THE USE OF SURROGATE REFERENCE STANDARDS IN QUANTITATIVE HPLC ANALYSIS: A CASE STUDY OF GLIBENCLAMIDE AND NAPROXEN TABLETS

A THESIS SUBMITTED IN
FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

MASTER OF PHILOSOPHY

In the

Department of Pharmaceutical Chemistry,
Faculty of Pharmacy and Pharmaceutical Sciences

by

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AUGUST, 2012

DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmaceutical Chemistry, KNUST. This work has not been submitted for any other degree.

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ABSTRACT

High Performance Liquid Chromatography (HPLC) is an indispensable tool in the pharmaceutical industry used in all stages of the drug discovery, manufacturing and control streamline process. HPLC analysis of pharmaceuticals hugely depends on reference standards for quantification, but the high purity and characterization requirements of these standards means they come at high costs. The possibility of doing HPLC analysis of the tablets of two essential drugs: naproxen and glibenclamide, without using their stipulated reference standards, but rather, the pure powders of common and easily available laboratory drugs to act as "surrogate standards" was explored in this study. This was possible as a constant, K, could be determined for each drug vs. surrogated standard pair that could be used in the assay of various tablet brands of the two drugs. For each drug, three surrogate standards were chosen and an HPLC method was developed for each drug-surrogate standard pair. Benzoic acid, paracetamol and prednisolone were the surrogate standards chosen for naproxen, whereas salicylic acid, indomethacin and chloramphenicol were chosen for glibenclamide. All methods adopted a Phenomenex Kromasil® fully porous silica C8 column and a flow rate of 1 ml/min for analysis. For each naproxen-surrogate standard pair, the method developed employed an isocratic water/methanol (35:65 %v/v) mobile phase system, whereas each glibenclamide-surrogate standard pair employed also an isocratic water/methanol (20:80 %v/v) mobile phase. The mean retention times were: naproxen: 4.716 ± 0.156; glibenclamide: 4.300 ± 0.041; benzoic acid: 2.624 ± 0.071; paracetamol: 3.214 ± 0.032; prednisolone: 7.708 ± 0.264; salicylic acid: 1.632 ± 0.016; indomethacin: 2.853 ± 0.096; and chloramphenicol: 3.242 ± 0.017. For naproxen against each one of its surrogate standards, the constants, K, determined were: 0.5967 ± 0.0059; 0.5626 ± 0.0102 and 0.9077 ± 0.0172 for benzoic acid, paracetamol and prednisolone respectively. Similarly, glibenclamide with each one of its surrogate standards yielded K constants of 1.278 ± 0.0013; 0.7798 ± 0.0171; and 0.3790 ± 0.0064 for salicylic acid, indomethacin and chloramphenicol respectively. Using these K constants to assay for various brands of naproxen and glibenclamide tablets yielded results that were comparable with those obtained using the respective pharmacopoeial methods, in most cases. The results have demonstrated that the various surrogate standards can successfully be employed in the HPLC analysis of naproxen and glibenclamide tablets, instead of their expensive reference standards, and would particularly be good alternatives under situations where the reference standards are unavailable.

DEDICATION

This work is dedicated to my late mother, Mrs. Esther Nana Serwah Afful, and my father Mr. David Afful.

ACKNOWLEDGMENT

The Lord is the beginning and the end of everything, and all that is contained in the printed pages of this dissertation, which gives an account of what the last two years of my life have been used for, would not have been possible without his guidance, favours and grace, and I wish to hence say: I am wholeheartedly grateful.

This dissertation holds far more than the culmination of two years of study, as it also reflects within its pages, the relationships with the many generous and inspiring people who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study. The list is long, but I cherish each contribution to my development, as I build my professional career.

I am truly indebted and thankful to my supervisor, Prof. J.K. Kwakye for his support, inspirational advices and encouragements. Same goes to Mr. Samuel Oppong Bekoe and all other lecturers in the Dept. of Pharmaceutical Chemistry, KNUST. My heartfelt gratitude goes to Mourne Training Services (Ireland, U.K), particularly Oona McPolin for the HPLC training which helped me in my HPLC analysis.

It is an honor for me to acknowledge my long list of mentors, their support and guidance without which reaching this stage of my life would not have been possible. To Prof. Karen Duca of the Dept. of Biochemistry, KNUST, I say I am sincerely and heartily thankful for the blessing you've been in my life. Same goes to Prof. (Mrs.) Margaret T. Frempong, Dr. W.K.B.A Owiredu, Dr. E.F. Laing, Dr. Christian Obirikorang, Dr. (Mrs.) Linda Ahenkorah Fondjo, Dr. Francis Agyemang Yeboah, Mr. James Osei Yeboah, Mr. Prosper Agbodaze all of the Dept. of Molecular Medicine, SMS, KNUST, and Dr. Nafui Amidu of the School of Medicine and Health Science, UDS, Tamale.

It is also a pleasure to acknowledge a long list of special people whose prayers, love, generosity and support has helped me survive these past two years. To Mame Soma Nyansah, Sylvia Owusu-Bempah, Emmanuel Kenneth Cudjoe, Ernest Obese, Kevin Osei Affram, Timmy Donkoh, Michael Klu, Eugene Fletcher, Daniel Sarfo Marfo (who provided my drug samples), and all friends, I say it's a big honour to have you contribute to my successful completion of my Masters program, and I'm very grateful. I also owe earnest thankfulness to all my course mates for their help in various forms throughout the program.

To my late mother, and my father whom this dissertation is dedicated to, I say: I am grateful for your unflagging love through everything. Finally, I am grateful to all my siblings, uncles and aunties for their love and unrelenting support.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
API	Active Pharmaceutical Ingredient
BDH	British Drug House
BMCT	Bonferroni's Multiple Comparison Test
BP	British Pharmacopoeia
CDS	Chromatographic Data System
CRM	Certified Reference Material
CRS	Chemical Reference Standard
DAD	Diode Array Detector
EcD	Electrochemical Detector
FDA	Food and Drug Administration
GC	Gas Chromatography
GEC	Gel Exclusion Chromatography
GPC	Gel Permeation Chromatography
HPLC	High Performance Liquid Chromatography
ICH	International Conference on Harmonization
ID	Internal Diameter
IE-HPLC	Ion Exchange HPLC
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation

MeOH	Methanol
MP	Mobile Phase
MPM	Mobile Phase Modifier
MS	Mass Spectroscopy
NCE	New Chemical Entity
NMR	Nuclear Magnetic Resonance
NP-HPLC	Normal Phase HPLC
NSAID	Non-Steroidal Anti Inflammatory Drug
ODS	Octyldodecylsilane
OTC	Over the Counter
PDA	Photodiode Array Detector
PEEK	Polyetheretherketone
RF	Response Factor
RI	Refractive Index
RM	Reference Material
RPLC	Reversed Phase Liquid Chromatography
RS	Reference Standard
RSD	Relative Standard Deviation
RSPP	Reciprocating Single Piston Pump
SD	Standard Deviation
SE-HPLC	Size Exclusion HPLC
SIM	Stability Indicating Method

SP	**Stationary Phase**
SRS	**Surrogate Reference Standard**
TES	**Tetraethoxysilane**
THF	**Tetrahydrofuran**
UHPLC	**Ultra HPLC**
USP	**United States Pharmacopoeia**
WHO	**World Health Organization**

Chapter 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Drug manufacturing control requires high level and intensive analytical and chemical support of all stages to ensure the drug's quality and safety (Velagaleti *et al.*, 2003). The pharmacopeia constitutes a collection of recommended procedures for analysis, and specifications for the determination of pharmaceutical substances, excipients, and dosage forms that is intended to serve as source material for reference or adaptation by anyone wishing to fulfill pharmaceutical requirements. The most important analytical technique used during the various steps of drug development and manufacturing is the separation technique: High Performance Liquid Chromatography (HPLC).

In the pharmaceutical industry, HPLC is the method of choice for a wide variety of samples and tests including checking the purity of new drug candidates, monitoring changes or the scale up of synthetic procedures, in-process testing for developing new formulations, and quality control/assurance of final drug products (Ahuja, 2005). Simply put, without HPLC, crucial tests such as identification, assay and content uniformity, dissolution, impurity and stability testing for many pharmaceutical products would not be possible.

The goal of HPLC analysis is to separate the analyte(s) from the other components in the sample in order to obtain accurate quantitation for each analyte. HPLC separates mixtures of compounds by exploiting the differences in their distribution equilibrium between two phases, the stationary phase packed inside a column and the mobile phase, delivered through the column by high pressure pumps. To achieve a separation, an analyte dissolved in a suitable solvent is introduced into an injection device, which allows the injected sample to be pumped onto the stationary

phase surface. This presents analytes in the solvent with two possibilities: either to remain dissolved or to associate with the stationary phase. The equilibrium partitioning of the analyte(s) between the two phases is upset by the pumping of a stream of mobile phase through the system. This leads to an elution of the particles based on their affinities as the analyte with the greatest affinity for the stationary phase spends the most time in the system and hence is eluted last. The various analytes of the sample are therefore separated based on their differential elution patterns, and the separated compounds appear as peaks shown by a detector, which usually contains a low volume cell through which the mobile phase passes carrying the sample components eluting from the column (Rasmussen, 2001).

There are various modes of HPLC operation and the classification depends on the analyte-stationary phase interaction mechanism. Reversed Phase Liquid Chromatography (RPLC) is the most widely used mode for the separation of pharmaceutical products, making use of a non-polar lipophilic stationary phase system made of alkylated/phenylated silica. Common mobile phases in use in RPLC include methanol, acetonitrile, water and tetrahydrofuran. The other modes of HPLC include normal phase, ion exchange and size exclusion HPLCs respectively (McPolin, 2009).

One central process in pharmaceutical analysis is the development and validation of HPLC methods for use in the assay of pharmaceutical products together with related products. Various factors that can call for a change in, or development of an HPLC method may include drug formulation modifications, changes with regards to the synthesis process of the drug, changes in scale-up, unreliability or unavailability of existing methods among others (Rasmussen *et al.*, 2005). The method development can be done both for single and multiple-analyte assays, and is usually grouped under one of three main method types: qualitative, quantitative and preparative (Dong, 2006). HPLC method development usually follows a common series of procedures, despite the considerable diversity that may be

exhibited during each individual's handling of the process. Sample considerations including separation goals and pretreatment is the first step to a good method development. This is followed by other considerations including choosing a detector and its settings, choosing a mobile phase and separation conditions, optimization of conditions and finally checking for problems. After a method is developed, the next thing that must be done before its release for routine laboratory use, as required by Good Manufacturing Practice (GMP) is validation, which involves determining the linearity, precision, accuracy, sensitivity, the reproducibility and robustness of the method and finally the limits of detection and quantification (Synder *et al.*, 1997).

HPLC has enjoyed widespread usage across all analytical fields owing to its remarkable quantitation capabilities. The detector produces a response; an electrical signal that is dictated by the amount of the analyte present. The two main detector response parameters used in HPLC quantitation are peak height and peak area. Both parameters can be used in quantitation, though the peak area approach is the most commonly used (Wang, 2002). Since both are only representative of the detector response, they must in a way be linked to the amount of the analyte present, and this task is accomplished by the use of calibration with a standard. Three main calibration methods are available: calibration using an external standard, using an internal standard and using standard addition. All three require a standard sample of the analyte for quantitation and hence under circumstances where a standard is not available, a fourth quantitation method that can be applied is the normalized peak area method (Synder *et al.*, 1997). The calibration by external standards has enjoyed widespread usage in HPLC quantitation and it's the most general method. Standard concentrations of the analyte are prepared and injected into the HPLC system to induce responses that correspond to the various concentrations. A linear calibration curve is then plotted and the concentration of the unknown sample can be deduced from the plot. Alternatively, a parameter called the response factor (RF) can be determined by dividing the detector response

(peak area or height) of the standard by its corresponding concentration. The unknown sample concentration can then be calculated by dividing the sample detector response by the RF (Wang, 2002).

The standards used for quantitation in HPLC are usually preparations from reference standards. A reference standard of a compound is its pure form with known purity. The FDA and other drug regulatory authorities the world over, including Ghana's Food and Drugs Board recognize two categories of reference standards: Pharmacopoeial and non-compendial reference standards. The second category is usually employed when compendial reference standards for the particular analyte does not exist (Richardson and Erni, 2005). The pharmacopoeias require that reference standards bear a high degree of purity as assay quantification and impurity level detection depends on their response parameters. This has made reference standards not only costly to obtain in a third world nation like Ghana but also hard to come by. In the light of these, this study seeks to experiment the possibility of doing HPLC quantitation of two widely used drugs, naproxen and glibenclamide without the use of their chromatographic reference standards, but rather using the pure powders of common and inexpensive laboratory drug compounds that share some similar properties with the drugs, to act as 'surrogate standards'.

1.2 STUDY RATIONALE

The detector response, indicated as the peak area 'A' is proportional to the concentration 'C' of the analyte (drug sample) injected.

This implies that: $A \propto C$

and therefore introducing a constant X

$$\Rightarrow A = XC$$

$$\Rightarrow X = \frac{A}{C}$$

If the pure reference standard of the drug sample is used together with the drug, then:

$$\frac{A\ sample}{C\ sample} = \frac{A\ std}{C\ std}, \quad \text{since X remains the same}$$

However if a surrogate compound is used as a standard (surr.std), then:

$$\frac{A\ sample}{C\ sample} \neq \frac{A\ surr.std}{C\ surr.std}, \quad \text{but rather,} \quad \frac{A\ sample}{C\ sample} = K\frac{A\ surr.std}{C\ surr.std}$$

Where, K is a constant which can be determined. Therefore upon injection of a pure drug sample of known concentration together with its pure surrogate standard also of known concentration into an HPLC system, K can be determined from the peak areas as follows:

$$K = \frac{A\ sample}{C\ sample} \times \frac{C\ surr.std}{A\ surr.std}$$

1.3 STUDY HYPOTHESIS

This study therefore hypothesizes that if K can be determined for a drug sample as shown above, then HPLC quantitation of a formulated product of the drug can be done without the use of its stated reference standard, but rather by the use of the pure powder of the surrogate standard that was used to determine the K, especially in cases where the stated reference standard cost high to obtain or is unavailable.

1.4 STUDY OBJECTIVE

This study aims to explore the possibility of using various chosen surrogate standards in the HPLC quantification of two formulated drug products: naproxen and glibenclamide tablets.

1.4.1 Specific Objectives

1. To select for each drug compound (naproxen and glibenclamide), three common laboratory drugs that are easily obtainable and less expensive to come by, to act as surrogate standards.

2. To establish working HPLC conditions that will allow the elution together of the surrogate standards with their drug samples, that gives appreciable retention times and hence can be used to assay formulated products of the two drugs.

3. To ensure traceability of the developed HPLC methods and also validate them using International Conference on Harmonization (ICH) approved method validation parameters including limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, sensitivity, robustness and reproducibility.

4. To determine the constant K that can be used together with its surrogate standard in the HPLC quantification of naproxen and glibenclamide tablets.

5. To determine using the K determined, and the method developed for each drug sample, the percentage content of active pharmaceutical product present in three different brands each of naproxen and glibenclamide tablets.

6. To compare the percentage content of active product in each brand of naproxen and glibenclamide tablets determined using the developed method, with the standard method for each drug described in a standard Pharmacopoeia.

7. To judge the overall acceptability of the developed methods for use in pharmaceutical analysis.

1.5 PROBLEM STATEMENT AND JUSTIFICATION

Drug analysis has always encompassed the various analytical investigations of bulk drug materials, intermediates in drug synthesis, drug formulations, impurities and degradation products of drugs and biological samples containing the drugs as well as their metabolites; with the overall aim to obtain data that will be critical to a high drug quality, maximum efficacy as well as maximum drug safety. Drug quality and efficacy describes the suitability of the drug for its planned use and encompasses three important attributes which are identity, purity and strength. The main aim of pharmaceutical analysts working in quality control laboratories in the pharmaceutical industry and regulatory agencies has always been to contribute to safe and effective drug therapy by controlling these attributes. Strength and sometimes purity testing of pharmaceutical products relies heavily on the various

assay methods available to the pharmaceutical analyst. The ICH defines an assay as a test "to provide an exact result which allows an accurate statement of the content or potency of the analyte in a sample" (ICH, 2000). Unfortunately, the classical assay methods (Titrimetry, UV spectrophotometry/colorimetry and gravimetry) have fell short in this fulfillment (Görög, 2012) mainly due to their lack of selectivity.

The paradigm shift in the second part of the 20th century was the result of the invention and rapid spread of highly selective, sensitive and robust chromatographic techniques especially HPLC. Hence the most characteristic feature of the development in the methodology of pharmaceutical and biomedical analysis during the past 25 years is that HPLC became undoubtedly the most important analytical method for identification and quantification of drugs, either in their active pharmaceutical ingredient or in their formulations during the process of their discovery, development and manufacturing. Clear evidence for this observation is provided by the numerous chromatographic papers captured in the over 18 journals entirely dedicated to chromatography; not to talk of the overwhelming majority of papers in the area published in other analytical and pharmacy-related journals. It is not surprising therefore that HPLC and other chromatographic methods are step by step replacing other assay methods in successive revisions of the major pharmacopoeias. The United States pharmacopoeia presents a typical case of this observation; the current edition (USP 34, issued in 2011) contains 3900 monographs of drug materials, medicinal plants, excipients and drug formulations of which a huge majority of 77% contain one or two chromatographic methods principally HPLC for identification, assay and purity testing (Görög, 2012). Comparing this percentage with the 3% for USP 16, the difference is obvious and the important role of HPLC is clear.

This paradigm shifting role played by HPLC in pharmaceutical analysis may however not be all good news for pharmaceutical industries and analysts in

developing countries due to two major limitations. One, the cost of and availability of chemical reference standards for quantification and purity check, could be a major problem in a country like Ghana where pharmaceutical industries are in their growing stages. The quality and purity of reference standards are critical and hence these materials are expected be well characterized and highly purified. Unfortunately this must come with a price; one that impedes effective quality control of pharmaceutical substances in the country. The two drugs chosen in this study, naproxen and glibenclamide are widely used drugs in their respective domains. Naproxen is a common non-steroidal anti-inflammatory OTC drug whereas glibenclamide is one of only two oral anti-diabetic drugs listed in the WHO Model List of Essential Medicines. Good HPLC quality control of the drugs as critical as it may be is hampered by the high cost of their respective reference materials; for instance, 100mg of the British Pharmacopoeia's naproxen and glibenclamide chemical reference standard (CRS) costs £111.00 each (details in Table 1.1 below).

Table 1-1 Cost of Naproxen and Glibenclamide BP Chemical Reference Standards

Catalogue No.	Drug Product	Batch No	Pack Size	Price (£)
435	Naproxen	2731	100mg	111.00
175	Glibenclamide	2663	100mg	111.00

Source: (BP, 2012)

Alternate ways of HPLC quality control of pharmaceuticals that circumvents around the use of reference standards will be a major milestone reached in Ghanaian pharmaceutical analysis and quality control. The possibility of the use of 'surrogate' references standards (SRS), which are pure powders of other drugs particularly common, inexpensive laboratory drugs in this regard has been a major research theme in the Dept. of Pharmaceutical Chemistry, KNUST for some time now, with successes obtained with the quantification of a number of drugs

including paracetamol, aspirin, diclofenac, indomethacin, diazepam, prednisolone, chlopherenamine, mebendazole, metronidazole, piroxicam and metformin. With a long term aim to create a database that will contain each drug and their surrogate reference standard(s) as well as the K constants that will aid in their quantification, there is a pressing need for the exploration to be done on as many drugs as possible especially the more commonly used ones, calling for more of studies such as this.

The second limitation faced in HPLC analysis of pharmaceuticals in developing countries is the fact that the methods described in all the pharmacopoeias, especially for RPLC, employ expensive solvents like acetonitrile and tetrahydrofuran. To address this limitation, the development of simple, robust and reproducible methods that employs more common and inexpensive solvents like methanol for HPLC analysis is paramount. An HPLC method is yet to be described in the major pharmacopoeias for naproxen though quite a number of papers have been published in this regard. Only a few of those publications however concern the quantification of naproxen in formulations, as the huge majority is focused on its quantification in serum. With glibenclamide, the current methods described in the pharmacopoeias for quantification of the formulated tablets are HPLC but as stated above utilizes acetonitrile as a solvent and hence will be expensive to run.

To summarize, the expensive nature and sometimes unavailability of both reference standards and solvents alike are major setbacks in HPLC analysis and quality control of pharmaceuticals in Ghana and other developing countries that calls for methods that both utilizes less expensive solvents as well as alternate, readily available and less expensive standards, all of which are addressed by this study.

1.6 BENEFITS OF THE STUDY

The study hopes to bring to bear the following benefits:

1. Present a cheaper, simpler and alternate way to do HPLC quantitation of naproxen and glibenclamide without the use of their respective expensive reference standards, but rather by the use of common, inexpensive laboratory drugs. This could also be beneficial under situations where the reference standards are unavailable for a reason.

2. The simple and efficient HPLC methods to be developed for both drugs would make laboratory analysis of the two drugs, quicker and less cumbersome, especially when the pharmacopoeial methods described falls short in one or more of these areas.

3. For glibenclamide, the method to be developed would be relatively cheaper compared to the described pharmacopoeial methods as adoption of less expensive solvents will be used.

4. The study will provide basic HPLC data on naproxen and glibenclamide as well as all six chosen drugs acting as surrogate standards; in this way leading to both new insights into HPLC analysis of the drugs or adding on to already existing scientific knowledge.

5. With regards to point (4) above, the study will be an informative source for pharmaceutical analysts to obtain baseline information that can be built upon during the method development process of other formulated products.

6. In pharmaceutical analysis, constants are widely used, making assays and identification of drugs easy; a common example is the A(1%, 1cm) in

spectrophotometry. The K value determined in this study will act in a similar regard as it will be specific for a given drug-surrogate standard pair and can be used as a means of identification, especially since no such constants exist in HPLC.

Chapter 2

LITERATURE REVIEW

2.1 CHROMATOGRAPHY AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Separation methods are employed widely not only in science but even in everyday life. A chromatographic method is simply a physical method of separation in which components of a mixture to be separated are distributed differentially between two non-miscible phases. One of the phases, called the stationary phase (SP) does not move whereas the other moves over the SP and is called the mobile phase (MP) (Ahuja, 2003a). Selection of the phases is made such that components to be separated have differing solubilities in each phase. In this regard, a component that is quite soluble in the SP will be retained longer during separation than one that is relatively more soluble in the mobile phase. This differing solubility is what accounts for the separation. The versatility and indispensability of chromatography as a separation technique lies in the ability of the SP and MP to be utilized in a number of innovative ways to provide enormous advantages that helps to resolve a large scope of compounds.

Various chromatographic methods and modes exist depending on the criteria under consideration. If classification is based on the geometry of the system, two types of chromatography that are worth mentioning are column and planner chromatography (e.g. paper chromatography and thin layer chromatography – TLC). Classification can also be by the type of retention mechanism, in which case the various types of chromatography include adsorption, partition, ion-exchange and size exclusion chromatographies. Classification by phases is usually the most common type of classification and it is based on the physical states of both the SP and MP. The two broad sub-categories that encompass this criterion of classification are liquid chromatography (LC) and gas chromatography (GC). Different types of

chromatographies that arise as a result of this classification and sub-classification include gas-liquid, gas-solid, liquid-liquid and liquid-solid chromatographies (Encyclopaedia Britannica, 2012).

High Performance Liquid Chromatography (HPLC) is a form of liquid-liquid chromatography that is today one of the most powerful tools of analytical chemistry, owing to its remarkable ability to separate, identity and quantitate compounds present in any sample that can dissolve in a liquid. For this reason, HPLC can be, and has been applied in the separation and quantification of just any sample including pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples, industry chemicals etc.

2.1.1 HPLC – Theory and Principles

HPLC is today one of the most useful and widely applied analytical techniques. Various improvements in the equipment, materials used for separation, technique as well as application of the theory particularly contribute to its exceptionality in comparison with other separation procedures. Aside it's major advantages in convenience, speed and the ability to carry out difficult separations (Ahuja, 2003a), HPLC is also endowed with the following advantageous characteristics (Synder *et al.*, 1997):

- Near universality in terms of applicability
- Remarkable assay precision
- Commercial availability of a wide range of equipment, columns and other accessories that allows its use for almost every application.

Separation in HPLC is possible courtesy a constantly flowing MP passing through a packed column at a finite rate. The tight packing of the column is made possible by the use of very small, finely divided spherical particles, usually porous silica. The stationary phase bonded phase i.e. the kind of chemical group attached to the silica or other packing material fills the inside of each pore between the particles. The

MP surrounds the particles as it flows through the column, and the analyte particles can also enter the pores by diffusion (Synder *et al.*, 1997). The interaction of an analyte with the chemical groups in the pores relative its interaction with the mobile phase surrounding the particle is the basis for separation.

The principle of HPLC separation is illustrated in Figure 2-1 below where a hypothetical separation of a mixture of three analytes, X, Y and Z is shown. The mobile phase flows in the direction shown and the sample solvent which leaves the column during separation is indicated by '+'. Moving from a – d, analyte X is the first to elute followed by Y and then Z indicating that Z spends the most time in the system or interacts the most with the SP. In this case, as with all HPLC separations, two clear-cut aspects encompass the separation theory: kinetic and thermodynamic, both of which result in the analytes in the mixture exhibiting two behaviours respectively. The kinetic aspect is solely responsible for band broadening and hence peak width, whereas the thermodynamics aspect is responsible for retention of the analytes in the column and hence peak position (Kazakevich, 2007). The two behaviours, molecular spreading and differential migration respectively, are hence responsible for the final separation observed in Figure 2-2(d). Differential migration forms the basis for HPLC separation as without a difference in the rate movement of analytes, a separation is not possible.

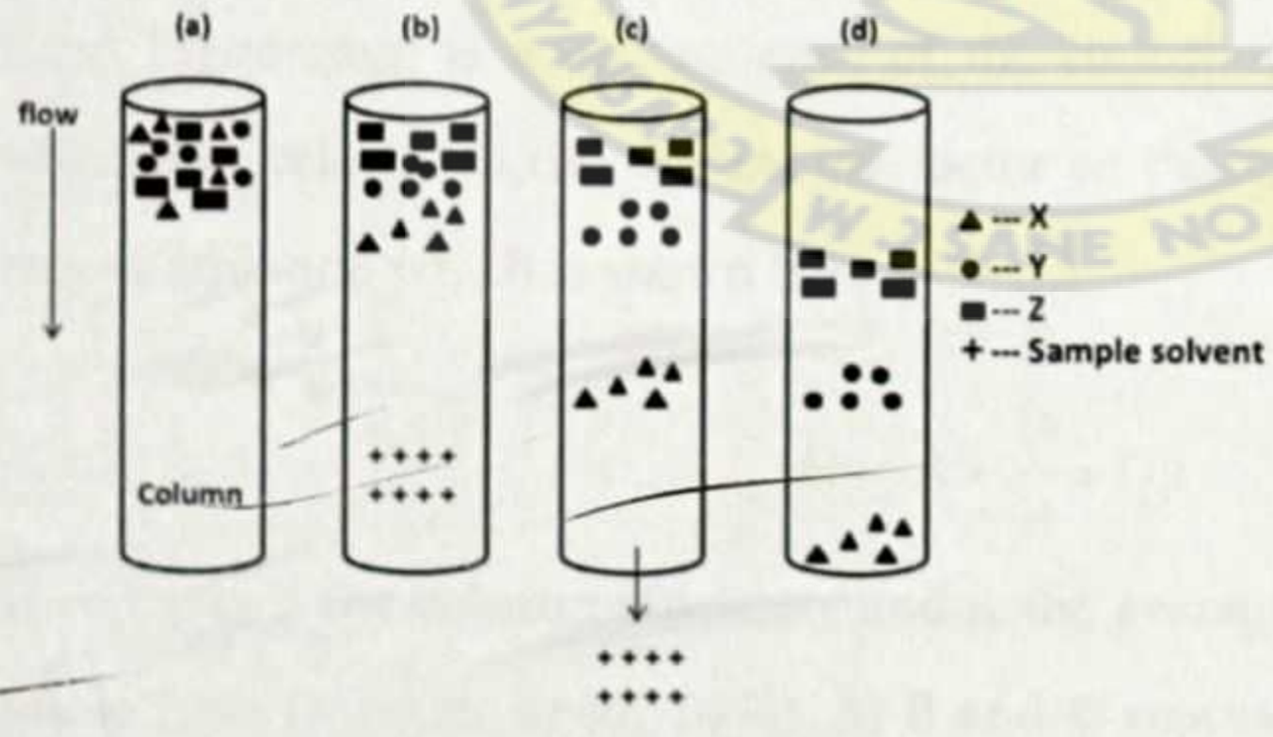


Figure 2-1 Sequential separation process of a hypothetical mixture of analytes, X, Y and Z in HPLC. (a-d) The different stages during separation (Source: Synder *et al.*, 1997)

It is critically related to the equilibrium distribution of the different analytes, in this case X, Y, Z between the SP and flowing MP. The strength of an analyte's interaction with the SP, hence governing its movement through the column ensures that the speed at which the analytes travel (μx) is dependent on its concentration in the MP (Ahuja, 2003b). The resultant effect of this is what is observed in Figure 2-1 where the molecules of analyte X which spend most of the time in the mobile phase moves through the column the fastest to Y and Z. The molecules of the sample solvent also travel the fastest through the column relative all three analytes which is the case in most types of HPLC, except in size exclusion where separation is based on molecular size.

In Figure 2-1, the analytes starts as a narrow band as with the case with all HPLC separations, and increasingly spread out as they get separated so as to occupy a larger volume in the column. For a particular analyte, a band results as a consequence of the volume its molecules occupy in the column and the width of this analyte-volume measured in the direction of flow of solvent, defines the band-width. A peak results after a band leaves the column and is picked up by the detector and recorded in the chromatogram. The identity of the peak can be determined from the time it leaves the column (retention time) and the concentration of analyte is proportional to the peak size.

Band broadening is a consequence of the contribution of three processes, all of which are related to the diffusional factor as captured by the van Deemter rate theory equation which is shown below:

$$H = A + \frac{B}{\mu} + C\mu$$

H represents the column efficiency and μ, the average linear velocity of the mobile phase (van Deemter *et al.*, 1956). A, B and C represents the contribution to band broadening by the three diffusional processes of eddy diffusion (Haky, 2009), longitudinal diffusion (Ahuja, 2003b) and resistance to mass transfer.

2.1.2 HPLC descriptors

In HPLC four principal descriptors are used to report on the characteristics of the chromatographic column, system as well as the particular separation. These include the:

1. Retention factor (k)
2. Efficiency (N)
3. Selectivity (α)
4. Resolution (Rs)

Retention factor, efficiency and selectivity are all used to modify the resolution of separation (Kazakevich, 2007).

2.1.2.1 Retention factor (k)

The time elapsed between the injection of an analyte into the column and its detection is known as the Retention Time (t_R). The time taken for an unretained analyte to leave the column is the column dead time, more specifically called void time (t_0), Figure 2-2 below illustrates the t_R and the t_0 as well as the width (Wb) and height (h) of the peak formed after time t_R.

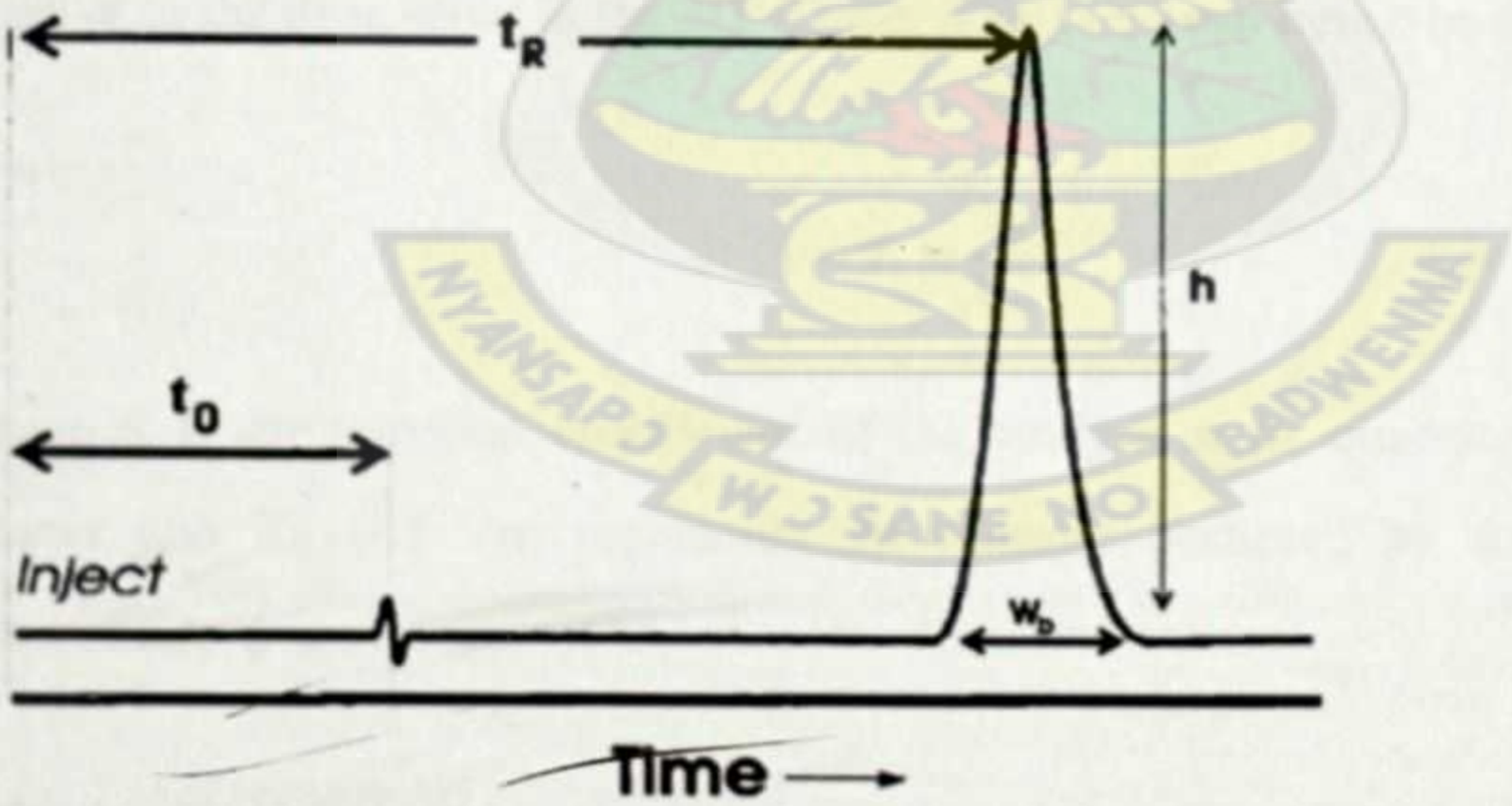


Figure 2-2 Chromatogram showing the retention time (tR), void time (t0), peak base width (Wb) and height h (Source: Ornaf and Dong, 2005)

The retention time of an analyte depends however on parameters like the flow rate, column specifications, temperature and others, and so is used solely for identification purposes only. The capacity factor (also called retention factor) on the other hand, is independent on those parameters and hence is a more fundamental term that measures the degree of retention of an analyte. It describes the thermodynamic basis of the separation and is calculated by normalizing the net retention time, $t_{R'}$ (i.e. $t_R - t_0$). The capacity factor is defined as:

$$k = \frac{t_R - t_0}{t_0} \quad \text{(Ornaf and Dong, 2005)}$$

If the equation is rewritten, $t_R = t_0(1 + k)$ indicating that retention time is proportional to the retention factor. A k value of zero is indicative of a non-retained analyte that elutes with the mobile phase. A value of 1 indicates slight retention whereas a value such as 20 means a highly retained analyte that spends much time in the column as a result of its strong interactions with the SP. Usually k values between 1 and 20 are satisfactory.

Retention factor can also be defined as the time spent by the analyte in the SP relative to the time spent in the MP (Dong, 2006), and can hence be defined as:

$$k = K \times \frac{V_s}{V_m}$$

where K is the partition co-efficient of the analyte or its quantity between both phases and Vs and Vm represents the volume occupied by the SP and MP respectively.

2.1.2.2 Selectivity (α)

Selectivity is a measure of the relative or differential retention of two analytes. Also called the separation factor, it is defined as the ratio of the capacity factors of the two peaks from the analytes and calculated as:

$$\alpha = \frac{k_2}{k_1}$$

where k_2 and k_1 are the retention factors of the two peaks. Selectivity must be > 1 for good peak separation. It depends on a number of factors including the nature of the SP i.e. the type of bonded phase groups attached, temperature as well as the MP composition (Haky, 2009).

2.1.2.3 Efficiency (N)

This is a measure of the degree of dispersion of the peaks in a particular column and hence is essentially a characteristic of the column. An efficient column produces sharp peaks and can separate many sample components in a relatively short time. In a chromatogram, the width of a peak depends on the degree to which a band of analyte molecules spreads out over the time it takes to pass travel through the column. This band separation is best defined in terms of the number of theoretical plates, N which is a measure of the efficiency of the column. Efficiency is related to the retention time (t_R) and width of the peak base (W_b) by the equation below:

$$N = 16\left(\frac{t_R}{W_b}\right)^2$$

A high N value means a lower degree of band broadening implying narrower peaks (Dong, 2006).

2.1.2.4 Resolution (Rs)

Resolution is a measure of the degree of separation of two peaks that are adjacent to each other. It is the ability of the column to resolve two analytes in two separate peaks which is actually the goal of most HPLC analyses. For best analytical results, little or no overlap must exist between peaks in the chromatogram. The resolution is a function of the distance between the peak maxima and their corresponding widths. It is hence defined as the ratio between the difference in the retention times

of two peaks t_1 and t_2 and the average of their base widths, Wb_1 and Wb_2 respectively, as illustrated by the equation below:

$$Rs = \frac{t_1 - t_2}{0.5(W_1 + W_2)}$$

A resolution between 1.5 and 2.0 indicates good baseline resolution and is ideal for an HPLC method.

From the Rs equation above, it implies that resolution of analytes is dependent on two things: 1) their relative retention on the chromatographic system and 2) their peak widths. A clear understanding of the nature of these parameters and the factors that affect them is hence paramount for a maximum resolution. The chromatographic descriptors of selectivity, retention and efficiency can all also be manipulated to achieve a good resolution and hence resolution can also be defined to encompass these parameters as well. Hence for two peaks of approximately equal widths with capacity factors k_1 and k_2 and a mean theoretical plate number N, the resolution Rs can be defined as:

$$Rs = \left(\frac{N^{1/2}}{4}\right)\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2}{1 + k_1}\right)$$

(1) (2) (3)

Where the (1) component represents the efficiency, the (2) component the selectivity and the (3) component the retention. These terms can be treated as independent of each other and hence variation of experimental conditions during method development could lead to their modification for optimized resolution.

2.1.3 Separation modes and types of HPLC

There are four principal types of HPLC namely partition, adsorption, ion-exchange and size-exclusion. Aside these, two major modes of HPLC exist namely reversed-phase and normal-phase HPLC. In general, three characteristics of chemical compounds results in these different types and modes of HPLC:

1. Polarity
2. Electrical charge
3. Molecular size

2.1.3.1 Separation based on polarity

A molecule's structure, activity, and physicochemical characteristics are determined by the arrangement of its constituent atoms and the bonds between them. Within a molecule, a specific arrangement of certain atoms that is responsible for special properties and predictable chemical reactions is called a functional group. This structure often determines whether the molecule is polar or non-polar. Organic molecules are sorted into classes according to the principal functional group(s) each contains. Using a separation mode based on polarity, the relative chromatographic retention of different kinds of molecules is largely determined by the nature and location of these functional groups. As shown in Figure 2-3 below, classes of molecules can be ordered by their relative retention into a range or spectrum of chromatographic polarity from highly polar to highly non-polar.

Figure 2-3 The different analyte functional groups and the resulting chromatographic polarity spectrum (Source: www.waters.com)

Molecules with similar chromatographic polarity tend to be attracted to each other; those with dissimilar polarity exhibit much weaker attraction, if any, and may even repel one another. This becomes the basis for chromatographic separation modes based on polarity (www.waters.com). To design a chromatographic separation system, competition for the various compounds contained in the sample is created by choosing a mobile phase and a stationary phase with different polarities. Then,

compounds in the sample that are similar in polarity to the stationary phase will be delayed because of their strong attraction to the particles. Compounds whose polarity is similar to that of the mobile phase will be preferentially attracted to it and move faster. Partition and adsorption HPLC are the two HPLC types, whereas normal phase (NP) and reversed phase (RP) HPLC are the two HPLC modes that separate analytes based on the characteristic of polarity.

2.1.3.1.1 Normal Phase HPLC (NP-HPLC)

Chromatographers in the early 1900s often adopted columns packed with polar, inorganic particles with less-polar solvents such as ligroin. This 'normal' way of chromatography had come to stay and was the common method adopted for the next 60 years. This led to the adoption of the term normal-phase chromatography: the use of polar SPs with non-polar or less polar MPs, which is still in use today. The use of NP-HPLC became less common after the discovery of reversed phase HPLC (RP-HPLC), but is still useful mainly for 1) Analytical separations by thin-layer chromatography; 2) Purification of crude samples; 3) Separation of very polar samples that are poorly retained and separated; and 4) Resolution of achiral isomers (Synder *et al.*, 1997).

In NP-HPLC, the polar SP is silica gel that has been bonded with a polar phase. The usual polar phases widely available from many manufacturers include cyano, amino, nitro, and diol phases (Rabel, 2002). Common solvents employed as mobile phase in NP-HPLC include hexane, methylene chloride, chloroform and ethyl acetate. The separation mechanism in NP-HPLC is illustrated in Figure 2-4(a) below. Polar analytes are retained longer in the column due to their strong interactions with the polar stationary phase.

