
3	100.44
4	100.13
5	100.00
MEAN	100.274
SD	0.187147001
RSD	0.186635619

Table 3.17: Assay of Lisinopril with Metronidazole as surrogate

INTRADAY PRECISION	
RUN #	MEAN ASSAY
1	99.60
2	99.42
3	99.54
4	99.61

5	99.60
MEAN	99.554
SD	0.071442284
RSD	0.071762344

Table 3.18: Assay of amlodipine with Metronidazole as surrogate

INTRADAY PRECISION	
RUN #	MEAN ASSAY
1	99.02
2	100.03
3	99.00
4	99.32
5	99.01
MEAN	99.276
SD	0.39570696
RSD	0.39859277

Table 3.19: Assay of amlodipine with ibuprofen as surrogate

INTRADAY PRECISION	
RUN #	MEAN ASSAY
1	99.40
2	99.80
3	99.05
4	100.40
5	100.25
MEAN	99.78

SD	0.51
RSD	0.51

Table 3.20: Assay of Amlodipine with Diclofenac sodium as surrogate

INTRADAY PRECISION	
RUN #	MEAN ASSAY
1	99.20
2	100.4
3	99.50
4	100.12
5	99.65
MEAN	99.77
SD	0.43
RSD	0.43

Inter-day Precision (Reproducibility)

Table 3.21: Assay of Amlodipine with metronidazole as surrogate

	INTER-DAY PRECISION	
RUN #	DAY 1	DAY 2
1	99.02	99.04
2	100.03	99.10
3	99.00	99.14
4	99.32	99.21
5	99.01	99.33
MEAN	99.276	99.16
SD	0.39570696	0.10

RSD	0.39859277	0.10

P- Value (F-test) = 0.3359

P-Value (Unpaired T-test) = 0.6320

Table 3.22: Assay of amlodipine with Ibuprofen as surrogate

	INTERDAY PRECISION	
RUN #	DAY 1	DAY 2
1	99.40	98.90
2	99.80	99.04
3	99.05	99.30
4	100.40	98.90
5	100.25	99.40
MEAN	99.78	99.11
SD	0.506557	0.21
RSD	0.507674	0.21

P- Value (F-test) = 0.1533

P-Value (Unpaired T-test) = 0.0827

Table 3.23: Assay of amlodipine with diclofenac sodium as the surrogate

	INTERDAY PRECISION	
RUN #	DAY 1	DAY 2
1	99.20	99.04
2	100.4	99.10
3	99.50	99.80
4	100.12	99.40
5	99.65	99.37
MEAN	99.774	99.34
SD	0.43162947	0.27
RSD	0.43260716	0.27

P- Value (F-test) = 0.3845

P-Value (Unpaired T-test) = 0.1280

Table 3.24: Assay of Lisinopril with diclofenac sodium as the surrogate

	INTER-DAY PRECISION	
RUN #	DAY 1	DAY 2
1	99.50	99.31
2	99.41	99.10
3	99.43	99.50
4	99.50	99.12
5	99.53	99.04
MEAN	99.474	99.21
SD	0.04586938	0.17
RSD	0.04611193	0.17

P- Value (F-test) = 0.1931

P-Value (Unpaired T-test) = 0.9066

Table 3.25: Assay of Lisinopril with Ibuprofen as the surrogate

	INTER-DAY PRECISION	
RUN #	DAY 1	DAY 2
1	100.50	99.94
2	100.30	100.04
3	100.44	100.13
4	100.13	99.84
5	100.00	100.30
MEAN	100.274	100.05
SD	0.187147001	0.16
RSD	0.186635619	0.158161243

P-Value (F-test) = 0.7530

P-Value (Unpaired T-test) = 0.1050

Table 3.26: Assay of Lisinopril with Metronidazole as the surrogate

	INTERDAY PRECISION	
RUN #	DAY 1	DAY 2
1	99.60	99.71
2	99.42	99.60
3	99.54	99.74
4	99.61	99.55
5	99.60	99.62
MEAN	99.554	99.644
SD	0.07144228	0.07059745
RSD	0.07176234	0.070849675

P- Value (F-test) = 0.9821

P-Value (Unpaired T-test) = 0.1109

Ruggedness

Table 3.27: Assay of Lisinopril with Diclofenac sodium as the surrogate

	Assay	
Run number	250mm column length	150mm column length
1	99.40	98.90
2	99.80	99.04
3	99.05	99.30
Mean	99.42 ± 0.36	99.08 ± 0.20
%RSD	0.3593	0.20488
P-Value (Unpaired)	0.2435	
P- Value (F-test)	0.4527	

Table 3.28: Assay of Amlodipine with Diclofenac sodium as the surrogate at different laboratories.

	Assay	
Run number	Lab 1	Lab 2
1	99.64	99.65
2	99.54	99.56
3	99.61	99.67
Mean	99.60 ± 0.05	99.63 ± 0.06
%RSD	0.0515	0.0588
P-Value (Unpaired)	0.5412	
P- Value (F-test)	0.8681	

3.3.5 Determination of Surrogate Constant (K), For Each Analyte Using the Surrogate Reference

Table 3.29: surrogate constant for Amlodipine using Metronidazole.

A(analyte)	C(standard)	C(analyte)	A(standard)	K
2779792.8	0.01	0.01	2273322.1	1.2227888
5047685.4	0.02	0.02	4109954.6	1.22816087
9782134.7	0.04	0.04	8055770.3	1.21430159
13723316.6	0.06	0.06	11298760	1.21458608
18976513.5	0.08	0.08	15310194.9	1.2394691
22722519	0.1	0.1	18680973.8	1.21634553
			MEAN ± SD	1.2226 ± 0.74

Table 3.30: Surrogate constant for Amlodipine using Diclofenac

A(analyte)	C(standard)	C(analyte)	A(standard)	K

2996532.13	0.01	0.01	4963087.23	0.60376374
5937990.46	0.02	0.02	8234680.74	0.72109541
10370717	0.04	0.04	13798173.2	0.75160073
14525277.1	0.06	0.06	19272662.2	0.75367258
19748145.7	0.08	0.08	24395032.6	0.80951504
24223471	0.1	0.1	28933367.1	0.83721576
			MEAN ± SD	0.7461 ± 0.07

Table 3.31: Surrogate constant for Amlodipine using Ibuprofen

A(analyte)	C(standard)	C(analyte)	A(standard)	K
3843807.1	0.01	0.01	2552222.4	1.506062755
6096825.8	0.02	0.02	3980567.7	1.531647307
10503600.3	0.04	0.04	7000329.6	1.500443679
15032973	0.06	0.06	9901935.2	1.518185354
18653619.9	0.08	0.08	12262756.4	1.521160438
22413393.5	0.1	0.1	14513793.4	1.544282248
			MEAN ± SD	1.5203 ± 0.97

Table 3.32: Surrogate constant for Lisinopril using Metronidazole

A(analyte)	C(standard)	C(analyte)	A(standard)	K
967897	0.01	0.01	1599437	0.60514856
1577021.7	0.02	0.02	2639294	0.59751649

2793317.4	0.04	0.04	4571162	0.61107381
3976564.3	0.06	0.06	6649366	0.59803661
5018199.6	0.08	0.08	8822987	0.56876425
6027637	0.1	0.1	10590582	0.56915069
			MEAN ± SD	0.5916 ± 2.82

Table 3.33: Surrogate constant for Lisinopril using Diclofenac

	A(analyte)	C(standard)	C(analyte)	A(standard)	K
0.01	795903.8	0.01	0.01	3384630.4	0.23515235
0.02	1484945.1	0.02	0.02	6430193.6	0.23093319
0.04	2801240.2	0.04	0.04	12782824	0.21914095
0.06	4145383.1	0.06	0.06	19129888.5	0.21669667
0.08	5296163	0.08	0.08	24029540.1	0.22040218
0.1	6499604.9	0.1	0.1	30884547.4	0.21044844
				MEAN ± SD	0.2221 ± 0.01

Table 3.34: Surrogate constant for Lisinopril using Ibuprofen

A(analyte)	C(standard)	C(analyte)	A(standard)	K
833763.9	0.01	0.01	2280320.8	0.365634476
1567507.4	0.02	0.02	4341433.0	0.361057605
2931386.6	0.04	0.04	8754680.8	0.334836491
4209987.0	0.06	0.06	12913028.8	0.326026300

5383125.0	0.08	0.08	16694974.2	0.322439851
6589218.5	0.10	0.10	20326396.9	0.324170512
			MEAN ± SD	0.3390 ± 0.01

3.4 ASSAY OF COMMERCIAL SAMPLE TABLETS USING METHOD DEVELOPED

3.4.1 Tablets Weight Uniformity Tests

Refer to Appendix A4 for results on weight uniformity tests on the sample tablets employed in the research.

3.4.2 Assay of Commercial Tablets of Amlodipine and Lisinopril with Surrogate Constants

Refer to appendix A10 for sample calculation

Table 3.35: Assay of Lisinopril with Diclofenac Sodium as the Surrogate

SAMPLE	L-AA	L-BB	L-CC
1	96.60	99.60	102.00
2	98.60	97.30	102.20
3	97.30	98.40	100.40
MEAN ± SD	97.50 ± 0.850	98.43 ± 0.954	101.53 ± 0.793

Table 3.36: Assay of Lisinopril with Ibuprofen as the Surrogate

	L-AA	L-BB	L-CC
1	97.820	98.812	101.200

2	97.810	98.814	100.900
3	97.830	98.816	101.400
MEAN ± SD	97.82 ± 0.008	98.81 ± 0.002	101.17 ± 0.203

Table 3.37: Assay of Lisinopril with Metronidazole as the Surrogate

SAMPLE	L-AA	L-BB	L-CC
1	97.106	98.291	102.020
2	97.400	98.350	102.200
3	97.550	98.241	101.110
MEAN ± SD	97.35 ± 0.189	98.29 ± 0.045	101.78 ± 0.469

Table 3.38: Assay of Amlodipine with diclofenac sodium as the surrogate

SAMPLE	A-AA	A-BB	A-CC
1	94.60	97.40	96.05
2	94.70	97.30	96.10
3	94.80	97.50	96.30
MEAN ± SD	94.7 ± 0.086	97.4 ± 0.084	96.15 ± 0.112

Table 3.39: Assay of Amlodipine with Ibuprofen as the Surrogate

SAMPLE	A-AA	A-BB	A-CC
1	94.66	96.99	96.40
2	95.82	98.54	97.02
3	94.93	98.34	96.99

MEAN ± SD	95.14 ± 0.521	97.96 ± 0.703	96.80 ± 0.295

Table 3.40: Assay of Amlodipine with Metronidazole as the Surrogate

	A-AA	A-BB	A-CC
1	95.6	98.4	95.9
2	95.8	98.06	96.8
3	96.1	98.34	96.8
MEAN ± SD	95.83 ± 0.214	98.27 ± 0.151	96.5 ± 0.440

3.4.3 Assay of Commercial Sample Tablets Using the Standard Methods

Amlodipine Tablets

Table 3.41: Official Assay of Amlodipine Samples

SAMPLE	1	3	3	MEAN ± SD
A-AA	96.21	95.90	95.40	95.83 ± 0.348
A-BB	98.40	98.88	97.70	98.33 ± 0.493
A-CC	96.90	96.23	96.70	96.61 ± 0.291

Lisinopril Tablets

Table 3.42: Official Assay of Lisinopril Tablets

SAMPLE	1	2	3	MEAN ± SD
L-AA	97.80	97.60	97.40	97.6 ± 0.167
L-BB	98.90	98.12	98.50	98.51 ± 0.323
L-CC	101.20	101.24	101.20	101.21 ± 0.019

3.5 STATISTICAL COMPARISON OF THE DEVELOPED METHOD TO THE STANDARD METHODS

Table 3.43: P-Values (Unpaired T-test): Assay of formulations of Lisinopril with Ibuprofen, metronidazole and diclofenac sodium as the surrogate.