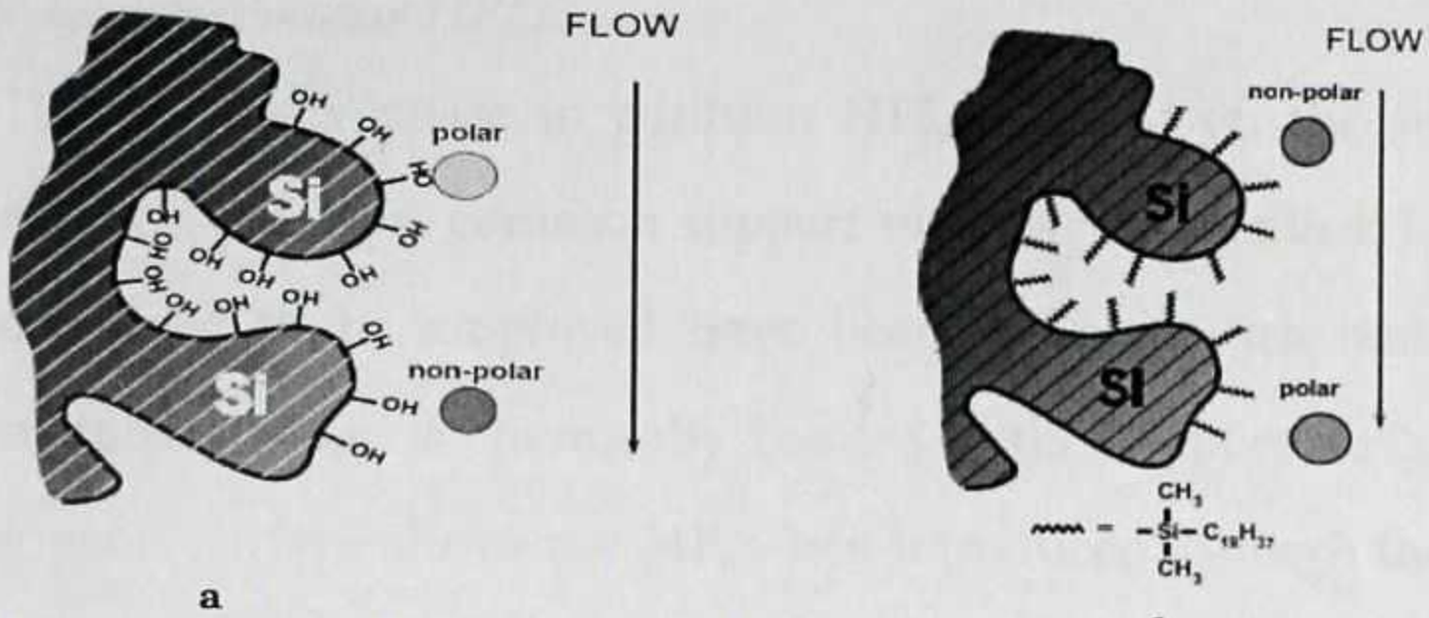


Figure 2-4 Separation modes of normal phase (a) and reversed phase (b) chromatography (Source: Dong, 2006)

2.1.3.1.2 Reversed Phase HPLC (RP-HPLC)

In RP-HPLC, the separation exploits the partition coefficients between a polar MP and a non-polar SP. The earliest stationary phases were solid particles coated with nonpolar liquids but have been replaced now with more permanently bonding hydrophobic groups such as C18 bonded groups. Figure 2-4(b) illustrates the separation mechanism in RP-HPLC. Polar analytes are retained less longer than non-polar ones which interact strongly with the stationary phase bonded phase.

Common stationary phases used include silica bonded to C4, C8, C18, phenyl, CN and phenyl-hexyl groups (McPolin, 2009). Mobile phases usually used in RP-HPLC include water, methanol, acetonitrile and tetrahydrofuran. RPC is usually a first choice for the separation of both neutral and ionic samples, and its present day popularity and widespread usage is largely due to the many strong points this form of HPLC holds. RP-HPLC separations are more convenient, robust and versatile. Columns for this type of HPLC tend to be more efficient and reproducible, offering a wide spectrum of options with regards to column specifications and dimensions. Moreover, RP-HPLC solvents tends to be less flammable, toxic and have appreciably low UV cut-offs that can allow detection even below 230nm. Generally, columns used for RP-HPLC equilibrate fast and easily, especially helpful when switching mobile phases, which is another desirable merit held by this mode of HPLC (Synder *et al.*, 1997).

2.1.3.1.3 Partition HPLC

The stationary phase in partition HPLC is held on the support particles on the packing, the most common support material being silica. Liquid stationary phases that used to be employed have been replaced with bonded phases where the stationary phase is chemically bonded to the support surface. The porosity of the support surface allows the MP, when introduced through the SP, to pass in and out of pores, with the polar mobile phase being repelled by the non-polar groups of the stationary phase in RP-HPLC. In NP-HPLC, the non-polar MP is repelled by the polar SP. When the analyte(s) of interest is introduced, an attraction from both the MP and SP is encountered and the equilibrium experienced is a partition between the two phases. When partitionings like this occur, a term that comes into consideration is the partitioning coefficient, i.e. the ratio of concentration of an analyte in two phases of a mixture of immiscible solvents at equilibrium. Since the partitioning coefficients of different molecules differ they will travel at different rates through the SP, ensuring a separation is possible (McPolin, 2009). RP partition HPLC is the most widely used mode of HPLC in the pharmaceutical industry.

2.1.3.1.4 Adsorption HPLC

Realistically, the original chromatographic process as developed by Tswett was based on separation by adsorption, also referred to as liquid-solid chromatography (Hurtubise, 2002). The SP is the polar surface of a finely divided solid such as silica or even alumina, though less column, that is combined with a non-polar MP. The analyte and MP both compete for adsorption sites on the surface of the mobile phase the resulting adsorptive forces created in what causes the separation. This form of HPLC is most suitable for non-polar solvent-soluble analytes that have very little solubility in aqueous solvents. It is particularly suited for the separation of isomeric mixtures. It is still however a not widely used HPCL technique owing to problems relating to the reproducibility of retention times, which is usually due to the hydration state of the silica caused by the presence of water or protic organic

solvents. Hence reversed-phase partition HPLC is usually favoured whenever possible.

2.1.3.2 Separations based on charge – Ion-exchange HPLC (IE-HPLC)

Unlike separations based on polarity where like is attracted to like and opposites may repel each other, separations based on ionic charge reverses this phenomenon and hence likes may rather repel while opposites may attract each other. The separation mechanism in IE-HPLC is backboned upon the exchange of ionic analytes with the counter-ions of ionic groups attached to the stationary phase support. The completion for attraction to the counter-ions on the SP between the analyte and the ions of the MP set the grounds for exchange equilibria between analyte ions in solution and those of similar sign on the SP surface (Swadesh, 2001).

Ion exchange stationary phase separations are characterized by the nature and strength of the acidic or basic functions on their surfaces and the types of ions that are attracted and retained by them. Cation exchange is used to retain and separate positively charged ions on a negative surface, whereas anion exchange is used to retain and separate negatively charged ions on a positive surface (Gooding, 2002). Anionic exchangers commonly contain quaternary amine groups, referred to as strong anion exchange (SAX) or primary amine groups, referred to as weak anion exchange (WAX). For cation exchange, two common bonded phases include the sulfonic acid group, referred to as strong cation exchange (SCX) and the weak cation exchange (WCX) carboxylic acid group (McPolin, 2009). The functional groups of strong ion exchangers whether ionic or cationic, are always ionized and are typically used to retain and separate weak ions. The weak ions may be eluted by displacement with a mobile phase containing ions that are more strongly attracted to the stationary phase sites or may be retained on the column, then neutralized by changing the pH of the mobile phase, causing them to lose their attraction and elute. Conversely weak ion exchangers whether anionic or cationic, may be neutralized above or below a certain pH value and lose their ability to retain ions by

charge. They are used to retain strong ions when charged and if the ions cannot be eluted by displacement, then the stationary phase exchange sites may be neutralized, shutting off the ionic attraction, and permitting elution of the charged analytes (Gooding, 2002).

Common applications of IE-HPLC include it use in the analysis of ions and biological components including amino acids, proteins or peptides and polynucleotides.

2.1.3.3 Separations based on size – Size-Exclusion HPLC (SE-HPLC)

Unlike the other two modes of separation above, separation based on size is the only one where no physical interaction between analytes and the SP and MP take place. Separation is entirely based on the molecular size differences of analytes that allow different rates of travels through a porous stationary phase (Dong, 2006). In this way, lower molecular weight analytes enter the pores on the column packing and hence take a longer travel path through the column than higher molecular weight ones whose little or no fit in the pore means little or no retention (McPolin, 2009). While in the pores, the analyte is effectively trapped and removed from the flow of the MP and the mean residence time in the pores depends on the effective size of the analyte molecules. Hence molecules having diameters that are significantly smaller than the pores can penetrate throughout the pore maze, leading to their higher entrapment times.

SE-HPLC packing consists of a polymer, generally polystyrene, which is chemically cross-linked so that varying size pores are created. Several separation model theories have been described by Barth *et al.* (1998). During separation, an analyte dissolved in the size exclusion MP is injected and the eluent is monitored by a mass-sensitive detector which responds to the weight concentration of polymer in the mobile phase; the most common detector used is a differential refractometer (Soneji, 2002).

Two major subtypes of SE-HPLC are available: gel permeation chromatography (GPC) which is analogous to NP-HPLC and therefore utilizes stationary phases that are used with non-polar mobile phases, and gel filtration chromatography (GFC) which is analogous to RP-HPLC and hence employs stationary phases that are used with polar (aqueous) mobile phases (McPolin, 2009). GPC is commonly used for the determination of molecular weight of organic polymers and GFC in the separation of water-soluble biological materials (Dong, 2006).

2.1.4 The HPLC stationary phase and column chemistry

The HPLC column that houses the fine support media for the SP is the heart of the HPLC system. A column is described by a number of parameters, one of the most critical being the type and surface chemistry of the packing material it houses, as this would determine a number of separation outcomes like the type and mode of HPLC that can be used, the selectivity of separation, retention, broadening among others. The packing material is hence recognized as the "media" that produces the separation making its properties of primary importance for successful separations (Kazakevich and LoBrutto, 2006).

The first packing materials used in a HPLC were beads of organic gel permeation resins used for size separations. These were commercially available resins and no attempt was made to optimize them for high pressure, except to select for a high degree of cross-linkage to prevent crushing. Silica-based particle technology became a growing field and the early fully porous silica-based beads had diameters between 35 - 60µm (McMaster, 2007). Today fully spherical microporous packings have been developed with particle size as small as 5µm.

HPLC separations are driven by the interactions between analytes and the base material surface groups. The packing is expected to be mechanically and chemically stable while ensuring that it's interaction with different analytes are specific (Kazakevich and LoBrutto, 2006). Variation of the adsorbent surface

chemistry is achieved via chemical modification of the base material surface which is done by chemical binding of specific ligands. This ensures two things: (1) that the surface of the base material is shielded (i.e. mechanical stability) and (2) specific surface interactions are introduced (i.e. chemical stability).

Packing material particles are subject to a significant level of mechanical stress under column packing procedures and even during operation. Material rigidity depends mainly on its surface tension which is a function of the surface chemistry of the material. Modification of the base material chemically serves to alter this characteristic significantly leading to an improved rigidity (Lisichkin, 2003). Chemical modification of the base material is also very important as it dictates the hydrolytic stability of the base material, especially since most HPLC separations employ water and other organic solvents with controlled pH (Kazakevich and LoBrutto, 2006).

Silica is the most common base material used in HPLC, dues to its physical robustness and chemical stability in all solvents and even at a low pH (McPolin, 2009). Other less common base materials include alumina which was common in the early HPLC days, zirconia which is solely used as a support material for bonded phases and polymers.

2.1.4.1 Silica

The use of silica as column packing find diverse uses that cut across all the types and modes of HPLC. It may be used as the separating material in adsorption HPLC or as a support material to hold a bonded phase in partition or ion-exchange HPLC (McPolin, 2009). Studies into the chemistry of silica, methods of its controlled synthesis, surface chemistry and its properties have existed for over two centuries. Aside its advantageous characteristics of mechanical and chemical stability and feasibility of chemical modification, silica is particularly desirable as a both base and separating material owing to the freedom with which surface area and pore size can be manipulated and tailored to specific uses (Neue *et al.*, 2007). Despite

these pluses however, the use of silica as stationary phase still has some drawbacks, two major being their water solubility at high pH and their extreme polarity (Kazakevich and LoBrutto, 2006).

2.1.4.1.1 Synthesis of silica

Different synthetic procedures have been described for silica. One, a patented process, is the colloidal sol–gel procedure involves the formation of spherical silica droplets by passage of the silica sol through a non-aqueous media. The spherical droplets rapidly solidify into hydrogel beads which are dried before their calcination at high temperatures (De Vries *et al.*, 1967). Slice produced in this way, called type A silica contains some relevant amount of impurities like sodium and iron metals and sometimes are irregular shaped. A second method, which involves the polycondensation of tetraethoxysiliane (TES) produces highly pure silica, classified as type B silica which is required for HPLC. The TES first undergoes a partial hydrolysis in a viscous liquid, then emulsified in an ethanol-water mixture before undergoing a further hydrolytic condensation. The formed solid beads of hydrogel are washed and dried into porous silica (Kazakevich and LoBrutto, 2006). Silica packed columns may also be produced as a single piece of continuous silica, called a monoliths (McPolin, 2009).

2.1.4.1.2 Silica chemical structure

Silica consists of silicon atoms bridged by oxygen atoms in a 3-dimensional manner. Figure 2-5 below shows the various surface chemical structures of silica as well as the different adsorption sites. The Si-OH or the silanol free groups constitutes the major portion of the silica surface and are the most active adsorption site. These groups are shown as (a) in the figure. During synthesis, calcination (at temperatures > 800°C) usually would lead to riding adjacent silanols of water molecules leading to the formation of a second adsorption site, the siloxane bonds (c). The process, called dehydroxylation, results in the formation of dehydroxylated silica which though very inert, can rehydroxylate when it absorbs

water (Synder *et al.*, 1997). Neighbouring silanols may also be involved in hydrogen bonding leading to the formation of hydrogen bonded silanols (d). Silanol groups exhibit some slight degree of acidity and the acidity is increased by metal ions (shown as Me^{+}) present in the silica (b).

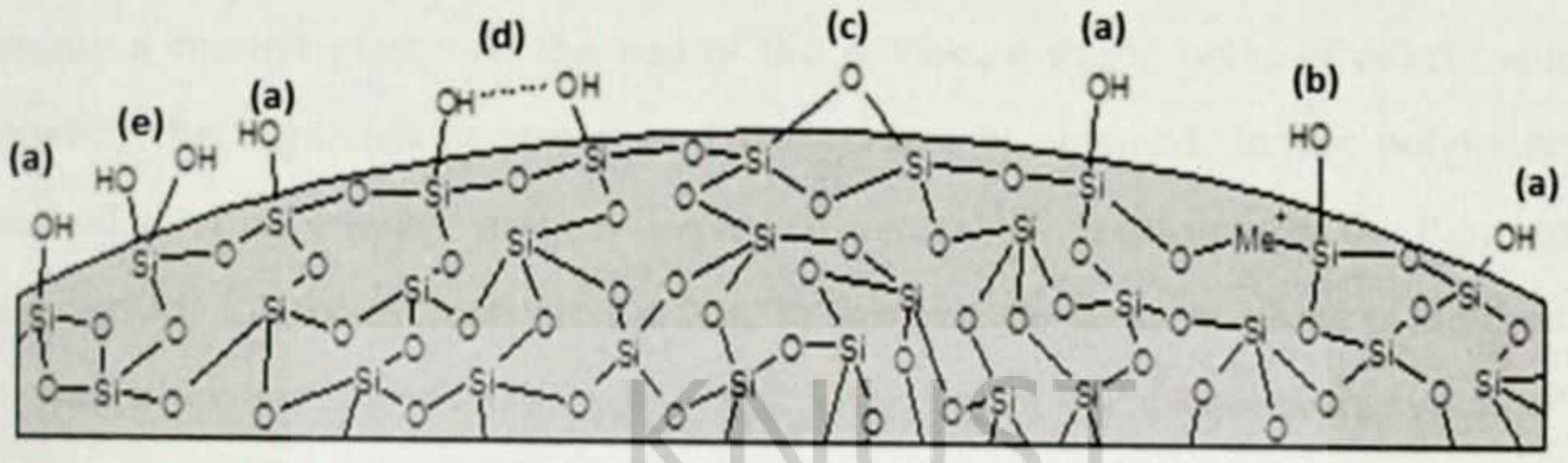


Figure 2-5 Surface structure of silica (Source: Mourne Training Services) showing surface silanol groups

A final adsorption site in silica is a hypothetical site called the germinal silanol group (e). Formed when silica becomes boned to two hydroxyl groups, this surface silanol group has only been reported in literature and their existence is yet to be confirmed (Kazakevich and LoBrutto, 2006).

The use of silica packing in an HPLC column may be either one of two ways: the silica could be the stationary phase itself or may be a matrix to which the bonded phase is attached. The use of silica as the SP itself is the practice in adsorption HPLC where it used in combination with a non-polar MP. Different columns and their manufacturers in this regime include Spherisorb® silica by Waters™, Kromasil® SIL by Eka Chemicals™ and ZORBAX® Rx-SIL by Agilent™ (McPolin, 2009). The use of silica as a matrix for bonded phases involves chemical modification of the surface silanol groups by derivatization. The resulting bonded phase may either be polar (for NP-HPLC) or non-polar (for RP-HPLC).

2.1.4.1.3 Reversed phase silica bonded phases

Organosilanization is the most common method of chemical modification of silica for RP applications. The process involves reaction of the organochlorosilane group

with the silica support material in an appropriate organic solvent (McPolin, 2009). The two methods of organosilanization available are the monomeric and polymeric methods. The monomeric method employs an organosilane reagent comprised of a reactive group X (usually a halide like Cl^-) and two organic groups, R and R'. R is the main group that endows the hydrophobic properties and R' is a small group, usually a methyl group. At the end of the process, a single point of attachment between the organosilane group and the surface is obtained. In the polymeric method, the organosilane reagent employs three reactive X groups and the R group as described for the monomeric method. In this case, the bonded phase is attached to the silica surface at one point and cross-linked to neighbouring bonded organosilanes through a siloxane linkage (Pesek and Matyska, 2002). Both methods are used in the production of commercially available SP for HPLC applications. It is the R group that dictates the overall hydrophobicity of the resulting stationary phase in both methods of organosilanization and the most important factors that control their hydrophobicity are the chain length, number of carbon atoms as well as their bonding densities. Alkyl groups are the most commonly used bonded phases for RP-HPLC and examples include the popular C18 (also referred to as octyldodecylsilane – ODS), octyl (C8) and other less popular groups like the methyl, butyl and the phenyl group. Generally, alkyl chains of length greater than 18 have been found to be undesirable.

A host of column manufacturers today produce a wide range of columns with different selectivities. Column manufacturers include Waters (Symmetry® columns), Thermo™ (Betabasic™ columns), Phenomenex (Luna® columns) and Eka Chemicals (Kromasil columns) (McPolin, 2009).

2.1.4.1.4 Normal phase silica bonded phases

Silica is the most widely used commercially available normal-phase packing in various forms. Bonded phases for NP-HPLC are polar, being combined with a non-

polar mobile phase. Typical chemically bonded phases include the aminoisopropyl, cyanopropyl, nitrophenyl, and diol bonded phases.

2.1.5 HPLC Solvents and Mobile phases

An appreciation of the key role played by stationary phases in the whole HPLC process is critical for a successful separation. However, equally influential in driving a separation is the solvent, an often overlooked variable despite its ability to affect parameters like retention time, peak shape, functional group specificity, backpressure among others. They are particularly of primary concern because their properties must fall within narrow limits for acceptable performance (Synder *et al.*, 1997). Solvents are used in HPLC for formulating mobile phases, for dissolving the sample, and for carrying out sample preparation.

Three factors must be considered before the choice of solvent is made for a given HPLC analysis (Sadek, 2002):

1. The solvent physical properties
2. The solvent chemical properties
3. The effects of the above two on the chromatographic process.

The mobile phase, which the liquid that is pumped through the column during separation is prepared from the solvent, which is the liquid from the manufacturer. Common solvents in use for HPLC include, in increasing order of polarity are: Hexane > methylene chloride > chloroform > ethyl acetate > tetrahydrofuran > isopropanol > acetonitrile > methanol > water (McPolin, 2009). Usually a blend of two or sometime more of the solvent is employed as the mobile phase during analysis.

2.1.5.1 Solvent UV cutoff

The consideration of the UV cutoff of a solvent only comes into play when UV absorption is the detection mechanism involved, which is the case with a majority of HPLC separations. The UV cutoff of a solvent is defined as "the wavelength at

which the absorbance of the solvent in a 1cm cell (against air as reference) is 1. Though this is not a typically critical parameter upon which solvent selection can be based, the UV cutoff is important in two respects: it is fast way to assess whether or not the characteristics of the solvent makes them an appropriate choice based on the systems operating wavelength and whether or not it has changed from lot to lot (Sadek, 2002). Usually, a 'perfect' solvent would not absorb at wavelengths greater than 195nm.

2.1.5.2 Solvent Viscosity

Viscosity in general is simply the resistance a fluid develops to forced flow through a constricted path. In HPLC, a consideration of the various mathematical relationships between the pressure drops on one side and the flow rate, column length and radius, viscosity, particle diameter and sometime the flow resistance factor all on another side (Neue, 1997), indicates that an increase flow rate or a decrease in column radius would lead to increases in the pressure drop (more commonly referred to as backpressure) in the column. An increase in mobile phase viscosity has generally been found to be associated with a decreased efficiency (Sadek, 2002). Viscosity however, decreases with increasing temperature and hence for most common HPLC solvents, increasing the temperature will result in a reduced backpressure.

2.1.5.3 Solvent Miscibility and Solubility

Two solvents are miscible if they can be mixed together in all components without forming separate phases where the maximum amount of a solvent that can dissolve in another solvent is its solubility in that solvent. Solubility considerations are particularly critical for the analyte(s) as their solubility in the solvent(s) will become an important factor during separation. Regarding two solvents as immiscible would not necessarily imply that they are not soluble to a chromatographically useful extent (Sadek, 2002). Single-phase ternary mixtures are

formed by addition of a miscible solvent to an immiscible pair of solvents and they provide a way around the obstacle of solvent immiscibility in HPLC.

2.1.5.4 Solvent Polarity and Selectivity

Solubility of an analyte in a solvent is dictated by its polarity. In this regard, analytes which are less polar tend to be soluble in correspondingly less polar solvents like ethyl acetate and hexane whereas more polar solvents like water and methanol tend to dissolve polar analytes. Different solvents also offer different degrees of selectivity even for the same analyte and therefore knowledge of the selectivity properties is vital for a successful separation (Synder *et al.*, 1997).

2.1.5.5 Other solvent characteristics

Other characteristics that must be considered of a solvent before its selection as part of a mobile phase system include safety, stability, boiling point and density.

2.1.5.6 RP-HPLC Solvents

The most common RP-HPLC solvents are water, methanol (MeOH), acetonitrile (ACN) and tetrahydrofuran (THF). Usually water is combined with one or more of the other three solvents to make a mobile phase for analysis (McPolin, 2009). ACN and MeOH are water miscible, safe to use and compatible with HPLC systems. With regards to UV cutoff, water has the lowest followed by ACN before methanol at 210nm. ACN is usually the first choice over methanol due to its low viscosity but its use is limited by its high costs and unavailability. THF is the most rarely used RP-HPLC solvent owing to its high UV cutoff and high viscosity leading to high backpressures. Moreover, its use is limited by its instability that leads to its degradation to form peroxides (Sadek, 2002). THF is also undesirable as a RP-HPLC solvent due to its ability to react with PEEK fittings of columns and other system parts.

2.1.5.7 NP-HPLC Solvents

Hexane, dichloromethane (methylene chloride), isopropanol and ethyl acetate are the four commonly used normal phase HPLC solvents. Hexane is the most frequently used among the four owing to its low UV cutoff, low background absorbance and its high chemical stability. Hexane is usually used in combination with any one or more of the remaining three to constitute the NP-HPLC mobile phase. This becomes necessary since hexane is highly non-polar and hence a relatively more polar solvent must be combined so polar analytes can be eluted. Dichloromethane is an unstable solvent as it degrades to through free radical formation. This coupled with its low water solubility makes it less used solvent for NP-HPLC. Isopropanol is on the other hand a widely used NP-HPLC solvent and sometimes even finds uses in RP conditions (Sadek, 2002). It has low UV cutoff of 205nm and is miscible in a wide range of solvents including water. The use of ethyl acetate as a NP-HPLC solvent is limited by its high UV cutoff of 256nm though it exhibits intermediate solvent characteristics to dichloromethane and isopropanol. Just like dichloromethane, ethyl acetate is immiscible in water and finds limited use in RP-HPLC unlike isopropanol (McPolin, 2009).

2.1.5.8 Mobile phase modifiers

Typically, mobile phases comprise major and minor components. Generally, minor components are present in <5% of the total MP composition and are commonly referred to as mobile phase modifiers (MPMs). Typical MPMs include diluted acids like phosphoric, acetic and triflouroacetic acids; bases like tri- and di- ethylamine and triethanolamine; buffer systems like the acetate and phosphate buffers; ion-pair reagents like sodium dodecylsulphate (Sadek, 2002). Buffers are among the most generally used MPMs, as among other things, their contribution to the overall HPLC separation when used is profound.

2.1.5.9 Isocratic vs. Gradient elution

The simplest and most common elution practice in HPLC is the isocratic elution where the mobile phase composition is held constant throughout the analysis period. This mode of elution though works well for many samples, is less suitable when multi-analyte samples are to be separated. Under such conditions, elution by a continuous change in the mobile phase composition during analysis, called gradient elution is the best option. Gradient elution is especially suited for a mixture of analytes that that differ widely in hydrophobicity such that the use of isocratic elution will result in an undesirably long analysis time (McPolin, 2009). Other reasons that may call for the use of gradient elution are high-molecular-weight samples, sample preparation and peak tailing (Synder *et al.*, 1997).

2.1.5.10 Mobile phase preparation

In chromatography, the strong solvent of a mobile phase solvent pair is designated as the 'B' solvent whereas the weak as the 'A' solvent. Solvent 'strength' as used in such situations refers to polarity, and hence relatively non-polar solvents become the strong solvent and the more polar ones the weak solvent. Therefore in RP-HPLC, water the always present mobile phase is the weak solvent and MeOH or ACN will be the strong B solvent (Synder *et al.*, 1997). During mobile phase preparation, the solvents used must of HPLC grade. These means they must be of the higher possible quality. Water for HPLC must be purified to remove all metals and contaminants; hence HPLC grade water is available from many manufacturers. Buffers must also be of HPLC grade.

2.1.5.10.1 Measuring and mixing the mobile phase

The standard practice during mobile phase measuring is to measure each solvent separately using an appropriate measuring cylinder and adding them together. It is hence bad practice to measure the first solvent and top it with the second in the same container. This is because of contraction effect which will make the overall mixture lower in volume than the expected volume being prepared (McPolin,

2009). All MPMs must be weighed or measured separately, any adjustments made in the aqueous phase before transferring and mixing with the organic phase. Mixing could be done manually in both isocratic and gradient modes by measuring each solvent separately into a container and shaking before use, or by online mixing using the HPLC pump, in which case programming of the system is required for the system to know how much of each solvent to take.

2.1.5.10.2 Mobile phase filtration and degassing

A 0.45 microns membrane filter is generally required for filtration of the sample before injection into the HPLC system. This process is essential as it removes any trace impurities and matter that could undermine successful separations. Air bubbles in mobile phases, if left uncheck could cause problems with both pump and detector function. It is hence essential that the mobile phase be rid of all air bubbles prior to their entering of the pump system. Modern HPLC are equipped with a vacuum degassing unit that degasses the mobile phase. Other methods of degassing include vacuum filtration, sonication and helium sparging (McPolin, 2009).

2.1.5.10.3 Mobile phase storage

Usually, the accepted practice is to prepare the mobile phase fresh each time it has to be used, especially applicable to buffers, as contamination as result of microbial growth could result. On occasions where the mobile phase has to be stored for further use, the expiry date will depend on among other things, its composition, conditions of storage as well as the local procedures of the laboratory where it is being used (McPolin, 2009).

2.1.6 HPLC instrumentation

HPLC has become a versatile analytical technique whose instrumentation has gone through various decades of refinement. Despite the many advances with instrumentation technology however, all HPLC systems contain the same basic

framework as shown in Figure 2-6 below. At the heart of the system is the column where the separation takes place. In modern systems, the column is housed in a compartment that allows temperature control to facilitate separation. The mobile phase reservoir is connected on one side of the column and the waste collection reservoir on other side. Due to the tight packing of the stationary phase in the column, a high pressure pump is needed to force the MP through the column. In most modern systems a vacuum degasser is connected in the way of the MP before it gets to the pump. Sample is introduced into the system by an injection device. After analytes have been separated they exit the column and are detected by a detector connected to the column. The detector output is displayed as peaks on a chromatography data system (CDS), which is a computer system attached to the detector.

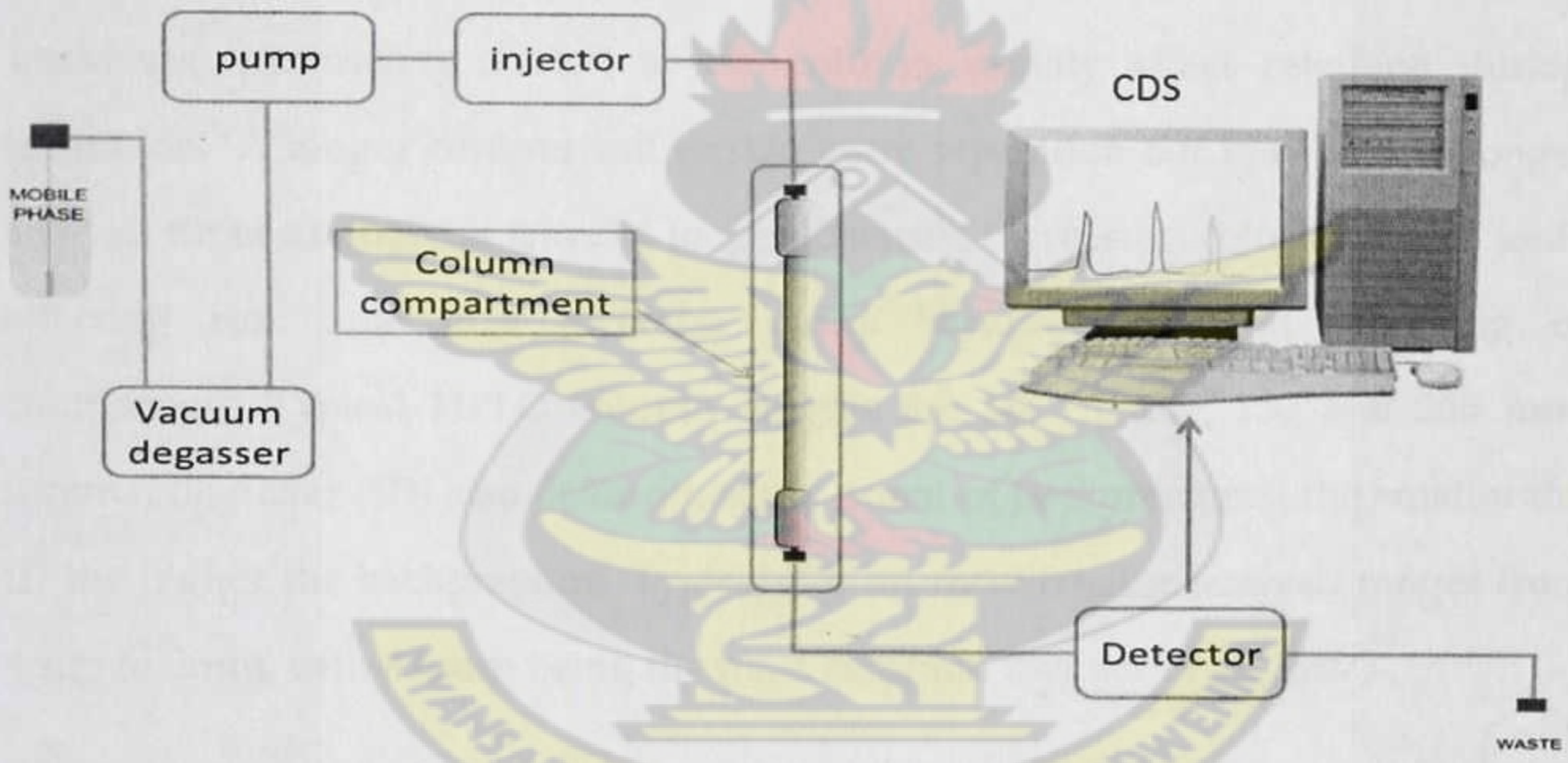


Figure 2-6 A typical HPLC system showing the various parts (Source: Mourne Training Services)

2.1.6.1 The column

Aside the SP packing type and chemistry as described above, other parameters are used to describe the column that have important bearings on separation outcome. These parameters usually refer to the size of the column contents as well as the dimensions of the column and the materials used in its construction.

2.1.6.1.1 Particle size

This is an important parameter of the column. Particle sizes typically range from 1.5 to 10µm. A small particle size means narrower peaks due less band broadening and a better efficiency as well as faster analysis. This however also means high operating pressures are needed to force MP through.

2.1.6.1.2 Pore size

This is the other parameter related to the particle of the column. Porosity is a characteristic of most HPLC particles as separation actually takes place inside the pores. Most columns for analytical separations have utilize 100Å pore sized particles whereas separations that are solely based on size like size exclusion HPLC employ particle pore sizes above 300Å (McPolin, 2009).

2.1.6.1.3 Column length and internal diameter

These two parameters related to the column usually affect retention during separation. A longer column will enable more separation but may lead to longer analysis times as analytes travel a longer distance. Increasing column length aside affecting run time also increases pump backpressures and spreading of components. Typical HPLC column lengths are 50, 75, 100, 150 and 250 mm. Internal diameter (ID) also determines the extent of backpressures; the smaller the ID the higher the backpressure. Typical IDs for most routine analysis ranges from 4mm to 5mm, with 4.6mm being the most common (Synder *et al.*, 1997).

2.1.6.1.4 Column hardware

This describes the material used to construct the external tubing as well as end fittings of the column. These are expected to be highly inert and tough and hence a majority of columns are constructed using stainless steel. Another material that can be used in this regard is polyetheretherketone (PEEK).

2.1.6.2 Mobile phase reservoir

MP reservoirs are typically made of glass and not plastic as additives could leach the MP and get into the HPLC system. Inertness is hence a very important reservoir

characteristic just as cleanliness is. Reservoirs must be of appropriate sizes so as to contain the MP and must have good lids. The lidding must be such that it is not so tight as to create a vacuum inside the reservoir when MP is being pumped (Synder *et al.*, 1997). A tubing system connects the reservoir to the rest of the system and it is usually made of PTFE or other inert material as it should not interact with MP solvent(s) in any way. Inside the reservoir the tubing is connected to inlet-line frit that serves two purposes: filtration of MP before entry (porosity ≥ 10) and acting as a sinker to hold tubing at the bottom of MP. The inlet-line frit is typically made of glass, stainless steel or PEEK (Kar, 2005).

2.1.6.3 Mobile phase degasser

Many solvents contain appreciable amounts of dissolved atmospheric gases which could introduce air bubbles into the HPLC system when MP is prepared from them. During the process, MP is made to pass through a piece of polymeric membrane tubing inside a vacuum chamber that is connected to a vacuum pump. Dissolved gases are hence pumped into across the membrane in the vacuum chamber and the degassed mobile phase is transferred to the pump by PTFE tubing or similar inert material (Synder *et al.*, 1997).

2.1.6.4 The HPLC pump

Being one of the most delicate parts of the HPLC system, the pump serves two major functions: passing MP through the column (1) at high pressures and (2) at a constant controlled flow rate (Kar, 2005). Another less commonly used function of the pump is for online mixing of mobile phase. The pump system in HPLC is the reciprocating single piston pump (RSPP) (Figure 2-7) that is based on a suction-discharge system and utilizes check valves that allow only one way flow of MP (McPolin, 2009). The RSPP comprises a piston which is moved in and out by the use of motor polymeric pump seals which prevent MP leakage out of the pump. The two valves, the inlet and outlet check valves control MP flow. During the suction part of the RSPP operation, pulling of the piston out of the pump head creates a low

pressure region inside it and the effect of this is the closure of the outlet check valve but opening of the inlet check valve. Once the MP has been sucked inside the pump head, the delivery or discharge phase begins. The piston is pushed inside the pump head increasing the pressure inside, resulting in the closure of the inlet and opening of the outlet check valve which forces mobile phase through the column (Synder *et al.*, 1997).

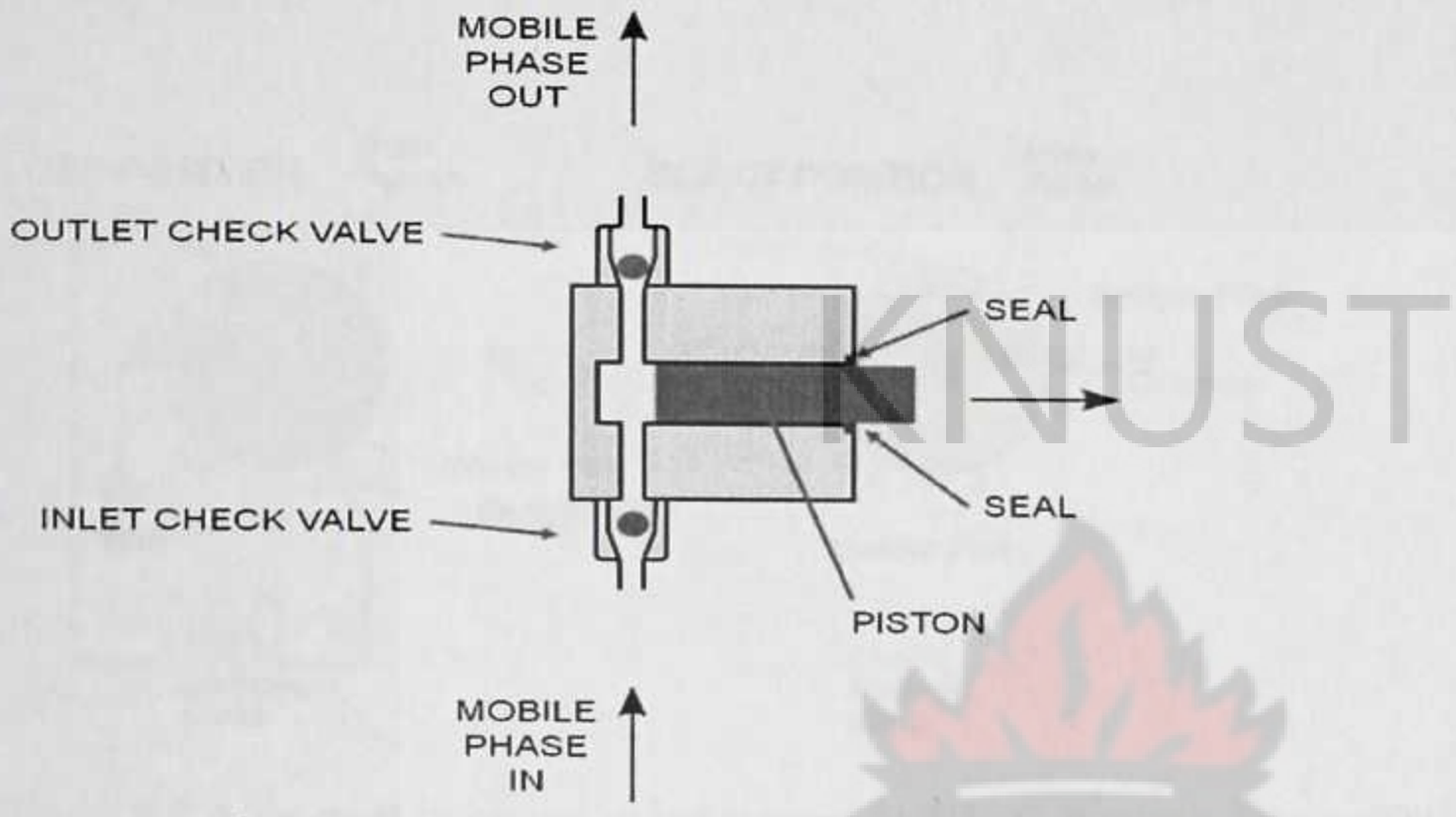


Figure 2-7 A reciprocating single piston HPLC pump (Source: Mourne Training Services)

The single piston pump system introduces pulsation flows during analysis as during suction no delivery of MP is made, a characteristic which is unsuitable for HPLC analysis. In most systems therefore, a dual-piston pumping system is used so that as one suck in MP the other discharges and vice versa, ensuring a constant flow of MP (McPolin, 2009).

2.1.6.5 Injector

An HPLC injector is used to introduce the sample to the column under high pressure. Most injectors employ a 6-port injection valve system that introduces sample into the HPLC system without stopping MP flow. The valve system and principle of operation is shown in Figure 2-8 below. With the valve in the load position as shown, the MP flows from position 2 through 3 into the column. The sample is introduced at the needed port position as shown and enters the sample loop where it replaces any contents in the loop which is connected to waste at this

point. When the injector moves to the inject position, the sample loop is now connected in the way of the MP and hence sample is pumped to the column (Dong, 2006). This manual form of injection is not possible when analysis involves a large number of injections. Under such situations an automatic injection system is needed which is achieved by the use of auto injectors, typically called autosamplers. Autosampler function is also based on the six-port injection system described above (Dong, 2005).

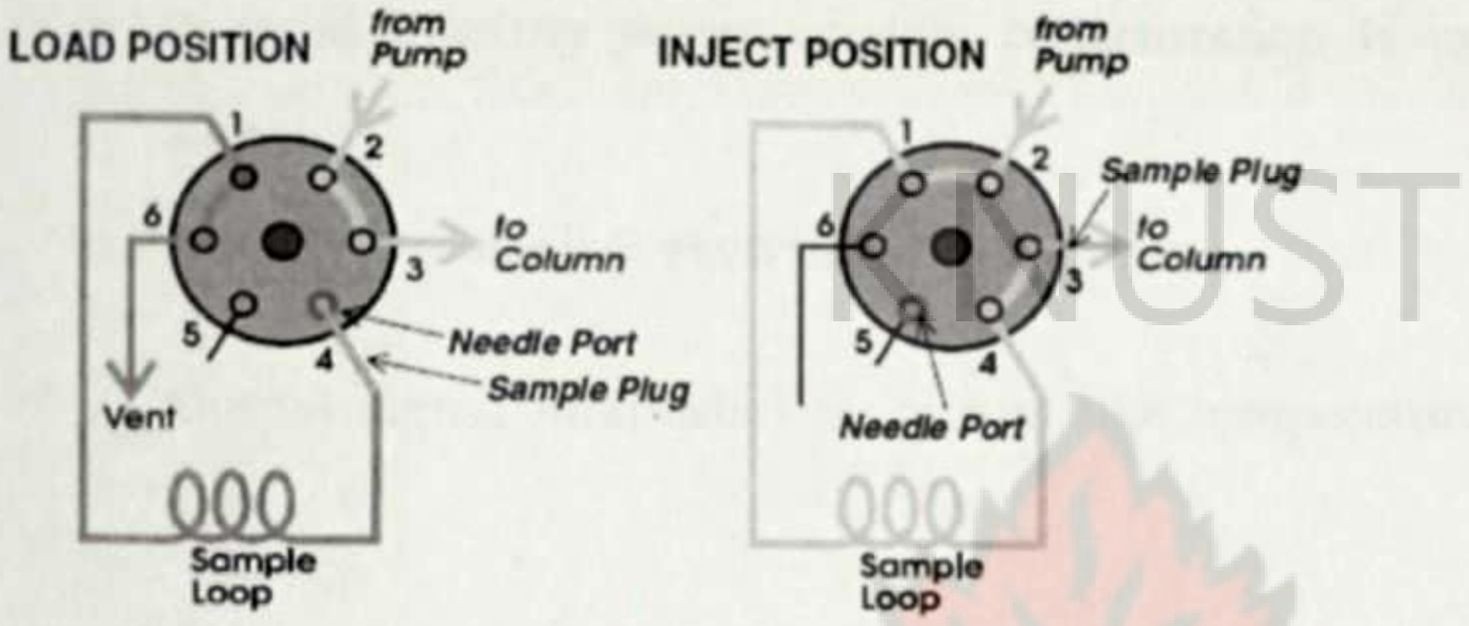


Figure 2-8 A six-port injection valve system in HPLC (Source: Dong, 2006)

2.1.6.6 HPLC detectors

The past 30 years has seen massive improvements in the evolution of detection principles in liquid chromatography and today most HPLC analyses are monitored by one of four detection principles: UV absorption, fluorescence, electrical conductivity and refractive index (Scott, 1986). Typically, a detector consist of two parts: the sensor determines the overall performance of the detector and associated electronics which usually serves as a signal modifier. They are often modified spectrophotometers equipped with a small flow cell that monitors analyte concentration.

Detectors are usually classified under one of two broad categories. Bulk property detectors usually measure some bulk property of eluents exiting the column like refractive index and conductivity include the refractive index and electrical conductivity detectors respectively. One the other hand, the solute property detector measures some physical or chemical property of the solute itself which is

expected to be independent of the MP (Kar, 2005). Examples under the solute property detectors category include the UV and the fluorescence detectors.

An ideal detector must exhibit among others, certain desirable characteristics as listed below (McPolin, 2009):

- Analyte detection in the mobile phase either by bulk property detection mechanisms or by solute property mechanisms (specificity and selectivity).
- A good linearity where analyte concentration is proportional to output signal.
- Detection of analyte even in trace amounts.
- Output signal unaffected by factors like temperature and composition of MP.
- Low noise levels

2.1.6.6.1 UV detectors

UV detection is the most popular and widely used mode of detection in HPLC. This popularity stems from the fact that a majority of pharmaceutical compounds absorb UV light. Depending on the design, three types of UV detectors are in use today: single wavelength, variable wavelength and diode array detectors. In all three, detection is based on the principle of UV absorption by the effluent emerging out of the column and passing through a photocell placed in the radiation beam (Kar, 2005). Absorption of UV light at a stated wavelength generally follows the Beer's law which is converted to a concentration-dependent signal. Typical detector cell volumes range from 5 – 10 mL and light path length from 6 to 10 mm (Jinno, 2002).

The fixed single wavelength detector is the most widely used and the most sensitive. It has a very simple design and is hence the least expensive among the three types of UV detectors. The light source is a mercury lamp that emits light at a

fixed wavelength of 254 nm. The strong emission from the lamp is what contributes to its widespread use as it makes the detector suited to a host of pharmaceutical compound, particularly those that have aromatic systems. Moreover, the intensity of the light beam entering the system allows for a wide linear response range and high sensitivity (Jinno, 2002).

The lamp in a variable-wavelength detector emits light over a wide range of wavelengths. With the help of monochromators, also present in the system the desired wavelength can be selected for analysis. Aside its obvious advantage at allowing the selection of different wavelengths to access the best one for analysis, the variable-wavelength detector also allows wavelength scan while analysis is going which one, but the process involves the cessation of MP flow. It can also be used to confirm peak purity as a spectrum of the analyte can be taken at different points across the analyte peak (Scott, 1986).

The diode array detector (DAD) or photodiode array detector (PDA) is also a multi-wavelength detector like the variable-wavelength detector, except it operates on an entirely different principle. In its operation, a deuterium or xenon lamp emits light over the whole analyte UV spectrum range which is focused on achromatic lens systems through the sample cell and onto a holographic grating. The dispersed light from the grating is subsequently arranged to fall on a linear diode array. A microprocessor then scans the array of diodes several times in one second and the resulting spectrum can be visualized (Scott, 1986). The detector can as a result take the UV spectrum of the eluent continuously throughout the complete development of the chromatogram, which is one of its strong points (Kar, 2005). The DAD is the most suitable UV detector for impurity testing and method development (especially for peak identification). Moreover, it facilitates purity evaluation during method validation (Dong, 2005).

2.1.6.6.2 Fluorescence detectors

Though one of the most sensitive HPLC detectors, the fluorescence detection is not a widely used detection principle especially in pharmaceutical analysis. This limitation is probably due to fact that the detector can only detect analytes that have some degree of fluorescence naturally or by derivitization (Scott, 1986). It however finds widespread usage in trace analysis in the biochemical, food, environmental analysis etc. the detector consists of a xenon source, an excitation and an emission monochromators, a square flow cell and a photomultiplier for amplification of emitted photons (Dong, 2005).

2.1.6.6.3 Refractive index detectors

This detects base on the refractive index (RI) of the bulk eluent from the column. It measures the RI between the eluting analyte in the mobile phase and that of a pure sample of the analyte in a reference cell (Dong, 2005). Though the detector offers the advantage of universal detection, its non-specificity and low sensitivity contributes to its limited use. Moreover it is prone to temperature and flow rate changes (Jinno, 2002). It use is therefore limited to analytes of low chromophoric activities like sugars, triglycerides, organic acids, polymers, and it's the predominantly used detector in size exclusion HPLC.

2.1.6.6.4 Electrochemical detectors (EcDs)

HPLC analysis by EcDs relies on the oxidation-reduction processes of analytes by an applied potential. The form of detection offers sensitivity but limited by the narrow spectrum of analytes that can be electrochemically detected. They are hence mainly employed in the clinical, food and environmental industries (Jinno, 2002).

2.1.6.6.5 Other detection methods

Other less commonly used detectors in HPLC include the evaporative light scattering detector (ELSD), corona-charged aerosol detector (CAD), chemiluminiscence nitrogen detector (CLND), conductivity detector and the radiometric detector.

2.1.6.7 Chromatographic data systems (CDSs)

These are computer software packages that allow control and manipulation of data output from the detector. CDSs are usually supplied by instrument manufacturers and other software specialists. Among other features, CDSs allow instrument control to manipulate various instrument parameters like the flow rate, wavelength, temperature, run time etc. Importantly, CDSs also allow data processing by providing platforms for data manipulation like peak integration with the overall aim to make sense out of the data. CDSs do not only aid in data processing but in their management as well (McPolin, 2009).

2.2 HYPHENATED TECHNIQUES AND SPECIALIZED HPLC SEPARATIONS

Despite the popularity of the four common HPLC detectors mentioned above and the widespread usage of for instance the UV detector, they still face a major limitation; one whose impact is felt most under situations where the molecular structural characterization of analytes is desired (Elipe, 2006). Two analytical techniques that can be called on under such situations to provide structural information on analytes are mass spectrometry (MS) and nuclear magnetic resonance (NMR), and the possibility of combining these two and other analytical techniques with liquid chromatography has led to the term, hyphenated liquid chromatography. The last two decades has seen such hyphenated techniques grow at an amazing rate and have even been successfully applied to solve problems in the drug industry. The two techniques, LC-MS and LC-NMR have hence become indispensable quantification and identification tools in the pharmaceutical industry.

2.2.1 LC-MS and LC-NMR

Due to its high HPLC compatibility and sensitivity, LC-MS has enjoyed a good level of popularity compared to LC-NMR, though recent developments in NMR technology have led to better LC-NMR compatibility and sensitivity. The two techniques have found numerous applications in the areas of natural products and

other drug discovery researches and drug metabolism where is has been applied for the identification of metabolites. They both have also been applied to other critical pharmaceutical industry areas including drug impurities and degradation products (Elipe, 2003).

2.3 HPLC IN THE PHARMACEUTICAL INDUSTRY

The past two to three decades has seen HPLC evolutionarize to such an extent that today, the pharmaceutical industry is non-existent without it. Figure 2-9 below shows the role HPLC play in the various stages of the pharmaceutical streamline which involves the process of discovery of a drug, its development and finally its manufacture. Applications in the drug discovery process involve high throughput screening of pharmaceutical ingredients of therapeutic value, their characterization as well as their metabolism and pharmacokinetic studies (Bleicher *et al.*, 2003). In the development stage HPLC play roles spanning from characterization of active pharmaceutical ingredients (APIs) awaiting chemical synthesis to their analysis for impurities and degradation products. Methods are developed for the API and validated, after which they are transferred to the manufacturing stage where they are used for quality control (QC) procedure.

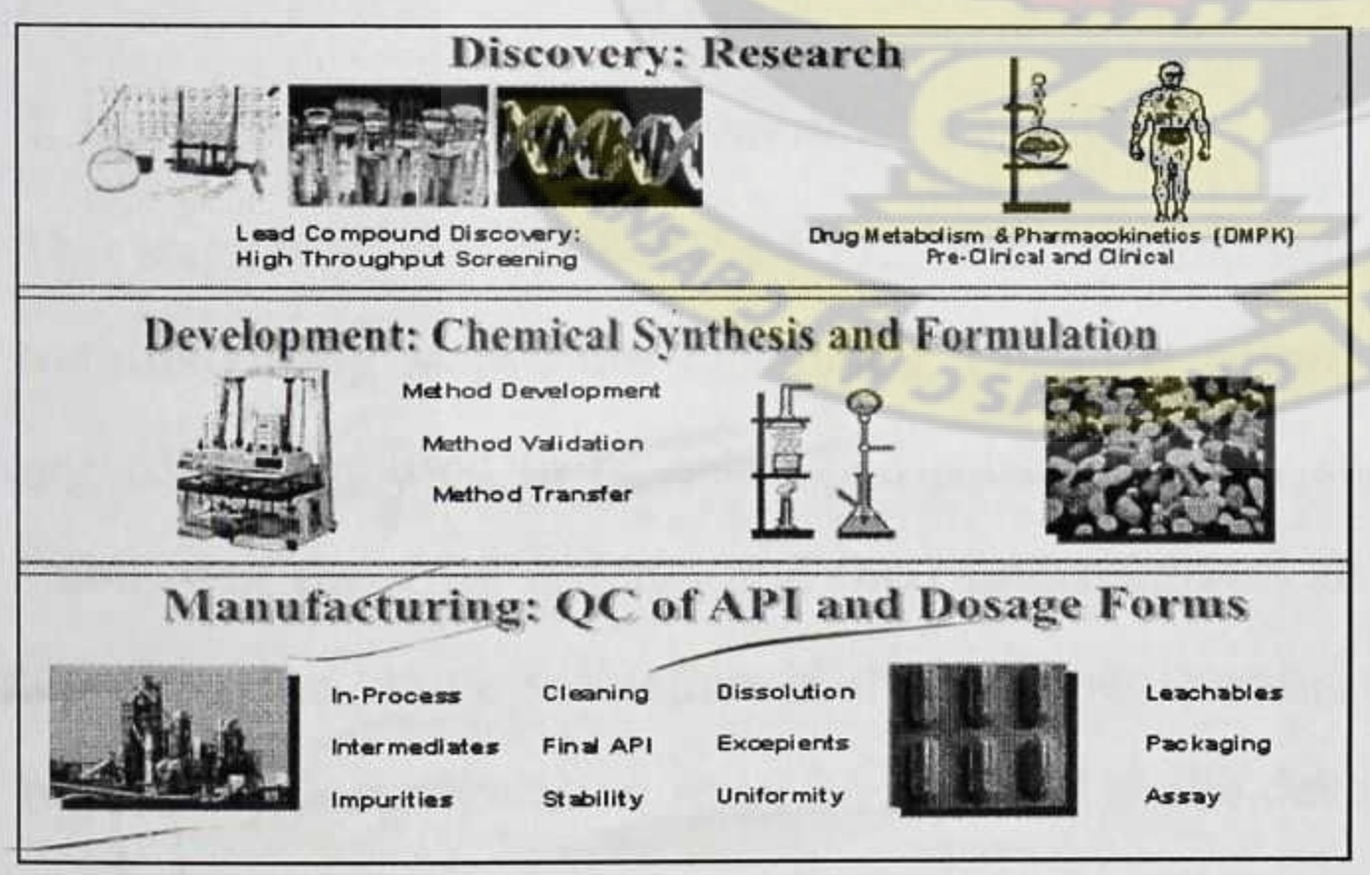


Figure 2-9 HPLC and the role it plays in the pharmaceutical streamline of drug discovery, development and manufacturing (Source: www.bioforum.org.il/HPLC)

2.3.1 Role in drug discovery

HPLC represents one of the most important analytical tools used in the different stages of modern drug discovery as illustrated in Figure 2-10 below.

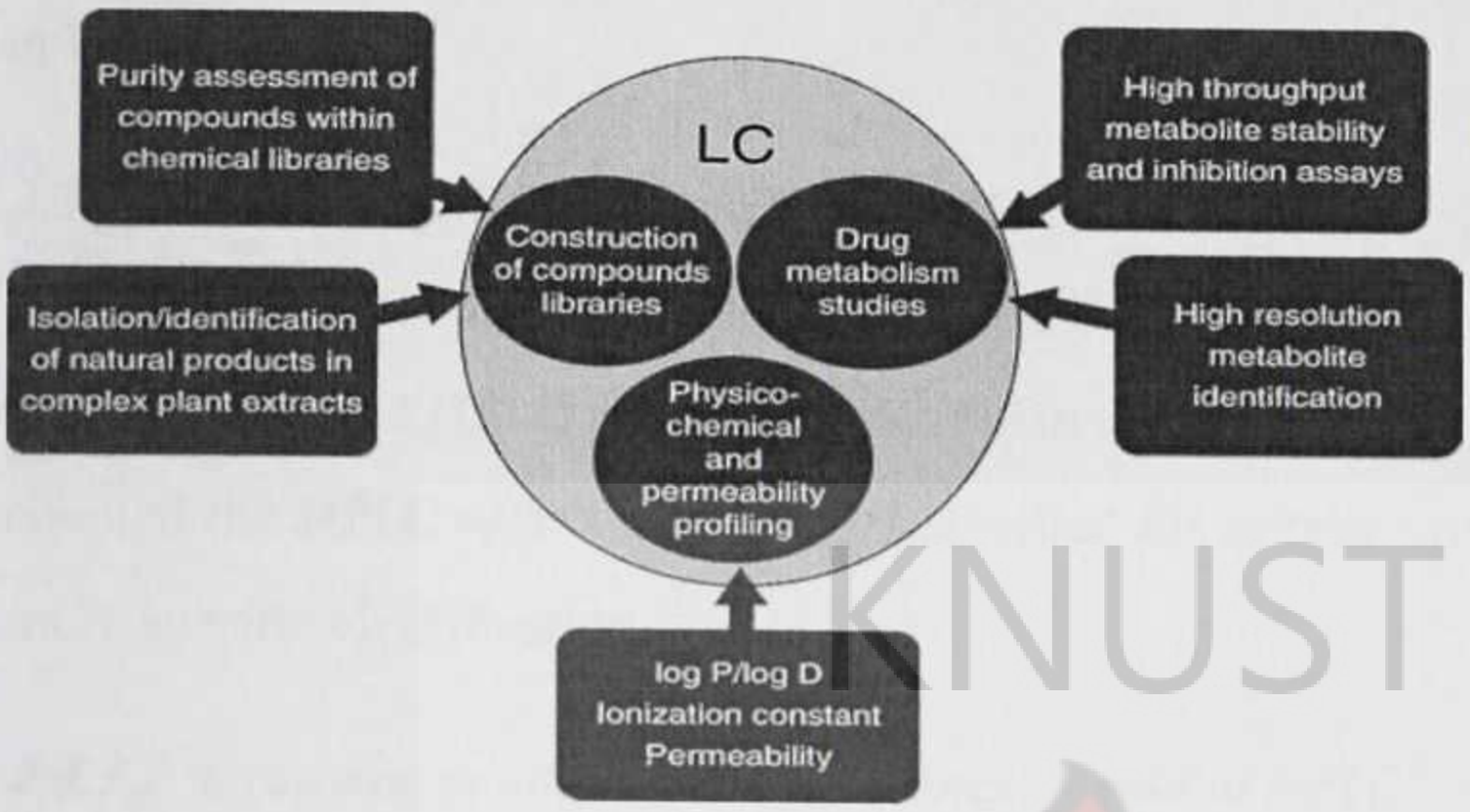


Figure 2-10 The various applications of HPLC in various process of drug discovery (Source: Nicoli *et al.*, 2010)

Recent developments in HPLC have led to the use of sub-2 μm porous particles that requires very high pressure to operate, better referred to as Ultra-HPLC (UHPLC). UHPLC, together with the hyphenated HPLC techniques of LC-MS and LC-NMR have been the major HPLC techniques used in the drug discovery process (Kassel, 2006).

2.3.2 Role in drug development

This stage is where some of the HPLC methods that will be used during subsequent manufacturing stages are developed, validated before being transferred. In this regard, the method to be developed must be fit for such concerns as purity and stability of the drug product and their intermediates all with the goal of aiding in identification. Aside validation of the method, specifications must also be set and regulatory documentation must be provided at the development stage (Thompson and LoBrutto, 2006).

2.3.3 Role in drug manufacturing

HPLC finds numerous uses in the various testing processes of interest under the drug manufacturing stage include identification, assay and content uniformity, dissolution, impurity, stability in-process control and cleaning validation as shown in Figure 2-9.

2.3.3.1 Identification tests – role of HPLC

This is aimed at confirming the identity of the API in either the drug substance or drug product and HPLC plays a pivotal role in the process. More typical application coupled the HPLC with a spectroscopic method for instance mass spectroscopy for more specific identification.

2.3.3.2 Drug assays and related substances – role of HPLC

Drug therapy encompasses three parameters of topmost importance: safety, quality and efficacy. The quality and efficacy are both assessed by the amount of API present in either a drug substance or a drug product. In most cases, products may also contain related products, which are impurities that result from the synthetic process or other processes in the manufacturing stage (Patel and LoBrutto, 2006). Under such circumstances the levels of such related products must be properly monitored and controlled as they may possess unwanted pharmacological and/or toxicological effects. Assay procedures allow judgments to be made on the three parameters of safety, efficacy and quality of drug products. An assay of a drug product measures the actual amount of the API and verifies it against the claim on the product label. In this way the amount of related substances can also be determined. The pharmacopoeias, which contain the official assay methods requires that 10-20 units of tablets or capsules of the drug product is taken, and the API extracted from it before a quantity equivalent to an average unit dosage form weight is taken for assay testing. Typical specifications for most drug products are 90 – 110% of the label claim. Content uniformity tests involve assays of individual solid dosage forms to assess the inter-tablet content variation. Due to its high

selectivity and sensitivity, HPLC has gradually become the first choice analytical method for the above testing procedures (test for related substances, assays and content uniformity).

2.3.3.3 Drug stability studies – role of HPLC

This type of testing is necessary to ensure that the drug substances or products do not vary significantly with time under the influence of some environmental factors like temperature, humidity and light (Carstensen and Rhodes, 2000). This becomes necessary to know the shelf life of the drug substance or product as well as the best storage conditions. The methods employed under such stability studies, more typically called Stability Indicating Methods (SIM) routinely employ HPLC techniques, making HPLC an indispensable tool for a good SIM.

2.3.3.4 Drug impurity testing – role of HPLC

Related substances in a drug substance or product could serve as a source of impurities. Other sources of impurities, the majority of which originate from the API manufacturing process including reagents, solvents, catalysts reaction by-products, may be present. HPLC together with hyphenated techniques like LC-MS have become major analytical tools in this regime.

2.3.3.5 Drug dissolution testing – role of HPLC

A variety of factors underlie the absorption of an API from a drug product after oral ingestion. These include its release from the product, its dissolution under physiological conditions and its permeability across the GIT. An in vitro simulation of dissolution of the product will hence be a good predictor of the in vivo performance. The dissolution test measures the release of the drug substance from its dosage form into a dissolution bath under standardized conditions, specified by the pharmacopeias. At specified time intervals, samples are taken for analysis to assess the release profile of the drug product. HPLC has been the preferred analytical tool for such dissolution testing due to its separation capabilities, leading to high specificities and sensitivities. This is necessary especially if excipients have

the tendency of interfering with analysis. HPLC methods are also the first choice method for dissolution testing particularly when dealing with a multi API drug product or one which is in a very low dose (Patel and LoBrutto, 2006).

2.3.3.6 In-process control and cleaning validation – role of HPLC

In-process control encompasses methods that the overall progress of the manufacturing process of the pharmaceutical substance or its product. Typical in-process control methods adopt HPLC techniques due their speed of analysis as well as their efficiency and sensitivity. Results from the analysis is a signal to the manufacturing unit to either proceed or halt a given unit operation.

Cleaning validation tests are performed to assure the cleanliness of the pharmaceutical manufacturing equipment, such as blender, tablet press, etc. This is necessary as there are regulations concerning the maximum allowable amounts of residues the equipment can habour. The analytical method to be employed under such situations are expected to be highly sensitive to record the smallest levels of any analyte residues, their degradation products and impurities, or even the cleaning reagents (Fountain *et al.*, 2007). With the entire manufacturing process dependent on the cleanliness of the equipment, the method to be adopted for cleaning validation is expected to be highly sensitive and short; characteristics that are offered better than no analytical method other than HPLC.

2.4 QUANTIFICATION IN HPLC ANALYSIS

The high popularity and widespread usage of HPLC techniques today is as a result of its remarkable quantitation abilities, one of its fundamental strongholds (Wang, 2002). Its quantitation capabilities span from being used to quantify major component in purified sample, to components of a reaction mixture and even trace impurities in a complex sample matrix. Quantitation is based on the response of the detector in relation to the concentration of the sample. The two main detector response parameters used in quantitation are the peak height and area. The peak

height method though the simplest way to measure the detector response is still a less common method. It is however the preferred method for trace analysis and also for well resolved, symmetrical peaks.

All quantitation procedures involve a calibration of the HPLC equipment with a standard. In this regard, three calibration techniques that arise: using an external standard, using an internal standard and using a standard addition.

2.4.1 Calibration by external standards

This is the preferred and most general calibration method especially for samples that do not require extensive preparation. Two quantification approaches are possible under this method. The first and most common approach involves using various prepared concentrations of the analyte, called external standards to make a calibration plot. The plot is obtained by plotting the concentrations against their respective peak responses. Next, the unknown sample is injected and analyzed in a likewise manner, after which its concentration is determined graphically from the plot using its peak response (Synder *et al.*, 1997). It is essential that such calibration plots must have a good linearity as well as encompass the concentration expected.

The second approach involves the use of what is referred to as response factors (RF). Also called a sensitivity factor, a response factor is calculated from a standard within the linear calibration range as:

$$\text{RF} = \frac{\text{Standard peak response}}{\text{Standard concentration}}$$

Once the RF has been determined, the concentration of the unknown analyte can then be calculated as:

$$\text{Sample concentration} = \frac{\text{Sample peak response}}{\text{RF}}$$

In the event that two or more sample concentrations are used, the average RF is preferable especially to minimize any uncertainties in the RF determination (Wang, 2002).

This method is referred to as 'external standards' method because the standards are analyzed in a separate chromatogram than that of the sample. Reproducibility of sample injections is hence one fundamental source of error that comes with this method of calibration, especially for manual injections. It is also imperative that all chromatographic conditions be the same for both samples and standards for good quantification results using this method.

2.4.2 Calibration by internal standards

This method calibration involves the addition of what is referred to as an internal standard (int.std) to both calibration solutions and samples. Such a standard is usually different compound from the analyte that can be well resolved from the analyte during separation. Especially necessary for samples requirement a high degree of pretreatment, this method is essential to compensate for any sample losses as a result of the pretreatment. The various pre-treatment procedures include derivatization, filtration, extraction etc. and the internal standard is expected to mimic the sample during these procedures, hence correcting for any losses (Synder *et al.*, 1997). The method also involves the generation of a calibration plot, this time by using varying concentrations of the standard each containing a fixed concentration of the internal standard. The standard-int.std peak area ratio is calculated for each concentration level and plotted against the various concentrations. The unknown sample-int.std area ratio is next determined after injection of the sample also containing the same fixed amount of the internal standard, and then its concentration is determined graphically from the plot (Vial and Jardy, 2002).

Just like in the external standards method, RFs can also be used here, under which case the RF is calculated as:

$$RF = \frac{X}{\text{Standard concentration}}$$

where X is the standard-int.std peak area ratio.

The sample-int.std peak area ratio is also determined and the sample concentration is determined from the RF as:

$$\text{Sample concentration} = \frac{\text{Sample-Int.Std are ratio}}{RF}$$

Internal standards are expected to meet certain basic criteria which include: similar retention (k) to the analyte; does not have to be chemically similar to analyte, must be commercially available in high purity; must be stable and inert to sample or mobile phase; should have a similar detector responses to analyte for the concentration used (Synder *et al.*, 1997). The most important and hence challenging requirement is the fact that the internal standard must be separated and well resolved from all analytes in the sample. This makes the method unsuitable for complex-analyte samples. The internal standard method with its advantages might not always produce improved results and hence the external standards method is still the preferred.

2.4.3 Calibration by standard addition

Often used in trace analysis, this method involves the addition of different weights of analyte(s) to a sample matrix that initially contains an unknown concentration of the analyte. The peak response for each standard addition concentration level is plotted against the amount added for each, and the concentration of the unspiked sample is extrapolated from the liner plot using its peak response (Synder *et al.*, 1997). This method is a powerful one that enables the quantification of an analyte present in a matrix susceptible to modify its behavior. It is however a tedious method of calibration it requires many preparations and injections to obtain enough points for a sufficient reliability (Vial and Jardy, 2002).

2.5 REFERENCE STANDARDS

All the calibration methods described above require standards of the analyte to operate making standards a very critical part of HPLC analysis. These standards are expected to be prepared from reference materials (RM) or standards (RS). Reference standards are hence highly characterized specimens of drug substances, excipients, impurities and degradation products that are used not only in quantification but also identity and purity checks (Konieczka and Namiesnik, 2009). HPLC reference standards are expected to bear a high degree of quality and purity. Certified Reference Material (CRMs) are those reference standards that are accompanied by documentation issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceabilities, using valid procedures (Konieczka and Namiesnik, 2009). In the pharmaceutical industry, such authoritative bodies are usually the Pharmacopoeial Commissions and regulatory agencies like the ISO and FDA. The various pharmacopoeial reference standards include the USP reference standards (USP RS), BP chromatographic reference standards (BPCRS), Ph. Eur chemical reference standards (Ph. Eur CRS) and the international pharmacopeia chemical reference standards (WHO CRS) (SFDA, 2010). These materials are usually highly pure and hence need no further characterization before their use. Three types of reference standards are recognized by the pharmacopoeias and other regulatory agencies: primary reference standard, secondary or working reference standard and house (non-compendial) reference standards.

The primary reference standards are those supplied by the pharmacopoeias as described above. These are usually very expensive due to their highly purified and characterized nature. A cheaper, though still expensive alternative is offered by secondary reference standards. These are supplied by reagent manufacturers and are developed by analyzing and validating a lot of the drug substance against the primary standard. Non-compendial reference standards are usually for new

chemical entities (NCE) that have yet to have a compendial standard developed for it. Under such circumstances, the material must be highly purified after manufacture and fully characterized as well as structurally elucidated (SFDA, 2010).

2.6 HPLC METHOD DEVELOPMENT

The four important reasons that will call for a new analytical method (Dong, 2006) are:

- Unavailability of existing methods (in the case of NCEs)
- Concerns about reliability, sensitivity/selectivity or cost effectiveness of existing methods
- Better performance for new method (instrumentation)
- The method is needed for regulatory compliance

In general, method development usually follows a common set of strategies as illustrated in Figure 2-11 below:

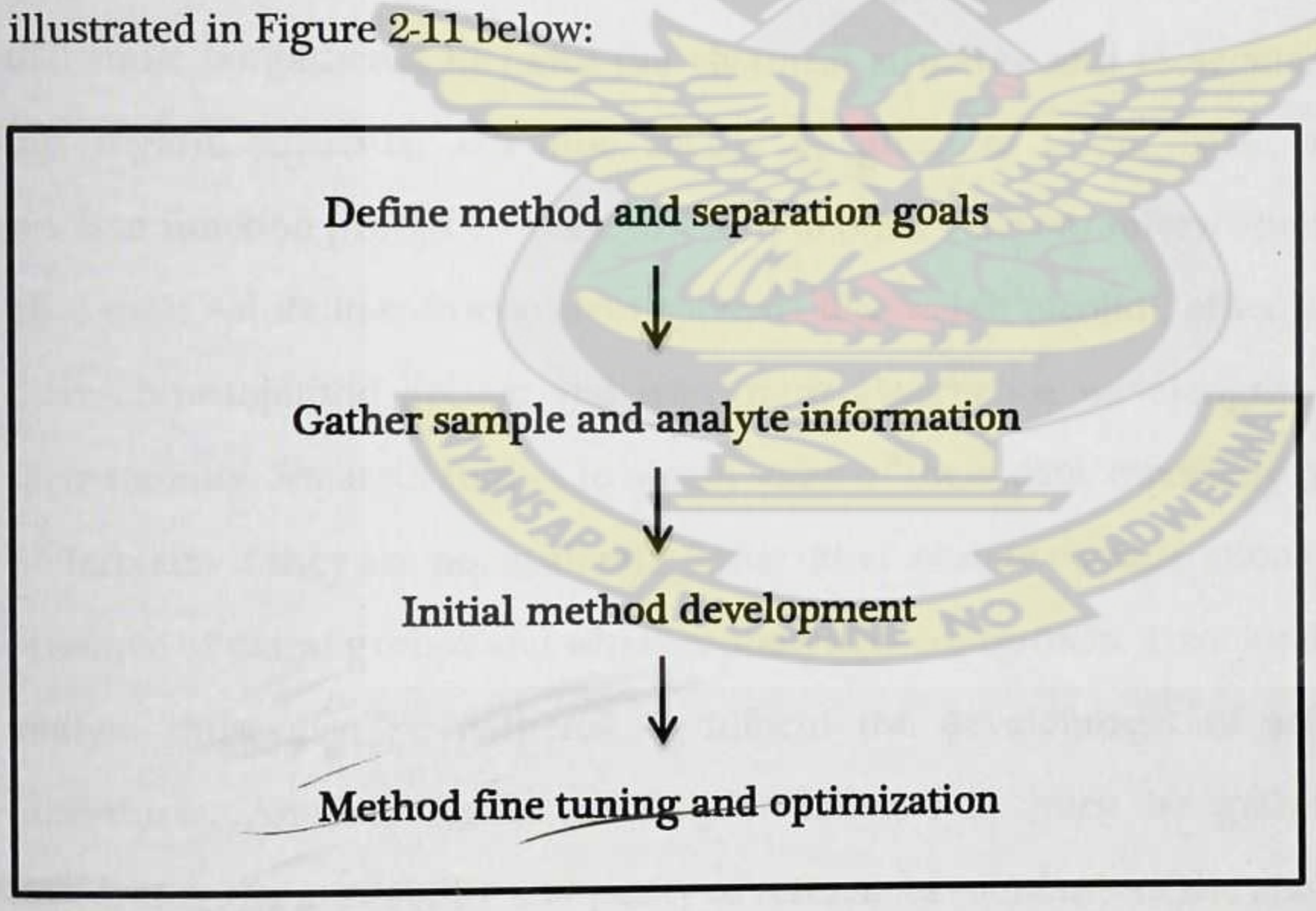


Figure 2-11 Typical steps involved in developing a new HPLC method

2.6.1 Defining method and separation goals

The type of assay desired, whether single-analyte or multi-analyte is considered as the method will have to be developed with all analytes in mind. Also whether the method to be developed is going to be qualitative, quantitative or preparative must also be considered.

The goals of the method with regards to type of resolution, precision, specificity and sensitivity desired must also be defined. Typical methods for assaying an API for instance must have ≥ 1.5 resolution between component peaks, retention time and peak area precision <1-2% RSD, linearity in the range of 50-150% of the label claim (Dong, 2006).

2.6.2 Gathering sample and analyte information

This is the next step after defining the goals of the method. It requires gathering as much information about the analyte(s) as possible with the aim of factoring all that in to develop the most effective method. With regards to the analyte(s), information that must be gathered includes the chemical structure and molecular weight. In this regard, particular attention should be given to acidic, basic, aromatic or reactive function groups as those will lead to other relevant information on analyte pKa, their solubilities in common solvents like water, alcohol, ether, and hexane, their chromophoric groups and maximum absorbance wavelength, and finally their stability. Separate studies to obtain some of these data, especially pKa must be undertaken if they are not available. Some other analyte considerations include the presence of chiral groups and whether analyte(s) are isomers. Toxicity data on each analyte must also be gathered to inform the development of safe handling procedures. Another fundamental information that must be gathered is that concerning the availability and purity of references standards of the analyte(s).

Sample preparation requirements are also an important concern under this stage of method development. The various types of preparation include solid handling

(grinding, milling, homogenization), extraction (shaking, ultrasonificaiton, liquid-liquid partitioning, solid phase extraction), liquid handling (pipetting, diluting, pH/ionic strength adjustment), phase separation (filtration, centrifugation, precipitation) and sometimes derivatization (Dong, 2006).

2.6.3 Initial method development

During this stage, a preliminary set of conditions are selected to obtain 'scouting chromatograms'. Considerations that must be made include detector selection, chromatographic mode and type selection, column selection and mobile phase selection. Whether an isocratic or gradient method of elution would be used must also be considered under this stage.

With most pharmaceutical compounds having one or more chromophoric groups, the UV detector is the first choice for most methods. The polarity and molecular weight of the analyte will inform the mode and type of HPLC method to be developed, with RP-partition HPLC being the most likely. A starting point for a column remains silica based C18 or C8. Starting point column specifications are a 5µm particle size, 100 – 250mm column length and 4.6mm ID. Mobile phases must be selected after consideration of all relevant parameters as discussed under mobile phase and solvents above. Buffered mobile phases must be used when ionizable analytes are present to control pH effects on retention and ion pairing agents can also be used to refine the separation (McPolin, 2009).

Usually the first step involves using a broad scouting gradient to obtain the first chromatograms. If such problems as insufficient retention, bad peak shape or poor sensitivity is encountered, other approaches like a change in HPLC mode, column, MP should be explored.

2.6.4 Method fine tuning and optimization

It is essential that maximum time as possible is spent on this stage to rule out any problems during the method validation phase. All HPLC methods aim at achieving

a good resolution, with sufficient precision and sensitivity in a reasonable time. As a result, fine tuning of the method after the initial scouting procedures is essential to achieve this goal. Various parameters are modified under this stage of method development to achieve specific outcomes.

2.6.4.1 Mobile phase parameters

Various mobile phase parameters that can be modified include percentage organic solvent content, buffer type and concentration, pH and solvent type. Retention and selectivity are the main descriptors that are affected by modifications in this regard. Chromatographers by convention, refer to the strong in a mobile phase combination as the 'B' solvent and the weak one as the 'A' solvent. Decreasing the %B content of the mobile phase increases the resolution of peak but results in an increased retention time compared to the same mobile phase with a high %B content (McPolin, 2009). A popular rule in HPLC analysis for small RP analysis is the 'Rule of 3' which states that a 10% increase in %B content will result in a retention time reduced by a factor of 3. Buffers are especially required when analytes contain ionizable groups. Typical buffer concentrations range between 10-25mM. The mobile phase pH is a powerful characteristic that controls the retention of analytes. Sometimes selectivity can be improved with a change in the B solvent, for instance from ACN to MeOH.

2.6.4.2 Operation parameters

Flow rate and temperature are the two main operation parameters that affect retention and to a less extent selectivity during a HPLC separation. Increasing column temperature and MP flow rate result in a reduced retention (Dong, 2006). In gradient elution two other operation parameters that can be modified to obtain an improved resolution are the gradient time and gradient range.

2.6.4.3 Colum parameters

This is usually a last resort that is turned to only when all other modifications of mobile phase and operation parameters do not lead to any improve resolution.

Under such situations, a complete change of column specifications like bonded phase type, length, particle size and internal diameter is recommended.

2.7 METHOD VALIDATON

Before a method's release for routine laboratory use, enough evidence must be provided that proves that the method is suited for its intended purpose. This evidence collection and documentation process is what method validation is about and involves a set of prerequisite tests usually prescribed by regulatory bodies (Samanidou and Ioannis, 2009). The various method validation parameters as stipulated by the ICH and the USP are discussed below.

2.7.1 Specificity

This is the methods ability to distinguish an analyte of interest from other components in a sample like impurities, degradants or even excipients (Dong, 2006). Demonstration of specificity can be done by injection of a blank sample to prove non-interference from reagents and contaminants. A placebo can also be injected to demonstrate non-interference from excipients. Finally, peak purity assessment can be done to demonstrate non-interference from other analytes using a PDA or even a MS.

2.7.2 Linearity and range

Linearity is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range. Linearity is generally reported as the variance of the slope of the regression line. The ICH stipulates a correlation coefficient (r) value ≥ 0.998 for APIs and ≥ 0.998 for impurities (ICH, 1996). The range describes the interval between the upper and lower concentrations of the analyte that have been demonstrated to be determined with precision, accuracy and linearity using the method. It is usually expressed in the same units as the test results obtained by the method. A minimum of five concentration levels

prepared from a stock is stipulated by the ICH to demonstrate linearity and range. Various minimum specified ranges are expected to be met for various tests; for assay testing, it is 80 – 120% of the target concentration (Swartz and Krull, 1997).

2.7.3 Precision

Normally expressed as the %SD for a statistically significant number of samples, precision measures the extent of agreement or repeatability of an analytical method. By ICH requirements, the measured SD can be subdivided under three major categories: repeatability, immediate precision, reproducibility (ICH, 1996).

Repeatability refers to results obtained by the performance of the analysis over a relatively short time-span by the same analyst in the same laboratory. It involves minimum of 6 measurements of the same sample or three repetitions each of 3 different concentration levels (80%, 100%, 120%) combined with accuracy. The determined relative standard deviations (RSD) must be $\leq$ 2% for acceptable repeatability of assays (Swartz and Krull, 1997).

Intermediate precision (considered a part of ruggedness) has been defined by the ICH as the long-term variability of the measurement process and is determined by comparing the results of a method run within a single laboratory over a number of weeks. A method's intermediate precision may reflect discrepancies in results obtained by different operators, from different instruments, with standards and reagents from different suppliers, with columns from different batches or a combination of these (Taylor, 1983). The RSD requirements for intermediate precision are the same as for repeatability.

Reproducibility, the less common of the three precision subcategories refers to the deviations determined when different laboratories perform the same analysis using the developed method. The objective is to verify that the method will provide the same results in different laboratories, preparing it for the transfer to other sites.

2.7.4 Accuracy

This is a measure of the extent to which test results are close to their true value. Accuracy is measured as the percentage of analyte recovered by assay, by spiking samples in a blind study. For the assay of a drug substance, accuracy measurements are obtained by comparison of the results with those of a standard reference material, or by comparison to a second, well-characterized method. For the assay of a drug product, accuracy is evaluated by analyzing synthetic mixtures spiked with known quantities of components. For impurities, accuracy is demonstrated by analyzing samples (drug substance or drug product) spiked with known amounts of impurities. The ICH stipulated methodology requires a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range. Data must be reported as the percent recovery of the known, added amount, or as the difference between the mean and true value with confidence intervals (Swartz and Krull, 1997)

2.7.5 Limit of detection (LOD)

This is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified. It is expressed as a concentration at a specified signal-to noise ratio, usually a 2- or 3-to-1 ratio. Aside this signal-to-noise ratio method, the ICH also recognizes a calculation method for the determination of LOD. It is based on the SD of the response (σ) and the slope (S) of the calibration curve at levels approaching the LOD as illustrated in the equation below:

$$\mathrm{LOD} = \frac{3.3\sigma}{S}$$

2.7.6 Limit of quantification (LOQ)

Unlike LOD, LOQ is the minimum injected amount that gives precise measurements. However, like LOD, LOQ is also expressed as a concentration, with the precision and accuracy of the measurement also reported. A signal-to-noise ratio of 10-to-1 is recognized by the ICH for the determination of LOD. However,

like LOD, a calculation method is also recognized as illustrated in the equation below:

$$\text{LOD} = \frac{10\sigma}{S}$$

For both LOD and LOQ the SD of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y intercepts of regression lines (Swartz and Krull, 1997).

2.7.7 Robustness

This is a methods ability to remain unaffected by small but deliberate variations in method parameters like pH changes, mobile phase composition, column changes, temperature and flow rate.

2.8 ANALYTICAL PROFILE: DRUG SAMPLES

2.8.1 Glibenclamide

Glibenclamide, also called glyburide is a sulfonylurea anti-diabetic drug developed in 1966 and has the IUPAC name 1-[4-[2-(chloro-2-methoxybenzamido)ethyl]-benzenesulphonyl]-3-cyclohexyl-urea. Glibenclamide is also called 5-chloro-N-[2-[4[[[(cyclohexyl(amino)carbonyl]-amino]sulfonyl]-phenyl]ethyl]-2-methoxy benzamide or 1-[[p-[-2-(5-chloro-o-anisamido)ethyl]phenyl]-sulphonyl-3-cyclohexylurea (Takla, 1981; Zaman *et al.*, 2006). The molecular structure of glibenclamide is shown below.

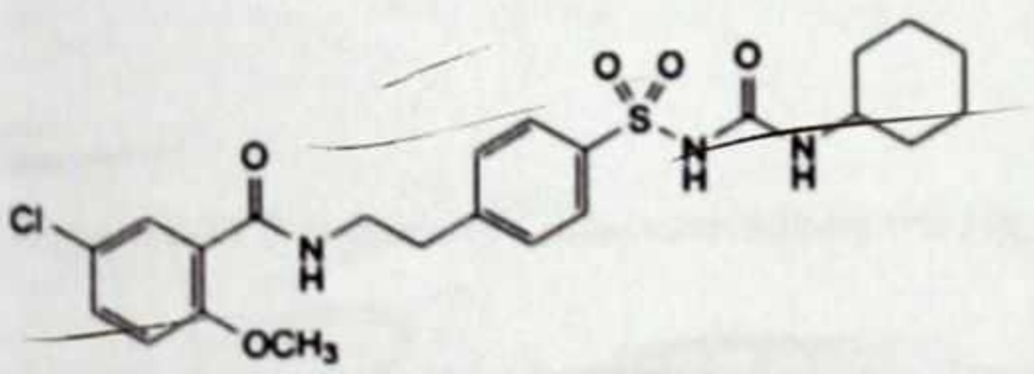


Figure 2-12 Structure of Glibenclamide (Source: BP, 2009)

Glibenclamide (MW = 494g/mol) is a white, crystalline, odourless powder; practically without taste that melts between 164°C and 174°C. With regards to solubility, it is virtually insoluble in water and ether; soluble in 330 parts of alcohol, in 36 parts of chloro-form, and in 250 parts of methanol (Moffat *et al.*, 2004). It has an octanol/water partition coefficient of 4.8 and forms water-soluble salts with alkali hydroxides. Glibenclamide is a weak acid and it has been concluded that it has the same dissociation constant as tolbutamide (5.3 ± 0.1), since both compounds show the same dissociation at half-neutralization in solvent mixtures such as methyl cellosolve and water or methanol and water. The direct determination of its pKa in water is impossible owing to its low solubility (Takla, 1981).

Various methods have been described and patented for the synthesis of glibenclamide. Described below (Figure 2-13) is one by (Hsi, 1973). In this method, the N-acetyl derivative of β-phenethylamine is reacted with chlorosulfonic acid to form the para sulfonyl chloride derivative, which is then subjected to ammonolysis, followed by base-catalyzed removal of the acetamide. The resulting product is then acylated with 2-methoxy-5-chlorobenzoic acid chloride to give the amide intermediate after which it is reacted with cyclohexyl isocyanate to yield the sulfonylurea glibenclamide.