Surrogate	L-AA	L-BB	L-CC
Ibuprofen	0.0849	0.1836	0.8155
Metronidazole	0.1611	0.3075	0.1228
Diclofenac sodium	0.8513	0.9009	0.5586

Table 3.44: P-Values (Unpaired T-test): Assay of formulations of Amlodipine with Ibuprofen, Metronidazole and Diclofenac sodium as the surrogate.

Surrogate	A-AA	A-BB	A-CC
Ibuprofen	0.1291	0.4969	0.4715
Metronidazole	> 0.9999	0.8501	0.7362
Diclofenac sodium	0.0991	0.1735	0.0630

Table 3.45: P-Values (F-test): Assay of formulations of Lisinopril with Ibuprofen, Metronidazole and Diclofenac sodium as the surrogate.

Surrogate	L-AA	L-BB	L-CC
Ibuprofen	0.3733	0.5543	0.9339
Metronidazole	0.8769	0.6800	0.2820
Diclofenac sodium	0.0743	0.1910	0.1086

Table 3.46: P-Values (F-test): Assay of formulations of Amlodipine with Ibuprofen, Metronidazole and Diclofenac sodium as the surrogate.

Surrogate	A-AA	A-BB	A-CC
Ibuprofen	0.6170	0.6593	0.9863
Metronidazole	0.5488	0.1715	0.6086
Diclofenac sodium	0.2894	0.5124	0.2580

CHAPTER FOUR

DISCUSSION, CONCLUSION AND RECOMMENDATION

4.1 DISCUSSION

4.1.1 Identification tests and assay of samples

Amlodipine

Amlodipine was identified by IR spectroscopy. Amlodipine was freely soluble in methanol, sparingly soluble in absolute alcohol and slightly soluble in water. This provides some evidence that the sample tested may be amlodipine. The melting point of the sample was 177-178^{o}C, which fall within the official meting point range of 178-179^{o}C confirming the identity of the sample as Amlodipine. An IR spectrum of the sample produced principal peaks at wavenumbers 1262.15, 1192.11, 1089.58cm $^{-1}$ further confirming the sample to be amlodipine. A percentage purity of 99.5% was obtained for the sample, which falls within the British Pharmacopoeia (2013) stipulated range of 97% – 102% for Amlodipine pure powder.

Lisinopril

Lisinopril was identified by TLC and IR spectroscopy. It was soluble in water and insoluble in ethanol. A thin layer chromatographic analysis carried out on the sample and the principal spot of the test sample observed in the chromatogram corresponded in position and colour to that of the standard which produced a retention factor of 0.717, which was similar to the standard R_f of 0.72. This provides some evidence that the sample tested may be lisinopril. The melting point of the sample was 146-147^{o}C, which fall within the official meting point range of 146-148^{o}C confirming the identity of the sample as Lisinopril.

An IR spectrum of the sample produced principal peaks at wavenumbers 1609, 1570, 1389.84; 748,739 cm $^{-1}$ with a broad peak around 3000cm^{-1}. This further confirms the sample to be amlodipine. A percentage purity of 100.6% was obtained for the sample, which falls within the United States Pharmacopoeia (USP 30) stipulated range of 98% - 102% for Amlodipine pure powder.

Diclofenac sodium

A blue coloration with the formation of a precipitate was observed and produced the reactions of sodium as per the B.P (2013), indicating the sample may be diclofenac sodium. A melting point of 280^{o}C (with decomposition) is quoted by the B.P (2013) as the melting point of Diclofenac sodium. Hence the experimental melting point range of 280-281^{o}C obtained falls within the required range of the standard melting point range indicating that the sample may be indeed diclofenac sodium.

An IR spectrum of the sample produced principal peaks at wavenumbers 1573.44, 765.49, 1498.07, 1282.1, 1304.34 cm $^{-1}$. This further confirms the sample to be Diclofenac sodium.

Diclofenac sodium powder as stated by the British Pharmacopoeia (2013) should have a percentage content ranging from 99% - 101%. The content obtained for the sample upon assay was 99.2%, which is within the permissible range.

Metronidazole

The melting point of 160 - 162^{o}C obtained for the sample also falls within the British Pharmacopoeia (2013) stipulated metronidazole melting point range of

159 -163^{o}C. These signify that the sample labeled Metronidazole is indeed Metronidazole.

The pure Metronidazole, scanned over a spectral range of 230-350 nm produced maximum and minimum absorption at 277 nm and 240 nm respectively. An IR spectrum of the sample produced principal peaks at wavenumbers 1185.07, 1533.83, 1072.93, 1263.83, 743.02, 1157.62 cm $^{-1}$. These further confirm the sample to be Metronidazole.

Metronidazole powder as stated by the British Pharmacopoeia (2013) should have a percentage content ranging from 99% - 101%. The content obtained for the sample upon assay was 99.33%, which is within the permissible range.

Ibuprofen

The melting point of 77-78^{o}C obtained for the sample also falls within the British Pharmacopoeia (2013) stipulated Ibuprofen melting point range of 76 78^{o}C.

Scanning the sample over a spectral range of 230-350 nm produced absorption maxima at 272 nm and 264 nm with a shoulder at 258 nm. The ratios of absorbance at 258nm/264nm and at 258nm/272nm were 1.22 and 1.00 respectively. An IR spectrum of the sample produced principal peaks at wavenumbers 1707.05, 1229.02, 779, 1183.11, 1267.78, 865.66 cm^{-1}. All confirming the sample to be Ibuprofen.

Ibuprofen as stated by the British Pharmacopoeia (2013) should have a percentage content ranging from 98.5% - 101%. The content obtained for the sample upon assay was 100.05%, which is within the permissible range.

Identification of Amlodipine and Lisinopril tablets

All brands of Amlodipine and Lisinopril tablets used in the study were tested to ensure they contain Amlodipine and Lisinopril respectively before their subsequent use for quantitative analysis.

All the brands after thin TLC analysis gave single spots with retention factors similar to the pure samples as seen in appendix A2. A single spot gives an indication of a single compound in the tablet interacting with the mobile/stationary phase. Hence, the TLC chromatogram in appendix A2 indicates that all brands selected contain Lisinopril in its pure form with no brand showing any degree of adulteration or breakdown products. Furthermore, the results suggest that all assay results obtained from it is mainly due to the active component present in the selected tablet samples.

The tablets were further assayed in accordance with the British Pharmacopeia (2013) method for Amlodipine tablets. The British Pharmacopoeia (2013) stipulates that the content of Amlodipine tablets should be 97 -102% of the stated amount. The results of the assay show all Amlodipine brands used had percentage content value within the range and hence passed the assay. Also, the United States Pharmacopoeia (30) stipulates that, the content of Lisinopril tablets should be 90 – 110% of the stated amount. The results of the assay show all Lisinopril tablet brands used in the study had content within the acceptable limit, hence passed the content test.

4.2 HPLC METHOD DEVELOPMENT

The thesis aimed at developing a simple, rapid and reliable reverse-phase HPLC method for assaying Amlodipine and Lisinopril in tablet dosage forms. This was achieved by employing different chemical surrogates to indirectly estimate the

content of the active components using reverse-phase HPLC methods. The final separation conditions were achieved by varying the polarities and pH of the mobile phases.

In selecting the surrogate reference standards, compounds that have similar physicochemical properties as the analytes were chosen. The selected surrogate reference standards used for the analysis of Amlodipine and Lisinopril tablets were Metronidazole, Diclofenac and Ibuprofen. Additionally, they all have ionizable or carboxylic acid functional groups. However, they possess different side chains leading to a variation in polarities and hence, will elute at different times from the test drugs. This therefore makes them good candidates as surrogate reference standards.

Chemical properties of eluting compounds are one of the most important determinants of efficient separation in HPLC. In the analysis of amlodipine tablets, a C18 stable bonded phase (LUMX Iu C18 5µm, 3.9 x 150mm Å~100) was selected and the best results were obtained using mobile phase of 0.1M Orthophosphoric Acid: Methanol in a ratio 15:85. A wavelength of 230 nm for amlodipine analysis showed appreciable absorption and detection by both analyte and surrogate reference standards employed. Also, in the HPLC method development using surrogate reference standards for the analysis of Lisinopril tablets, the same chromatographic conditions were employed except for the detection wavelength, which was 219 nm.

The column selection was based on the fact that both amlodipine and Lisinopril have a bulky hydrophobic group in relation to the hydrophilic group and thus when a less polar stationary phase is used, retention time will be increased,

which subsequently affects time required for the assay. Moreover, the difference in retention times of the analyte and surrogate reference standard is due to the fact that they have different side chains.

4.2.1 Analytical Performance Parameters

Stability

Every drug is known to breakdown over time. However, the rate of breakdown occurs at different rates based on different environmental conditions. The breakdown process occurs relatively quicker when in solution; hence the time for sample analysis is important. Stability of the analytes in solution was studied to evaluate the duration of sample handling. This was done to ascertain the duration under which the sample is stable to produce the same results under the developed analytical method.

From the graph in figure 3.12, the peak area of the analyte and standards remained quite stable within the time of study. It can be inferred that the analyte and surrogate reference standards will produce reproducible results within the analytical run time (12h).

Linearity

Linearity of an analytical method is the capacity of the method to produce measured responses that is directly proportional to the concentration of analytes in the sample over a specified range of analyte concentration.

The linearity of the method for Amlodipine and its surrogate reference standards was assessed over a working concentration range of 0.01 to 0.1%w/v. From the corresponding plot, response against concentration, the coefficient of correlation for Amlodipine, Diclofenac sodium, Metronidazole and Ibuprofen

were 0.9983, 0.9978, 0.9993 and 0.9963 respectively suggestive of adequate linearity for the assay method.

The linearity of the method for Lisinopril was also evaluated over the working concentration range of 0.01 to 0.1%w/v for both analyte and surrogate reference standards. The coefficients of correlation were 0.9985, 0.9986, 0.9986 and 0.9951 for Lisinopril, Diclofenac sodium, Metronidazole and Ibuprofen respectively. The coefficients obtained suggest that the response is linear and hence the absorbances (peak areas) are directly proportional to concentration range studied.

Specificity

Specificity demonstrates the capability of the method to measure accurately the analyte in the presence of other components. Since the method developed is aimed at assaying the amount of a particular compound (Lisinopril or Amlodipine) in tablets, it stands to reason that, the method developed should be able to distinguish between the active principal ingredient and the excipients. The method developed in this project was specific for the analytes since comparable peaks with similar retention times were produced in concordance to the pure samples. Furthermore, there was no interference of spectra from excipients. In addition, the solvent system produced no peak, at the specified wavelength, when injected alone. This indicates that peaks or responses obtained from the formulations are precisely due to analytes, not the solvent system or excipients in the formulation and hence the method developed is thus specific for the analytes and can be used for the assay of the analyte in the presence of sample matrix, such as those found in the tablet formulation.

Precision

Intra-day precision (Repeatability)

Precision is an essential component of method development since it ensures that repetition of the test under similar conditions will produce results that are not significantly different. The precision of the method was assessed at different times during the day in an attempt to test its repeatability.

The degree of spread of the percentage contents obtained at each time of assay within the day was evaluated by calculating the relative standard deviation (RSD) of the data. The relative standard deviations obtained at these different times in tables 3.16 – 3.21 were all ≤ 2% (the accepted range). Hence the method developed can produce similar results irrespective of time of analysis.

Inter-day precision (Reproducibility)

Although precision assesses inter-day reliability, the ability of the method to produce repeatable quantitative assay values over different days is represented in reproducibility. This is achieved by performing assays of the analytes with the developed method at different times on separate days by two independent analysts. Comparing differences between the means of assayed contents using calculated RSD, F-test and T-test statistical methods, there was no significant difference between data. *P*-values obtained from the F-test and T-test in tables 3.22 – 3.27, for all comparisons were less than 0.05 indicating no significant differences between assay values on different test days. Also, the RSD values obtained were also below 2%, which is within the acceptance criteria of ≤ 2%. This demonstrates the reproducibility of the developed method.

Ruggedness

Analyzing samples under different laboratory and analytical conditions is a means of evaluating the robustness of developed methods. This determines the performance of the method in the presence of small changes. First, the assay was replicated in different laboratories. Secondly, the column length and flow rate were varied between 150-250 cm and 1.0-1.3ml/min respectively. The RSD obtained for the varied and original conditions was 0.3593 and 0.2488 for change in column and 0.0515 and 0.0588 for change in laboratory respectively, which complies with the acceptance criteria of ≤ 2%.

Analysis of variance also revealed no significant differences between results obtained under the varied conditions as well as the variation in results with the surrogate reference standard used. This provides conclusive evidence that the method developed when adapted will be to able produce reliable results with some degree of tolerance to changes or errors.

4.2.2 Determination of Surrogate Constant, K

The K value is dependent on the absorption of the surrogate reference standard in relation to the analyte at the wavelength of detection. This in turn is dependent on the chromophore and auxochromes present in the chemical structure of the surrogate reference standard which influence the absorption at the wavelength of study.

The concentration and peak area of analyte and surrogate reference standards injected were used in the calculation of the k value using the relation stated earlier.

The concentration was varied to determine its effect on the k value. Results obtained were analyzed by ANOVA test. The comparison revealed that there is no significant difference in the k value with change in concentration as the calculated F-values were lesser than the critical F-values.

4.3 ASSAY OF COMMERCIAL SAMPLE TABLETS USING METHOD DEVELOPED

4.3.1 Uniformity of Weight Test

The British pharmacopoeia (2013) stipulates that, for film coated or uncoated tablets weighing more than 80mg and less than 250mg; not more than two of the weights should deviate from the average weight of the twenty (20) tablets by more than ± 7.5% and none of the tablets should deviate by more than twice the permissible deviation.

Based on the above premise, it can be inferred that all batches of the three brands of Amlodipine and Lisinopril as seen in appendix A5 passed the uniformity of weight test except sample A- BB. This is because none of the brands deviated by more than ±7.5% and by more than twice the permissible deviation apart from the A-BB sample. The highest percentage deviation for

Amlodipine was 14.7135% and that of Lisinopril was 13.101426%.

4.3.2 Assay of Commercial Tablets of Amlodipine and Lisinopril with Surrogate Constants

Three brands each of Amlodipine and Lisinopril were analyzed with their respective developed method. Percentage contents obtained fell within 97 - 102 % (Amlodipine) and 90 - 110 % (Lisinopril) stipulated by the BP and USP respectively as seen from tables 3.36 – 3.41. This shows that the results from

the developed method are comparable to those obtained from the standard methods. The percentage content (mean ± SD) obtained for A-AA, A-BB and A-CC with Metronidazole as surrogate reference standard were 95.83 ± 0.214, 98.27 ± 0.151, and 96.5 ± 0.440.

The percentage content (mean ± SD) obtained for A-AA, A-BB and A-CC with Ibuprofen as surrogate were 95.14 ± 0.521, 97.96 ± 0.703, and 96.80 ± 0.295.

The percentage content (mean ± SD) obtained for A-AA, A-BB and A-CC with Diclofenac sodium as surrogate were 94.7 ± 0.086, 97.4 ± 0.084, 96.15 ± 0.112.

The percentage content (mean ± SD) for L-AA, L-BB and L-CC with Metronidazole as surrogate reference standard were 97.35 ± 0.189, 98.29 ± 0.045, and 101.78 ± 0.469.

The percentage content (mean ± SD) obtained for L-AA, L-BB and L-CC with Ibuprofen as surrogate reference standard were 97.82 ± 0.008, 98.81 ± 0.002, and 101.17 ± 0.203.

The percentage content (mean ± SD) obtained for L-AA, L-BB and L-CC with Diclofenac sodium were 97.5 ± 0.850, 98.43 ± 0.954, and 101.53 ± 0.793.

4.3.3 Assay of Commercial Sample Tablets using the Standard Methods

The three brands of Amlodipine were analyzed using the method specified by the BP (2013). The results obtained for A-AA, A-BB and A-CC were 95.83 ± 0.348, 98.33 ± 0.493 and 96.61 ± 0.291 respectively in table 3.42. The results were within the stipulated range 97 - 102 % of the BP (2013).

The brands of Lisinopril, L-AA, L-BB and L-CC were analyzed with the method specified in the USP. The percentage contents obtained were 97.6 ± 0.167, 98.51 ± 0.323 and 101.21 ± 0.019 as in table 3.43. They were within 90 to 110%. The

brands of both Amlodipine and Lisinopril passed by the standards of the USP and BP respectively.

4.4 COMPARISON OF THE STANDARD METHOD TO THE DEVELOPED METHOD

The developed methods and the standard methods were compared statistically to determine if there is a significant difference between the assay outcomes of both methods. The results were subjected to the paired t-test. There is no significant difference between the outcomes of the developed method and the standard BP and USP method at 95 % confidence level.

4.5 CONCLUSION

A reverse- phase HPLC method for the quantitative assessment of Amlodipine and Lisinopril tablets has been successfully developed using Diclofenac sodium, Metronidazole and Ibuprofen as surrogates reference standards. This method has an added advantage of being fast, efficient and reliable. Statistically, there was no significant difference between the accuracy of the standard method and the method developed using all the surrogate reference standards. Furthermore, there was no significant difference between the precisions of the two methods making it a suitable alternative for assay.

4.6 RECOMMENDATION

The surrogate method of drug analysis and assay could be adopted by the Ghanaian pharmaceutical and regulatory industries to assay most of their

products. This could expand the bracket of drugs to include frequently used drugs especially those listed in the Ghana essential medicines list.

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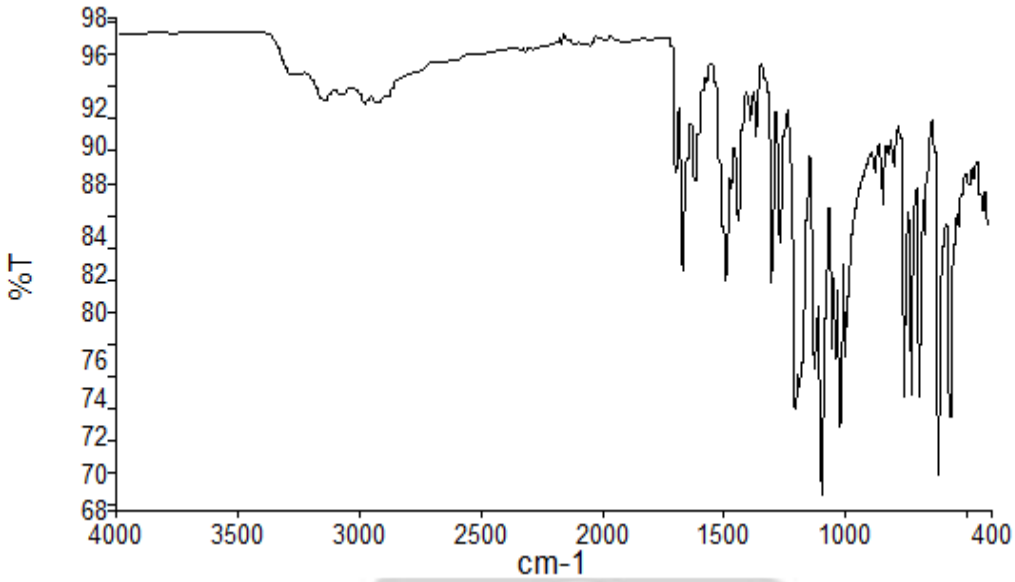
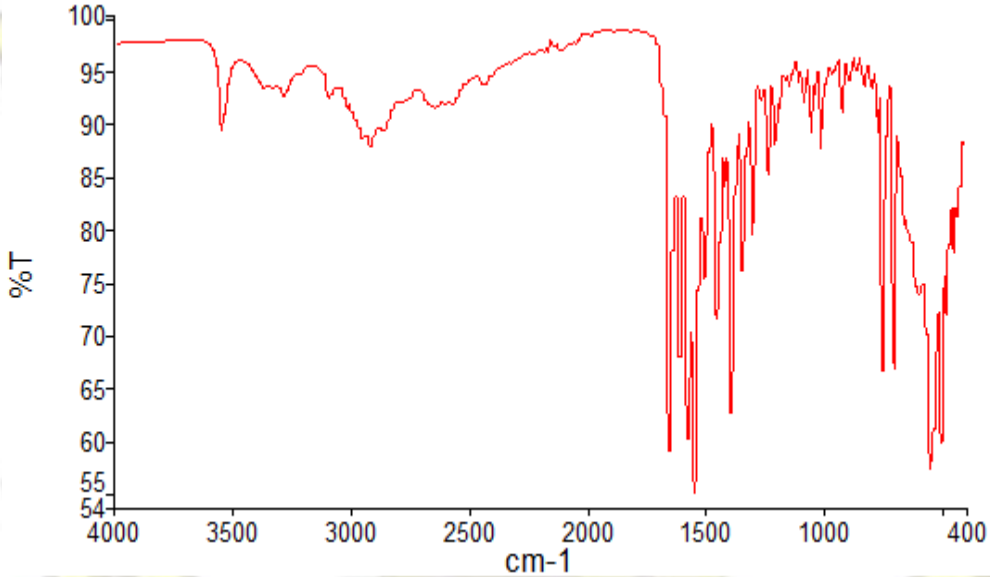
APPENDIX