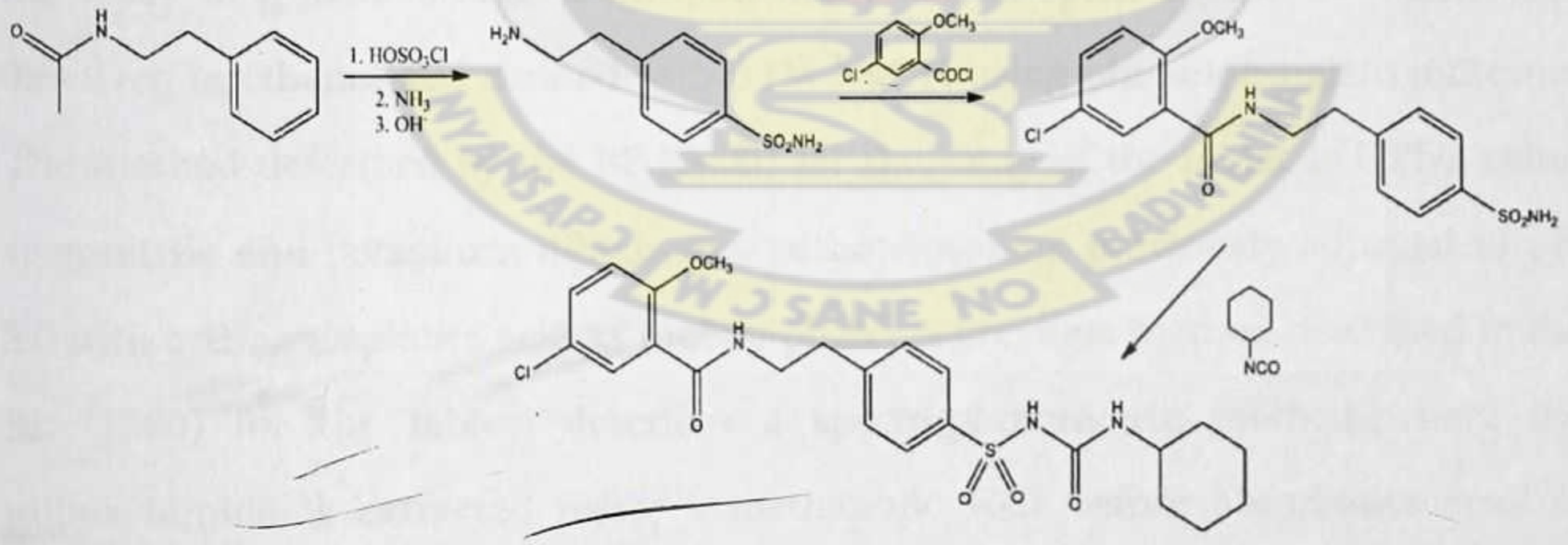


Figure 2-13 Synthesis of Glibenclamide by the Hsi method

Listed as one of only two anti-diabetic drugs in the WHO list of essential medicines, glibenclamide works by inhibiting ATP-sensitive potassium channels in pancreatic

beta cells. This inhibition causes cell membrane depolarization opening voltage-dependent calcium channel which in turn results in an increase in intracellular calcium in the beta cell and subsequent stimulation of insulin release (Serrano-Martín *et al.*, 2006). Hence it lowers blood glucose concentration principally by stimulating secretion of endogenous insulin from the beta cells of the pancreas. Other mechanisms of the hypoglycemic action associated with short-term glibenclamide therapy appear to include reduction of basal hepatic glucose production and enhancement of peripheral insulin action at postreceptor site(s). Glibenclamide may have extrapancreatic effects as well as it seems to enhance peripheral sensitivity to insulin and also reduce basal hepatic glucose production.

The various methods that have been used in the analysis of glibenclamide include polarography, non-aqueous titration, spectrophotometry and the most popular of all, chromatography (Takla, 1981). Identification tests for glibenclamide as given in the BP (2009) depend upon a) its melting point; b) its infrared absorption spectrum; c) its light absorption in the range 230 to 350nm; d) its coloured product formation when reacted with sulphuric acid and subsequently with chloral hydrate. For the tablet, identification as given in the BP (2009) depend upon its retention time in comparison with that of a glibenclamide BPCRS as well as TLC on silica plate. In the assay of glibenclamide, a titration method is adopted by the BP where it is dissolved in ethanol and titrated with 0.1M NaOH using phenolphthalein indicator. The method described by the BP (2009) for the assay of the tablet is HPLC using acetonitrile and potassium dihydrogen orthophosphate previously adjusted to pH 3.0 with orthophosphoric acid as mobile phase. A previous method described in the BP (1980) for the tablets describes a spectrophotometric method where the glibenclamide is extracted using a methanolic HCl before absorbance read at 300nm.

2.8.2 Naproxen

Naproxen, a propionic acid derivative, is an over-the-counter (OTC) non-steroidal anti-inflammatory drug (NSAID) that was developed in 1976. The drug is structurally and pharmacologically related to fenoprofen and ibuprofen (Uysal and Tunçel, 2004). Its various chemical names include (S)-6-Methoxy-α-methyl-2-naphthalene acetic acid; d-2-(6-methoxy-2-naphthyl)propionic acid; 2-naphthalenacetic acid; 6-methoxy-α-methyl-,(+); (+)-2-(6-Methoxy-2-naphthyl) propionic acid; and (+)-6-Methoxy-α-methyl-2-naphthaleneacetic acid (Al-Shammary *et al.*, 1992). The structure of naproxen (MW = 230.26g/mol) is shown below.

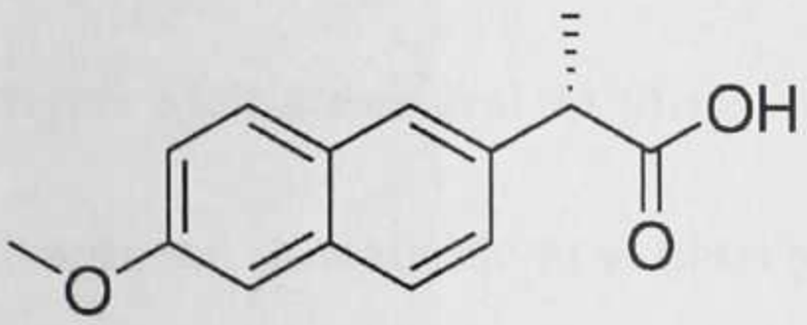


Figure 2-14 Structure of Naproxen (Source: BP, 2009)

Naproxen is an odourless or almost odourless white to off white crystalline powder with a bitter taste and has a melting point range of 152 – 156°C. With regards to solubility, it is practically insoluble in water, soluble in 25 parts of ethanol (96%), in 20 parts of methanol, in 15 parts of chloroform and in 40 parts of ether (Moffat *et al.*, 2004). It has a dissociation constant at 25°C to be 4.2 and an octanol/water partition coefficient of 3.2.

Various methods have been described for the synthesis of naproxen (Harrington and Lodewijk, 1997). The method described here is the original method by Harrison *et al.* (1970). In the method, naproxen is prepared by the acylation of substituted naphthalenes by AcCl forming the 2-acetyl derivative, which is further converted to 2-naphthyl acetic acid. Esterification and alkylation of 2-naphthyl acetic acid in the presence of H_2S0_4, MeOH, NaH, MeI and with NaOH gave after hydrolysis the naphthylpropionic acid. Resolution of 2-(6-methoxy-2-naphthyl) propionic (Naproxen) is readily achieved by crystallization of the cinchonidine salt.

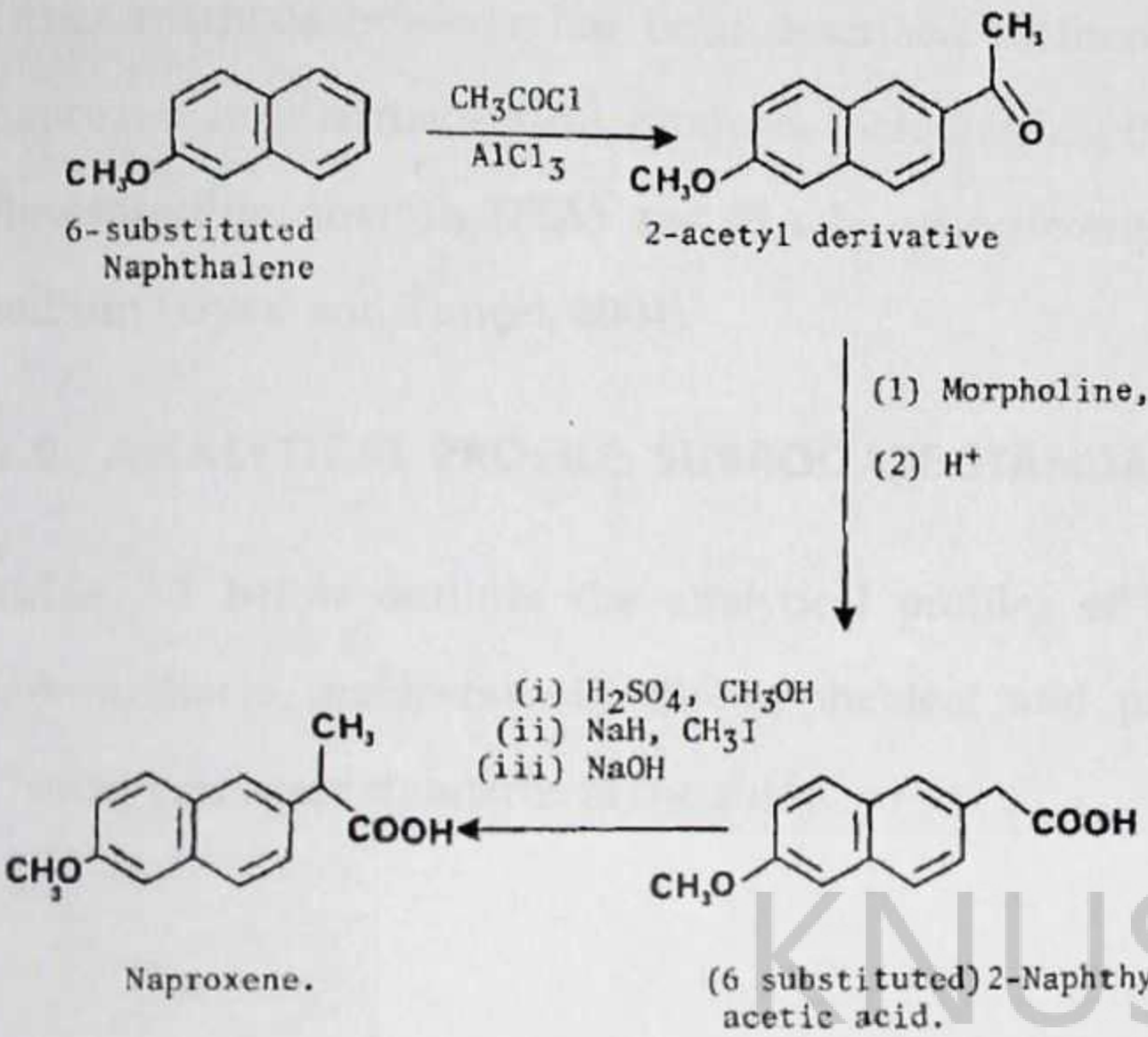


Figure 2-15 Harrison et al (1970) method for synthesis of naproxen

Naproxen is available in various preparations including tablets, oral suspension and suppositories (BP, 2009).

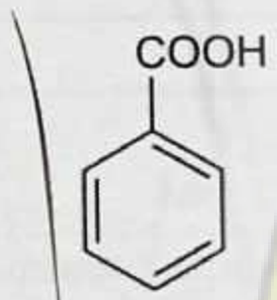
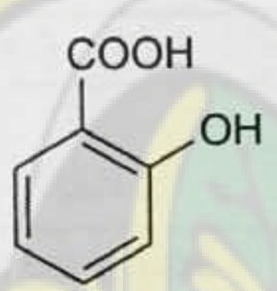
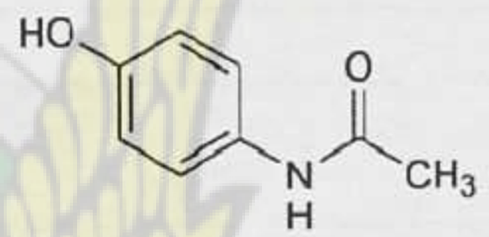
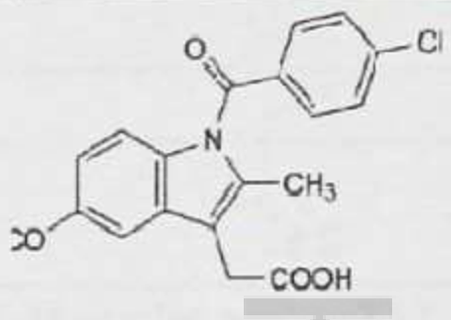
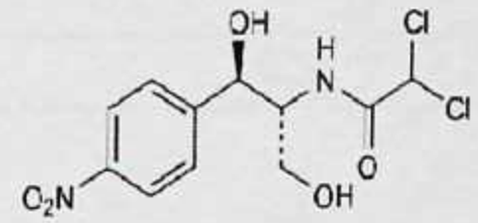
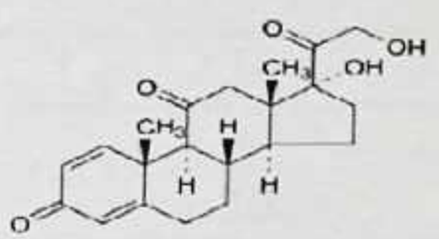
Naproxen possesses analgesic, anti-inflammatory and antipyretic activities and is indicated for the relief of symptoms of rheumatoid arthritis, osteoarthritis and ankylosing spondylitis (Idowu *et al.*, 2009). Like all NSAIDs, it acts by reducing the levels of prostaglandins, chemicals that are responsible for pain, fever and inflammation. Naproxen blocks the enzyme that makes prostaglandins (cyclooxygenase), resulting in lower concentrations of prostaglandins.

Identification of naproxen as recognized by the BP (2009) depends on 1) its infrared absorption spectrum; 2) its UV absorption between 250 – 300nm; 3) it melting point; and finally 4) it behaviour in colour reaction tests like the Liebermann's, Marquis and sulphuric acid. For the tablets, identification is by infrared spectroscopy and UV absorption. The official BP method for the assay of the API adopts a titration with 0.1M NaOH using phenolphthalein as indicator. The naproxen in dissolved in a water-methanol solution prior to titration. For the tablets, the method described is by UV spectroscopy at a wavelength of 331nm.

Other methods however has been described in literature for the estimation of naproxen in pharmaceutical products including capillary electrophoresis, HPLC, flow-injection analysis (FIA) and FIA by using complex formation of naproxen sodium (Uysal and Tunçel, 2004).

2.9 ANALYTICAL PROFILE: SURROGATE STANDARDS

Table 2-3 below outlines the analytical profiles of benzoic acid, salicylic acid, indomethacin, paracetamol, chloramphenicol and prednisolone, which are the chosen surrogate standards in the study.

	Benzoic acid	*Salicylic acid*	*Paracetamol*	*Indomethacin*	*Chloramphenicol*	*Prednisolone*
Chemical Name (S)	Benzenecarboxylic acid	2-Hydroxybenzenecarboxylic acid	N-(4-Hydroxyphenyl) acetamide	1-(4-Chlorobenzoyl)-5–methoxy–2–methyl–1H-indole–3–acetic acid	2,2-Dichloro-N-[(1R,2R)-2–hydroxy–1-(hydroxymethyl)-2-(4–nitrophenyl)ethyl]acetamide	11β,17,21-Trihydroxypregna-1,4-diene-3,20-dione
Structure						
EF	$C_7H_6O_2$	$C_7H_6O_3$	$C_8H_9NO_2$	$C_{19}H_{16}ClNO_4$	$C_{11}H_{12}Cl_2N_2O_5$	$C_{21}H_{26}O_5$
Molecular wt.	122.1	138.1	151.2	357.8	323.1	358.4
Melting point	122.4 °C	159°C	169.0 - 170.5°C	155 - 162 °C	149 - 153 °C	233 - 235°C
Appearance	White or almost white, crystalline powder or colourless crystals	Colourless, feathery crystals or a white crystalline powder	White or almost white, crystalline powder	White to yellow–tan, crystalline powder	Fine, white to greyish–white or yellowish–white crystals.	White or almost white, crystalline, hygroscopic powder

Solubility	Soluble 1 in about 350 of water, 1 in 20 of boiling water, 1 in 3 of ethanol, 1 in 5 of chloroform, and 1 in 3 of ether; freely soluble in acetone	Soluble 1 in about 550 of water, 1 in about 4 of ethanol, 1 in 45 of chloroform, and 1 in 3 of ether.	Sparingly soluble in water, freely soluble in alcohol, very slightly soluble in methylene chloride	Practically insoluble in water; soluble 1 in 50 of ethanol, 1 in 30 of chloroform, and 1 in about 40 of ether; soluble in acetone	slightly soluble in water, freely soluble in alcohol and in propylene glycol	Practically insoluble in water; soluble 1 in about 150 of ethanol, 1 in 300 of dehydrated alcohol, and 1 in 200 of chloroform; slightly soluble in methanol
pKa (25°C)	4.2	3.0, 13.4	9.5	4.5	5.5	-
Octanol/Water Partition Coeff.	1.9	2.3	0.5	1.0	1.1	1.5
UV absorption (nm)	Aqueous acid - 230 273 methanol – 227, 280	Aqueous acid – 236, 303 Aqueous alkali – 298 Methanol – 298	Aqueous acid – 245 aqueous alkali – 257	Methanolic acid – 318 Aqueous alkali – 230, 279	Water – 278	Ethanol - 240
References	Indrayanto *et al.*, 1999; Moffat *et al.*, 2004; Rowe *et al.*, 2006, BP, 2009	Abounassif et al, 1994; Moffat *et al.*, 2004; BP, 2009	BP, 2009 Moffat *et al., 2004*	BP, 2009 Moffat *et al., 2004*	Moffat *et al., 2004 ;* BP, 2009	BP, 2009 Moffat *et al., 2004*

Chapter 3

MATERIALS AND METHODS

3.1 REAGENTS AND CHEMICALS

Benzoic acid (BDH), salicylic acid (BDH), methanol (BDH), ethanol (BDH), sodium hydroxide (BDH), hydrochloric acid (BDH), acetone(BDH), potassium dichromate (BDH), orthophosphoric acid (BDH), nitric acid (BDH), acetic acid (BDH) and sulphuric acid (BDH) were provided by the Department of Pharmaceutical Chemistry, KNUST. Other chemicals provided included phenolphthalein and phenol red indicators and sulphamic acid.

3.2 PURE DRUG SAMPLES

Pure powders of naproxen (BDH) and glibenclamide (BDH) APIs were obtained from Ernest Chemists Ltd, Ghana, whereas pure powders of paracetamol (BDH), indomethacin (BDH), chloramphenicol (BDH) and prednisolone (BDH) APIs were obtained from Aryton Drugs Ltd, Ghana. The drug samples and their profile are shown in Table 3-1 below.

Table 3-1 Profile of pure drug samples used in the study

Drug	Batch Number	Manufacture date	Expiry date
Naproxen	110211 - 5	03-03-2011	01-02-2016
Glibenclamide	20110509A	23-05-2011	22-05-2015
Paracetamol	1164125	20-04-2011	19-04-2015
Indomethacin	T10 - 023	05-02-2010	04-01-2014
Prednisolone	X2 - 110825A.2	17-02-2011	01-02-2014
Chloramphenicol	C08 - W110813	19-05-2011	19-05-2015

3.3 FORMULATED DRUG PRODUCTS

Glibenclamide tablets manufactured by Ernest Chemists Ltd, Ghana, Hovid Inc, Malaysia and Sanofi Aventis Inc, as well as naproxen tablets manufactured by Roche Inc and Ernest Chemists Ltd were purchased at pharmacy retail shops in and around the Kumasi metropolis. The tablet names and profile are shown in Table 3-2 below.

Table 3-2 Glibenclamide and Naproxen tablets used in the study

Tablet	Manufacturing Company	Batch Number	Manufacture date	Expiry date
Daonil (DA)	Sanofi-Aventis Inc	147331G	03-03-11	01-02-15
Glibenil (GB)	Ernest Chemists Ltd	I009L	12-06-11	15-06-15
Clamide(CD)	Hovid Inc, Malaysia	BB09633	11-05-10	01-04-14
Naprosyn (Ns)	Roche, UK	N23863S	01-02-11	01-02-16
Naprox (Nx)	Ernest Chemists Ltd, Ghana	Q507K	01-07-11	01-07-16

3.4 INSTRUMENTATION

3.4.1 HPLC Instrumentation and Column

The HPLC equipment used was a Shimadzu HPLC LC Workstation from Shimadzu Corporation, Kyoto, Japan. The system consisted of the components listed in Table 3-3 below. The column used was a Kromasil® fully porous silica C8 column by Phenomenex, Inc. USA and had specifications as listed in Table 3-3.

Table 3-3 HPLC instrumentation used and column specification

HPLC	Colum
LC-20AB pump system	Length: 250 mm
GDU-20A3 degassing unit	I.D: 4.6 mm
SPD-20A UV detector	Particle size: 5 microns
LC Solution CDS Software	Pore size: 100 Å

3.4.2 Other Instrumentation

- Adam PW / 24 Analytical weighing balance
- Cecil CE 2041 2000 Series UV Spectrophotometer
- Stuart Melting Point SMP 10 Apparatus
- FS 28H Fisher Scientific Sonicator
- Büchi rotary evaporator

3.5 STUDY DESIGN

The flow of procedures and activities in the study is illustrated in Figure 3-1 below:

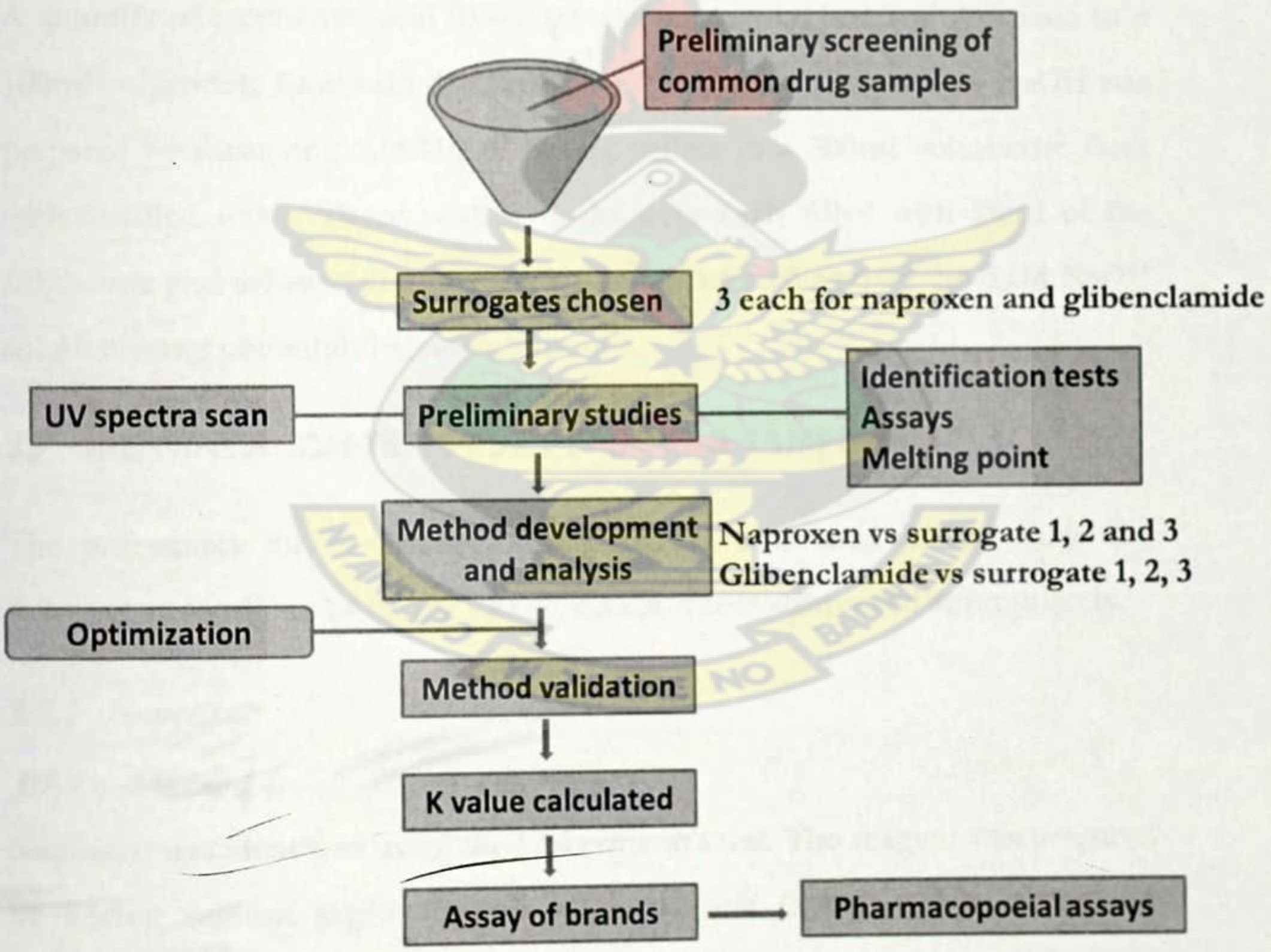


Figure 3-1 Design of the study

3.6 STANDARDIZATION OF SOLUTIONS

3.6.1 Standardization of 0.1M sodium hydroxide

Sulphamic acid (H_2NSO_3H, MW = 97.09g/mol) was used to standardize sodium hydroxide (NaOH). The two react according to the equation:

$$H_2NSO_3H + NaOH \rightarrow H_2NSO_3Na + H_2O$$

Hence 97.09g of H_2NSO_3H in 1000ml of solution $\equiv$ 1M NaOH

Therefore 0.9709g in 1000ml of solution $\equiv$ 0.1M NaOH

But percentage purity of H_2NSO_3H = 99.8%

$$\text{Hence nominal weight} = \frac{0.9709 \times 100}{99.8} = 0.9728\text{g}$$

A quantity of sulphamic acid (0.9800g) was hence weighed and dissolved in a 100ml volumetric flask with distilled water. Then a 0.1M solution of NaOH was prepared by dissolving 2.0831g of NaOH pellets in a 500ml volumetric flask with distilled water. Three conical flasks were each filled with 25ml of the sulphamic acid solution and then each one was titrated against the 0.1M NaOH solution using phenolphthalein as indicator.

3.7 IDENTIFICATION TESTS FOR PURE DRUG SAMPLES

The procedures for the identification (colour) tests used in the study are followed as described by the BP (2009), except where referenced appropriately.

3.7.1 Naproxen

3.7.1.1 Method 1

Naproxen was identified using the Liebermann's test. The reagent was prepared by adding sodium nitrite (5g) to sulphuric acid (50ml) with cooling and swirling. A quantity of naproxen (0.050g) was then weighed and transferred into a white tile, after which about 3 drops of the reagent were added (Stevens, 1986).

3.7.1.2 Method 2

A 0.004%w/v solution of naproxen was prepared in methanol, which was then examined between 230 nm and 350 nm.

3.7.2 Glibenclamide

3.7.2.1 Method 1

0.020g of glibenclamide was dissolved in 2ml of sulphuric acid and 0.1g of chloral hydrate was added. Observations were made within 5 minutes of the addition as well as after 20 minutes.

3.7.2.2 Method 2

0.0501g of glibenclamide was dissolved in 10ml of methanol with the aid of a sonicator. The dissolved solution was then diluted to 50ml with methanol. 1ml of hydrochloric acid (103g/L) was then added to 10ml of the glibenclamide solution and it absorption of UV light was examined between 230nm and 350nm.

3.7.3 Benzoic acid

Benzoic acid (0.5g) was weighed and dissolved with 10ml of ethanol. To 1 ml of this solution 0.5 ml of ferric chloride solution was added. An amount of ether was added to the precipitate obtained and observed.

3.7.4 Salicylic acid

Salicylic acid (0.030g) was dissolved in 0.05 M sodium hydroxide (5ml) and diluted to 20ml with water. To 1ml of the resulting solution, 0.5ml ferric chloride solution was added.

3.7.5 Paracetamol

To Paracetamol (0.1030g), 1M hydrochloric acid (1ml) was added. The mixture was heated to boil for 3 minutes and distilled water (1ml) was added. It was then

cooled in an ice bath. Potassium dichromate (0.049g) was dissolved in distilled water (10ml) and 0.05ml of this solution was added to the Paracetamol solution.

3.7.6 Indomethacin

Indomethacin (0.104g) was dissolved in 10ml of ethanol. A mixture consisting of 1ml of a 250g/L solution of hydroxylamine hydrochloride and 3ml of sodium hydroxide (dilute) was prepared. 2ml of this solution was then added to 0.1ml of the indomethacin solution and then 2ml of hydrochloride acid as well as 1ml of ferric chloride solutions are added and mixed.

3.7.7 Chloramphenicol

Chloramphenicol (0.0501g) was weighted into a porcelain crucible and 0.5g of anhydrous sodium carbonate was added. The crucible was then heated over an open flame for 10 minutes and allowed to cool. 5ml of dilute nitric acid was then added to the residue and the solution was filtered. 1ml of water was then added to 1ml of the filtrate.

3.7.8 Prednisolone

A quantity of prednisolone powder (0.050g) was weighed and transferred onto a white tile. About 4 drops of sulphuric acid were added directly to the prednisolone and observed. The solution was diluted further with more sulphuric acid and then observed under UV light at 350nm. The identity of the prednisolone was further observed with the naphthol-sulphuric acid test. The reagent is prepared by mixing 2-naphthol (1.0g) with 40ml of sulphuric acid with heating at 100°C until complete dissolution. A quantity of the prednisolone (0.1g) was then transferred into a test tube and 1ml of the reagent was added. The test tube was then heated in a water bath at 100°C for 2 minutes and the observed (Stevens, 1986).

3.8 MELTING POINT DETERMINATION

A small quantity of each pure drug sample was fed into a capillary tube to occupy a height of about 2mm. The tubes were then sealed and fed into the melting point apparatus where the melting range for each was determined.

3.9 ASSAY OF PURE SAMPLES (BP, 2009)

3.9.1 Naproxen

Naproxen (0.202g) was accurately weighed into a conical flask containing a mixture of 25ml of water and 75ml of methanol. It was then titrated with standardized 0.1M sodium hydroxide, using phenolphthalein solution as indicator, until a pink colour was observed. [1ml of 0.1 M NaOH is equivalent to 23.03mg of $C_{14}H_{14}O_3$]

3.9.2 Glibenclamide

Glibenclamide (0.402g) was dissolved with heating in 100ml of methanol. The solution was then titrated with 0.1M NaOH using 1ml of phenolphthalein solution as indicator, until a pink colour was obtained. [1ml of 0.1 M NaOH is equivalent to 49.40mg of $C_{23}H_{28}ClN_3O_5S$]

3.9.3 Benzoic acid

Benzoic acid (0.201 g) was accurately weighed into a conical flask and 20 ml of ethanol was added. It was then titrated with standardized 0.1 M sodium hydroxide, using phenol red solution as indicator until the colour changed from yellow to violet-red. [1 ml of 0.1 M NaOH is equivalent to 12.21 mg of $C_7H_6O_2$]

3.9.4 Salicylic acid

Salicylic Acid (0.1201g) was dissolved in ethanol (30ml). Water (20ml) was subsequently added. It was then titrated with standardized NaOH (0.1M), using phenol red solution (0.1ml) as indicator.

[Each ml of NaOH is equivalent to 13.81 mg of $C_7H_6O_3$]

3.9.5 Paracetamol

Paracetamol (0.1200g) was dissolved in methanol (10ml). The solution was then diluted to 500ml with water. 5ml of this solution was again diluted to 100ml with water. The absorbance of the solution was determined at 244nm. The specific absorbance of paracetamol was taken to be 715.

3.9.6 Indomethacin

Indomethacin (0.302 g) was weighed accurately into a conical flask. Acetone (75 ml) was added. Few drops of phenolphthalein solution was added as indictor and titrated with standardized 0.1 M sodium hydroxide. A blank determination was carried out.

[1 ml of 0.1 M NaOH is equivalent to 35.78 mg of $C_{19}H_{16}ClNO_4$]

3.9.7 Chloramphenicol

Chloramphenicol (0.100g) was accurately weighed and dissolved in water and diluted to 500ml in a conical flask. 10ml of this solution was diluted to 100ml with water. The absorbance of the diluted solution was then read at 278nm and the percentage content was calculated. The specific absorbance of chloramphenicol was taken to be 297.

3.9.8 Prednisolone

Prednisolone (0.0505g) was dissolved in ethanol and diluted to 50.0 ml with the same solvent. 1.0 ml of the solution was diluted to 50.0 ml with ethanol. The absorbance was measured at the maximum at 243.5 nm. The specific absorbance of prednisolone was taken to be 415.

3.10 IDENTIFICATION TESTS FOR COMMERCIAL SAMPLES

3.10.1 Naproxen tablets

20 tablets of each brand were powdered and a quantity containing 0.020g of naproxen was extracted in methanol to produce 100ml of solution which was then filtered. 10ml of this solution was diluted to 100ml with methanol and its light absorption was observed between 250nm and 350nm.

3.10.2 Glibenclamide tablets

A quantity of powdered tablets containing 0.050g of glibenclamide was dissolved with the aid of a sonicator in methanol to 100ml and filtered. 0.5ml of hydrochloric acid (103g/L) was added to 5ml of the glibenclamide solution and it was examined between 230nm and 350nm

3.11 HPLC METHOD DEVELOPMENT

3.11.1 Wavelength of maximum absorption of pure samples

A quantity of each pure sample (0.05g) was dissolved to 100ml with methanol. 1ml of each solution was then taken and diluted to 50ml with the same solvent, to prepare a concentration of 0.001%(w/v) of each pure sample. Scanning for the absorbance of UV light by each 0.001% solution was then done between 220nm to 350nm, and the wavelength of maximum absorbance was determined from the spectrum.

3.11.2 Mobile phase preparation

A mobile phase system comprising of water and methanol were used in the HPLC analysis of each drug sample– surrogate standard pair. For glibenclamide and its surrogate standards, a mobile phase composition of water (20%) and methanol (80%) were used; for naproxen and its surrogate standards, the composition was water (35%), methanol (65%). For each analysis a total mobile

phase volume of 500ml was prepared by measuring separately, the appropriate volumes of water and methanol into a conical flask and mixing thoroughly.

3.11.3 HPLC analysis

Six HPLC methods were developed, one each for a drug-surrogate standard pair. For each drug-surrogate standard pair, analysis was done by preparing stock solutions of both drug and the surrogate standard, before a volume of each is taken into a volumetric flask and diluted to 25ml using the mobile phase system developed for the drug. The preparation was made such that the concentration of the surrogate standard in the final 25ml was half that of the naproxen so that the corresponding areas would make identification of each analyte on the chromatogram easy. After the preparation, 20µL of the mixture was injected into the HPLC system.

3.12 METHOD VALIDATION

3.12.1 Linearity and range

For naproxen, a stock solution of 0.01% (w/v) concentration was prepared in a 100ml volumetric flask, and then stock solutions of all three surrogate standards each with concentration 0.005% (w/v) were prepared in 50ml volumetric flasks. Various volumes (10ml, 5ml, 2.5ml, 2ml, 1ml, 0.5ml and 0.1ml) of the naproxen stock solution was pipetted into 25ml volumetric flasks and subsequently, the same volumes of the stock solutions of each surrogate standard was pipetted into the their respective conical flasks containing the naproxen. The volumetric flasks were then diluted to 25ml with the mobile phase (35% water, 65% methanol). The final concentrations in the seven volumetric flasks are shown in Table 3-3 below:

Table 3-4 Serial dilutions for linearity determination of analytes

Volumetric flask	Volume of stock (mL)	Naproxen		Glibenclamide	
		Drug conc. (w/v)	Surr. Std conc. (w/v)	Drug conc. (w/v)	Surr. Std conc. (w/v)
1	10.0	0.00400	0.00200	-	-
2	5.0	0.00200	0.00100	0.00100	0.00050
3	2.5	0.00100	0.00050	0.00050	0.00025
4	2.0	0.00080	0.00030	0.00040	0.00015
5	1.0	0.00040	0.00015	0.00020	0.00075
6	0.5	0.00020	0.00006	0.00010	0.00003
7	0.1	0.00004	0.00002	0.00002	0.00001

For glibenclamide, the stock solution had a concentration 0.05% (w/v), whereas each one of the three surrogate standards had a concentration of 0.0025% (w/v). Table 3-3 again shows the various concentrations of the glibenclamide and surrogate standard in the final prepared samples before injection. After all the solutions were prepared, 20µL of each was injected into the HPLC system and the peak areas were obtained for assessing the linearity and range of the method for each one of the 8 analytes.

3.12.2 Limit of detection and limit of quantitation

Data from the calibration curves (residual S.D and slope) were used to calculate the LOD and LOQ using the appropriate equations.

3.12.3 Precision

3.12.3.1Repeatability

For each drug-surrogate standard pair, 10 injections of a single concentration level was made to assess the intra-day precision. The concentration of drug vs. surrogate standard in the injected sample was 0.001% vs. 0.0005% (w/v) respectively for naproxen and 0.0005% vs. 0.00025% (w/v) respectively for glibenclamide. The RSD of the peak areas were then calculated.

3.12.3.2Intermediate precision

The variation of the peak areas between different days was assessed in a 5-day study. Two concentration levels were prepared for each drug sample-surrogate standard pair and each was determined twice each day for all five days. For naproxen and its surrogate standards, the first concentration level contained 0.0008% (w/v) of naproxen and 0.0004% (w/v) of surrogate standard. The second level contained 0.0004% (w/v) of naproxen and 0.0002% (w/v) of surrogate standard. For glibenclamide, the first level contained 0.0005% (w/v) of glibenclamide and 0.00025% (w/v) of surrogate standard. The second level was prepared to contain 0.00025% (w/v) of glibenclamide and 0.000125% (w/v) of surrogate standard. The mean areas for each day were used to determine the RSD of the areas between the days.

3.13 DETERMINATION OF CONSTANT K

The surrogate standards used for naproxen were benzoic acid, paracetamol and prednisolone whereas that used for glibenclamide were salicylic acid, indomethacin and chloramphenicol. To determine the K value for each drug-surrogate standard pair, the injected sample contained the two analytes in different concentrations. To prepare the sample solution for naproxen-surrogate determinations, a stock solution of naproxen (0.01% (w/v)) was prepared whereas a stock solution each of benzoic acid (0.005%), paracetamol (0.005%) and prednisolone (0.005%) was also prepared. For each naproxen-surrogate standard analysis, 2.5ml each of the stock solutions of the naproxen and the standard were pipetted and diluted to 25ml with the mobile phase system developed for naproxen. For the glibenclamide-surrogate standard determinations, the glibenclamide stock solution had a concentration of 0.005%(w/v) whereas each surrogate standard stock had a concentration of 0.0025%(w/v). For each glibenclamide-surrogate standard analysis, 2.5ml the glibenclamide stock and 2.5ml of standard were pipetted and diluted to 25ml

with the mobile phase developed for glibenclamide. For each analysis, 20μL of sample was injected several times. The mean areas were obtained and together with their respective concentrations were used to calculate the K values for each surrogate standard.

3.14 ANALYSIS OF COMMERCIAL SAMPLES

3.14.1 Glibenclamide tablets

Tablets (20) of each brand of glibenclamide were obtained and powdered. A quantity of powder containing 0.025g of glibenclamide was accurately weighed and dissolved into 10mL, after which filtration was done. A volume of this solution (2ml) was pipetted and diluted to 100ml with methanol to prepare a glibenclamide stock solution of concentration 0.005% (w/v). For each glibenclamide-surrogate standard analysis, 2.5ml of the glibenclamide stock solution and 2.5ml of the 0.0025% (w/v) stock surrogate standard solution were pipetted and diluted to 25ml with the mobile phase to produce a glibenclamide vs. standard concentration of 0.0005% and 0.00025% (w/v) respectively. For each sample 20μL was injected and the areas were obtained.

3.14.2 Naproxen tablets

Just as for glibenclamide, 20 tablets of each brand were obtained and powdered. A quantity of powder containing 0.01g of naproxen was accurately weighed and dissolved to 100ml with methanol after which it was filtered. Stock solutions (0.005%) of each surrogate standard were also prepared. For the analysis, 2.5ml of the naproxen stock solution and 2.5ml of the surrogate standard was pipetted and diluted to 25ml with the mobile phase, producing a final naproxen concentration of 0.001% (w/v) and a surrogate standard concentration of 0.0005% (w/v). This was done for all surrogate standards. For each sample 20μL was injected and the areas were obtained.

3.15 B.P ASSAY METHOD FOR COMMERCIAL SAMPLES

3.15.1 Naproxen tablets (BP, 2009)

For each brand of naproxen, 20 tablets were obtained and a quantity of the grounded powder containing 0.05g (0.0579g) was accurately weighed and dissolved in 70ml of methanol for 30 minutes, after which sufficient methanol was added to produce 100ml before filtration was done. A quantity of the filtrate (10ml) was diluted to 50ml with methanol, to prepare a 0.01% naproxen solution and the absorbance was determined at a wavelength of 331nm. A similar 0.01% solution was prepared, but this time using a naproxen chromatographic reference standard and its absorbance was also read at 331nm. The percentage content of naproxen was then calculated.

3.15.2 Glibenclamide tablets (BP, 1980)

For each brand of glibenclamide, 20 tablets were obtained and a quantity of the grounded powder containing 0.01g of glibenclamide was accurately weighed and dissolved in 100ml of a solution of methanolic HCl. A quantity of this solution (5ml) was diluted to 50ml with methanol and the absorbance was read at 300nm. The operation was repeated this time with a glibenclamide reference standard with concentration as prepared for the tablets.

3.16 DATA ANALYSIS

The data was expressed percentages, Mean ± S.D and Mean ± S.E.M. Comparison of the mean assays obtained by the developed method vs. the pharmacopoeial method were done using student's t-test, whereas analysis of variance (ANOVA) together with "Bonferroni's Multiple Comparison Test" (BMCT) as a post test was used to compare the mean assays obtained by the various surrogate standards as well as the various brands of the tablets. In all a p-value ≤ 0.05 was considered statistically significant at a 95% confidence interval. Linear regression was used to assess the linearity of the method for each analyte and a

correlation coefficient (r^2) ≥ 0.998 was considered linear. RSD was calculated to assess the precision of the developed method, with RSD ≤ 2% indicative of good precision.

Statistical analysis was done using Microsoft Excel Spreadsheet Analysis Tool Pack, as well as Graph Pad Prism Software Version 5 (San Diego, California).

Chapter 4

RESULTS AND CALCULATIONS

4.1 IDENTIFICATION TESTS FOR PURE SAMPLES

For all pure samples, melting point and colour tests were used in their identification. For the two main drug samples however, an absorption maxima test was also done before identification was deduced. In the case of naproxen, a methanolic solution gave a spectrum with 4 absorption maxima: 262nm, 271nm, 316nm and 331nm whereas for glibenclamide, the spectrum yielded two absorption maxima: a very intense one at 300nm and a less intense one at 275nm. The results of the colour tests as well as the melting range for all eight pure samples, naproxen and glibenclamide inclusive is shown in Table 4-1.

For glibenclamide, addition of sulphuric acid gave a colourless solution that showed blue fluorescence in UV light at 365nm. Dissolving chloral hydrate in the solution gave a yellow colour within 5 minutes and then developed a brownish tinge after 20 minutes. Naproxen gave a black-green colour in the Lieberman's test whereas all the other pure samples gave the colours indicated respectively in Table 4-1. For salicylic acid, the violet colour still persisted after addition of acetic acid (0.1ml), whereas for paracetamol, the violet colour (which did not change to red) was formed only after cooling on ice for about 45 minutes, with no precipitate observed in the initial stages of cooling. Prednisolone gave an orange-brown colouration under the sulphuric acid test whereas under the naphthol-sulphuric acid test, it gave a brown colouration.

Table 4-1 Colour tests and melting ranges of pure samples used in the study

Pure sample	Melting point (°C)	Colour test
Naproxen	154 - 156	Black-green
Glibenclamide	169 - 175	Deep yellow within 5 minutes and brown after 20 minutes
Benzoic acid	122 - 124	Dull-yellow precipitate which dissolves in ether
Salicylic acid	158 - 161	Violet
Paracetamol	167 - 172	Violet
Chloramphenicol	150 - 153	White
Prednisolone	230 - 235	1. Orange-green 2. Brown
Indomethacin	158 - 160	Violet-pink

4.2 ASSAY OF PURE SAMPLES

4.2.1 Standardization of 0.1M sodium hydroxide

Nominal weight of sulphamic acid = 0.9728g

Actual weight taken = 0.9800g

Hence factor of $H_2HSO_3H = \frac{0.9800}{0.9728}g$

$\Rightarrow F(H_2NSO_3H) = 1.0074$

But $F(H_2NSO_3H) \times V(H_2NSO_3H) = F(NaOH) \times V(NaOH)$

where F = factor and V = volume

Hence factor of $NaOH = \frac{F(H_2NSO_3H) \times V(H_2NSO_3)H}{V(NaOH)}g$

Volume of sulphamic acid taken for titration = 25.0ml

Average titre of NaOH = 25.1ml

$$\text{Hence factor of NaOH} = \frac{1.0074 \times 25\text{ml}}{25.1\text{ml}}\text{g}$$

$$\Rightarrow \text{F(NaOH)} = 1.0039$$

4.2.2 Assay of naproxen, glibenclamide, benzoic acid, salicylic acid and indomethacin

Assay of the above pure samples all involved titration with 0.1M NaOH. Standardization of the 0.1M NaOH with sulphamic acid yielded a factor of NaOH of 1.0039. Table 4-2 shows the corrected (actual) volumes of NaOH used up in each titration.

Table 4-2 Corrected volumes of NaOH that reacted during titration of the respective pure samples

Pure sample	Corrected titre (mL)	
	1	2
Naproxen	8.834	8.734
Glibenclamide	8.132	8.132
Benzoic acid	16.464	16.464
Salicylic acid	8.634	8.734
Indomethacin	8.433	8.433

Each value is calculated as F(NaOH) × V(NaOH) (titre); 1 and 2 = various determinations

The equivalence in mg of each drug sample to 1ml of the 0.1M NaOH (as stated in the assay method for each analyte) was used to calculate the amount of drug sample equivalent to each titre.

Hence actual weight of drug sample = titre × weight equivalent to 1ml NaOH

$$\text{Hence percentage purity (assay)} = \frac{\text{Actual weight}}{\text{Weight taken (nominal weight)}} \times 100$$

Table 4-3 shows the actual weights of the above pure samples and their %purities.

Table 4-3 Titrimetric assay of naproxen, glibenclamide, benzoic acid, salicylic acid and indomethacin

	Naproxen		Glibenclamide		Ben. acid		Sal. acid		Indomethacin	
	WT = 202.0mg		WT = 402.0mg		WT = 201.0mg		WT = 120.1mg		WT = 302.0mg	
	1	2	1	2	1	2	1	2	1	2
Actual weight (mg)	203.5	201.1	401.7	401.7	0.2010	0.1998	119.2	120.6	301.7	301.7
Assay (%)	100.72	99.58	99.93	99.93	100.01	99.40	99.36	100.43	99.91	99.91

WT = weight taken (nominal weight); 1 = first determination; 2 = second determination

The mean ± SD %purities were: naproxen (100.15 ± 0.8061); glibenclamide (99.93); benzoic acid (99.71 ± 0.4313); salicylic acid (99.85 ± 0.7566); indomethacin (99.91).

4.2.3 Assay of chloramphenicol, prednisolone and paracetamol

UV spectrophotometry was the assay method used for the above pure samples. For each assay, the nominal concentration (%w/v) was calculated from the weights taken for analysis. The BP stipulated specific absorbances, together with the absorbances obtained for each analysis were then used to calculate the actual concentrations according to the relation:

$$\text{Actual concentration} = \frac{\text{Absorbance}}{\text{specific absorbance}} (\%w/v)$$

The percentage purity was hence calculated as:

$$\text{Percentage purity (assay)} = \frac{\text{Actual concentration}}{\text{Nominal concentration}} \times 100$$

Table 3-4 shows for each pure sample, the absorbance obtained, the nominal and the calculated concentrations as well as the percentage purities.

The mean ± SD percentage purities were: paracetamol (99.71 ± 0.0778); prednisolone (101.28 ± 0.1697); chloramphenicol (100.5 ± 0.2404).

Table 4-4 UV spectrophotometric assay of paracetamol, prednisolone and chloramphenicol

	Paracetamol NC = 1.200 %(w/v)		**Prednisolone** NC = 2.020 %(w/v)		**Chloramphenicol** NC = 2.000 %(w/v)	
	1	2	1	2	1	2
Abs	0.856	0.855	0.850	0.848	0.598	0.0596
Actual Conc. % (w/v)	1.197	1.196	2.048	2.043	2.014	2.001
Assay (%)	99.76	99.65	101.4	101.16	100.67	100.33

NC = Nominal concentration; 1 = first determination; 2 = second determination

4.3 HPLC METHOD DEVELOPMENT

4.3.1 Wavelength of maximum absorption

UV light scanning between 220nm and 350nm for all pure samples prepared in their respective mobile phases was done. Three absorption maxima were obtained for naproxen: 262nm, 271nm and 331nm, whereas three (at 225nm, 275nm and 300nm) were obtained for glibenclamide. The results obtained for the surrogate standards are shown in Table 4-5 below.

Table 4-5 UV wavelength(s) of maximum absorbance of surrogate standards

Pure sample	**λmax (nm)**
Benzoic acid	227
Salicylic acid	298; 234; 302
Paracetamol	247
Chloramphenicol	278
Prednisolone	245
Indomethacin	230, 320

4.3.2 Method conditions

A method was developed for each glibenclamide – surrogate standard pair as well as each naproxen – surrogate standard pair. A mobile phase flow rate of 1 mL/min was used for all the methods. The rest of the method conditions like the wavelength chosen for each method, the mobile phase and stationary phase are indicated in Table 4-6 below.

Table 4-6 Method conditions for each analysis

Drug sample	Surrogate standard	Mobile phase	Flow rate (mL/min)	Wavelength (nm)	Stationary phase
Glibenclamide	Salicylic acid	A(20%) B(80%)	1	225	Silica - C8 bonded phase
	Chloramphenicol			260	
	Indomethacin			225	
Naproxen	Benzoic acid	A(35%) B(65%)	1	225	Silica - C8 bonded phase
	Paracetamol			280	
	Prednisolone			245	

A = water; B = methanol

4.3.3 Retention times

The mean retention times (minutes ± SD) of all pure samples is shown in Table 4-7, together with the number of runs used for each determination.

Table 4-7 Mean retention times for pure samples

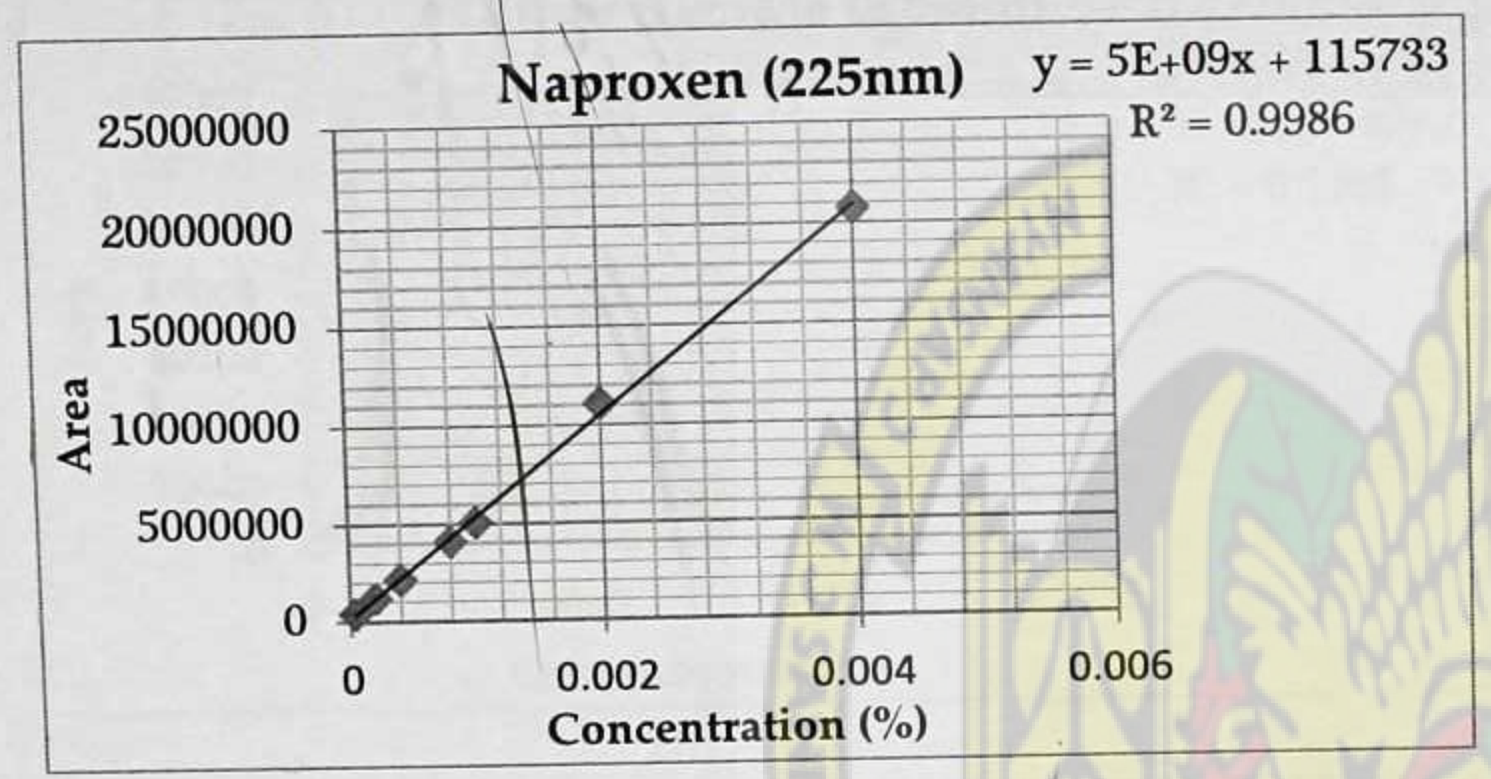
Drug	Retention time n (Minutes ± SD)
Glibenclamide	38(4.300 ± 0.041)
Naproxen	38(4.716 ± 0.156)
Salicylic acid	24(1.632 ± 0.016)
Indomethacin	24(2.853 ± 0.096)
Chloramphenicol	24(3.242 ± 0.017)
Benzoic acid	24(2.624 ± 0.071)
Paracetamol	24(3.214 ± 0.032)
Prednisolone	24(7.708 ± 0.264)

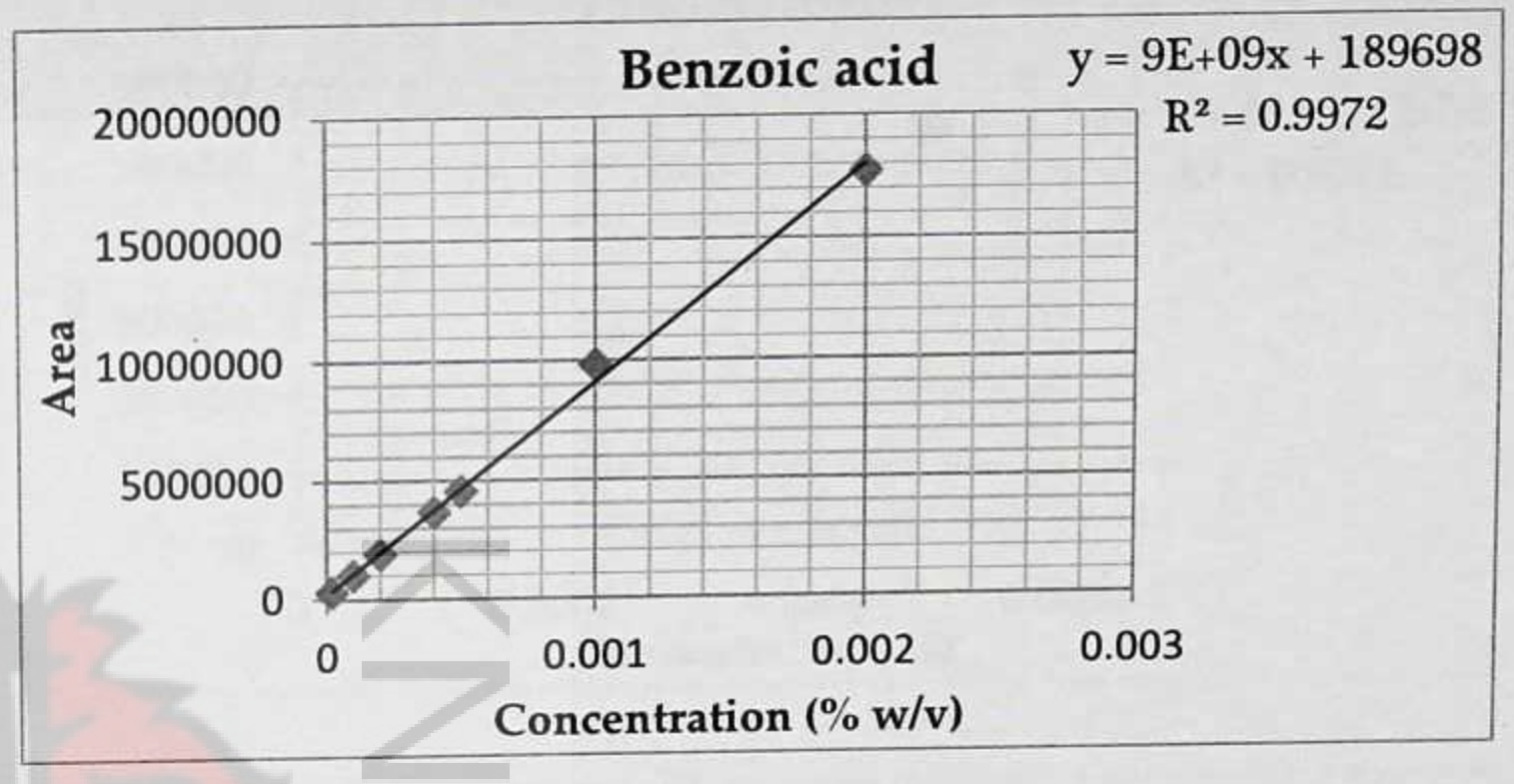
n = number of runs

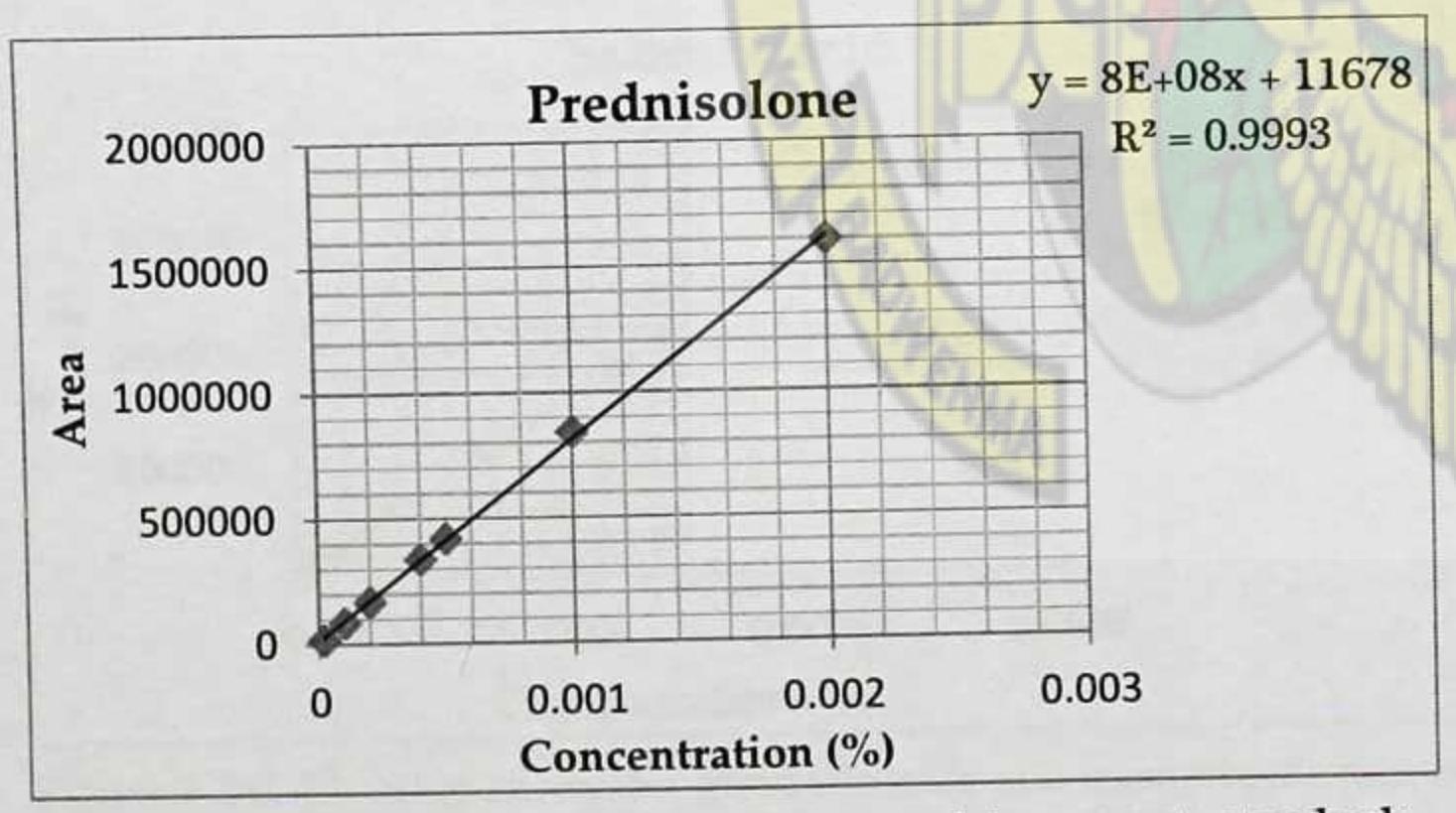
4.4 METHOD VALIDATION

4.4.1 Calibration curves

The linearity of the method for each analyte is shown by their respective calibration plots in Figures 4-1 and 4-2.







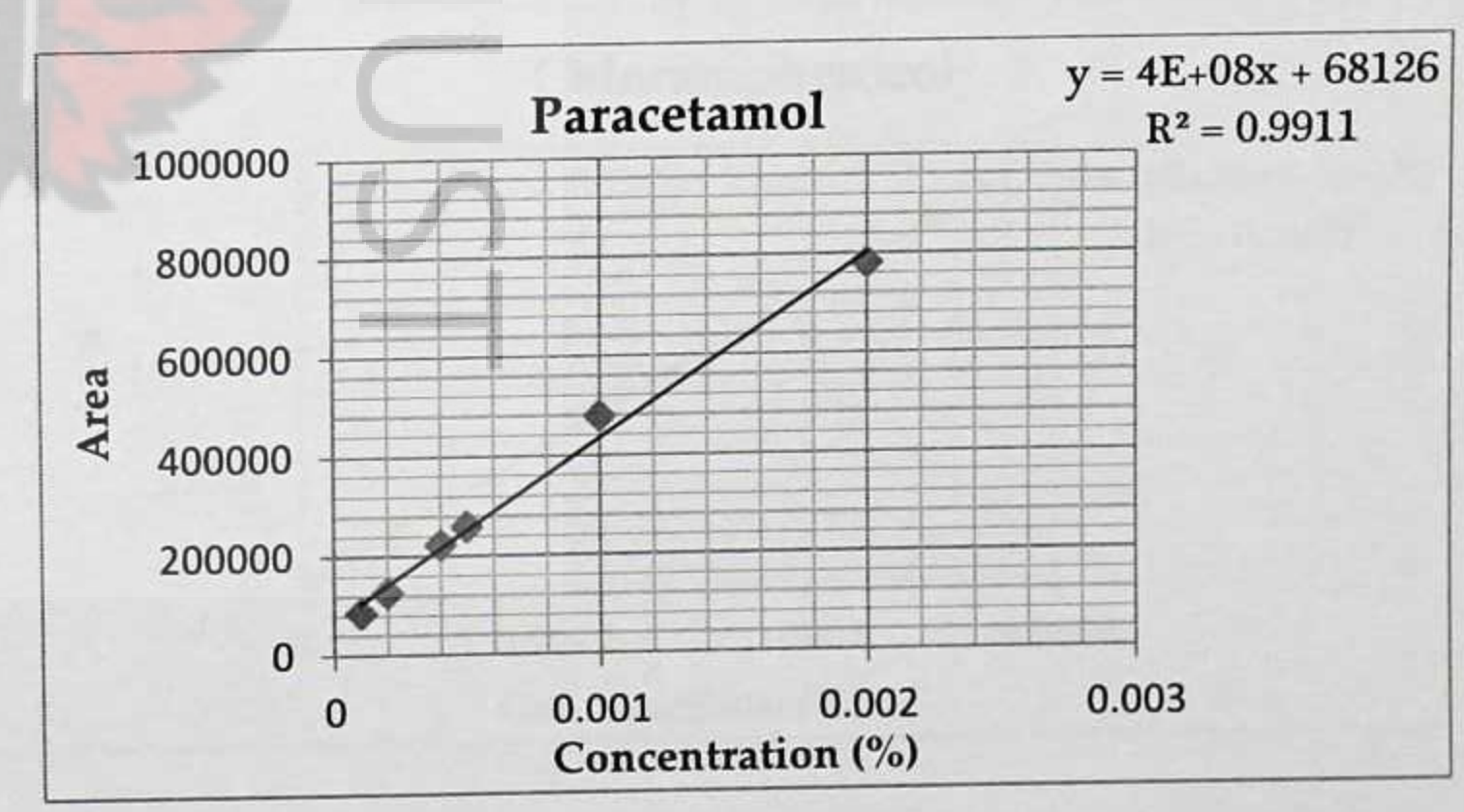
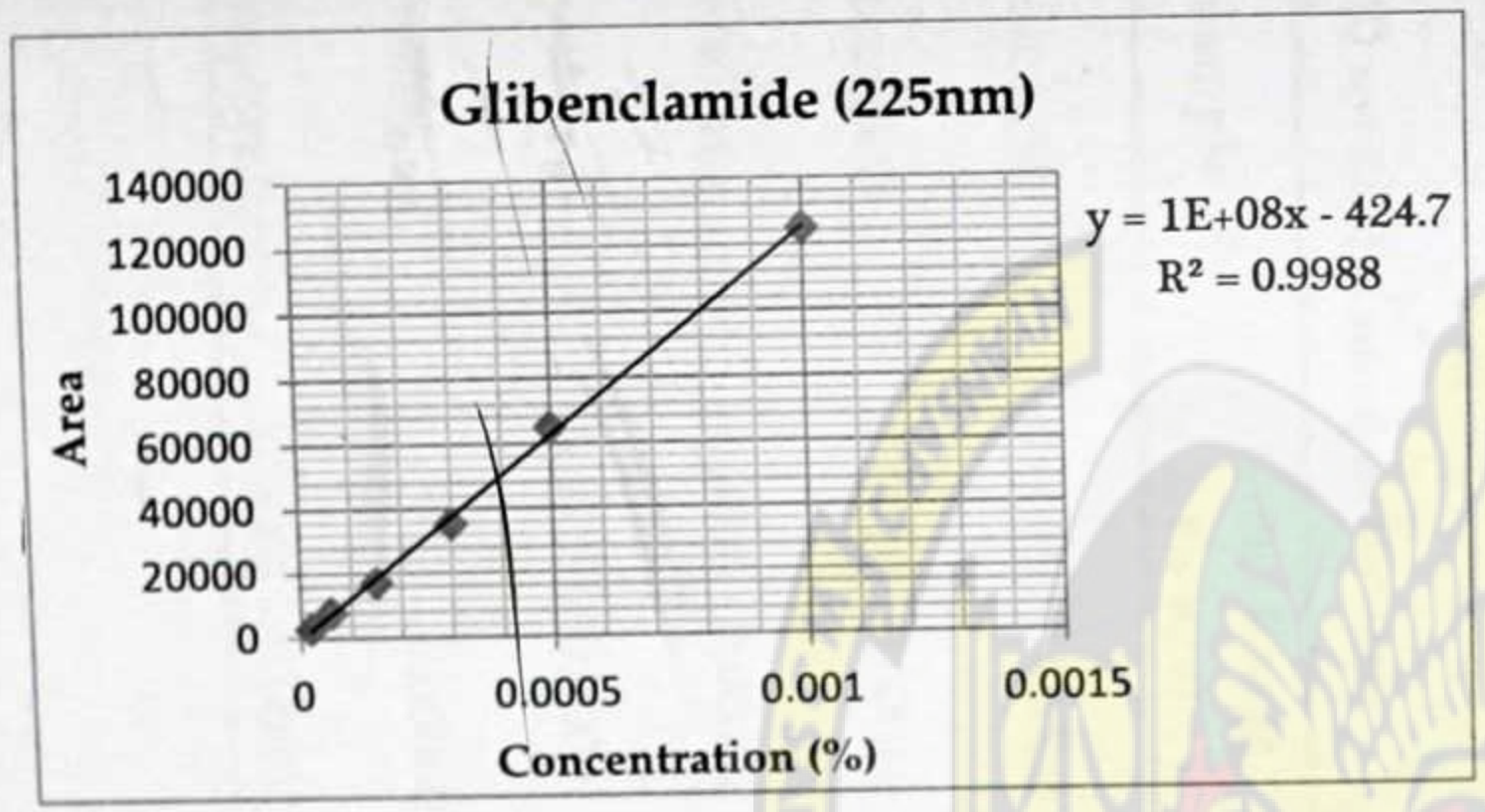
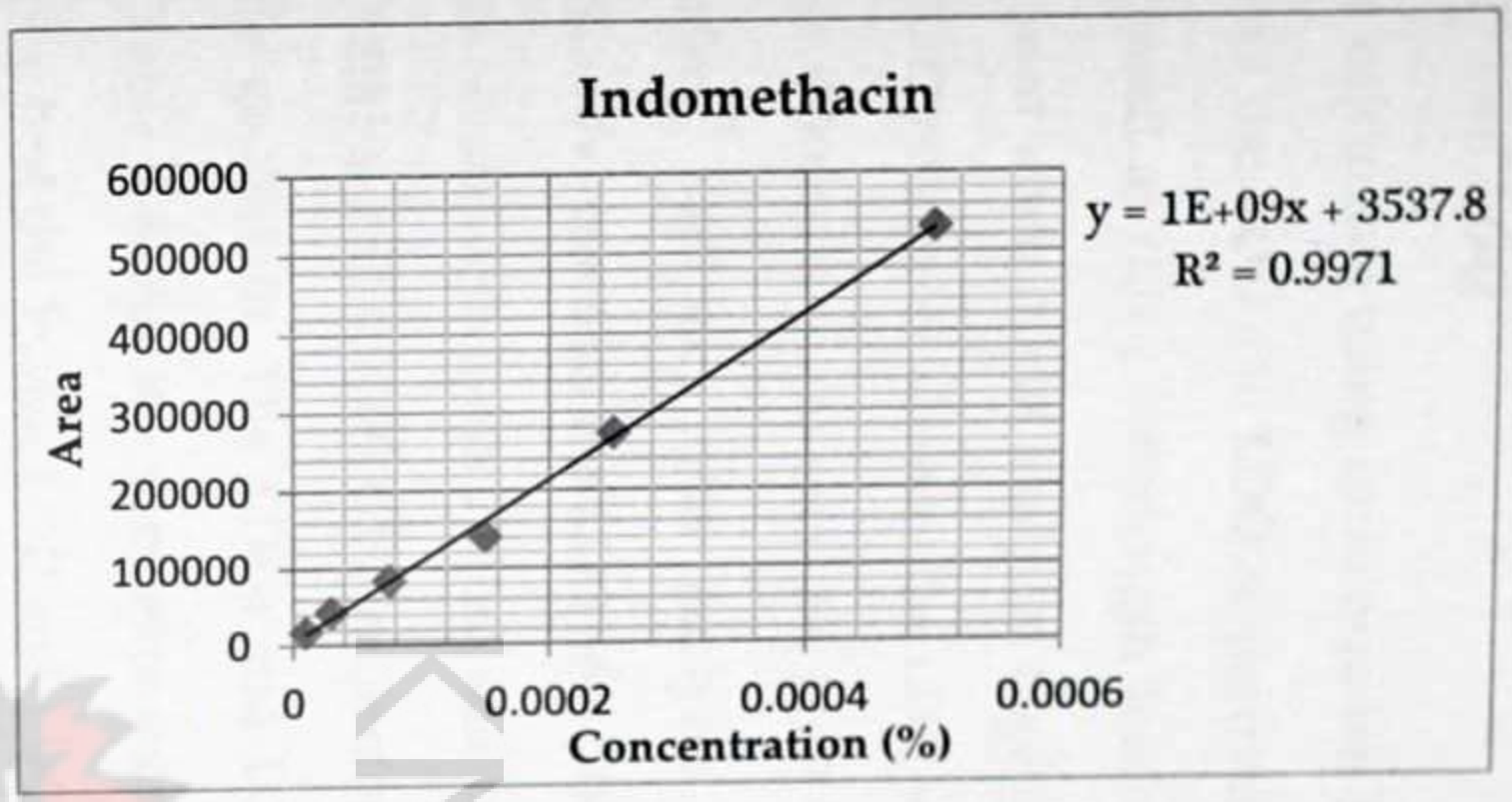
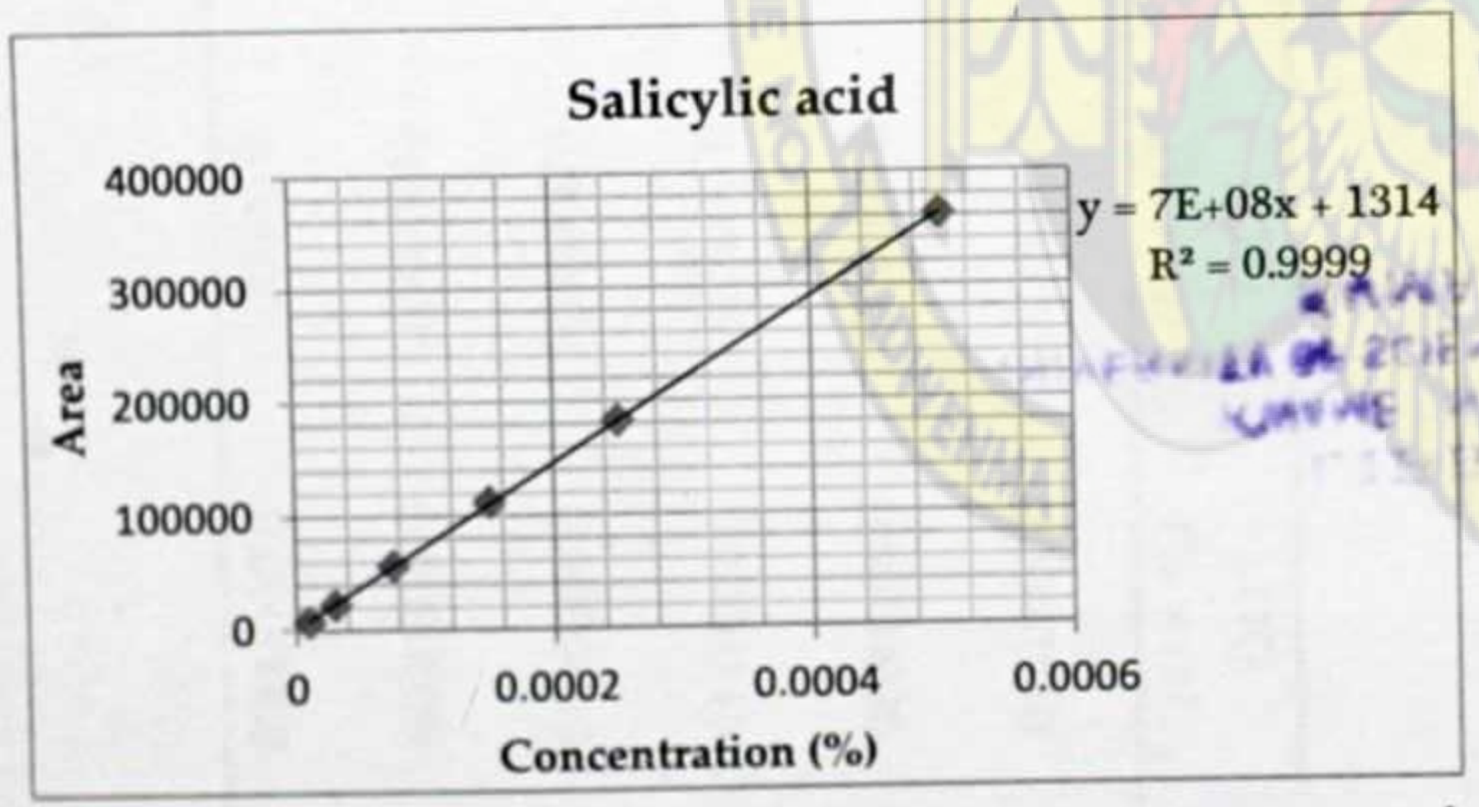


Figure 4-1 Calibration curves for naproxen and it surrogate standards

More than one calibration curve, apart from those shown in Figures 4-1 and 4-2 was plotted for naproxen and glibenclamide as their analysis was done at three and two different wavelengths respectively (Appendix).







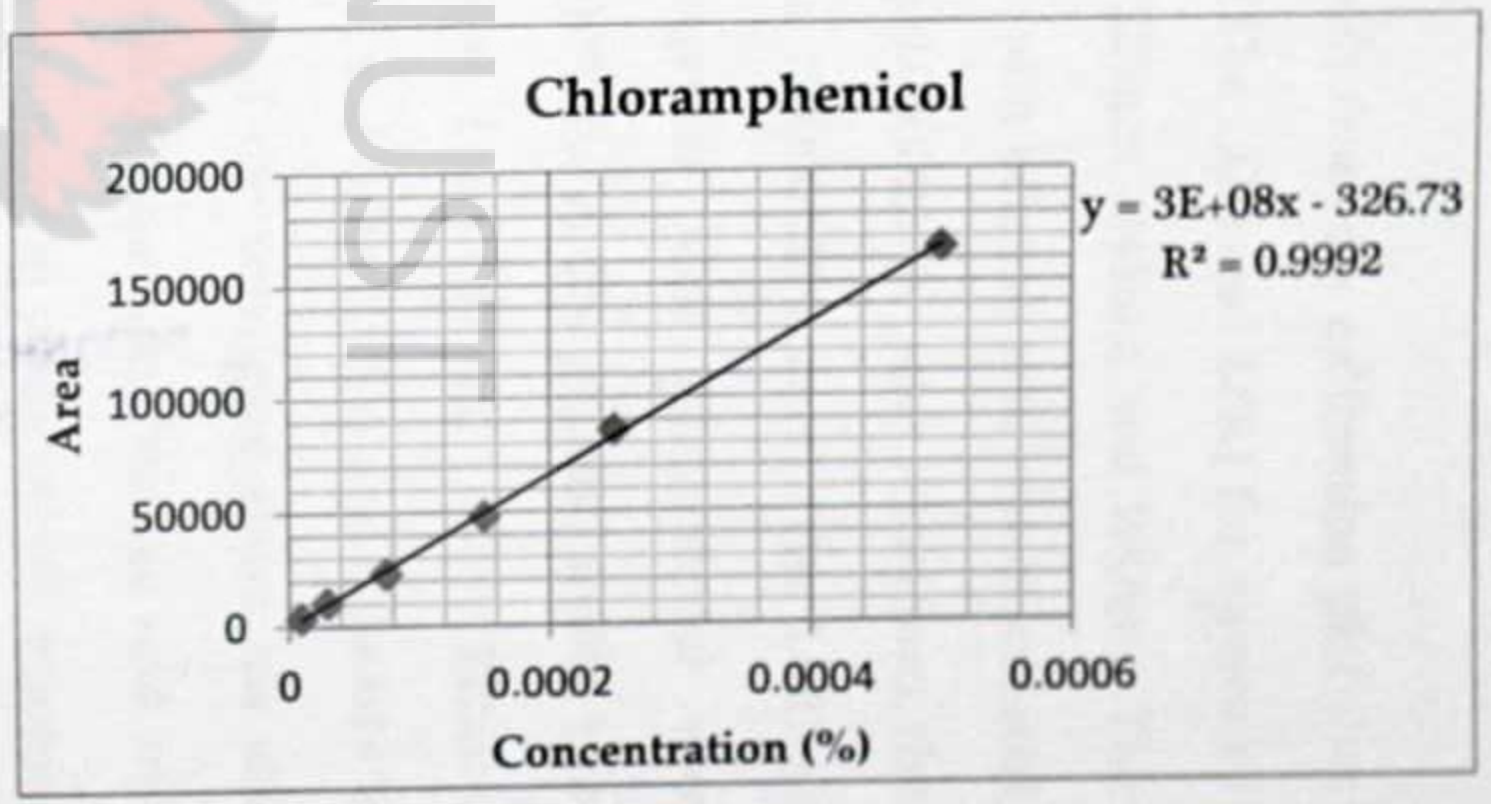


Figure 4-2 Calibration curves for glibenclamide and its surrogate standards

4.4.2 LOD and LOQ

These were calculated using concentration levels from the calibration plot that approximates the LOD and LOQ respectively. The LOD and LOQ for naproxen were calculated at three wavelength levels: 225nm, 245nm and 280nm. The LOD at 225nm when it was analyzed together with benzoic acid was calculated to be 0.00000783% (w/v) whereas the LOQ was 0.0000237% (w/v). At 245nm, the LOD was 0.0000061% (w/v) and the LOQ was 0.0000180% (w/v). The LOD at 280nm, when naproxen was analyzed together with paracetamol was 0.0000581% (w/v) whereas the LOQ was 0.00018% (w/v). For glibenclamide, two wavelength levels were used: 225nm and 260nm. The LOD and LOQ at 225nm were 0.0000021% and 0.0000063% respectively, and 0.0000045% and 0.000014% respectively at 260nm. The LODs and LOQs of the surrogate standards are shown in Table 4-8. For the surrogate standards of naproxen, benzoic acid and prednisolone had the lowest LOD and LOQs compared to paracetamol, whereas salicylic acid had the lowest LOD and LOQ among the surrogate standards of glibenclamide, followed by indomethacin.

Table 4-8 LOD and LOQ of surrogate standards

Pure sample	Conc. Range (% w/v)	LOD (% w/v)	LOQ (% w/v)
Benzoic acid	0.0005 - 0.00002	0.0000037	0.0000110
Paracetamol	0.0010 - 0.00010	0.0000303	0.0000920
Prednisolone	0.0005 - 0.00002	0.0000035	0.0000110
Salicylic acid	0.0005 – 0.00001	0.0000011	0.0000033
Indomethacin	0.00015 – 0.000005	0.0000013	0.0000039
Chloramphenicol	0.0005 – 0.00001	0.0000021	0.0000062

4.4.3 Repeatability and intermediate precision

The intra-day precision results for 10 injections of each drug sample-surrogate standard pair measured at a single concentration level are shown in Table 4-8, with RSD ranging between 0.33% (benzoic acid) and 1.91% (chloramphenicol).

Table 4-9 Repeatability of each analyte using the developed method

Sample	Mean area n = 10	SD	RSD (%)
Naproxen	4066440	16706	0.41
Benzoic acid	3688296	12039	0.33
Prednisolone	339160	1806	0.53
Paracetamol	227790	3430	1.51
Glibenclamide	459722	2092	0.46
Salicylic acid	179319	1844	1.03
Indomethacin	459557	2210	0.48
Chloramphenicol	86126	1644	1.91

n = number of injections

The 5-day intermediate precision results for two concentration levels of each sample are shown in Table 4-10.

Table 4-10 Intra-day precision of the method for each analyte

Drug sample	Conc. 1			Conc. 2		
	Mean Area* n = 5	SD	RSD (%)	Mean Area* n = 5	SD	RSD (%)
Naproxen	4065992	9338	0.23	2180122	19808	0.91
Benzoic acid	3689211	9511	0.26	1908259	9336	0.49
Prednisolone	339113	1006	0.30	167134	2694	1.61
Paracetamol	228246	2027	0.89	227041	2227	0.98
Glibenclamide	458790	2185	0.48	232547	2886	1.24
Salicylic acid	179662	1736	0.97	91155	1605	1.76
Indomethacin	460296	1256	0.27	230808	2556	1.11
Chloramphenicol	86277	1189	1.38	44260	689	1.56

*This was calculated from the mean area for each day

4.5 DETERMINATION OF CONSTANT K

The chromatograms obtained for glibenclamide and its surrogate standards are shown in Figures 4-3, 4-4 and 4-5 below.