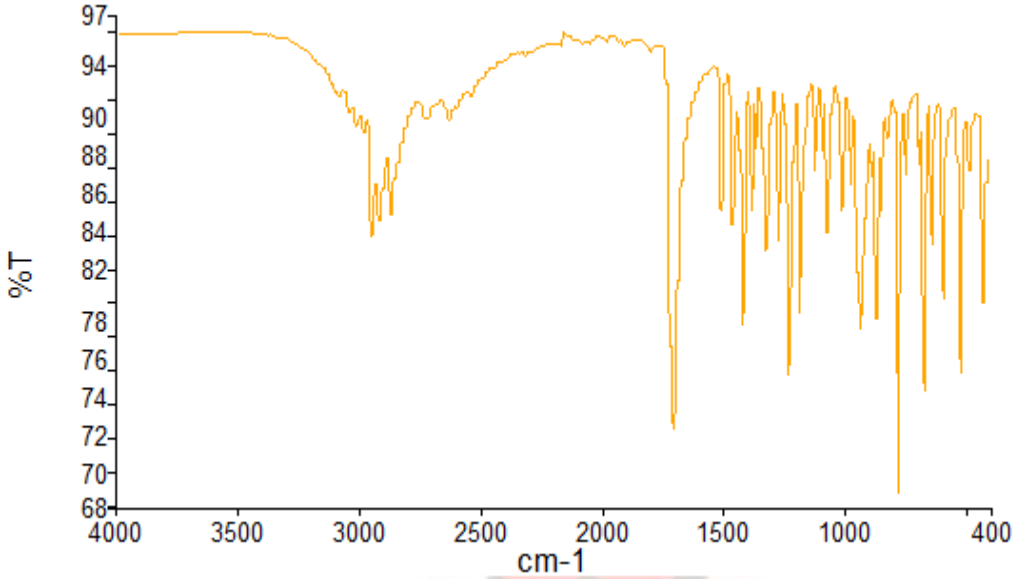
Appendix A1



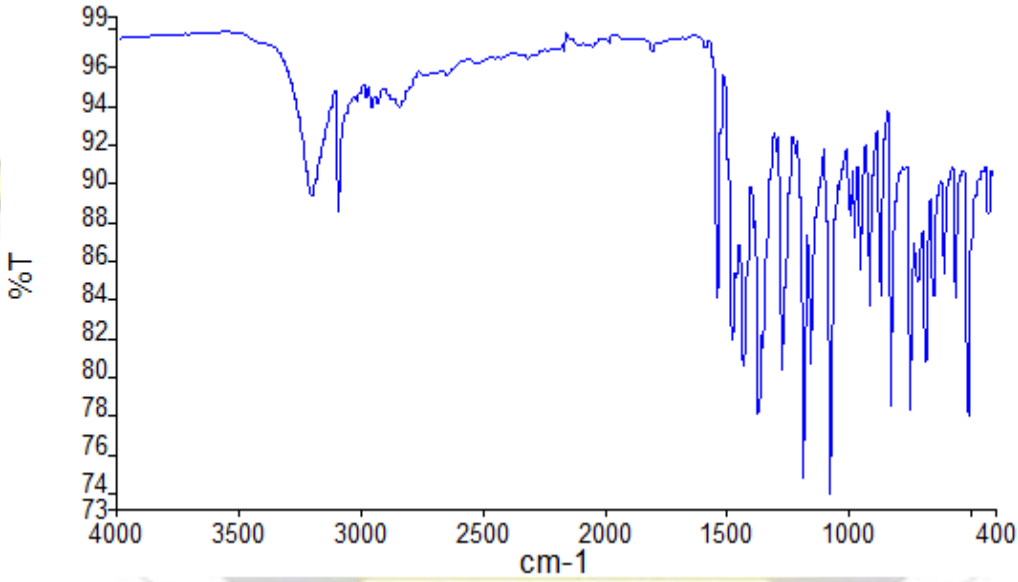
FTIR spectrum of Amlodipine



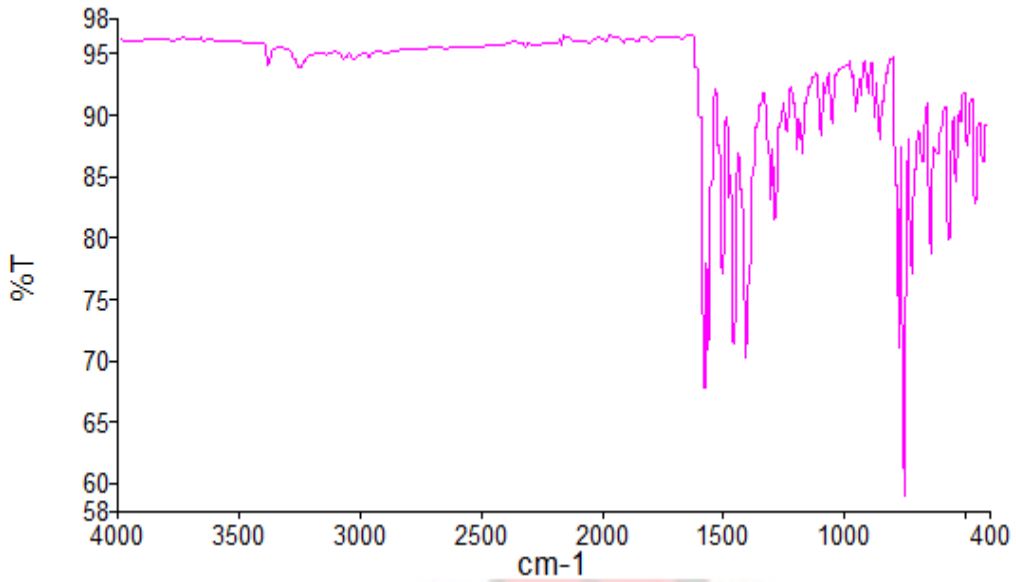
FTIR spectrum of Lisinopril



FTIR spectrum of Ibuprofen



FTIR spectrum of Metronidazole



FTIR spectrum of Diclofenac sodium

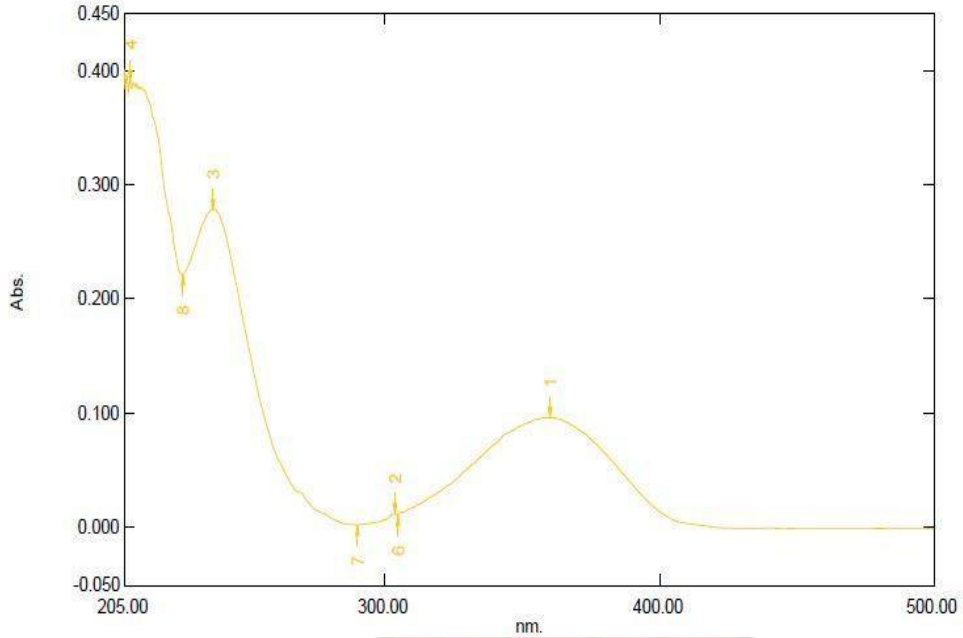
Appendix A2

Sample L- AA TLC Chromatogram

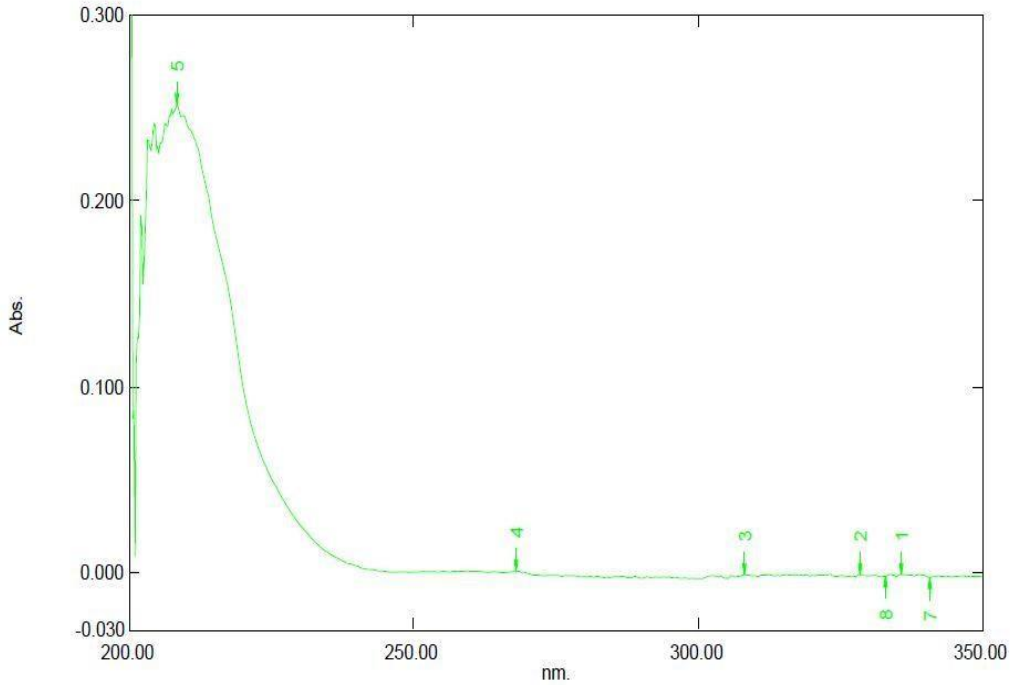
Sample L- BB TLC Chromatogram

Sample L- CC TLC Chromatogram

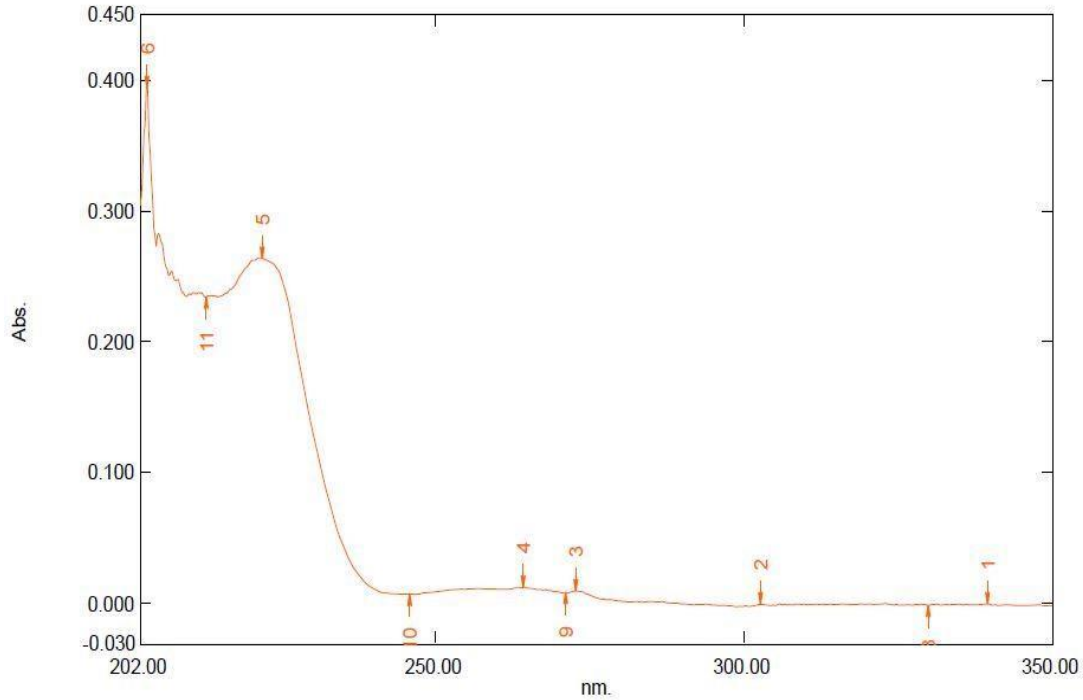
Appendix A3



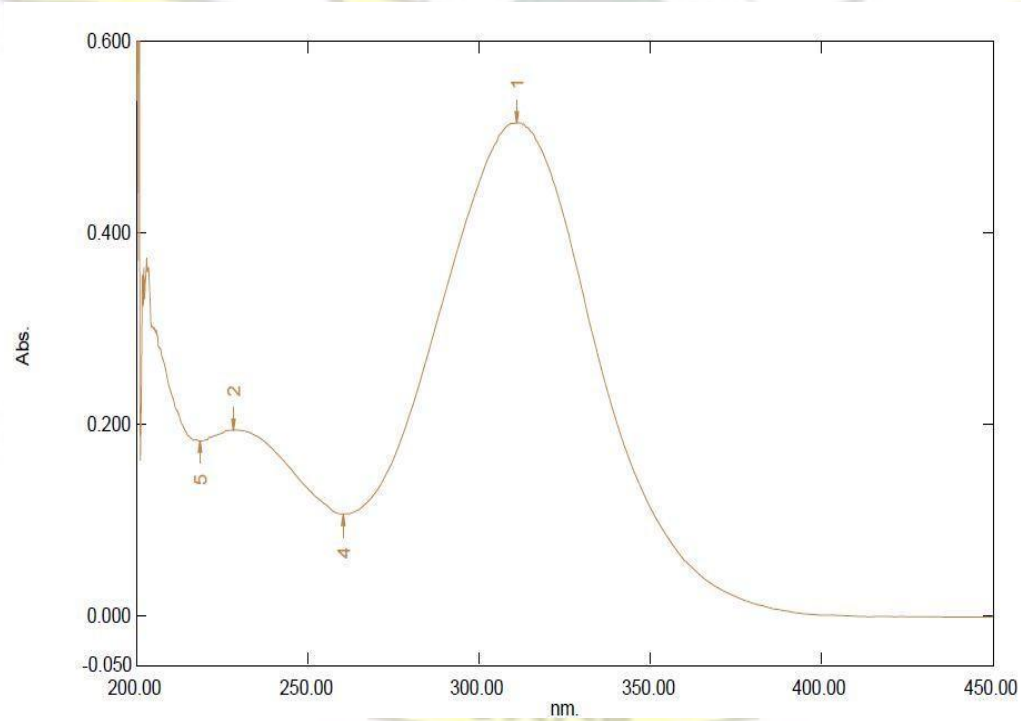
UV/Visible spectrum of Amlodipine



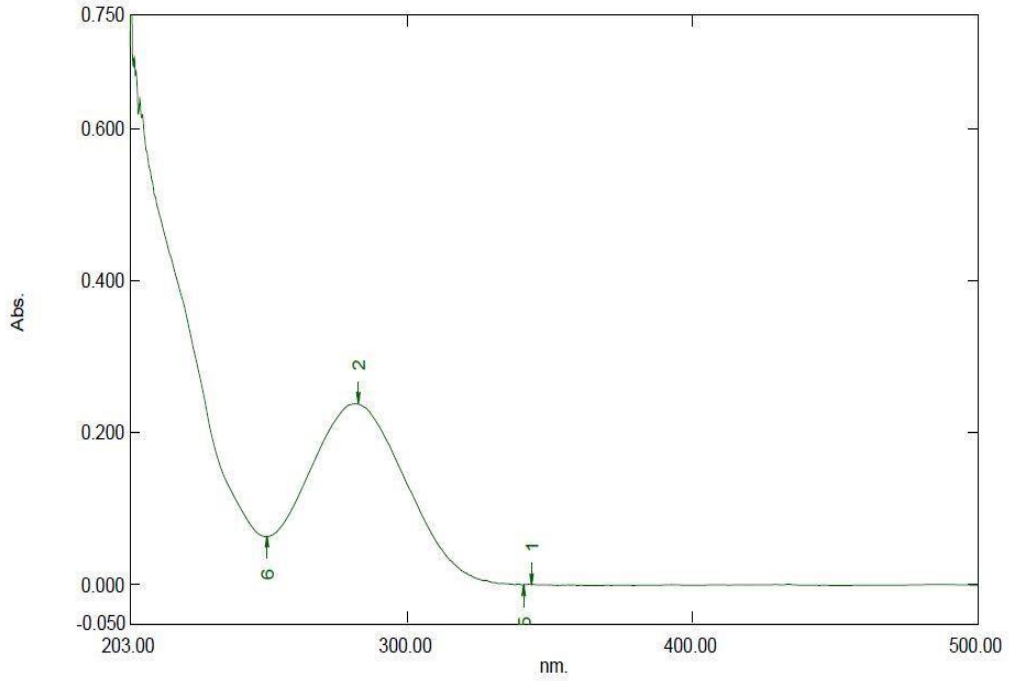
UV/Visible spectrum of Lisinopril



UV/Visible spectrum of Ibuprofen



UV/Visible spectrum of Metronidazole



UV/Visible spectrum of Diclofenac sodium **Appendix A4**

PREPARATION OF 1000ML OF 0.1M NaOH

40g NaOH ≡ 1000ml ≡ 1M NaOH

4.0g NaOH ≡ 1000ml ≡ 0.1M

4.0g NaOH ≡ 1000ml ≡ 0.1M NaOH

Assay = 99%

If 99% = 4.000

$$100\% = \frac{100\% \times 4.0000}{99} = 4.0404\text{g}$$

Table 1: Standardization of 0.1M NaOH results

Burette reading	I	II	III
Final reading	20.20	40.50	20.10
Initial reading	0.10	20.50	0.00
Titre	20.20	20.00	20.10

Nominal weight of H_2NSO_3H= 0.9$\frac{10g}{17g}$

Actual weight of H_2NSO_3H = 0.9

Factor (H_2NSO_3H) = $\frac{0.9717}{0.9710} = 1.0007$

F (NaOH) = $\frac{20 \times 1.0007}{20.10} = 0.9957$

Table 2: lisinopril assay results

Weight of Lisinopril	0.3500	0.3510	0.3500	0.3500	0.3510
Burette reading	I	II	III	IV	V
Final reading	9.20	18.10	27.50	38.90	8.90
Initial reading	0.00	9.10	18.50	300	0.00
Titre	9.200	8.90	8.70	8.90	8.70

For the first determination (I)

Weight of Lisinopril = 0.350g

Volume of 0.1M NaOH = 9.20ml

Actual volume of 0.1M NaOH used = 9.20 × F (NaOH)

Actual volume of 0.1M NaOH used = 9.20 × 0.9957

= 9.16ml

From the milliequivalent;

1 ml of 0.1 M sodium hydroxide is equivalent to 40.55 mg of $C_{21}H_{31}N_3O_5$.

Hence, 9.16ml of 0.1M NaOH $\equiv \frac{9.16 \times 0.04055}{1} = 0.3714g$

Actual weight of Lisinopril = 0.3714g

Percentage purity of Lisinopril sample = $\frac{\text{Actual weight}}{\text{Weight taken}} \times 100\%$