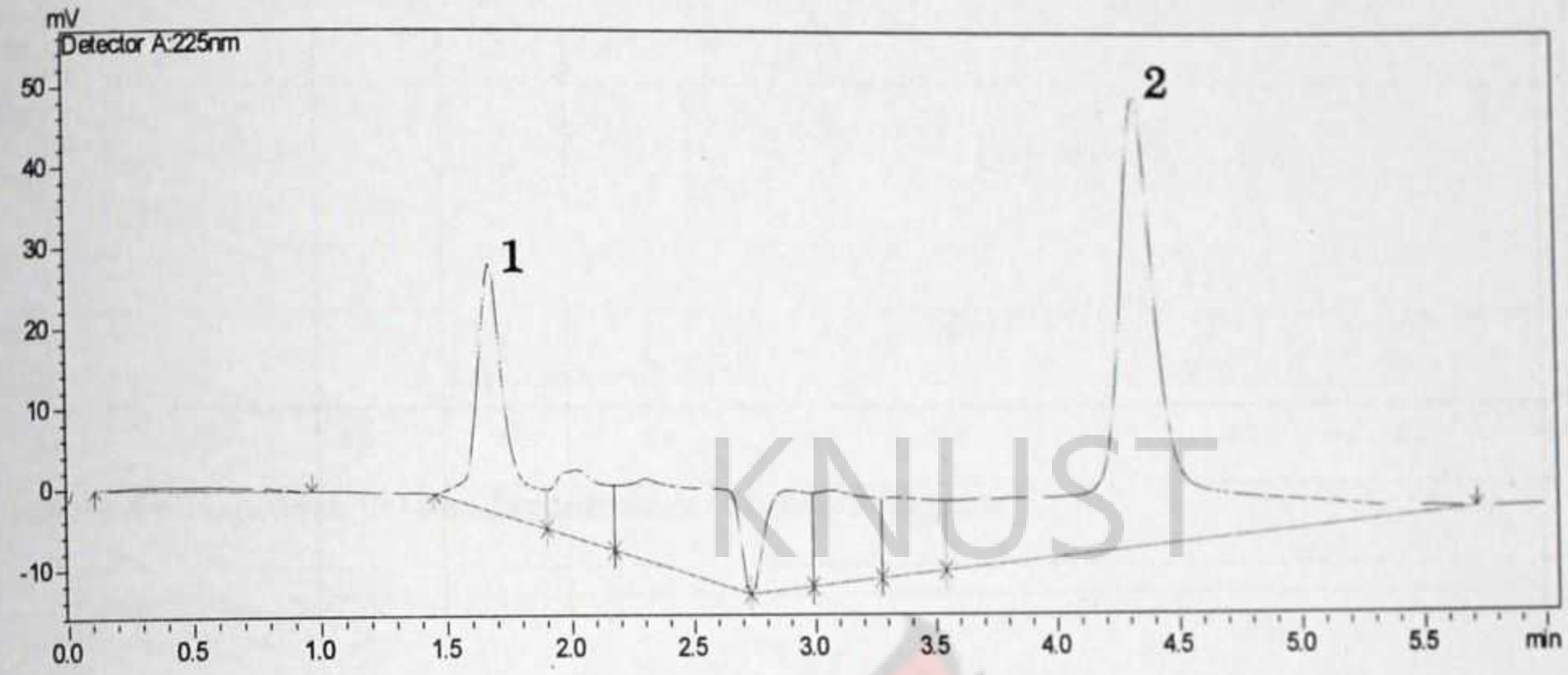


Figure 4-3 Glibenclamide (2) and Salicylic acid (1) chromatogram

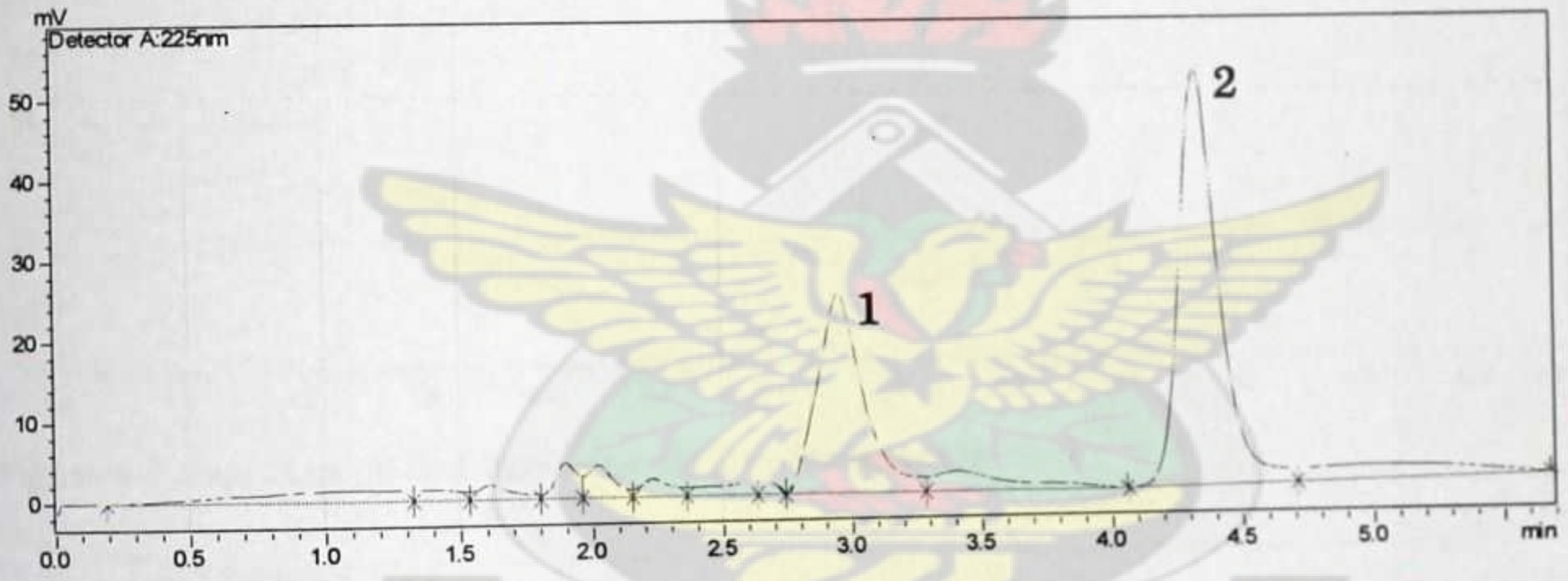


Figure 4-4 Glibenclamide (2) and Indomethacin (1) chromatogram

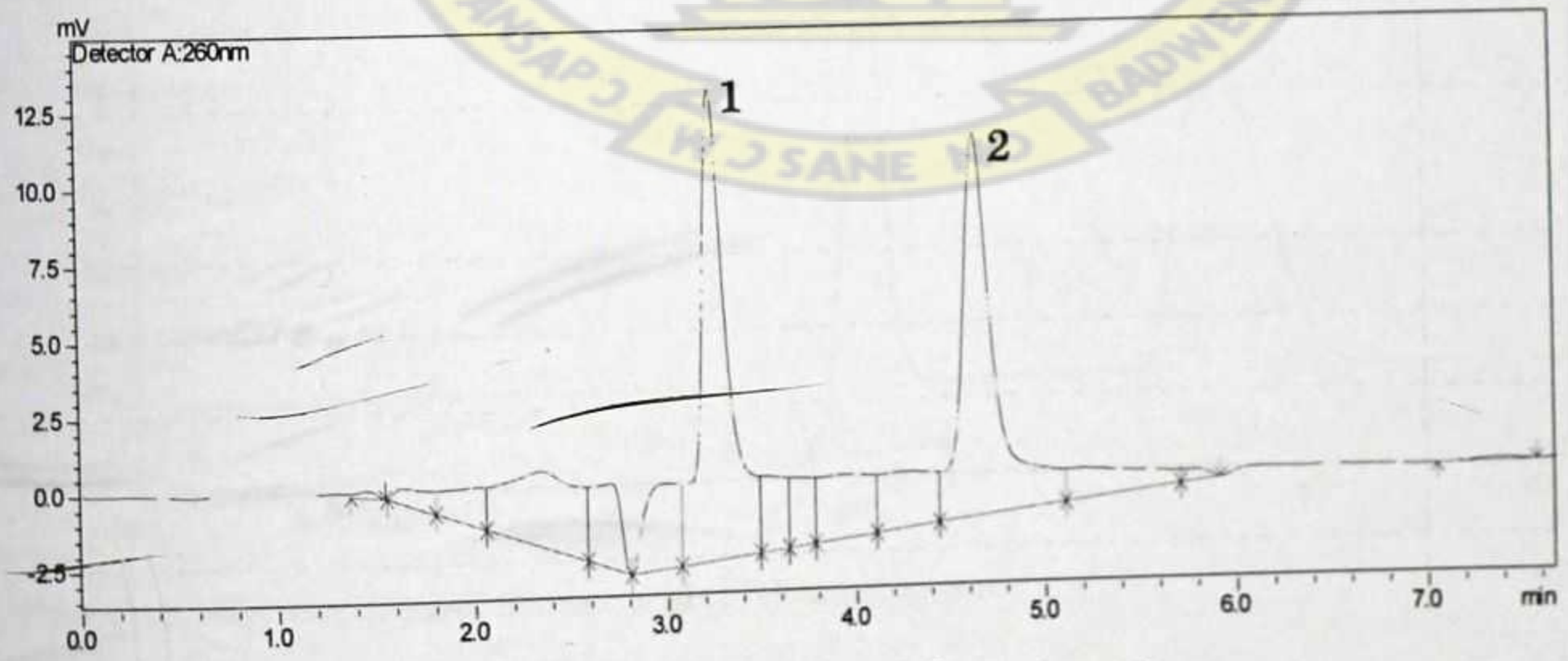


Figure 4-5 Glibenclamide (2) and Chloramphenicol (1) chromatogram

Figures 4-6, 4-7 and 4-8 shows the chromatograms for naproxen and its surrogate standards analyzed at their respective chosen wavelengths.

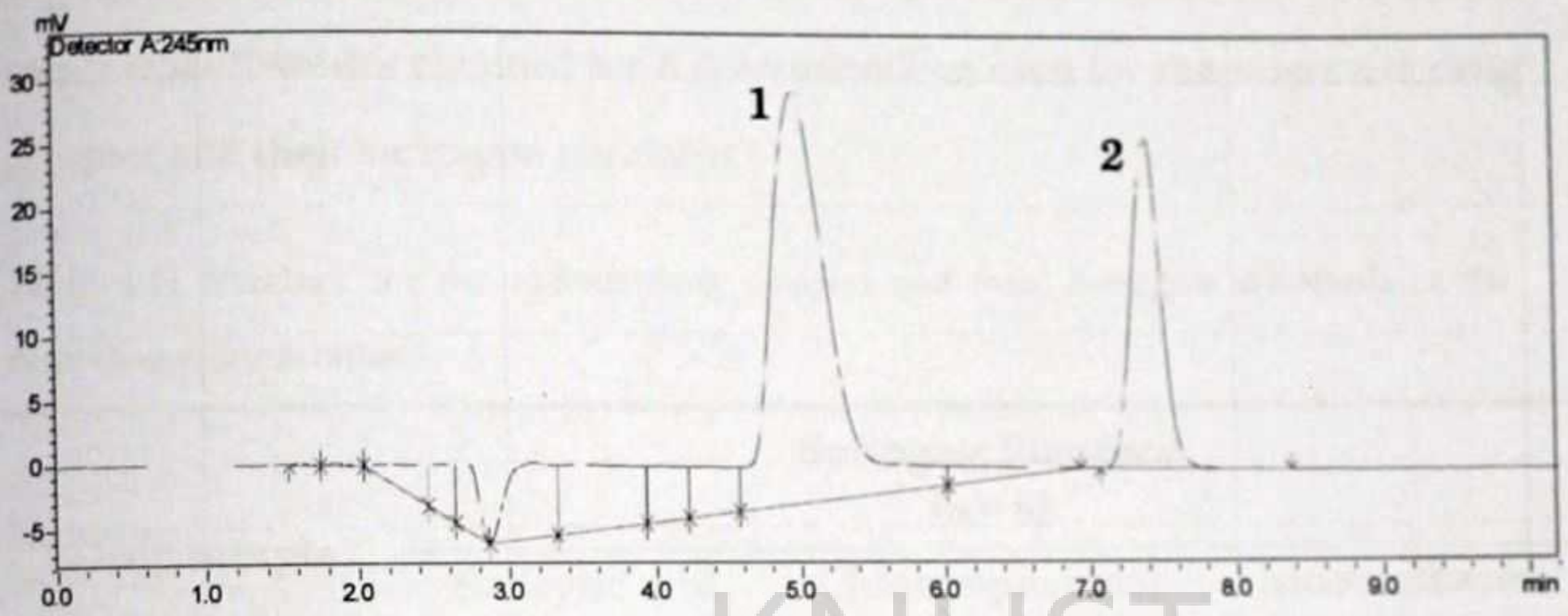


Figure 4-6 Naproxen (1) and Prednisolone (2) chromatogram

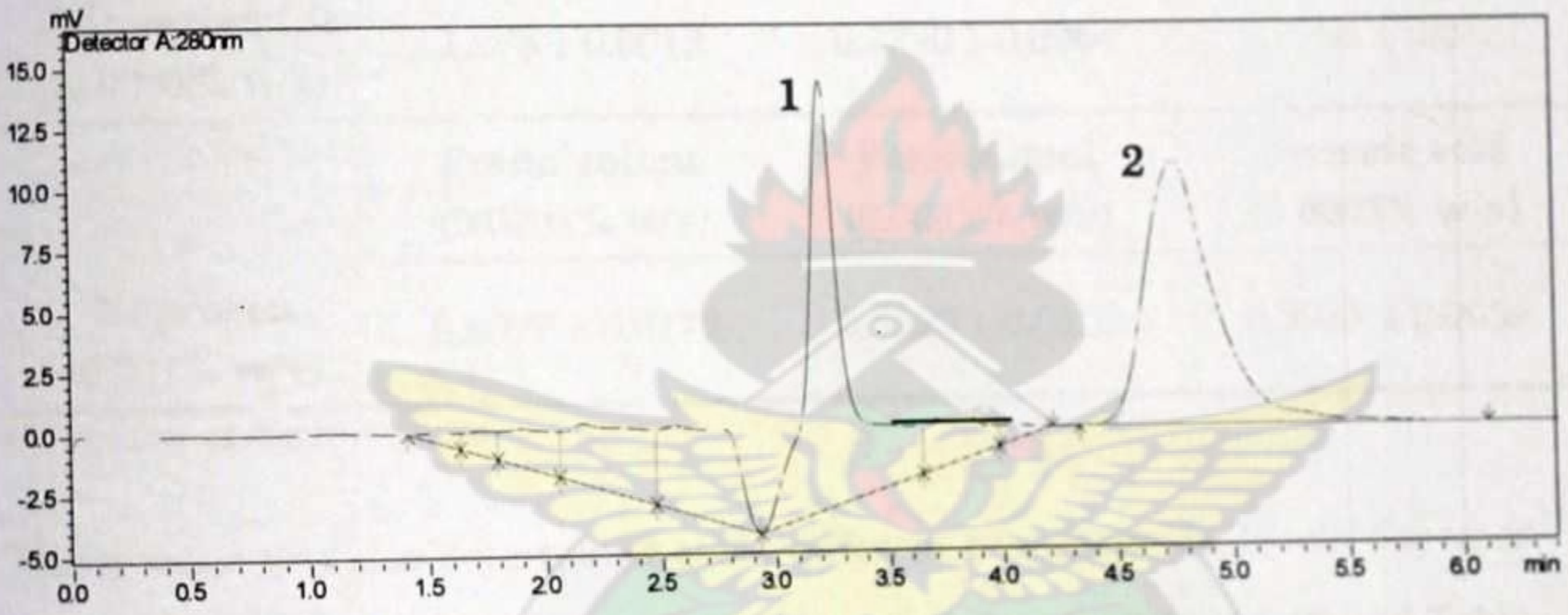


Figure 4-7 Naproxen (2) and Paracetamol (1) chromatogram

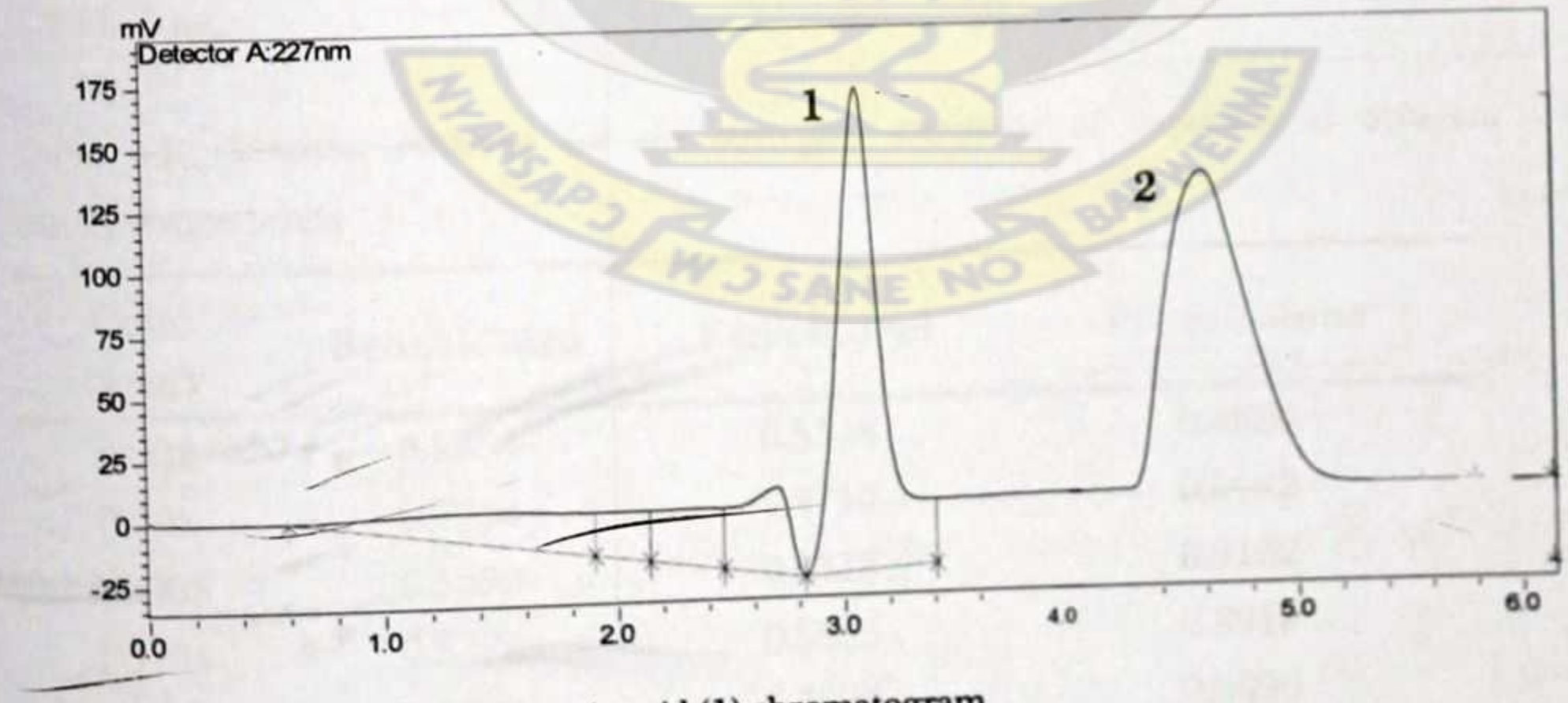


Figure 4-8 Naproxen (2) and Benzoic acid (1) chromatogram

The areas obtained for each drug sample and the surrogate standard in the pair was used to determine the K-value for each pair, as described by the equation deduced for its determination in the study rationale. Table 4-11 shows the mean ± SD K-values obtained for 6 determinations each for the respective drug samples and their surrogate standards.

Table 4-11 K-values for the various drug samples and their surrogate standards at the respective concentrations

Drug sample	Surrogate Standard (n = 6)		
	Salicylic acid (0.00025% w/v)	Chloramphenicol (0.00025% w/v)	Indomethacin (0.00025% w/v)
Glibenclamide (0.0005% w/v)	1.278 ± 0.0013	0.3790 ± 0.0064	0.7798 ± 0.0171
	Prednisolone (0.0005% w/v)	Paracetamol (0.0005% w/v)	Benzoic acid (0.0005% w/v)
Naproxen (0.001% w/v)	0.9077 ± 0.0172	0.5626 ± 0.0102	0.5967 ± 0.0059

n = number of determinations

The K-value was also determined at six different concentration levels, the ones used in Table 4-11 inclusive and the results are shown in Tables 4-12 and Table 4-13 below.

Table 4-12 K-values obtained for the surrogate standards of naproxen at different concentration levels

Conc. % w/v	Benzoic acid	Paracetamol	Prednisolone
0.002	0.5854	0.5546	0.8889
0.001	0.5524	0.5719	0.8982
0.0005	0.5339	0.5515	0.9162
0.0004	0.5428	0.5365	0.8918
0.0002	0.5899	0.5627	0.9090
0.0001	0.6324	0.5390	0.9047
0.00002	0.6283	0.5859	0.8924

For each concentration level of surrogate standard in Tables 14-12 and 14-13, the respective concentrations of naproxen and glibenclamide used were: 0.004%; 0.002%; 0.001%; 0.0008%; 0.0004%; 0.0002%; and 0.00004% for naproxen, and 0.001%; 0.005%; 0.003%; 0.00015%; 0.00006%; and 0.00002% for glibenclamide respectively. The mean K-values from the Tables 4-12 and 4-13 were determined to be: 0.5807 ± 0.0397 for benzoic acid; 0.5575 ± 0.0177 (paracetamol); 0.9002 ± 0.0101 (prednisolone); 1.275 ± 0.0060 (salicylic acid); 0.8084 ± 0.0447 (indomethacin) and 0.3762 ± 0059 for chloramphenicol.

Table 4-13 K-values obtained for the surrogate standards of glibenclamide at different concentration levels

Conc. % w/v	Salicylic acid	Indomethacin	Chloramphenicol
0.0005	1.2720	0.7744	0.3793
0.00025	1.2739	0.7853	0.3771
0.00015	1.2799	0.7830	0.3680
0.000075	1.2664	0.7764	0.3828
0.00003	1.2836	0.8638	0.3794
0.00001	1.2738	0.8679	0.3874

4.6 IDENTIFICATION TESTS FOR TABLETS

Examination of the absorbance spectrum of a quantity of naproxen tablets extracted in methanol between 250nm and 350nm yielded four absorption maxima at 262nm, 271nm, 316nm and 331nm; similar to that obtained for the pure naproxen. Similarly, for the various brands of glibenclamide tablets, extraction was done in methanol and UV scanning was done between 230nm and 250nm to produce two absorption maxima at 300nm and 275nm. In addition, colour tests done for each brand of the two drugs were very comparable to that obtained for the pure powders of the drugs.

4.7 ASSAY OF TABLETS USING THE DETERMINED K-VALUES

4.7.1 Naprosyn EC and Naprox tablets

For each brand of naproxen tablets, HPLC analysis was done by injecting a solution containing 0.001% w/v of naproxen and 0.005% w/v of one of its three surrogate standards. The obtained peak areas, together with the determined K-value for the particular surrogate standard was then used to assay for each brand of the tablet according to the equation deduced in the study rationale (Appendix). The obtained chromatograms are shown in the Figures below.

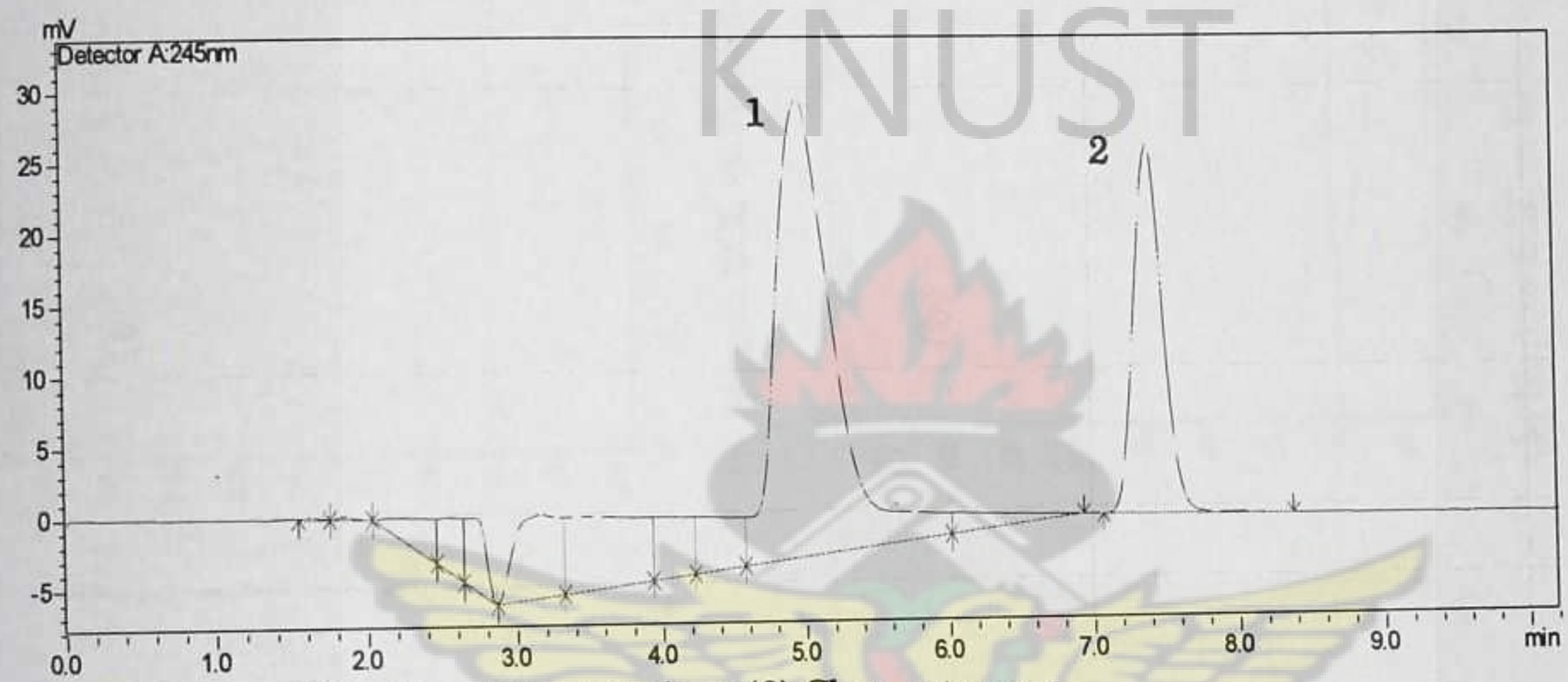


Figure 4-9 Naprox ECL (1) and Prednisolone (2) Chromatogram

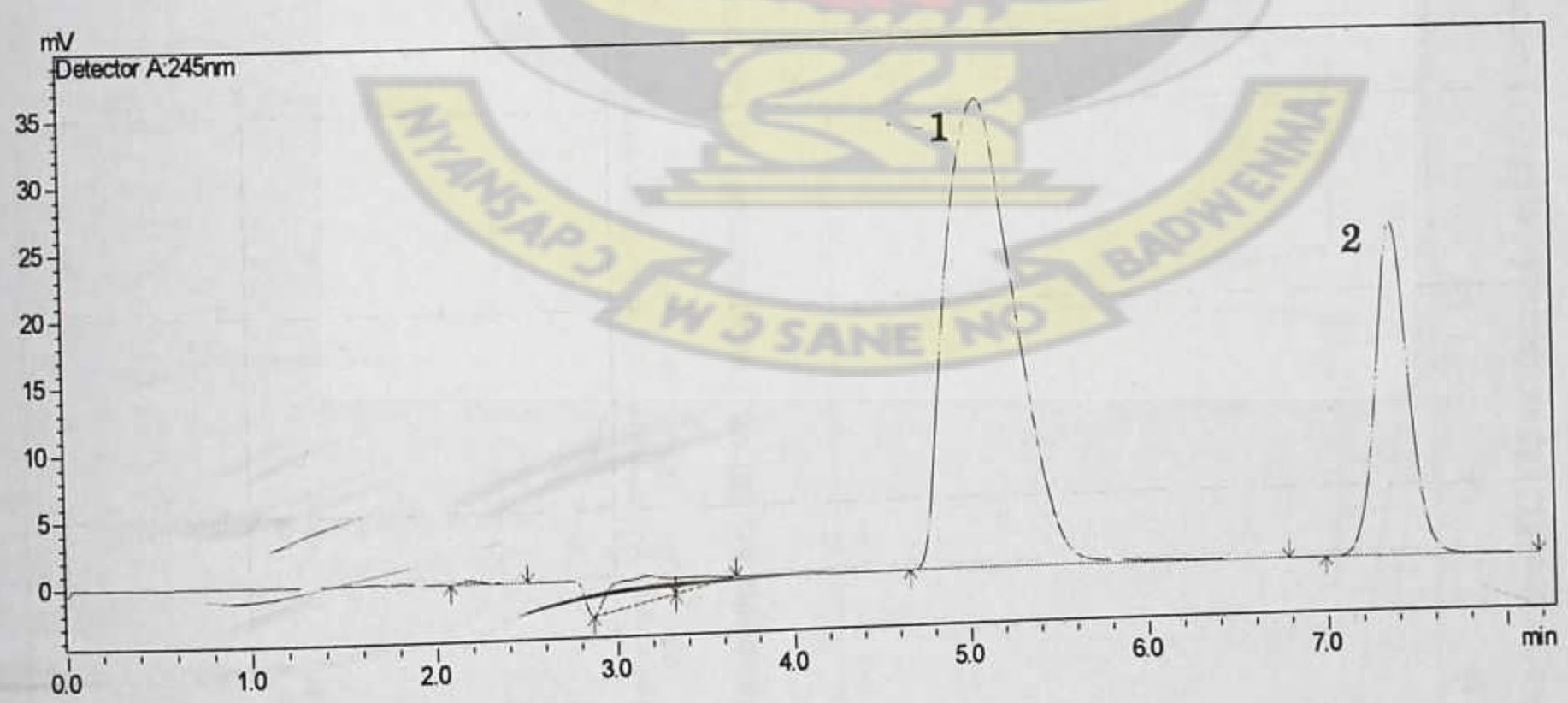


Figure 4-10 Naprosyn EC (1) and Prednisolone (3) Chromatogram

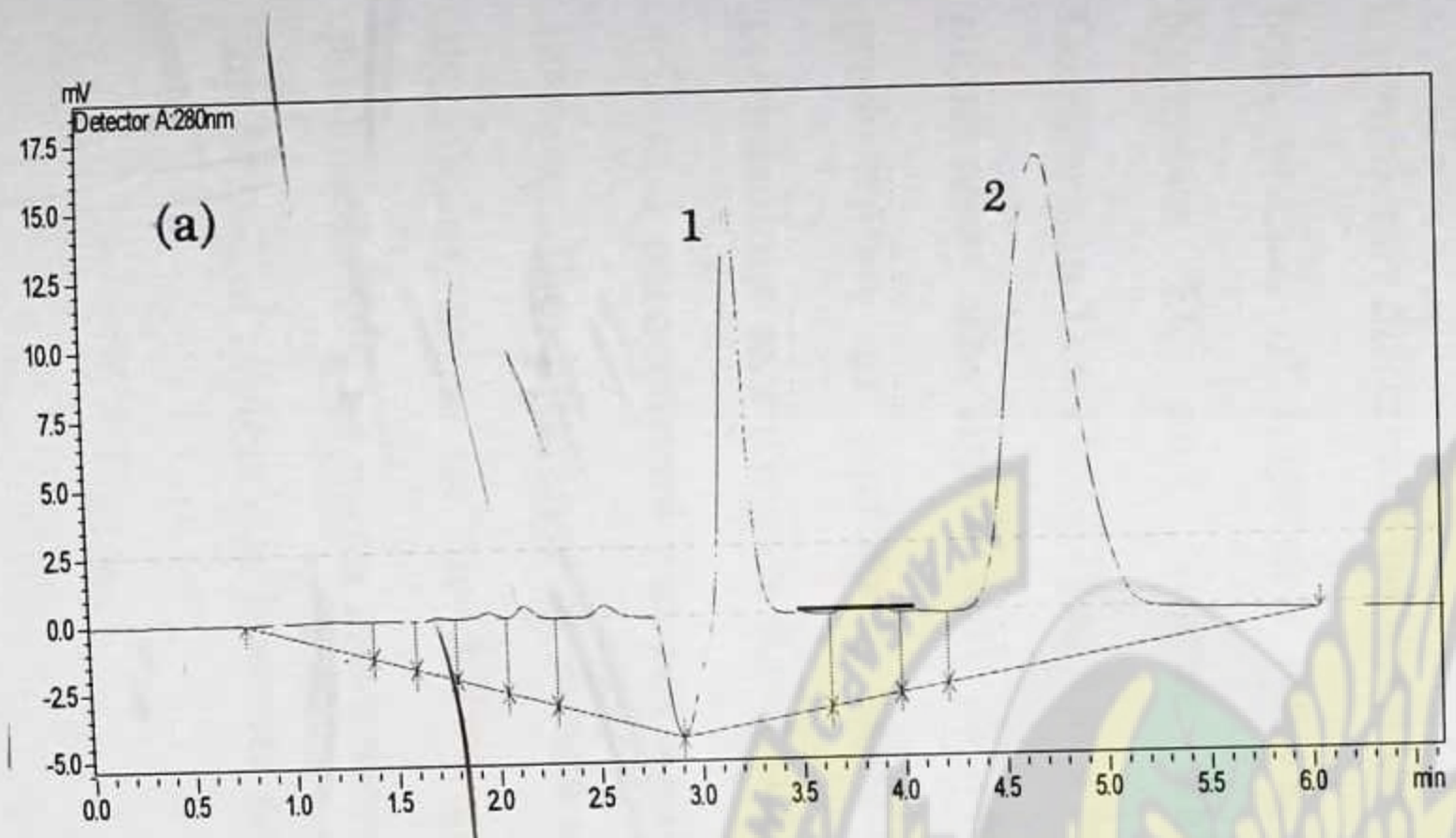
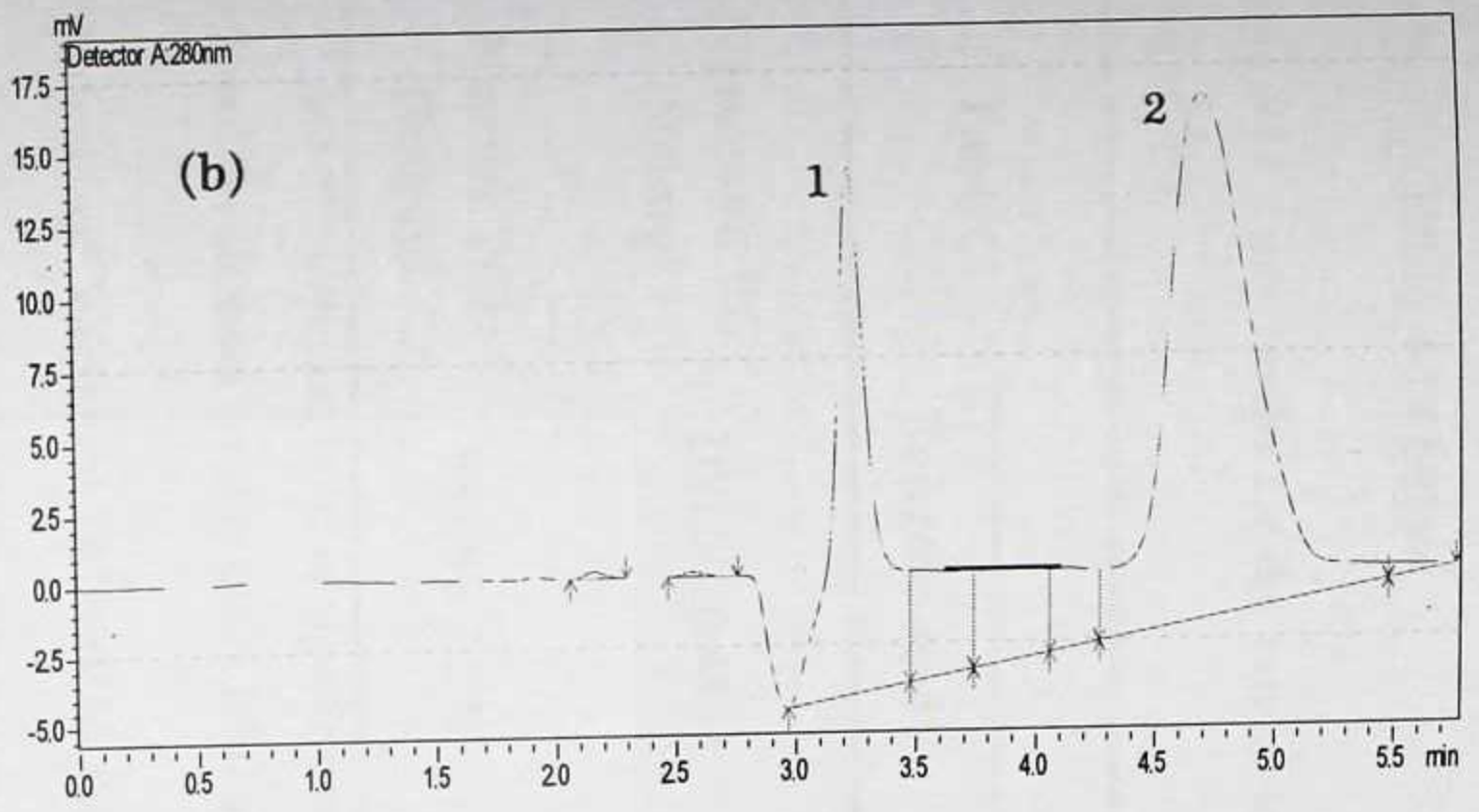


Figure 4-11 (a) Naprosyn EC (2) vs. Paracetamol (1) Chromatogram



(b) Naprox ECL vs. Paracetamol Chromatogram

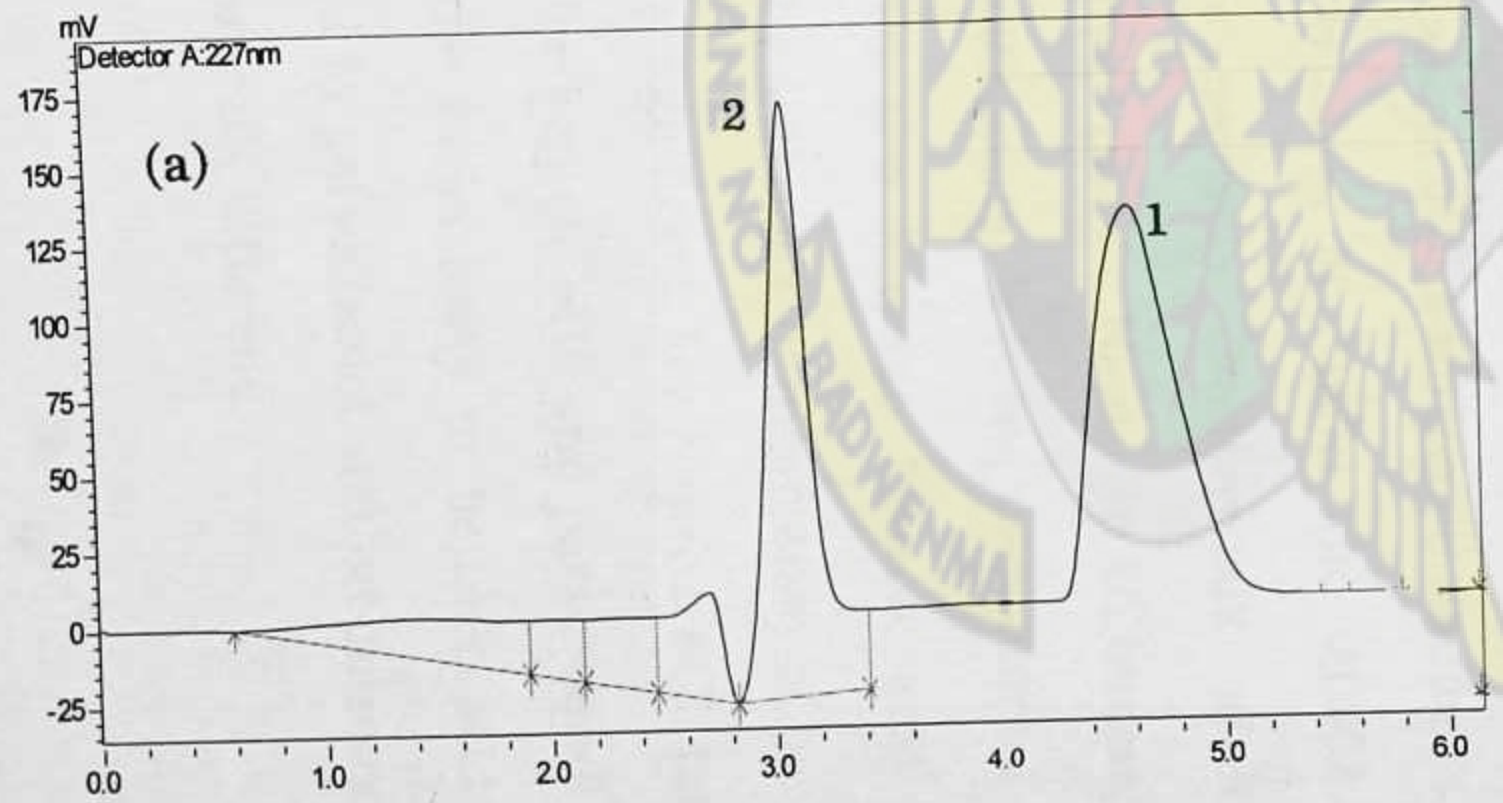
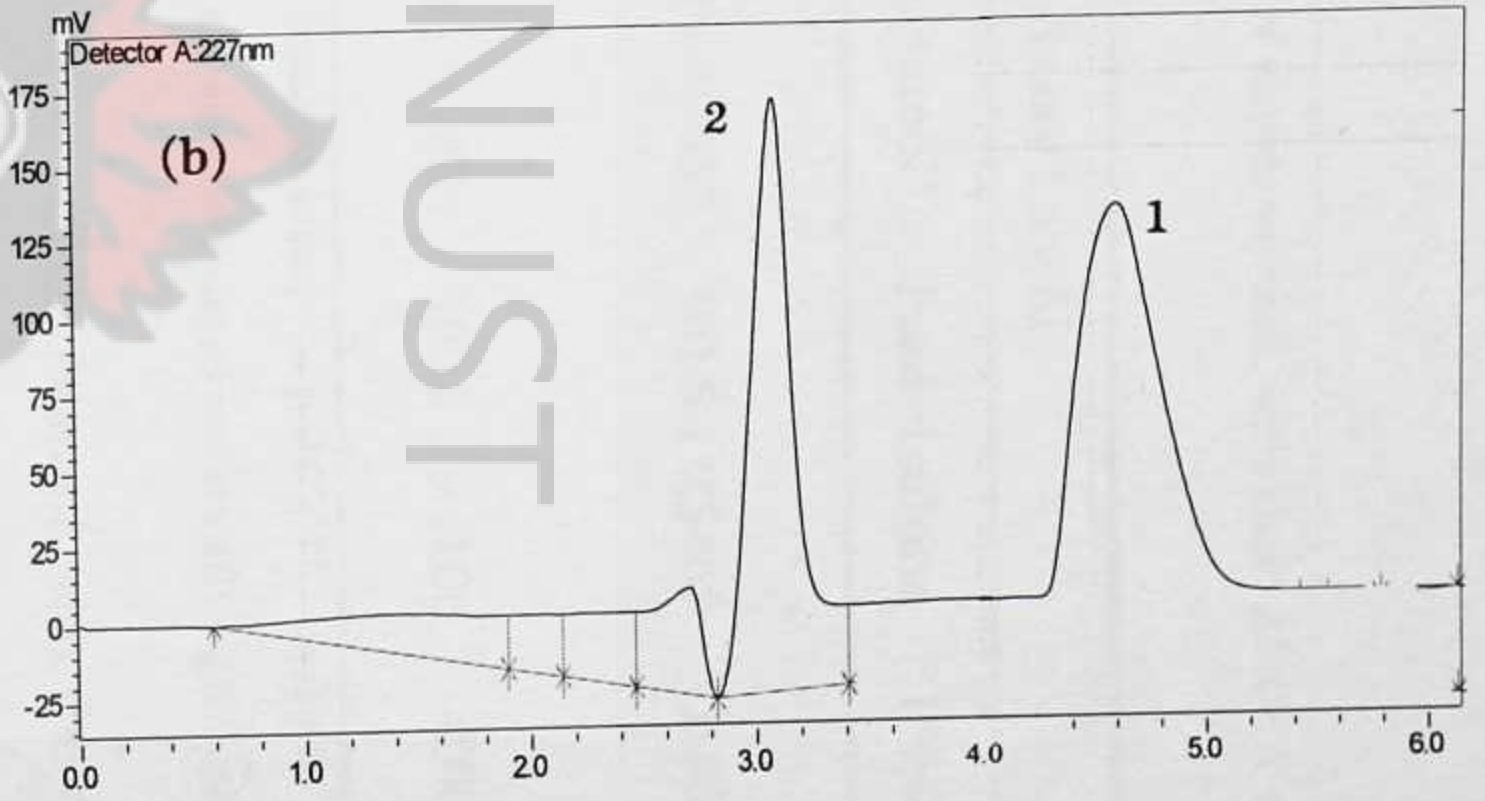


Figure 4-12 (a) Naprosyn EC (2) vs. Benzoic acid (1) Chromatogram



(b) Naprox ECL vs. Benzoic acid Chromatogram

The assay results obtained for the two brands of naproxen tablets used are shown in Table 4-14 below.

Table 4-14 Assay results for the various brands of naproxen used, with their ANOVA and t-test results

Tablet	Assay (n = 6)			
	Benzoic acid**	Paracetamol*	Prednisolone	P value
Naprosyn EC (500mg)	100.5 ± 0.4824	100.2 ± 0.7357	101.5 ± 0.5463	0.0073
Naprox ECL (500mg)	98.48 ± 1.272	101.3 ± 0.9360	100.9 ± 0.4160	0.0002

*, ** and *** indicates level of significance after students t-test. * = $p<0.05$, ** = $p<0.001$, *** = $p<0.0001$ (in all cases, p value < 0.05, 0.001 and 0.0001 were considered statistically significant)

Analysis of Variance (ANOVA) on the mean assays obtained with the three surrogate standards with respect to each brand shows, as indicated in Table 4-14, a significant difference between the mean assays of the surrogate standards for both brands of naproxen tablets, with p-values of 0.0073 and 0.0002 for Naprosyn EC and Naprox tablets respectively. Bonferroni's Multiple Comparison Test (BMCT) as a post test showed that for Naprosyn EC tablets, the mean assay was significantly different (statistically) between paracetamol and prednisolone as surrogate standards and also between benzoic acid and prednisolone as surrogate standards, but the difference in mean assay of benzoic acid and paracetamol was not statistically significant. For Naprox ECL tablets however, the difference in means assay for benzoic acid and paracetamol was significant, same as the differenee in the mean assay of benzoic acid and prednisolone. The mean assay determined by paracetamol and prednisolone in this brand of tablets was however not statistically different.

Student t-tests were also done between the assay results obtained for the two brands with each surrogate standard (Table 4-14). For benzoic acid as a surrogate standard, the difference in mean assay obtained for Naprosyn EC and Naprox ECL was statistically significant, same as for paracetamol as a surrogate standard. For prednisolone as a surrogate standard however, the mean assay obtained for two brands were not statistically different.

4.7.2 Daonil, Clamide and Glibenil tablets

The areas obtained after injection of a solution containing 0.0005% w/v of glibenclamide extracted from each brand of tablets, and 0.00025% w/v of one of the surrogate standards were used together with the determined K-value for the surrogate standard to assay to each three brand of glibenclamide tablets (Appendix). The chromatograms obtained for all brands and their surrogate standards are shown Figure 4-13 – 4-21 below.

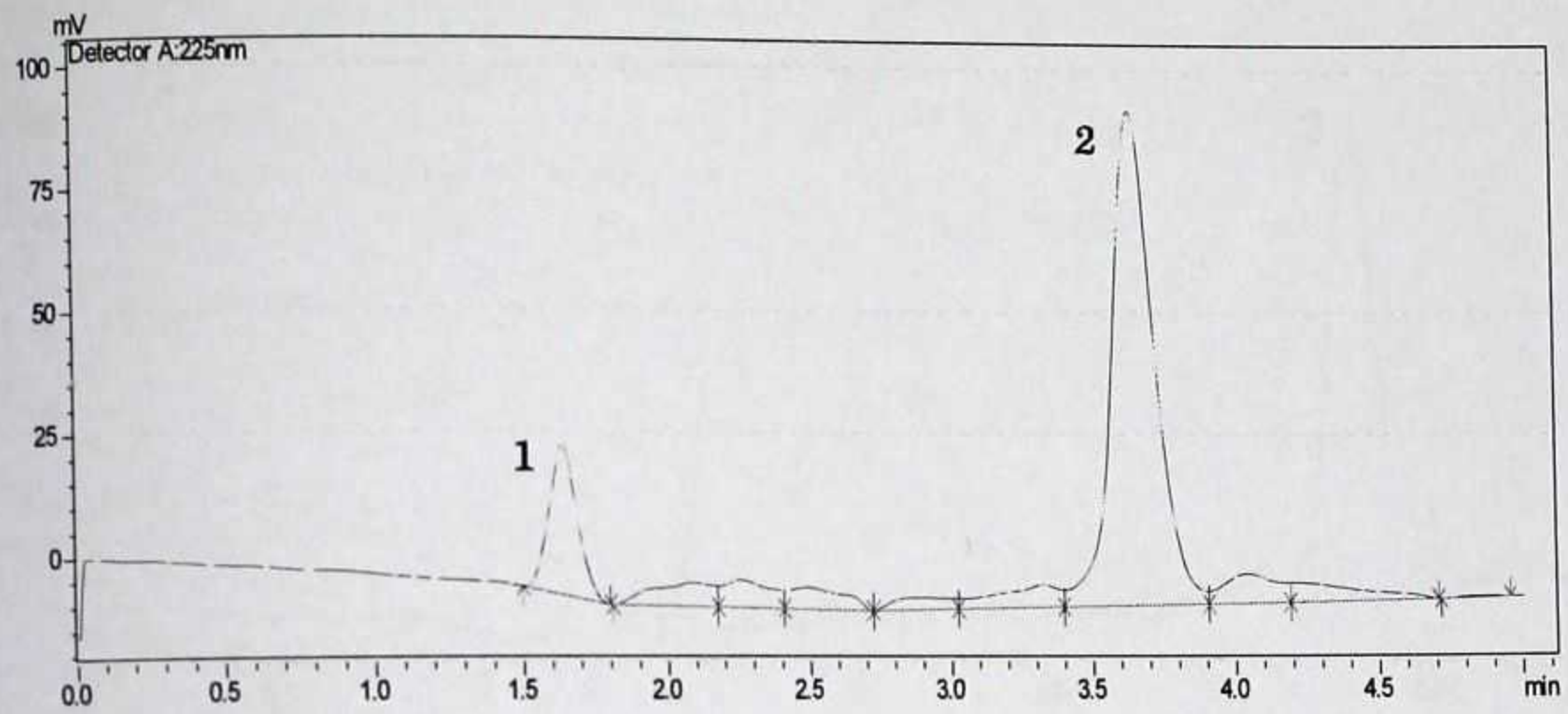


Figure 4-13 Daonil (2) and Salicylic acid (1) Chromatogram

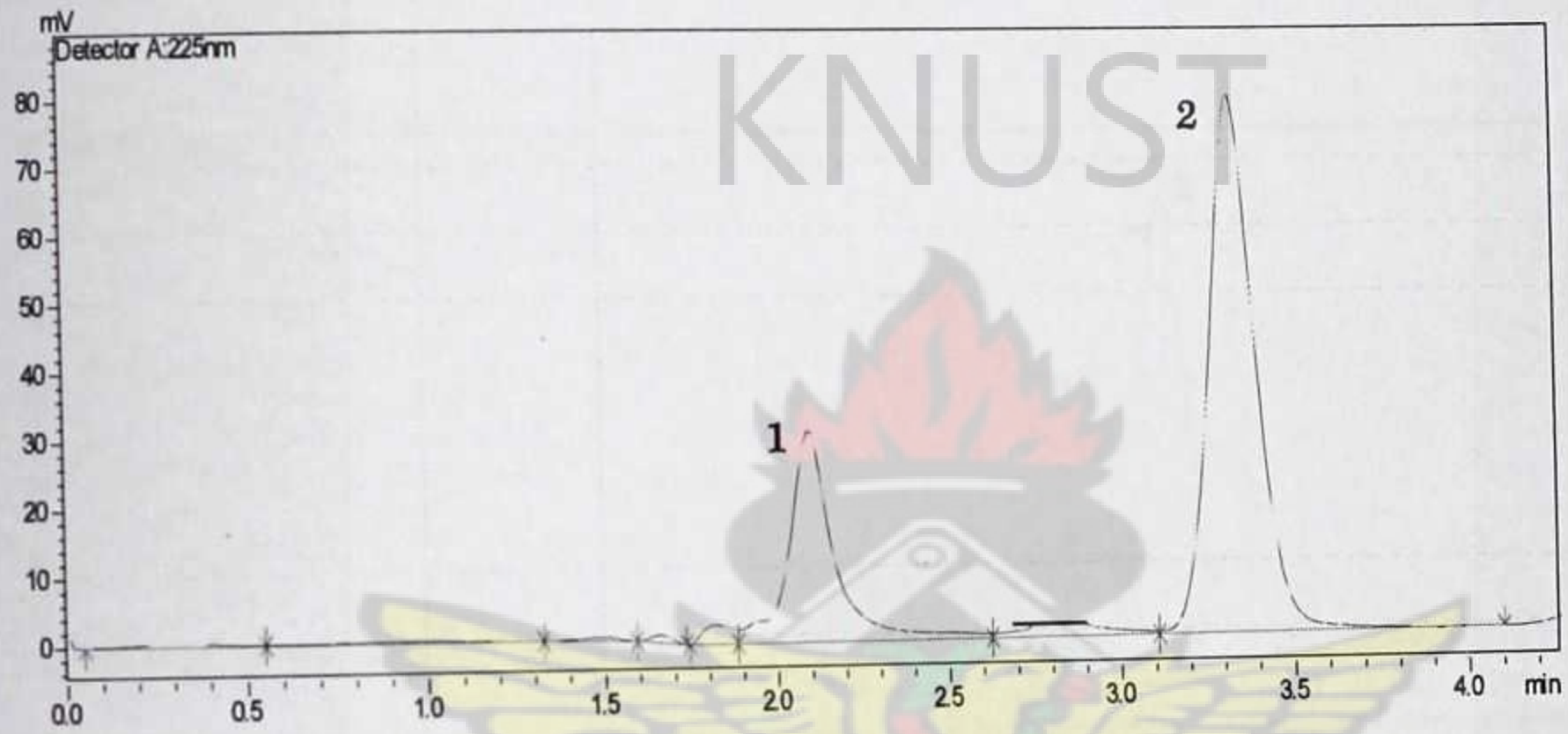


Figure 4-14 Daonil (2) and Indomethacin (1) Chromatogram

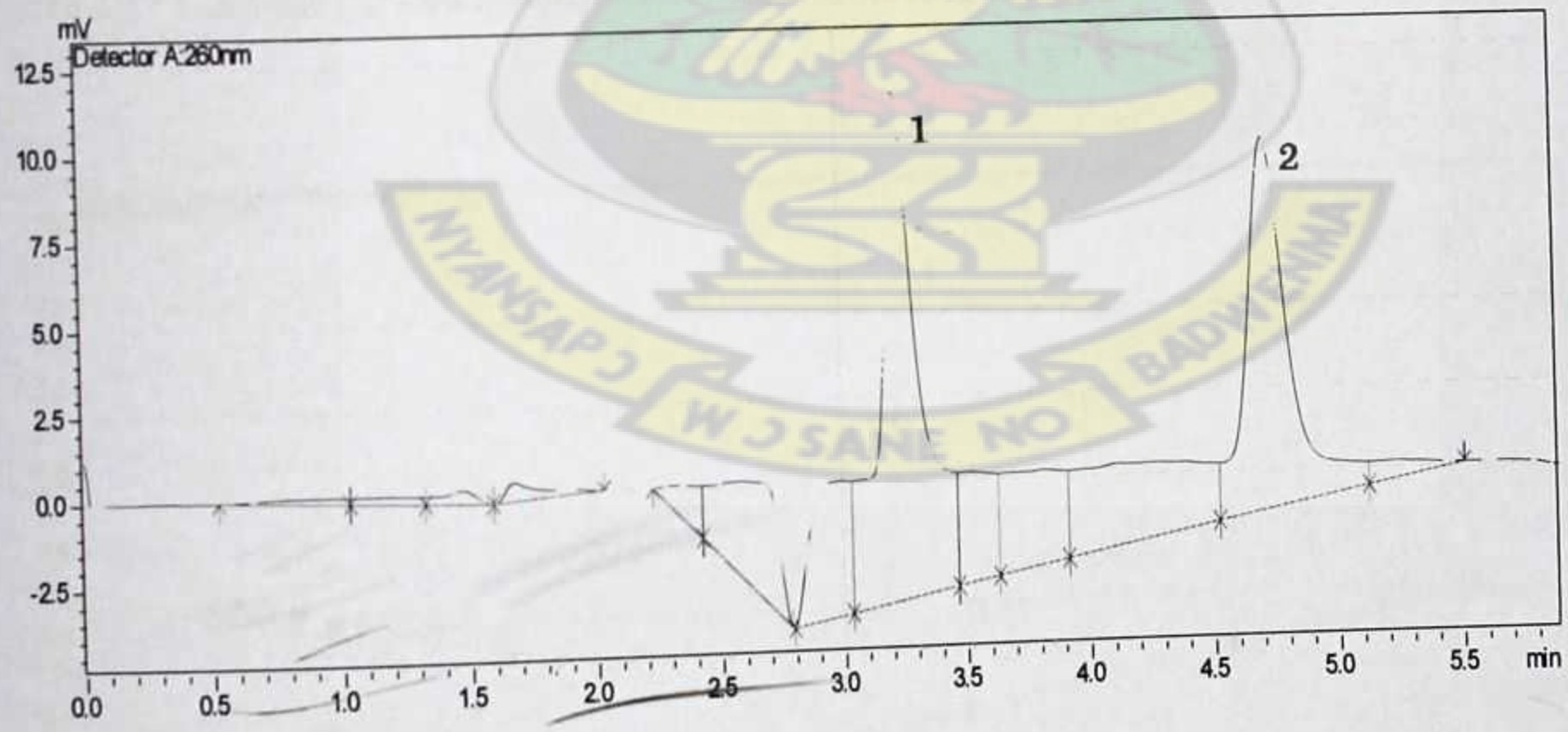


Figure 4-15 Daonil (2) and Chloramphenicol (1) Chromatogram

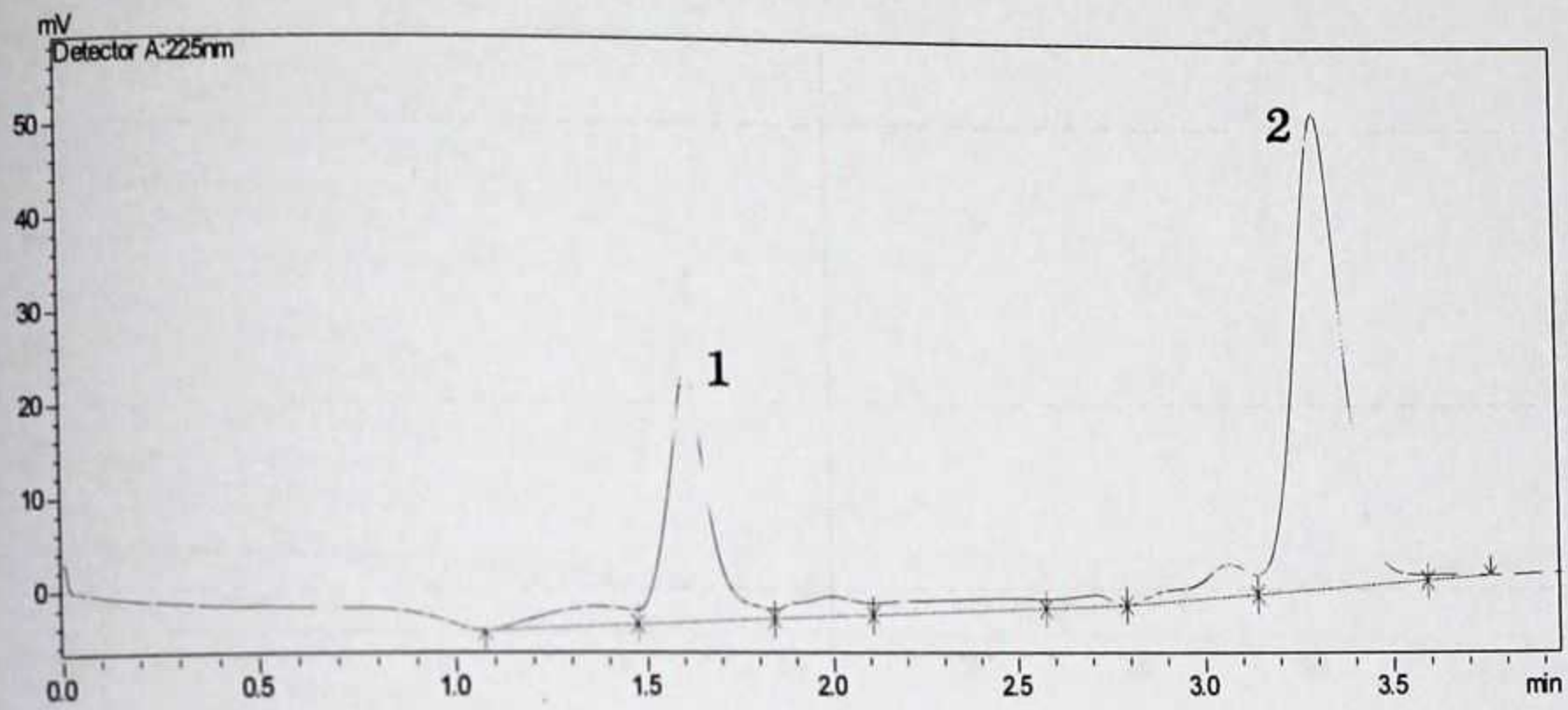


Figure 4-16 Clamide (2) and Salicylic acid (1) Chromatogram

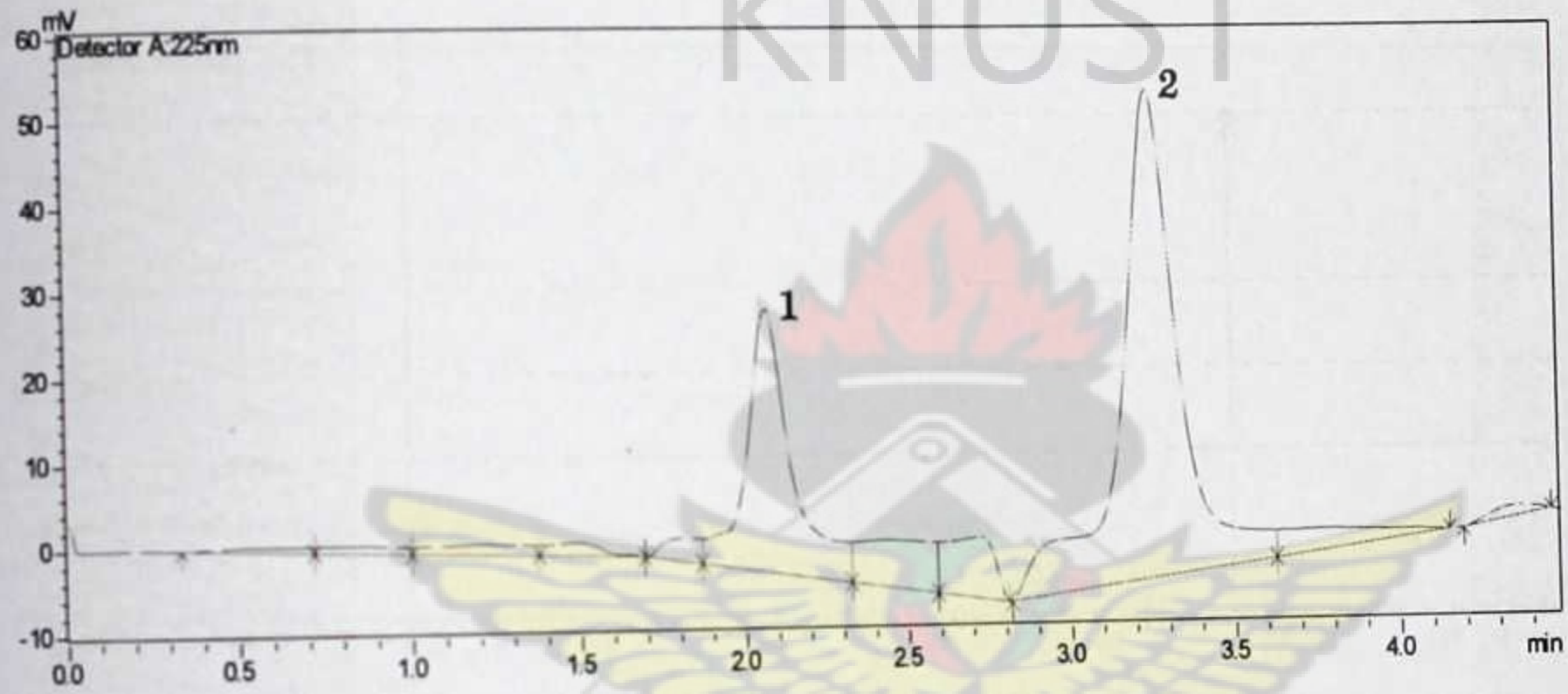


Figure 4-17 Clamide (2) and Indomethacin (1) Chromatogram

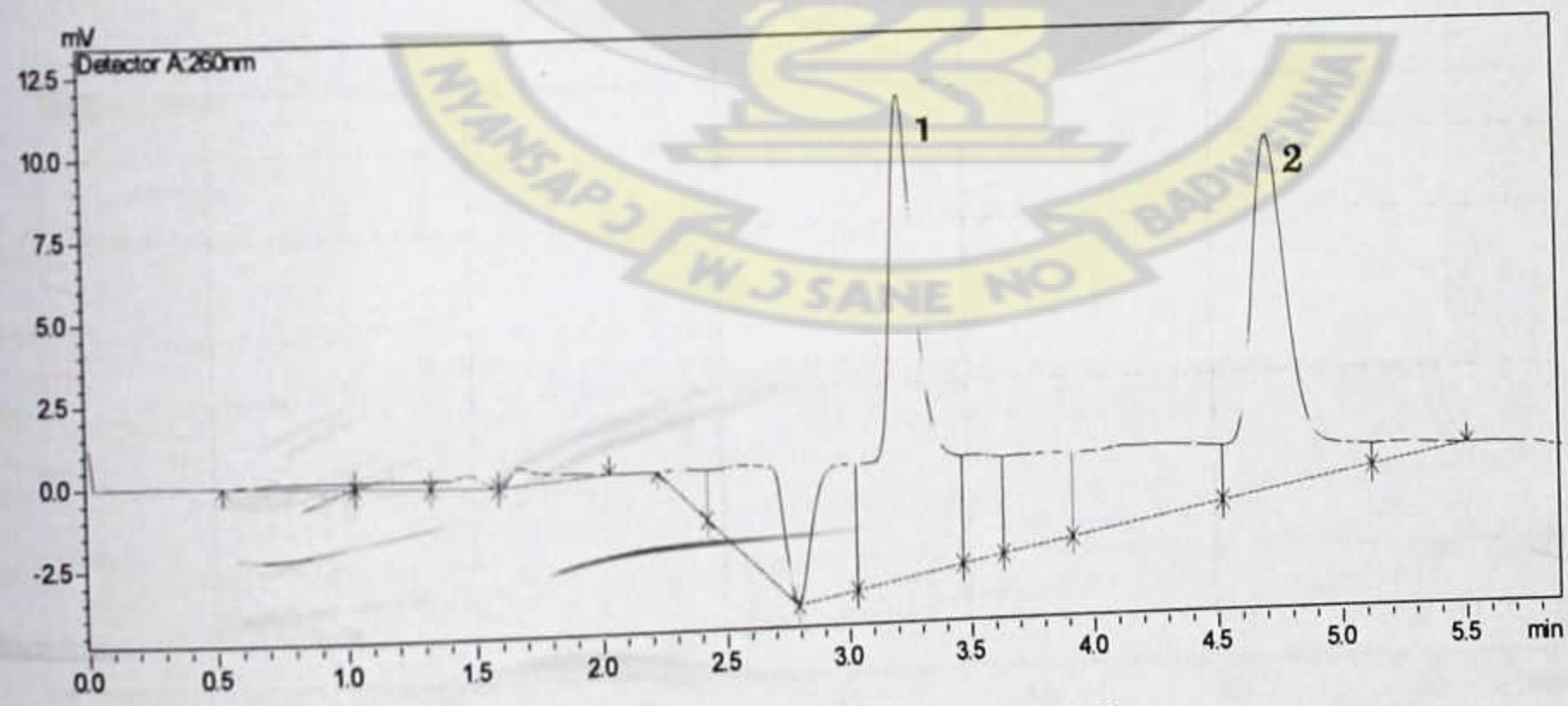


Figure 4-18 Clamide (2) and Chloramphenicol (1) Chromatogram

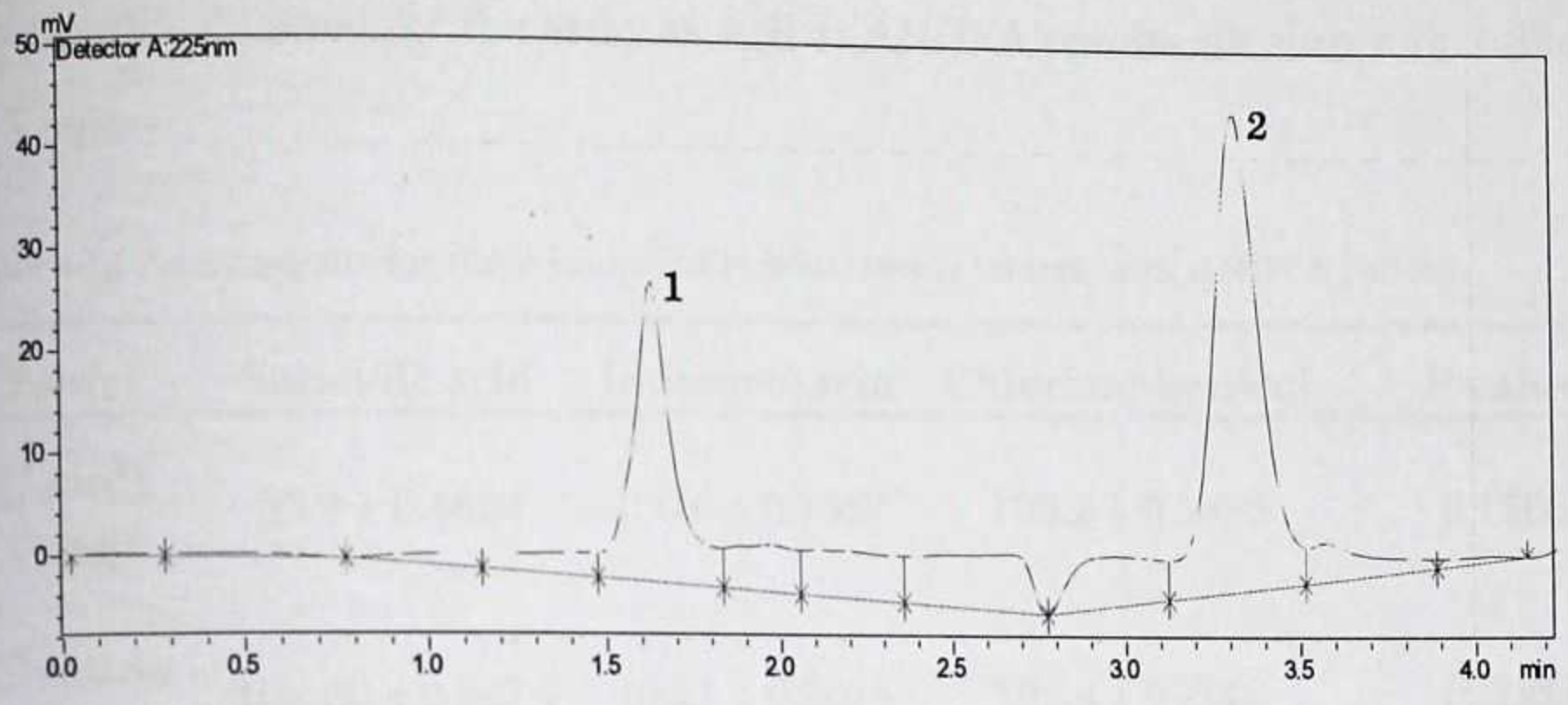


Figure 4-19 Glibenil (2) and Salicylic acid (1) Chromatogram

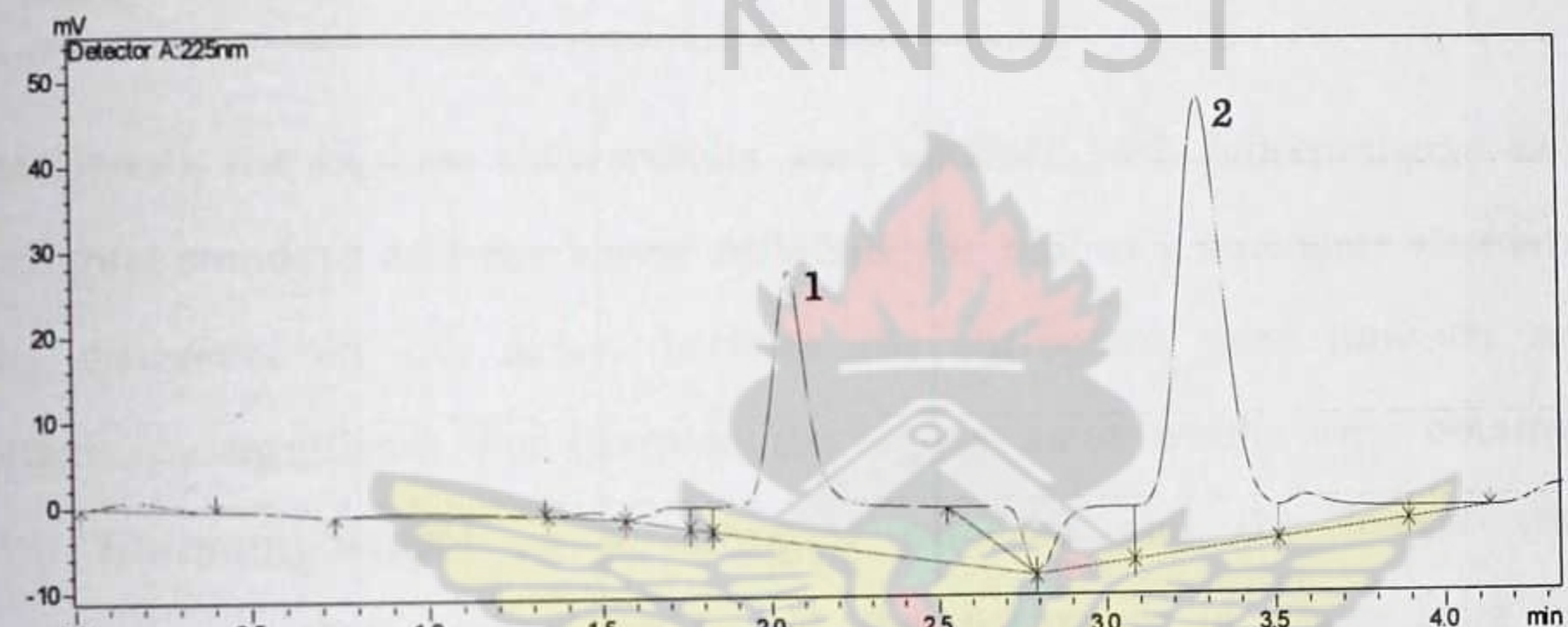


Figure 4-20 Glibenil (2) and Indomethacin (1) Chromatogram

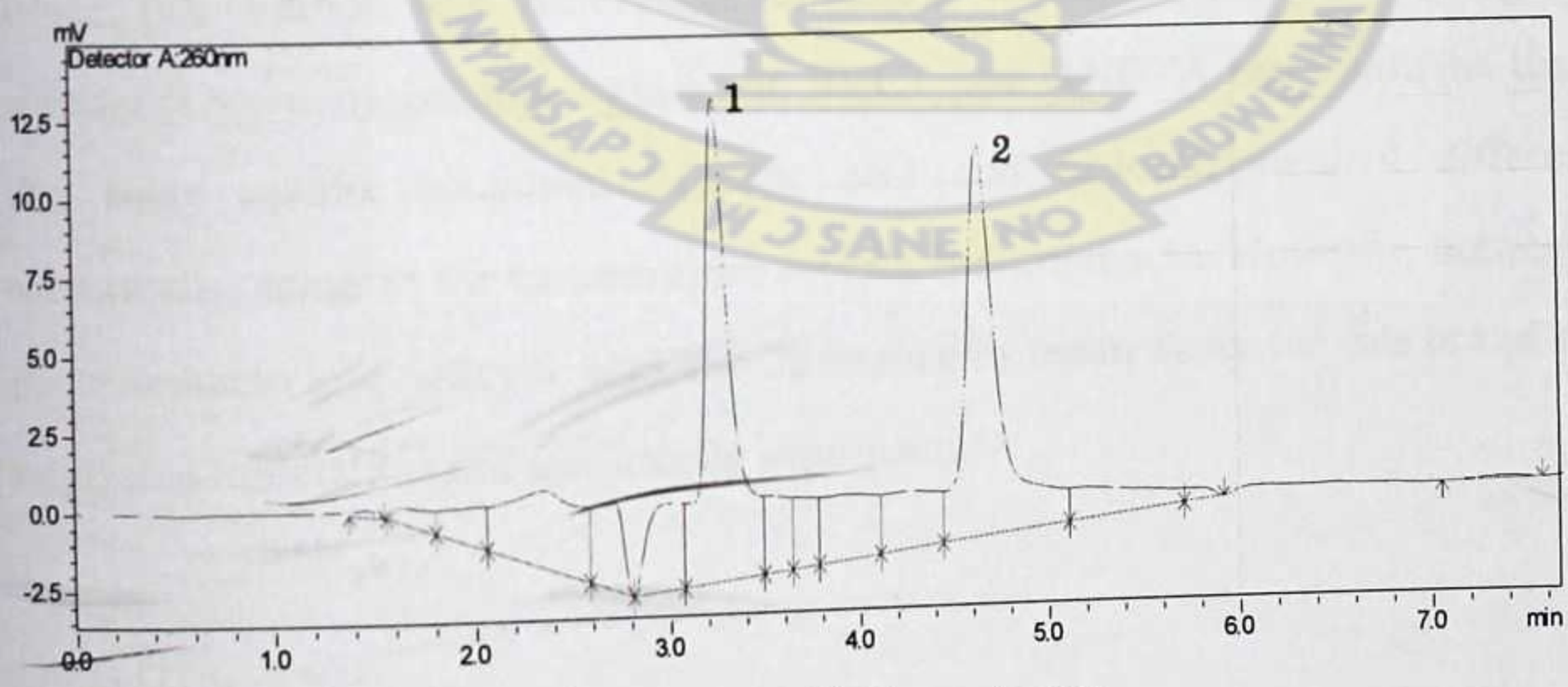


Figure 4-21 Glibenil (2) and Chloramphenicol (1) Chromatogram

The results obtained for the assay as well as ANOVA results are shown in Table 4-15 below.

Table 4-15 Assay results for three brands of glibenclamide tablets, with ANOVA results

Tablet	Salicylic acid	Indomethacin	Chloramphenicol	P value
Daonil (5mg)	99.9 ± 0.4824	100.6 ± 0.7357	100.2 ± 0.5463	0.1566
Clamide (5mg)	102.20 ± 0.8674	102.1 ± 0.9015	102.4 ± 0.7001	0.7666
Glibenil (5mg)	98.01 ± 1.794	98.27 ± 1.220	103.6 ± 0.9380	< 0.0001

For Daonil, the highest assay results were obtained with Indomethacin as a surrogate standard and the lowest with Salicylic acid as a surrogate standard. The difference in the assays between the surrogates were however not statistically significant. For Clamide, the highest assay results were obtained with Chloramphenicol as a surrogate standard and the lowest with Indomethacin, though just like Daonil, the differences in assays were not statistically significant. For Glibenil, the highest assay results were obtained with Chloramphenicol and the lowest with Salicylic acid. However, unlike the other two brands, the differences in assays obtained with the surrogate standards were statistically significant. BMCT post ANOVA test confirms that the assay results contained Salicylic acid and Chloramphenicol differed statistically, same as for Chloramphenicol vs. Indomethacin. However, between Indomethacin and Salicylic acid, the difference in mean assay for this brand of naproxen tablets was not statistically significant.

4.8 ASSAY OF TABLETS BY PHARMACOPOEIAL STIPULATED METHODS

4.8.1 Naprosyn EC and Naprox ECL tablets (BP, 2009)

For each brand, three determinations were done and the absorbance obtained for both the sample and the standard together with the assay results are shown in Table 4-16 below.

Table 4-16 UV-Spectrophotometric assay results of the two brands of naproxen tablets

Tablet	Determination	Abs Std	Abs Sample	Assay
Naprosyn EC (500mg)	1	0.822	0.82	99.76
	2	0.821	0.819	99.76
	3	0.820	0.819	99.88
Naprox (500mg)	1	0.822	0.815	99.15
	2	0.830	0.816	98.31
	3	0.831	0.817	98.32

The mean assays obtained by the BP method against the developed method using the various surrogate standards are shown Table 4-17 below, with the ANOVA results also showing the p-values.

Table 4-17 Summary of assay results obtained by the BP method against the developed method by each surrogate standard for the two brands of naproxen tablets

Tablet	BP	Benzoic acid	Paracetamol	Prednisolone	P-value*
Naprosyn EC (500mg)	99.8 ± 0.0693	100.5 ± 0.4824	100.2 ± 0.7357	101.5 ± 0.5463	0.0018
Naprox (500mg)	98.59 ± 0.4821	98.48 ± 1.272	101.3 ± 0.9360	100.9 ± 0.4160	<0.0001

*p-value < 0.05 was considered significant at 95% confidence interval

Overall, the percentage contents obtained with the BP method were lower than that obtained with the developed method for each surrogate standard, with the exception of the method by benzoic acid for Naprox tablets, which produced

slightly lower percentage content. Analysis of Variance also showed the differences in the percentage contents by all four methods for each brand of tablets were statistically significant.

Comparing each method with singly in the BP method by the student t-test yielded the p-values shown in Table 4-18 below. For both brands of tablets, the difference in assay result between the BP method and prednisolone as a surrogate standard were statistically significant. For benzoic acid as surrogate standard, the method yield results that were not significantly different from that of the BP method for Naprox ECL and Naprosyn EC (after BMCT post ANOVA test). For Paracetamol as a surrogate standard, the developed method yielded results that were significantly different from the results by the BP method for Naprox ECL tablets but not for Naprosyn EC tablet.

Table 4-18 P-values obtained for comparison of BP method with method for each surrogate standard for all brands of naproxen tablets

	Benzoic acid	Paracetamol	Prednisolone
	Naprosyn EC		
BP method	0.0374	0.3477	0.0014
	Naprox ECL		
	0.8888	0.0023	0.0001

p-value < 0.05 was considered significant at 95% confidence interval

4.8.2 Daonil, Clamide and Glibenil tablets (BP, 1980)

Just like the brands of naproxen tablets, three determinations were made of each prepared 0.001% w/v solution of tablets and standard. The absorbances of the sample and standards as well as percentage contents are shown in Table 4-19.

Table 4-19 UV-Spectrophotometric assay results of three brands of glibenclamide tablets

Tablet	Determination	Abs Std	Abs Sample	Assay
Daonil (5mg)	1	0.743	0.744	100.13
	2	0.742	0.736	99.19
	3	0.740	0.741	100.14
Clamide (5mg)	1	0.740	0.751	101.49
	2	0.742	0.748	100.81
	3	0.744	0.756	101.61
Glibenil (5mg)	1	0.741	0.758	102.29
	2	0.740	0.762	102.97
	3	0.745	0.755	101.34

A compilation of the mean assays obtained for the BP methods as well as the developed method for each surrogate standard with respect to all three brands is shown in Table 4-20 below, together with their respective p-values.

Table 4-20 Summary of assay results obtained by the BP method against the developed method by each surrogate standard for the three brands of glibenclamide tablets

Tablet	BP	Salicylic acid	Chloramphenicol	Indomethacin	P-value
Daonil (5mg)	99.82 ± 0.5456	99.9 ± 0.4824	100.6 ± 0.7357	100.2 ± 0.5463	0.1824
Clamide (5mg)	101.3 ± 0.4314	102.20 ± 0.8674	102.1 ± 0.9015	102.4 ± 0.7001	0.2861
Glibenil (5mg)	102.2 ± 0.8187	98.01 ± 1.794	98.27 ± 1.220	103.6 ± 0.9380	<0.0001

p-value < 0.05 is considered significant at 95% confidence interval

For both Daonil and Clamide tablets, the BP method yielded results that were slightly lower than that developed for all three surrogate standards. For Glibenil tablets, the results yielded by the BP method were higher than that developed

for two of the surrogate standards: Salicylic acid and Chloramphenicol, but not for Indomethacin. ANOVA results indicated, as shown in Table 4-20 that the difference in assay results obtained all four methods of Daonil and Clamide tablets independently were not statistically different ($p = 0.1824$ and 0.2861 respectively). For Glibenil tablets on the other hand, this was not the case as the difference in mean assay values of among all four methods were highly statistically significant, with $p < 0.001$.

Table 4-21 shows the p-values after t-test comparison of the BP method singly with each developed method. For both Daonil and Clamide tablets, the differences in percentage content results by the BP vs. all three methods singly were not statistically different. For Glibenil however, this trend was only observed in the method developed for Chloramphenicol but not the other two surrogate standards.

Table 4-21 P-values obtained for comparison of BP method with method for each surrogate standard for all brands of glibenclamide tablets

	Salicylic acid	Chloramphenicol	Indomethacin
	Daonil (5mg)		
BP method	0.7531	0.5554	0.093
	Clamide (5mg)		
	0.1307	0.0444	0.2263
	Glibenil (5mg)		
	0.0072	0.0707	0.0016

P-value < 0.05 is considered significant at 95% confidence interval

Chapter 5

DISCUSSIONS

The last decade has seen HPLC replace numerous spectroscopic, titrimetric and GC methods in both the quantitative and qualitative analysis of pharmaceutical substances (Nikolin *et al.*, 2004). It comes as no surprise therefore that HPLC has been used to solve no less than 50% of problems in pharmaceutical analysis (Misiuk, 2010). Its widespread applications in the field of drug analysis encompasses the analytical investigations of bulk drug materials, the intermediates in their synthesis, products of drug research, drug formulations, impurities and degradation products, and biological samples containing the drugs and their metabolites, which are all very important areas of research. Setbacks in the use of HPLC however still abound, all emanating from the high cost implications of using this analytical technique. The need for the development of simple inexpensive HPLC methods is now a major research theme in the pharmaceutical industry.