$= \frac{0.3714}{0.3500} \times 100\%$

% Purity = 100.6 %

The same calculation was done for the other determinations and the average calculated.

Average percentage purity = 100.6%

PREPARATION OF 1000ML OF 0.1M $HClO_4$

100.5g $HClO_4$ ≡ 1000ml ≡ 1M $HClO_4$

10.05g $HClO_4$ ≡ 1000ml ≡ 0.1M $HClO_4$

2.5125g $HClO_4$ ≡ 1000ml ≡ 0.1M $HClO_4$

Assay = 70%

If 70% = 2.5125

Then 100% = $\frac{100 \times 2.5125}{70}$

= 3.5892

Table 3: Standardization of Perchloric acid using analar potassium phthalate

Weight of $C_8H_5KO_4$	0.501	0.500	0.500	0.501	0.501
Burette reading	I	II	III	IV	V
Final reading	20.30	40.70	20.20	40.80	20.20
Initial reading	0.10	20.50	0.00	20.50	0.00
Titre	20.20	20.20	20.20	20.30	20.20

Calculation of factor for first determination (I),

Nominal weight of $C_8H_5KO_4$ = 0.5000g

Actual weight of $C_8H_5KO_4$ = 0.5010g

Factor ($C^{8}H_5KO_4$) = $\frac{0.501}{0.500} = 1.0020$

F ($HClO_4$) = $\frac{20 \times 1.0020}{20.20} = 0.9941$

The same calculation was repeated for the remaining determinations (i.e. II to V) to determine the factor of $HClO_4$, and the average calculated.

Average F ($HClO_4$) = 0.9941

Table 4: metronidazole assay results

Weight of Metronidazole	0.150	0.151	0.151	0.150	0.150
Burette reading	I	II	III	IV	V
Final reading	8.80	18.10	27.50	38.90	8.90
Initial reading	0.00	9.10	18.50	300	0.00
Titre	8.80	8.90	8.70	8.90	8.70

For the first determination (I)

Weight of Metronidazole = 0.150g

Volume of 0.1M $HClO_4$ = 8.80ml

Actual volume of 0.1M $HClO_4$ used = 8.80 × F ($HClO_4$)

Actual volume of 0.1M $HClO_4$ used = 8.80 × 0.9941

= 8.75ml

From the milliequivalent;

1ml of 0.1M Perchloric is equivalent to 17.12mg of $C_{17}H_{20}N_2S.HCl$

Hence, 8.75ml of 0.1M $HClO^4 \equiv \frac{8.75 \times 0.01712}{1} = 0.1498g$

Actual weight of Lisinopril = 0.1498g

Percentage purity of Lisinopril sample = $\frac{\text{Actual weight}}{\text{Weight taken}} \times 100\%$

$$= \frac{0.1498}{0.1500} \times 100\%$$

% Purity = 99.8 %

The same calculation was done for the other determinations and the average calculated.

Average percentage purity = 99.33%

Table 5: diclofenac sodium assay results

Weight of Diclofenac	0.2500	0.2504	0.2502	0.2502	0.2503
Burette reading	I	II	III	IV	V
Final reading	7.90	18.10	27.50	38.90	8.90
Initial reading	0.00	9.10	18.50	300	0.00
Titre	7.90	8.90	8.70	8.90	8.70

For the first determination (I)

Weight of Diclofenac = 0.2500g

Volume of 0.1M $HClO_4$ = 7.90ml

Actual volume of 0.1M $HClO_4$ used = 7.90 × F ($HClO_4$)

Actual volume of 0.1M $HClO_4$ used = 7.90 × 0.9941

= 7.85ml

From the milliequivalent;

1 mL of 0.1 M Perchloric acid is equivalent to 31.81 mg of $C_{14}H_{10}Cl_2NNaO_2$

Hence, 7.85ml of 0.1M $HClO^4 \equiv \frac{7.85 \times 0.03181}{1} = 0.2497$ g

Actual weight of Diclofenac sodium = 0.2497g

Percentage purity of Diclofenac sodium sample = $\frac{\text{Actual weight}}{\text{Weight taken}} \times 100\%$

$= \frac{0.2497}{0.2510} \times 100\%$

% Purity = 99.5 %

The same calculation was done for the other determinations and the average calculated.

Average percentage purity = 99.2%

Appendix A5

Uniformity of weight determination for selected brands of lisinopril and amlodipine

A5.1: Weight distribution of L-AA tablets

Tablet number	Weight/g	Deviation	% Deviation
1	0.2227	-0.00042	-0.1882395
2	0.2274	0.00428	1.91825027
3	0.216	-0.00712	-3.1911079
4	0.2246	0.00148	0.66332019
5	0.2213	-0.00182	-0.8157046
6	0.2244	0.00128	0.57368232
7	0.2235	0.00038	0.17031194
8	0.2207	-0.00242	-1.0846181
9	0.2189	-0.00422	-1.8913589
10	0.2275	0.00438	1.9630692
11	0.2284	0.00528	2.36643958
12	0.2262	0.00308	1.38042309
13	0.2242	0.00108	0.48404446
14	0.2254	0.00228	1.02187164
15	0.2222	-0.00092	-0.4123342

16	0.2218	-0.00132	-0.5916099
17	0.2227	-0.00042	-0.1882395
18	0.2183	-0.00482	-2.1602725
19	0.222	-0.00112	-0.501972
20	0.2242	0.00108	0.48404446
Mean tablet weight	**0.22312**		

A5.2: Weight distribution of L-BB tablets

Tablet number	Weight/g	Deviation	% Deviation
1	0.2229	-0.00022	-0.0986
2	0.2234	0.00028	0.125493
3	0.2216	-0.00152	-0.68125
4	0.223	-0.00012	-0.05378
5	0.2194	-0.00372	-1.66726
6	0.2227	-0.00042	-0.18824
7	0.2216	-0.00152	-0.68125
8	0.2248	0.00168	0.752958
9	0.2214	-0.00172	-0.77089
10	0.2225	-0.00062	-0.27788
11	0.2184	-0.00472	-2.11545
12	0.2195	-0.00362	-1.62245
13	0.2242	0.00108	0.484044
14	0.2207	-0.00242	-1.08462
15	0.2208	-0.00232	-1.0398
16	0.228	0.00488	2.187164
17	0.2217	-0.00142	-0.63643
18	0.2223	-0.00082	-0.36752
19	0.2233	0.00018	0.080674
20	0.2227	-0.00042	-0.18824
Mean tablet weight	**0.22312**		

-

A5.3: Weight distribution of L CC tablets

Tablet number	Weight/g	Deviation	% Deviation
1	0.2294	0.00431	1.91478964
2	0.2265	0.00141	0.6264161
3	0.2434	0.01831	7.13452397
4	0.2144	-0.01069	-4.7492114
5	0.2212	-0.00389	-1.7281976
6	0.2246	-0.00049	-0.2176907
7	0.2445	0.01941	6.62321738
8	0.1956	-0.02949	**-13.101426**
9	0.2138	-0.01129	-5.0157715
10	0.2116	-0.01349	-5.9931583
11	0.2127	-0.01239	-5.5044649
12	0.2253	0.00021	0.09329601
13	0.2087	-0.01639	-7.2815318
14	0.2269	0.00181	0.8041228
15	0.2285	0.00341	1.51494958
16	0.2355	0.01041	4.62481674
17	0.2543	0.02921	**12.9770314**
18	0.2213	-0.00379	-1.6837709
19	0.2319	0.00681	3.02545648
20	0.2316	0.00651	2.89217646
Mean tablet weight	**0.225085**		

-

A5.4: Weight distribution of A AA tablets

Tablet number	Weight/g	Deviation	% Deviation
1	0.1477	0.00049	0.332858
2	0.1439	-0.00331	-2.24849
3	0.1449	-0.00231	-1.56919
4	0.153	0.00579	3.933157
5	0.1486	0.00139	0.944229
6	0.1436	-0.00361	-2.45228
7	0.1446	-0.00261	-1.77298
8	0.1513	0.00409	2.778344
9	0.1491	0.00189	1.28388
10	0.1415	-0.00571	-3.87881
11	0.1421	-0.00511	-3.47123
12	0.144	-0.00321	-2.18056
13	0.1531	0.00589	4.001087
14	0.1508	0.00359	2.438693
15	0.148	0.00079	0.536648
16	0.1586	0.01139	**7.737246**
17	0.1405	-0.00671	-4.55811
18	0.1423	-0.00491	-3.33537
19	0.1445	-0.00271	-1.84091
20	0.152	0.00479	3.253855
Mean tablet weight	**0.147205**		

-

A5.5: Weight distribution of A BB tablets

Tablet number	Weight/g	Deviation	% Deviation
1	0.1993	0.00159	0.80420818
2	0.1782	-0.01951	**-9.8679885**
3	0.1753	-0.02241	**-11.334783**
4	0.1946	-0.00311	-1.573011
5	0.1884	-0.00931	-4.7089171
6	0.2268	0.02909	**14.7134692**
7	0.1819	-0.01581	-7.9965606
8	0.2174	0.01969	**9.9590309**
9	0.1902	-0.00751	-3.7984927
10	0.1944	-0.00331	-1.6741692
11	0.2011	0.00339	1.71463254
12	0.1883	-0.00941	-4.7594962
13	0.1773	-0.02041	**-10.323201**
14	0.2142	0.01649	**8.34049871**
15	0.2084	0.01069	5.40690911
16	0.2125	0.01479	7.48065348
17	0.2108	0.01309	6.62080825
18	0.2033	0.00559	2.82737343
19	0.1877	-0.01001	-5.062971
20	0.204	0.00629	3.18142734
Mean tablet weight	**0.197705**		

-

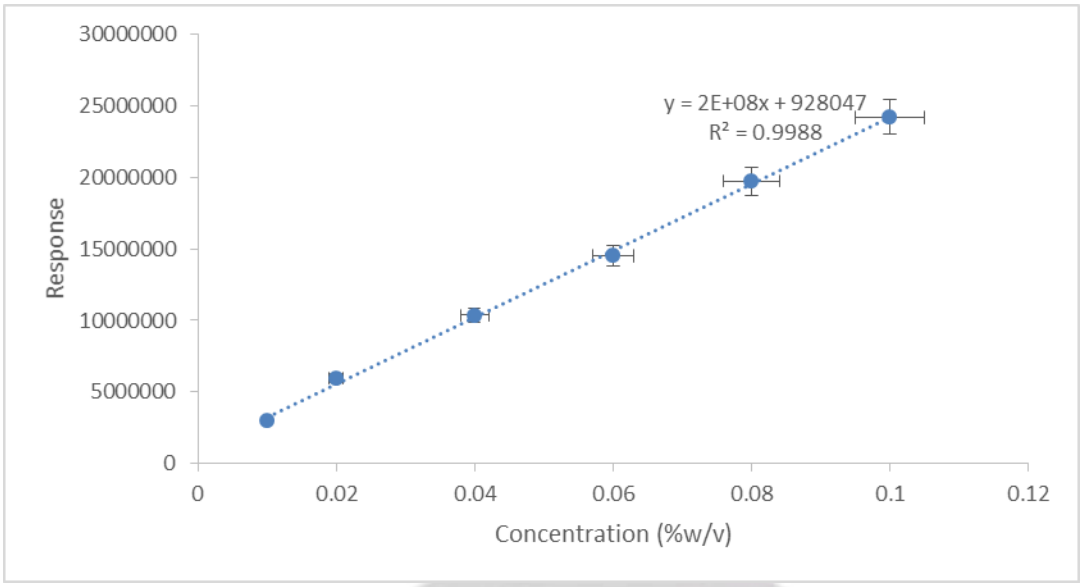
A5.6: Weight distribution of A CC tablets

Tablet number	Weight/g	Deviation	% Deviation
1	0.163	-0.00036	-0.22037
2	0.1615	-0.00186	-1.13859
3	0.1634	4E-05	0.024486
4	0.1647	0.00134	0.820274
5	0.1599	-0.00346	-2.11802
6	0.1644	0.00104	0.636631
7	0.1598	-0.00356	-2.17924
8	0.1629	-0.00046	-0.28159
9	0.1643	0.00094	0.575416
10	0.1708	0.00744	4.554358
11	0.1672	0.00384	2.350637
12	0.1593	-0.00406	-2.48531
13	0.164	0.00064	0.391773
14	0.161	-0.00236	-1.44466
15	0.1621	-0.00126	-0.7713
16	0.1633	-6E-05	-0.03673
17	0.1615	-0.00186	-1.13859
18	0.1646	0.00124	0.75906
19	0.167	0.00364	2.228208
20	0.1624	-0.00096	-0.58766
Mean tablet weight	**0.163355**		

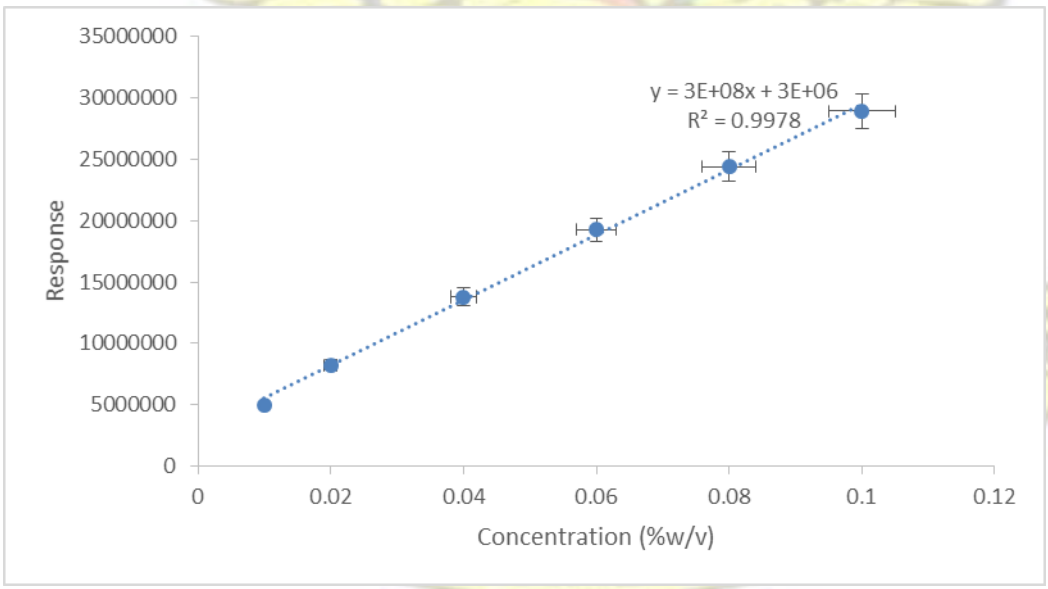
-

Appendix A7

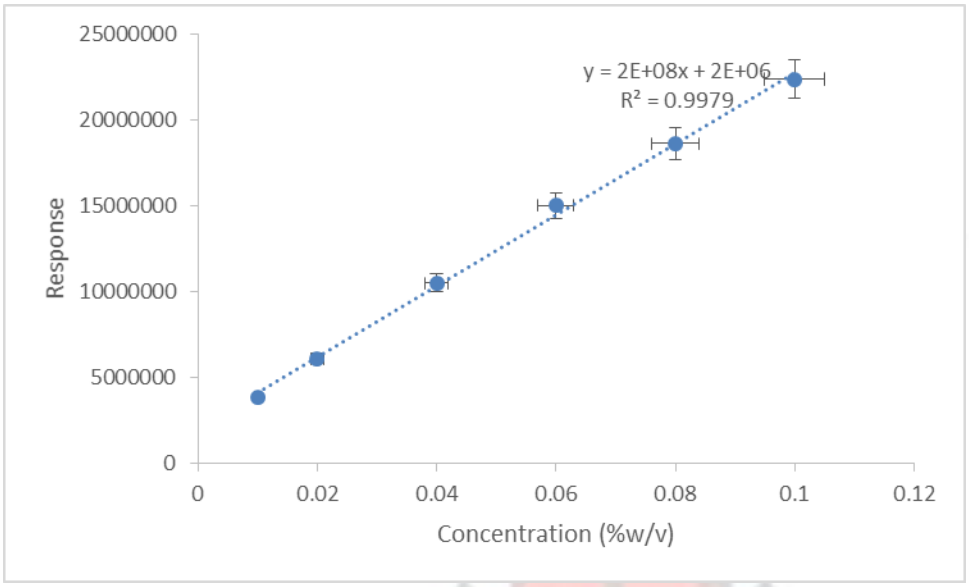
Calibration curves for pure samples employed in the thesis using the method developed



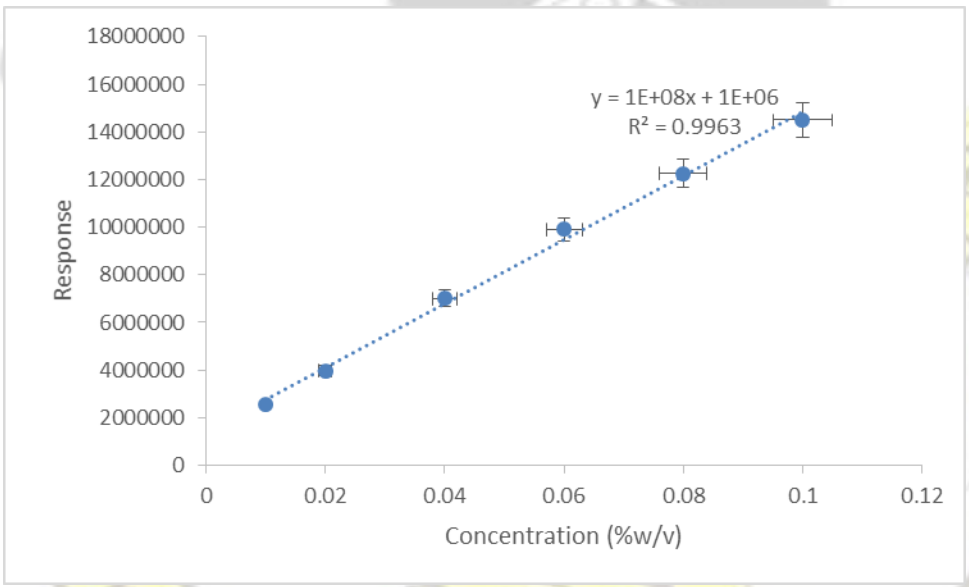
Calibration curve for Amlodipine (using Diclofenac as surrogate)



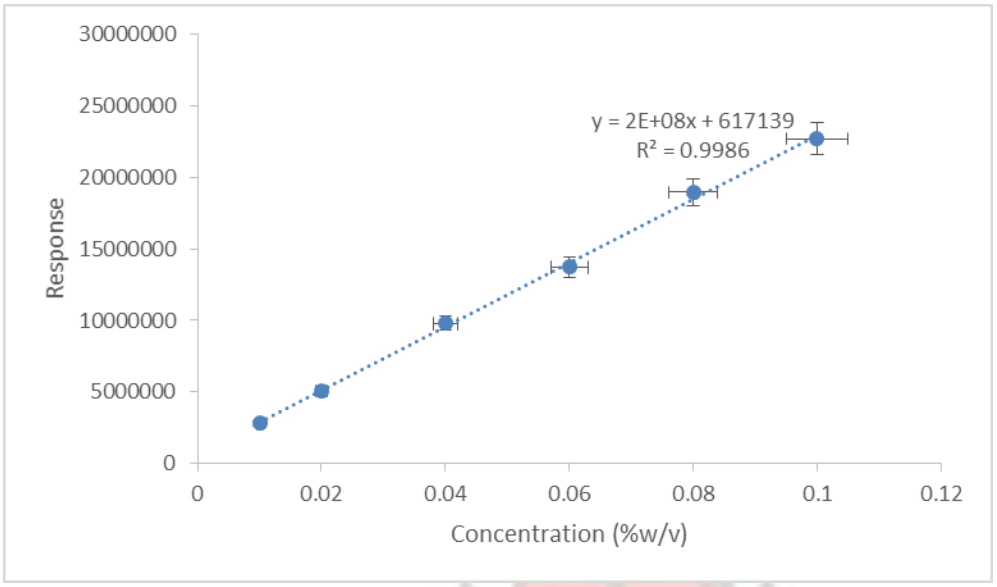
Calibration curve for diclofenac (using Amlodipine as analyte)



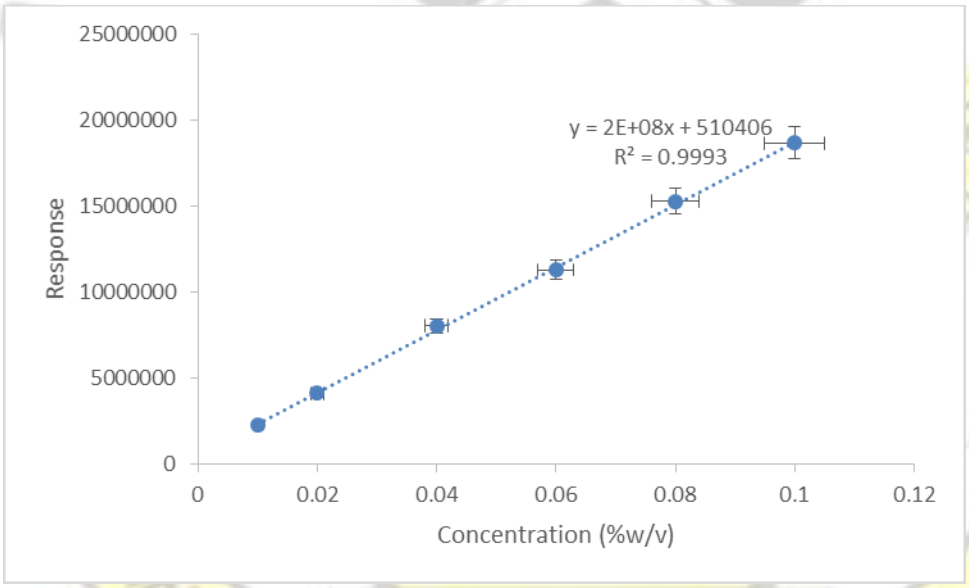
Calibration curve for Amlodipine (using Ibuprofen as surrogate)



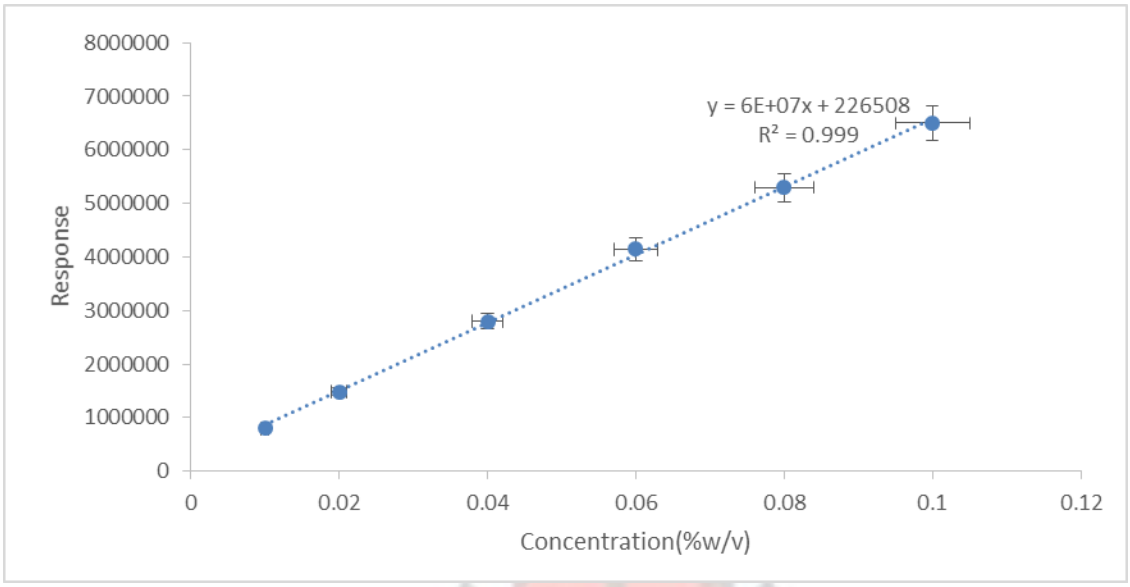
Calibration curve of Ibuprofen (using Amlodipine as analyte)



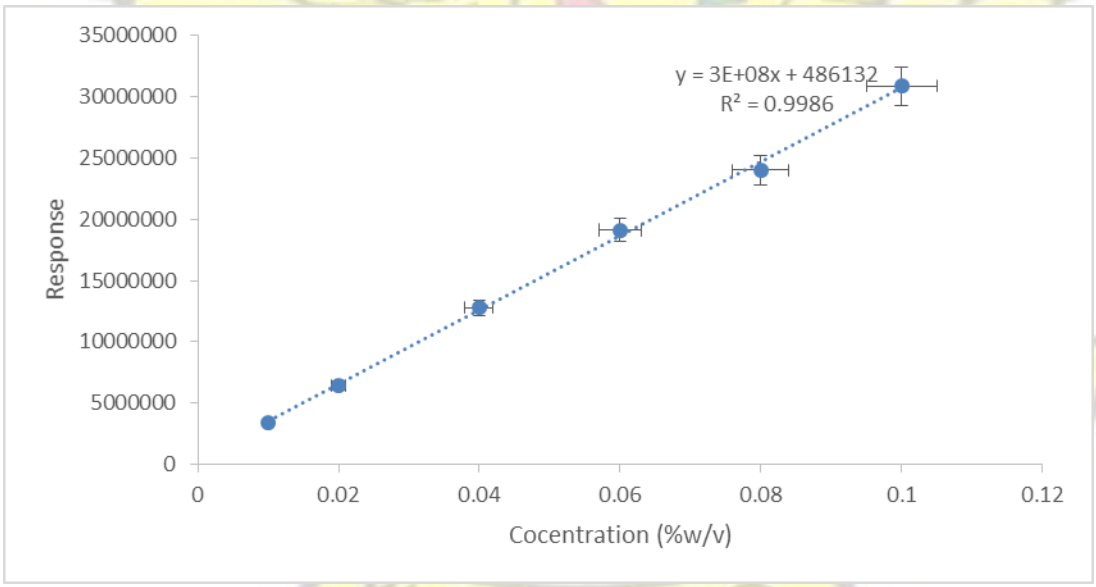
Calibration curve of Amlodipine (using Metronidazole as surrogate)



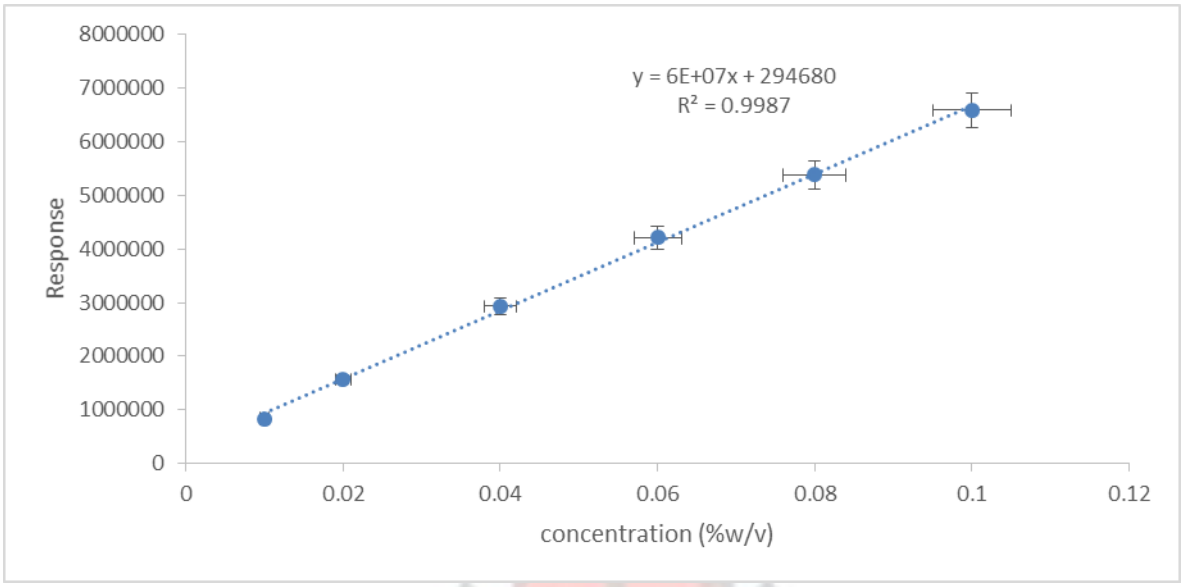
Calibration curve for Metronidazole (using Amlodipine as analyte)



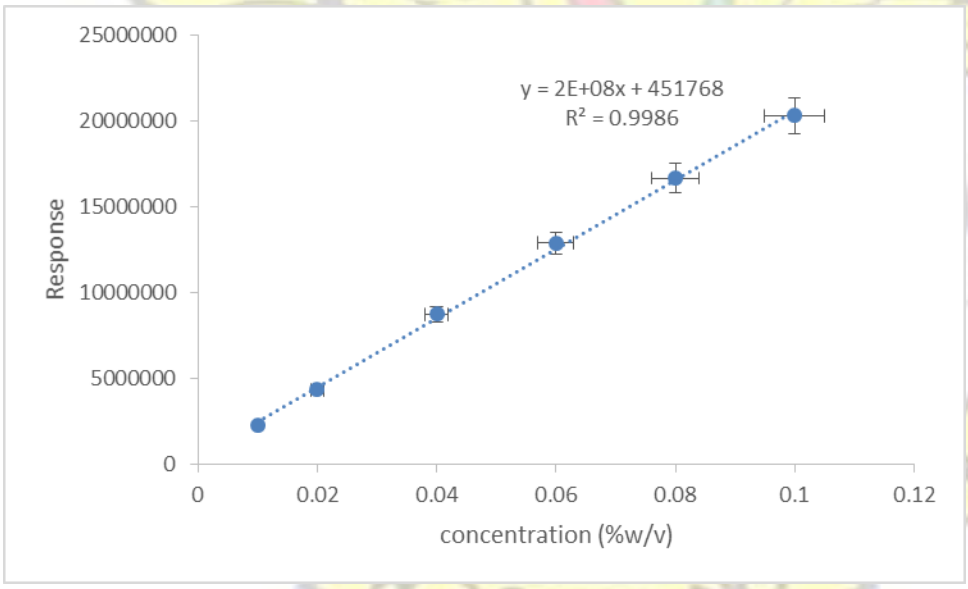
Calibration curve for Lisinopril (using Diclofenac as surrogate)



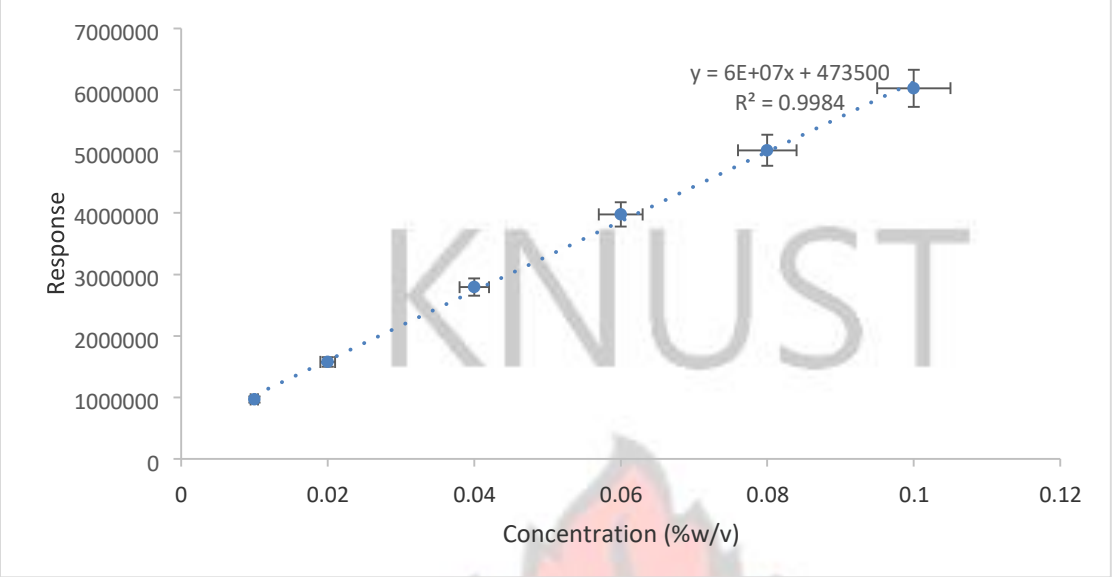
Calibration curve for Diclofenac (using Lisinopril as analyte)



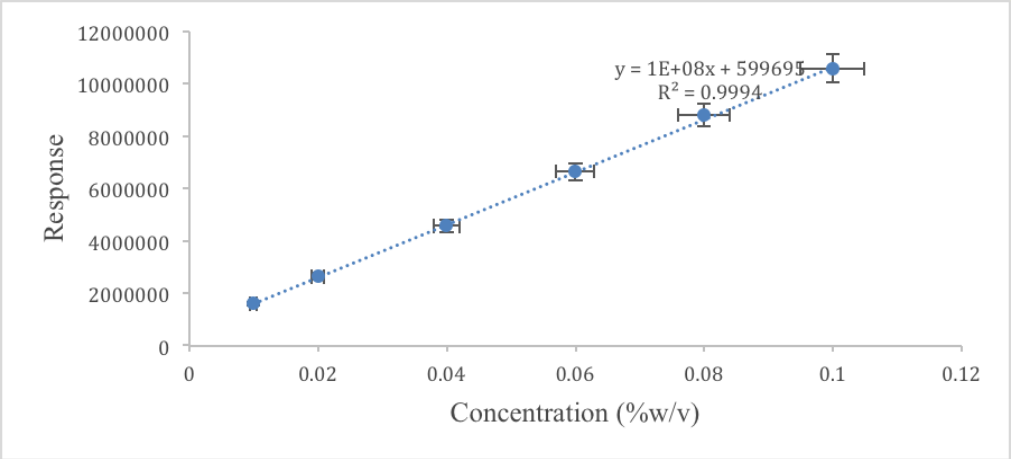
Calibration curve for Lisinopril (using Ibuprofen as surrogate)



Calibration curve for Ibuprofen (using Lisinopril as analyte)



Calibration curve for Lisinopril (using Metronidazole as surrogate)



Calibration curve for Metronidazole (using Lisinopril as analyte)

APPENDIX A8

Determination of surrogate constant (K)

Metronidazole was employed as the surrogate reference standard for amlodipine. A solution of approximately 0.001 %w/v each of Metronidazole and Amlodipine were prepared, injected and analyzed using the new method developed. The respective peak areas of 2779792.8 and 2273322.1 were obtained for Amlodipine and Mtronidazole. The actual concentration of the analyte and surrogates were then calculated using their respective linearity curves. The actual concentrations were 0.009670144 and 0.009644207 for amlodipine and metronidazole respectively.

$$\mathrm{K} = \frac{\mathrm{A(Analyte)} \times \mathrm{C(Standard)}}{\mathrm{A(Standard)} \times \mathrm{C(Analyte)}}$$

$$\mathrm{K} = \frac{2779792.8 \times 0.009644207}{0.009670144 \times 2273322.1} = 1.216$$

$$\mathrm{K} = 1.216$$

Sample calculation of the percentage content of Amlodipine tablets using the K of metronidazole

Average weight of Amlodipine tablet = 0.147205 g

Each tablet of Amlodipine tablet contain = 0.005 g Amlodipine

If 0.147205 g of powdered tablets = 0.005 g Amlodipine Then

$$0.7396 \text{ g of powder} = \frac{0.005 \times 0.7396}{0.147205} = 0.02512\ g$$