This study sought to develop each for naproxen and glibenclamide formulated products, three simple and robust HPLC methods, each of which employed common laboratory drug substances as the reference standard for use in quantification. These 'surrogate standards' as they are called in this study, are pure powders of easily obtainable drugs and hence would provide ready alternatives for pharmaceutical analysts in the event that a reference standard is unavailable. These standards were made to act in the capacity of an internal standard for each of the drugs and hence their selection had to take into consideration a number of factors. The most critical requirement of an internal standard is a good peak shape and clear resolution from other analytes on a chromatogram (Magee and Herd, 1999). This was hence an important consideration in the selection of the surrogate standards for each drug

substance. In this regard, the pKa of the various surrogate standards, whether they were acidic or basic and the extent of their polarity, all of which could directly affect their behavior in an HPLC system were paramount to their selection. Other important requirements for an ideal internal standard which were also taken into consideration were their stability in solution, commercial availability in a high pure form, ready solubility in the diluent required, possession of acceptably high UV activity at a desired wavelength, low costs and low toxicity. Out of the many drugs that were initially screened, benzoic acid, paracetamol and prednisolone were chosen as surrogate standards for naproxen, whereas salicylic acid, chloramphenicol and indomethacin were chosen for glibenclamide. Successes have previously been obtained with earlier studies in the Dept. of Pharmaceutical Chemistry as far as some of these drug substances are concerned; the drugs were either used as surrogate standards (benzoic acid, salicylic acid, paracetamol, indomethacin, naproxen) or as the drug of interest (indomethacin, paracetamol, prednisolone) (Mohammed, 2008; Tuani, 2009; Amegadzie, 2010; Oppong-Danquah, 2010).

5.1 IDENTIFICATION TESTS AND ASSAY OF PURE SAMPLES AND TABLETS

Identification tests for pharmaceutical substances and products are a critical part of pharmaceutical streamline process and cuts across all stages of the streamline. More importantly, identification is required before any analysis is done. A number of approaches can be adopted for the true identification of a drug: determination of physical constants; chromatographic tests; and finally the chemical (colour) tests. The physical constants essentially include melting point, boiling point, refractive index, weight per millilitre, specific optical rotation, light absorption, viscosity, specific surface area, swelling power and infra-red absorption (Kar, 2005). Melting point determination is important to knowing the purity of a substance. It is usually quoted as a range (melting

range) due to the different manufacturing processes available for a particular drug. The melting ranges obtained for all the drug samples (Table 4-1) used in the study fell within their pharmacopoeial stipulated ranges. The BP (2009) stipulated melting ranges are given as follows: naproxen (154 – 158 °C); glibenclamide (169 - 174 °C); benzoic acid (121 – 124 °C); salicylic acid (158 - 161 °C); paracetamol (168 - 172 °C); indomethacin (158 - 162 °C); prednisolone (231 - 234 °C); chloramphenicol (149 - 153 °C). A number of identified limitations as far as melting point determination as an identification test mean that they must be used in conjunction with another identification test method.

Chemical tests are the most specific and reliable identification tools for pharmaceutical substances (Kar, 2005) and are usually combined with physical tests to make a proper identification. Chemical tests may be categorized separately under tests for inorganic substances and organic substances. The former may be carried out by well-defined general inorganic analysis and the latter by specific reactions of one or more of the functional moieties present in a drug molecule (Kar, 2005). All pure samples used in the study passed their respective colour and chemical tests as described in the pharmacopoeias and drug handbooks. For the main drug samples naproxen and glibenclamide, another physical constant, UV absorbance was used in addition to melting point and the chemical test to make identification. This approach was also applied to all brands of tablets of the two drugs. The obtained absorption maximas conformed to what is stipulated in the pharmacopoeias for the respective drugs.

Once identification was done, assay of the analytes had to be done before their use in the HPLC analysis. This was necessary to ensure traceability of the HPLC results that will be obtained. Assay of the pure samples were done by their respective pharmacopoeial procedures and the percentage contents were reported. All analyte assays conformed to their stipulated ranges in the pharmacopoeia used (BP, 2009). Hence the assay for naproxen (100.15%),

glibenclamide (99.93%) and paracetamol (99.71%) all were within the BP's range of 99% – 101%. Similarly, benzoic acid (99.71%) and salicylic acid (99.85%) all conformed to the 99% – 100.5% specification. In a likewise manner, prednisolone (101.28%) conformed to its 97% – 103%, indomethacin (99.91%) to its 98.5% – 100.5%, and chloramphenicol (100.5%) to its 98% – 102% specification. All pure samples therefore passed their individual assay tests.

5.2 HPLC METHOD DEVELOPMENT

This study developed six simple HPLC methods, three for the analysis of glibenclamide and the remaining three for the analysis of naproxen. Having gathered information on all analytes that aided the selection of surrogate standards, UV scanning was done to help make deductions on a suitable wavelength for analysis of each drug-surrogate standard pair. For both naproxen and glibenclamide, UV scanning showed a wide range of wavelengths of absorbance of UV light. This suggested a high UV absorbance for the two drugs, possibly due to the high number of chromophoric groups present in both compounds. This flexibility offered by the two drugs meant that the wavelength to be chosen for their analysis with their surrogate standards depended more on UV absorbance behaviour of the surrogate standard. Hence for a surrogate standard like benzoic acid that absorbed only around low wavelengths, a wavelength of 227nm was chosen for its analysis with naproxen. Likewise, for prednisolone, its very bad UV absorption below 240 and above 250 meant analysis had to be done at its wavelength of maximum absorption (245nm). For paracetamol, wavelengths less than 240 resulted in very poor UV absorption whereas wavelengths around its absorption maxima gave so very high absorption that the peak for naproxen, whose concentration in the injected sample was twice that for the paracetamol was very small. Hence a wavelength of 280nm which gave appreciable absorption for both drugs was used. Analysis of glibenclamide against both indomethacin and salicylic acid was done at a

wavelength of 225nm as the two surrogate standards absorbed quite well at this wavelength, just like glibenclamide. For chloramphenicol against glibenclamide a wavelength of 260nm was chosen also because both groups absorbed quite well at this wavelength.

With the aim to develop a simple HPLC method, methanol was chosen to be the organic component of the mobile phase and initial scouting runs were made to select the best MP combination for optimum resolution. With the exception of prednisolone, all the analytes used in this study were polar, and this informed the selection of a relatively polar C8 column (compared to a more non-polar C18 column), though it was also borne in mind that the polar column could lengthen the run time for the polar analytes. Settling on a flow rate of 1ml/min, scouting runs revealed the ideal mobile phase combination for the surrogate standards of naproxen to be a 35:65 %v/v (water : methanol) combination. With this, prednisolone had the highest retention time, then paracetamol before benzoic acid. Numerous HPLC methods have been developed for the analysis of these analytes both in bulk and in formulations, either singly or in combination with other drugs (Lunn and Schmuff, 1997). Retention times for paracetamol for most of these methods range between 3 – 6 minutes depending on the mobile phase vs. column type used (Lunn and Schmuff, 1997; Süzen *et al.*, 1998; Altun, 2002; Moffat *et al.*, 2004; Battu and Reddy, 2009) which encompasses the retention time obtained in this study. Due to its non-polarity, prednisolone analysis on a C18 column takes a longer run time and hence most studies report retention times greater than 10 minutes (Moffat *et al.*, 2004; Rojanarata, 2012). It is however worth noting that very few studies have developed HPLC methods for the analysis of prednisolone. For naproxen, the retention time of about 5 minutes obtained in this study compares with that obtained with other studies, with most retention times between 4 and 8 minutes (Chakrabarti and Southard, 1996; Lunn and Schmuff, 1997; Moffat *et al.*, 2004).

With the same flow rate of 1ml/min, a mobile phase combination of 20% water and 80% methanol was settled on for the analysis of glibenclamide against its surrogate standards after initial scouting runs. The highest retention time was obtained with glibenclamide, followed by chloramphenicol, then indomethacin before salicylic acid at 1.6 minutes. Varying retention times have been reported as far as HPLC analysis of glibenclamide is concerned, with most reporting times between 2 – 8 minutes (Bagool *et al.*, 2009; Rayanm *et al.*, 2011; Jayanthi *et al.*, 2012). Most of the various studies that have focused on the HPLC analysis of indomethacin have reported retention times above 7 minutes, with very few reporting times between 4 and 5 minutes (Plakogiannis *et al.*, 1981; Shimek *et al.*, 1981; Moffat *et al.*, 2004; Tsvetkova *et al.*, 2012). The low retention time of 2.8 minutes obtained in this study could be as a result of the use of the relatively slightly polar C8 column compared to the C18 that almost all of these studies have employed. The high organic composition of the mobile phase could also be a contributing factor to the low retention time observed for indomethacin. For chloramphenicol, the retention time reported in this study (3.2 minutes) compares with that observed in other studies on its HPLC analysis (Tyczkowska *et al.*, 1988; Shadoul *et al.*, 2011), as most of the studies reported retention times between 3 – 6 minutes.

It must be noted however that most of the studies reported above for the analysis of the various analytes especially for naproxen and glibenclamide, have employed complex and expensive solvent systems (Lunne and Schmuff, 1997), in contrast to this study that used a simple water/methanol system. A case in point is the USP 30 HPLC method described for the assay of both analytes that adopts the expensive acetonitrile as the organic solvent.

5.3 VALIDATION OF THE HPLC METHODS

Each method was validated according to ICH guidelines on method validation (ICH, 1996). Linearity of the method developed for each of the 8 analytes used in this study conformed to the stipulated specification of a correlation coefficient ≥ 0.998 for pure APIs, with the exception of benzoic acid (0.9972), indomethacin (0.9971) and paracetamol (0.991) which deviated only slightly.

For the surrogate standards of naproxen, benzoic acid and prednisolone had the lowest LODs and LOQs, since their analysis with naproxen was done at their respective wavelengths of maximum absorption, compared to that of paracetamol that was done at 280nm, where absorption wasn't so high. In a likewise manner, the LODs and LOQs for indomethacin and salicylic acid, both of which were analyzed together with glibenclamide were lower than that of chloramphenicol as the wavelength used for their analysis with glibenclamide (225nm) was one they absorb UV light highly at. Among the three LODs and LOQs for naproxen, the LOD and LOQ was lowest when it was analyzed with prednisolone at 245nm, followed by its analysis with indomethacin at 225nm before with paracetamol. For glibenclamide, two LODs and LOQs were obtained at 225nm and 260nm. The lowest results were obtained with salicylic acid and indomethacin (225nm) before chloramphenicol at 260nm.

Precision of the developed method for each analyte was monitored both within and between days. With a requirement of a RSD not more than 2% by the ICH (2000), the method was judged to be precise for all eight analytes. Among naproxen and its surrogate standards, paracetamol had the highest intra-day RSD of 1.51% as well as inter-day RSD (0.89%). Benzoic acid had the lowest, with an intra-day RSD of 0.33% and together with naproxen (0.23%) had also the lowest inter-day RSD of 0.26%. Glibenclamide and indomethacin gave the lowest RSD for the intra-day precision compared to the other surrogate standards of glibenclamide, with chloramphenicol having the highest RSD. A

similar trend was obtained for the inter-day RSDs, with salicylic acid joining chloramphenicol as the analytes with the highest RSDs.

5.4 DETERMINATION OF K CONSTANT AND ITS USE IN THE ASSAY OF TABLETS

This study determined a constant, K, for each drug sample vs. surrogate standard pair by using areas obtained for the drug samples and the surrogate standards, and their injected concentrations. The rationale for the determination of the constant followed the general rationale behind the use of internal standards in HPLC analysis, as the surrogate standards could be considered as internal standards. Hence the ratio of the response factor of the drug sample to that of the surrogate standard, which would have been 1.0 should the drug and surrogate standard be the same compounds, gave the K constant.

The K constants obtained from previous studies in the department (unpublished data) has ranged from as low as 0.035 (phenacetin) to as high as 18.23 (Aspirin). Two of such studies have so far used naproxen as a surrogate reference standard. In one study where it was used as a surrogate standard for indomethacin, the K constant obtained was 1.674, whereas in the other study where it was used as a surrogate standard for prednisolone, the obtained K constant was 1.693. For benzoic acid, one study reported a high value of 11.66 when used in the assay of paracetamol, whereas another study reported a value of 0.646 when used in the assay of aspirin. Still another study reported a value of 3.426 when benzoic acid was used to assay indomethacin; whereas this study reported a value of 0.5967. The remaining two surrogate standards for naproxen, paracetamol (K constant = 0.5626) and prednisolone (K constant = 0.9077) have yet to be employed as surrogate standards in any study, though they have been used as the main drug sample in two previous studies as mentioned above. Among glibenclamide and its surrogate standards, only

indomethacin has been used in previous studies (two) as surrogate standards. In one where it was used as a surrogate standard for diazepam, the reported K constant was 0.323, whereas in the second, it was reported to be 1.83 as a surrogate standard of prednisolone. The reported K constant obtained for indomethacin in this study was 0.7798. Glibenclamide, salicylic acid and chloramphenicol have yet to be used as surrogate standards for any analysis. With the K constant values directly dependent on the areas of the drug vs. the standard, these wide differences in the K constants are clearly attributed to the difference in the HPLC behaviours of the respective drugs used. Moreover, the different method conditions developed for each method like MP combination could also play a role in the observed wide differences in K constants. When the analysis was done at different concentration levels (7 for naproxen vs. surrogates and 6 for glibenclamide vs. surrogates) the K constant varied only little or not at all (Tables 4-12 and 4-13). This seems to suggest that concentration have very little effects on the value of the constant. Further studies may however be required to fully confirm this as well as the effect of other conditions like temperature and pH on the K constant.

Once the K constants were obtained, the next step was the main focus of the work: to determine if they could successfully be used in the assay of formulated naproxen and glibenclamide tablets. One important determining factor of whether the K constant will work in this regard is the RF obtained for the tablet vs. surrogate standard. This RF must be very comparable to that obtained with the pure drug sample vs. the same surrogate standard. This means that the ratio of the areas of either naproxen or glibenclamide tablets vs. surrogate standard must be equal to, of be very close to the ratio of pure naproxen or glibenclamide vs. surrogate standard used to determine K constant. Hence with comparable RFs, the value of the K constant would not matter, and that is why no specification of an ideal K constant value can be made. Using the K constant as well as the RFs for both pure drug vs. surrogate standard and tablet vs.

surrogate standard, the actual concentration determined by the method was calculated which was compared with the nominal concentration expected in each injection and the percentage content in each brand of tablet was determined. Next the pharmacopoeial stipulated assay procedures for naproxen and glibenclamide were done so that comparison with the assays obtained with the developed method could be done.

As required by the pharmacopoeias and other regulatory bodies, assay of the tablets of any drug must begin with a weight uniformity test after identification is done. Regulatory bodies require that for uncoated and film coated tablets, no more than two tablets should deviate by more than 10% for a drug of strength 80mg or less, or 5% for a drug of strength 250mg (BP, 2009). A look at the weight uniformity results (Appendix) shows that all three brands of glibenclamide (Daonil, Clamide and Glibenil) used and the two brands of naproxen tablets (Naprosyn EC, Naprox ECL) passed the uniformity of weight test.

For the tablets of both drugs, the BP (2009) specification is that the assay must yield between 95% and 105% of the stated amount. A consideration of the percentage content obtained by each surrogate standard for the various brands of naproxen tablets (Table 4-14) shows that all methods yielded acceptable results, as for Naprosyn EC tablets, the lowest assay (100.2%) was obtained with paracetamol as surrogate standard, and the highest (101.5%) was obtained with prednisolone as surrogate standard. A similar trend was observed with Naprox ECL tablets but in this case the lowest assay (98.48%) was obtained with benzoic acid and the highest with paracetamol as a surrogate standard. Notwithstanding this however, statistical analysis showed that the differences observed among the assays obtained with the various surrogate standards for a particular brand was significant. The p-values obtained after ANOVA, 0.0073 and 0.0002, for Naprosyn EC and Naprox ECL tablets respectively confirms this. Post-test

analysis further confirms that between some surrogate standards, (benzoic acid vs. prednisolone and paracetamol vs. prednisolone for Naprosyn EC tablets, and benzoic acid vs. prednisolone as well as benzoic acid vs. paracetamol for Naprox ECL tablets) the difference in mean assay was statistically significant. Moreover, for each surrogate standard, with the exception of prednisolone, differences existed as far as comparing the mean assay obtained with that surrogate standard between the two brands was concerned. These significant differences in assay according the statistical analysis seems to favour prednisolone more as the most ideal of the three surrogate standards but further studies may be needed to clarify this assertion. When statistical comparison between these assay results and the BP-method-obtained results (Table 4-17 and 4-18) was done, there was a general significant difference between results by all four methods for both brands. The assay results by all three surrogate standards were higher than that obtained with the BP method for Naprosyn EC tablets, with a similar trend being observed for Naprox ECL with the exception of the method using benzoic acid. Hence for Naprosyn EC tablets, the higher assay results obtained with benzoic acid and prednisolone were statistically different hence the observed p-values. The difference in mean assay obtained between paracetamol vs. the BP method was however not significant. For Naprox ECL tablets however, a significant difference existed between the mean assays of paracetamol vs. BP method as well as prednisolone vs. BP method but not with benzoic acid vs. BP method.

The assay results obtained for each surrogate standard of glibenclamide for all three brands of tablets used, were acceptable as they were all within the BP's 95% to 105% specification. For Daonil tablets, the highest assay (100.6%) was obtained with indomethacin and the lowest (99.9%) with salicylic acid. For Clamide tablets, the highest assay (102.4%) was obtained with chloramphenicol and the lowest (102.1%) with indomethacin. For Glibenil tablets however, the highest assay result (103.6%) was obtained with chloramphenicol and the lowest

(98.01%) with salicylic acid. However, just like for naproxen, some statistical difference was observed, especially among the results obtained for Glibenil tablets due to the wide difference between its lowest and highest assay value. This trend was not observed with Daonil and Clamide tablets pointing to the implication that unlike the case of naproxen all the surrogate standards of glibenclamide are the same in terms of the results they offer. The trend was repeated when the assay result by each surrogate standard was compared to the assay obtained with the BP method for each brand. For both Clamide and Daonil tablets, no statistically significant difference was observed between the assay result obtained with the BP method vs. that by each surrogate standard. For Glibenil tablets however, this trend was only observed with chloramphenicol vs. BP method, not with salicylic acid vs. BP method and indomethacin vs. BP method, the p-value obtained with these two was significant.

It is however important to mention again that these statistical comparisons may not matter at all as all assays for all brands fell within the specified limits.

Chapter 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

This study has successfully demonstrated that the three surrogate standards selected for naproxen and glibenclamide can each be used in the HPLC analysis of tablets of the two drugs. For naproxen, an HPLC method was developed for each one of the surrogate standards (benzoic acid, paracetamol and prednisolone). The developed method employed a 35% : 65% (water/methanol) mobile phase system and C8 silica column for analysis. An HPLC method was also developed for each glibenclamide vs. surrogate standard pair, with the chosen surrogate standards being salicylic acid, indomethacin and chloramphenicol. Each pair employed a 20% : 80% (water/methanol) mobile phase system and also a C8 silica column for analysis. A constant K, was determined for each drug vs. surrogate standard pair and together with the surrogate standard, was used in the HPLC assay of two brands of naproxen and three brands of glibenclamide tablets. The percentage content of API determined in this way for each brand of tablet conformed to their required specifications as stipulated by the pharmacopoeias. Notwithstanding this however, statistical difference existed between the results obtained for some surrogate standards with some brands, in comparison with the pharmacopoeial (BP) methods.

This study has therefore demonstrated simple, cheaper and alternate methods of HPLC analysis of naproxen and glibenclamide tablets using common and easily available laboratory drugs as the reference standard.

6.2 RECOMMENDATIONS FOR FURTHER WORK

Further investigations into factors that have a tendency of affecting the K constant like pH and temperature must be carried out. There is also the need for the study to be extended to other unexploited drugs, and its feasibility of application to the combination therapies must be determined.

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APPENDIX

App.1 Preparation Of Solutions

App.1.1 Preparation of 0.1M sodium hydroxide (NaOH)

40g of NaOH in 1000ml of solution ≡ 1M solution

20g of NaOH in 500ml of solution ≡ 1M solution

:. 2g of NaOH in 500ml of solution ≡ 0.1M solution

But %purity of NaOH pellets used = 96.0%

Hence nominal weight = $\frac{2 \times 100}{96.0}$ = 2.0833g

NaOH pellets (2.0833g) were hence accurately weighed into a beaker and about 50ml of distilled water was added. The solution was allowed to cool before transferring into a 500ml volumetric flask and adding distilled water to the mark.

App.2 Titration Tables And Assay Information

App.2.1 Standardization of 0.1M NaOH with H_2NSO_3H

Determination	Initial reading (ml)	Final reading (ml)	Titre (ml)
1	0.0	25.2	25.2
2	0.0	25.1	25.1
3	0.0	25.1	25.1
Average titre			25.1

App.2.2 Assay of Naproxen with 0.1M NaOH

Determination	Initial reading (ml)	Final reading (ml)	Titre (ml)	Actual weight (g)
1	0.0	8.8	8.8	0.2035
2	10.0	18.7	8.7	0.2011

App.2.3 Assay of Glibenclamide with 0.1M NaOH

Determination	Initial reading (ml)	Final reading (ml)	Titre (ml)	Actual weight (g)
1	0.0	8.1	8.1	0.4017
2	10.0	18.1	8.1	0.4017

App.2.4 Assay of Benzoic acid with 0.1M NaOH

Determination	Initial reading (ml)	Final reading (ml)	Titre (ml)	Actual weight (g)
1	0.0	16.4	16.4	0.2010
2	20.0	36.3	16.3	0.1998

App.2.5 Assay of Salicylic acid with 0.1M NaOH

Determination	Initial reading (ml)	Final reading (ml)	Titre (ml)	Actual weight (g)
1	0.0	8.6	8.6	0.1192
2	10.0	18.7	8.7	0.1206

App.2.6 Assay of Indomethacin with 0.1M NaOH

Determination	Initial reading (ml)	Final reading (ml)	Titre (ml)	Actual weight (g)
1	0.0	8.4	8.4	0.3017
2	10.0	18.4	8.4	0.3017

App.3 Uniformity Of Weight Determination

App.3.1 Naprosyn EC tablets, 500mg (Roche, Inc.)

Tablet No.	Weight (g)	Deviation	% Deviation
1	0.619	-0.0099	-1.63
2	0.5982	0.0109	1.79
3	0.6056	0.0035	0.57
4	0.6036	0.0055	0.90
5	0.6047	0.0044	0.72
6	0.6153	-0.0062	-1.02
7	0.6076	0.0015	0.25
8	0.6076	0.0015	0.25
9	0.6149	-0.0058	-0.95
10	0.6146	-0.0055	-0.90
11	0.6146	-0.0055	-0.90
12	0.6079	0.0012	0.20
13	0.6147	-0.0056	-0.92
14	0.6035	0.0056	0.92
15	0.6153	-0.0062	-1.02
16	0.6074	0.0017	0.28
17	0.5982	0.0109	1.79
18	0.6189	-0.0098	-1.61
19	0.6055	0.0036	0.59
20	0.6048	0.0043	0.71
Average wt	0.6091		

App.3.2 Naprox tablets, 500mg (Ernest Chemists Ltd.)

Tablet No.	Weight (g)	Deviation	% Deviation
1	0.5691	0.0100	1.73
2	0.5703	0.0088	1.52
3	0.5765	0.0026	0.45
4	0.5889	-0.0098	-1.69
5	0.5744	0.0047	0.81
6	0.5853	-0.0062	-1.07
7	0.5757	0.0034	0.59
8	0.581	-0.0019	-0.33
9	0.5871	-0.0080	-1.38
10	0.573	0.0061	1.05
11	0.5834	-0.0043	-0.74
12	0.5773	0.0018	0.31
13	0.5765	0.0026	0.45
14	0.5884	-0.0093	-1.61
15	0.5885	-0.0094	-1.62
16	0.5767	0.0024	0.41
17	0.5715	0.0076	1.31
18	0.5778	0.0013	0.22
19	0.5748	0.0043	0.74
20	0.5848	-0.0057	-0.98
Average wt	**0.5791**		

App.3.3 Daonil tablets, 5mg (Sanofi Aventis Inc.)

Tablet No.	Weight (g)	Deviation	% Deviation
1	0.1616	-0.0013	-0.81
2	0.163	-0.0027	-1.68
3	0.1599	0.0004	0.25
4	0.156	0.0043	2.68
5	0.1596	0.0007	0.44
6	0.1607	-0.0004	-0.25
7	0.1565	0.0038	2.37
8	0.1634	-0.0031	-1.93
9	0.16	0.0003	0.19
10	0.1596	0.0007	0.44
11	0.1594	0.0009	0.56
12	0.1598	0.0005	0.31
13	0.1601	0.0002	0.12
14	0.1618	-0.0015	-0.94
15	0.1609	-0.0006	-0.37
16	0.1602	0.0001	0.06
17	0.1607	-0.0004	-0.25
18	0.1613	-0.0010	-0.62
19	0.1595	0.0008	0.50
20	0.1612	-0.0009	-0.56
Average wt	**0.1603**		

App.3.4 Glibenil tablets, 5mg (Ernest Chemists Ltd.)

Tablet No.	Weight (g)	Deviation	% Deviation
1	0.1998	0.0014	0.70
2	0.1979	0.0033	1.64
3	0.2044	-0.0032	-1.59
4	0.1947	0.0065	3.23
5	0.2047	-0.0035	-1.74
6	0.2006	0.0006	0.30
7	0.2009	0.0003	0.15
8	0.2007	0.0005	0.25
9	0.2048	-0.0036	-1.79
10	0.1996	0.0016	0.80
11	0.2	0.0012	0.60
12	0.2001	0.0011	0.55
13	0.2022	-0.0010	-0.50
14	0.2008	0.0004	0.20
15	0.2036	-0.0024	-1.19
16	0.1978	0.0034	1.69
17	0.2039	-0.0027	-1.34
18	0.2044	-0.0032	-1.59
19	0.1973	0.0039	1.94
20	0.2055	-0.0043	-2.14
Average wt	0.2012		

App.3.5 Clamide tablets, 5mg

Tablet No.	Weight (g)	Deviation	% Deviation
1	0.1622	0.0006	0.37
2	0.1649	-0.0021	-1.29
3	0.1626	0.0002	0.12
4	0.1652	-0.0024	-1.47
5	0.1612	0.0016	0.98
6	0.1603	0.0025	1.54
7	0.1631	-0.0003	-0.18
8	0.1621	0.0007	0.43
9	0.161	0.0018	1.11
10	0.1649	-0.0021	-1.29
11	0.1648	-0.0020	-1.23
12	0.163	-0.0002	-0.12
13	0.1624	0.0004	0.25
14	0.1612	0.0016	0.98
15	0.1627	0.0001	0.06
16	0.164	-0.0012	-0.74
17	0.1639	-0.0011	-0.68
18	0.1619	0.0009	0.55
19	0.1626	0.0002	0.12
20	0.162	0.0008	0.49
Average wt	0.1628		

App.4 Data And Calculation for LOD and LOQ

App.4.1 Naproxen at 225nm, 245nm and 280nm

Conc. (%w/v)	Wavelength		
	225nm	245nm	280nm
0.002	-	-	562786
0.001	5058998	-	289809
0.0008	4092861	586826	243116.5
0.0004	2143679	300607	136020
0.0002	1197058	154582	88882
0.00004	396113	38653.5	-
	Slope and Standard Error of Y and X values		
STEYX (σ)	11512.1299	1331.9052	4011.3825
Slope (S)	4849318658	721304084	437227846

App.4.2 Surrogate standards of Naproxen

Conc. (%w/v)	Prednisolone	Paracetamol	Benzoic acid
0.001	841897	478158	-
0.0005	420355.5	258341	4536499
0.0004	336862.5	222602	3667125
0.0002	166998	126405	1902411
0.0001	84984	85034	1008092
0.00002	15678.5	-	291805
	Slope and Standard Error of Y and X values		
STEYX (σ)	897.0219	4011.3825	9878.6582
Slope (S)	842448177	437227846	8844515683

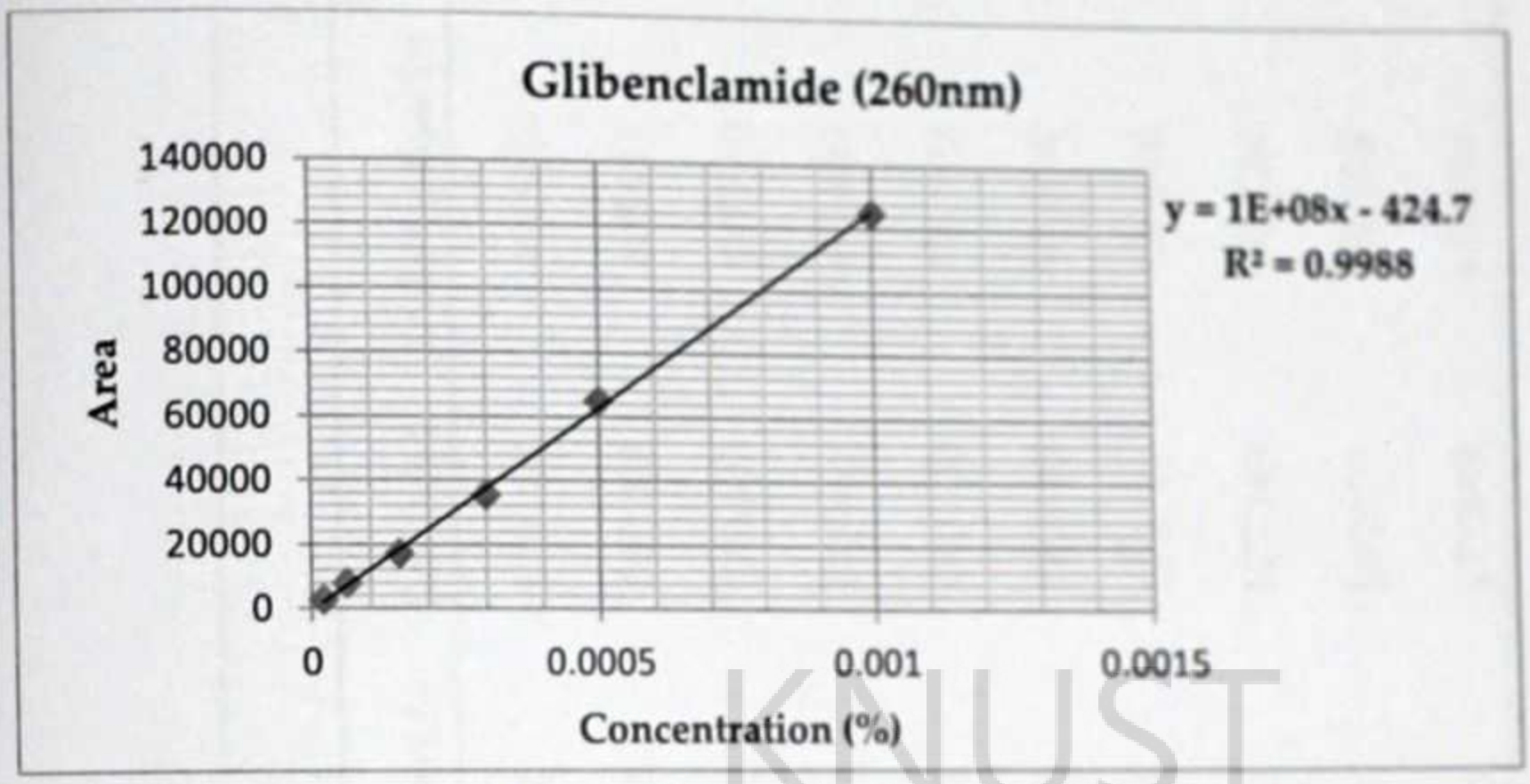
App.4.3 Glibenclamide at 225nm and 260nm

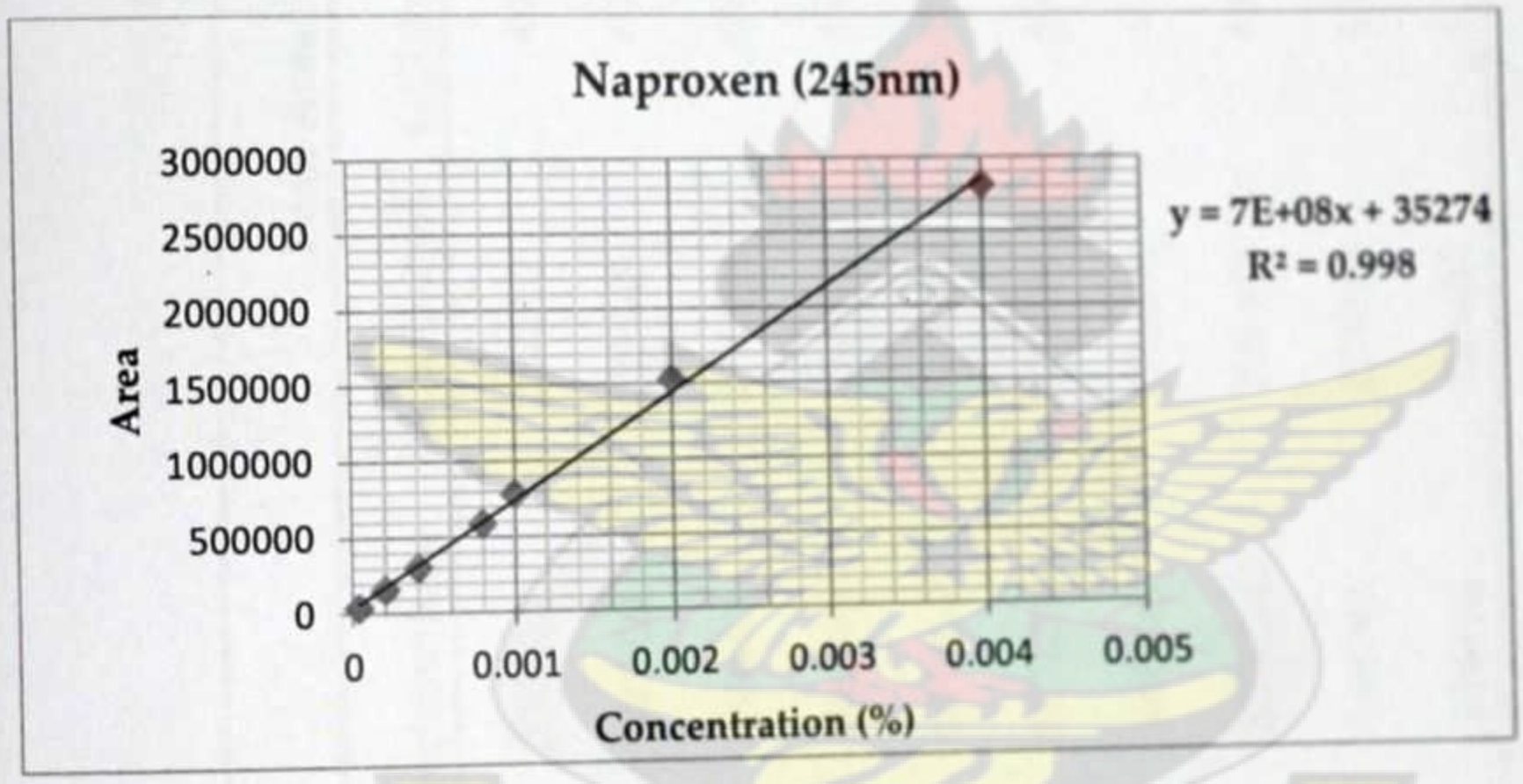
Conc. (%w/v)	Wavelength	
	225nm	260nm
0.001	925656	120898
0.0005	462878	60579
0.0003	280234	35890
0.00015	142031	17985
0.00006	57214	7045
0.00002	21235	2201
	Slope and Standard error of Y and X values	
STEYX (σ)	941.547106	163.46727
Slope (S)	922682352	121178951

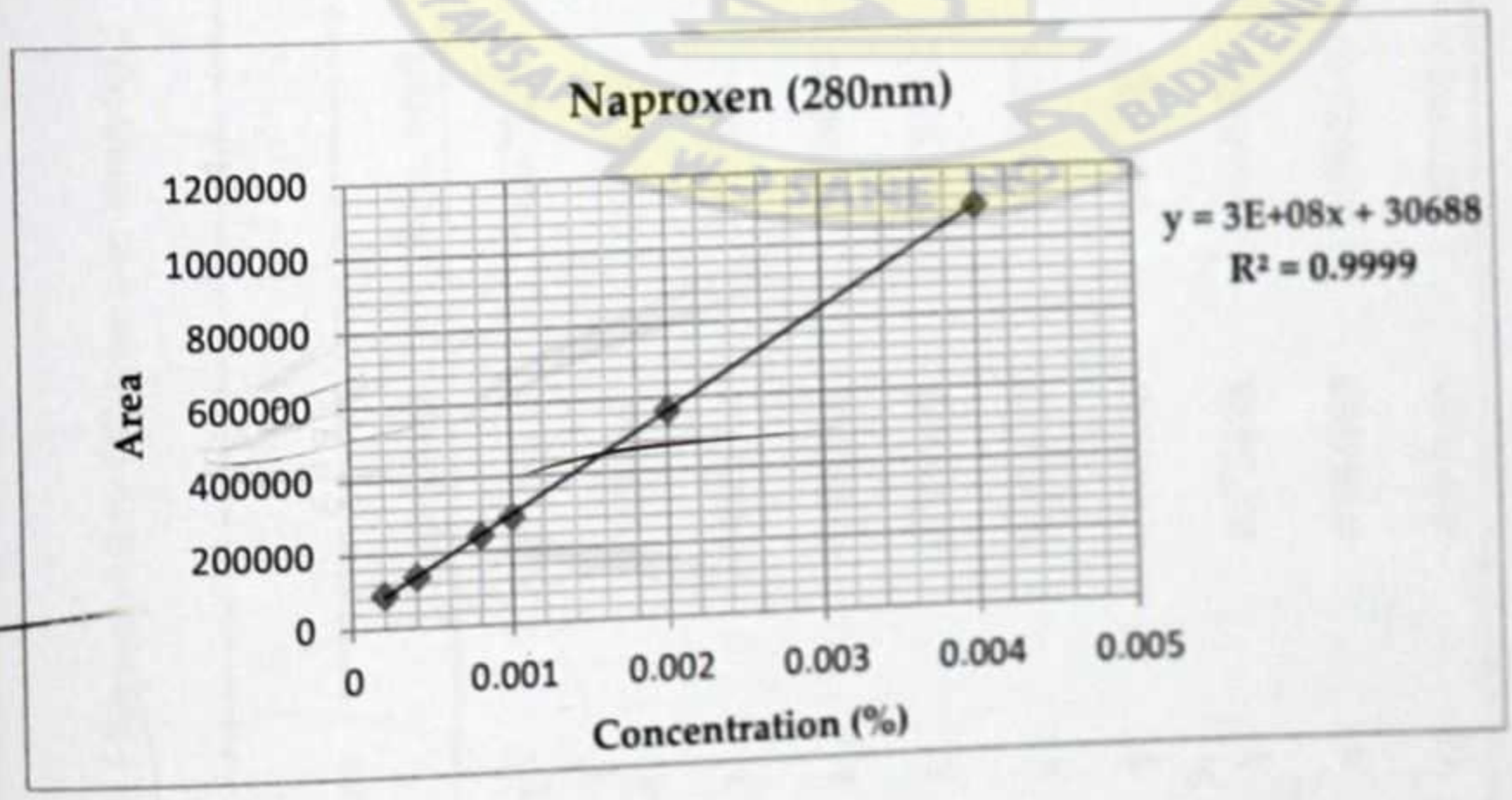
App.4.4 Surrogate standards of glibenclamide

Conc. (%w/v)	Salicylic acid	Indomethacin	Chloramphenicol
0.0005	368711	-	171020
0.00025	184662	-	84760
0.00015	111126	178781	49915
0.000075	56076	93186	24338
0.00003	22287	42893	8811
0.00001	7956	19157	2218
0.000005	-	13928	-
	Slope and Standard error of Y and X values		
STEYX (σ)	239.6139	445.8373	213.8811
Slope (S)	736404061	1136929993	344882892

App.5 Calibration Curve For Naproxen (245nm and 280nm) And Glibenclamide (260nm)







App.6 Repeatability and Intermediate Precision

App.6.1 Repeatability (10 runs per analyte)

Run	Peak Areas							
	Naproxen	Ben. acid	Prednisolone	Paracetamol	Glibenclamide	Sal. acid	Indomethacin	Chloramphenicol
1	4072861	3667125	336863	222602	459628	179762	460802	85610
2	4040944	3700260	340709	232548	460202	180102	463411	86222
3	4082672	3695041	338489	228589	462102	179460	457760	88102
4	4089532	3689090	336509	230701	457242	179768	459403	84820
5	4054569	3679842	341301	229543	456280	180100	456399	85668
6	4041190	3688075	337658	223234	460802	176345	460908	83865
7	4060198	3671268	341195	226486	461685	182708	456842	88222
8	4075438	3696685	339654	229468	458432	177349	461224	84508
9	4069898	3692546	340743	224324	462401	180250	460485	85684
10	4077095	3703028	338476	230405	458444	177349	458334	88555

App.6.2 Immediate precision (two concentration levels, 5 days, 2 runs each day per analyte)

Concentration level 1

	Run	Peak Areas							
		Naproxen	Ben. acid	Prednisolone	Paracetamol	Glibenclamide	Sal. acid	Indomethacin	Chloramphenicol
Day1	1	4072861	3667125	336863	222602	459628	180100	460802	85610
	2	4040944	3703028	340743	230701	460202	176345	463411	86222
		4056902.5*	3685076.5*	338803*	226651.5*	459915*	178222.5*	462106.5*	85916*
Day2	1	4054569	3689090	341301	232548	456280	180100	460908	85684
	2	4069898	3671268	337658	230405	458444	176345	456842	88555
		4062233.5*	3680179*	339479.5*	231476.5*	457362*	178222.5*	458875*	87119.5*
Day3	1	4040944	3671268	341301	226486	461685	177349	460485	84820
	2	4082672	3692546	338476	229468	462401	180250	458334	83865
		4061808*	3681907*	339888.5*	227977*	462043*	178799.5*	459409.5*	84342.5*
Day4	1	4060198	3695041	338489	228589	460234	179768	459403	88102
	2	4075438	3703028	341301	224324	455700	182708	461224	85684
		4067818*	3699034.5*	339895*	226456.5*	457967*	181238*	460313.5*	86893*
Day5	1	4072861	3696685	338489	229543	454890	180450	463450	88120
	2	4089532	3703028	336509	227790	458432	183205	458102	86103
		4081196.5*	3699856.5*	337499*	228666.5*	456661*	181827.5*	460776*	87111.5*

* = Mean peak area for each day

Concentration level 2

	Run	Peak Areas							
		Naproxen	Ben. acid	Prednisolone	Paracetamol	Glibenclamide	Sal. acid	Indomethacin	Chloramphenicol
Day1	1	2143679	1920525	162998	222602	229850	90820	230458	42839
	2	2171715	1902411	166998	230539	235300	89881	234909	43898
		2157697*	1911468*	164998*	226571*	232575*	90351*	232684*	43369*
Day2	1	2184050	1902346	168979	228112	228300	91234	229430	43301
	2	2203450	1935680	169395	225190	230049	93450	230301	44501
		2193750*	1919013*	169187*	226651*	229175*	92342*	229866*	43901*
Day3	1	2212401	1893456	169060	230980	231309	87609	226101	43103
	2	2198882	1934037	166490	223109	230109	90801	228129	45156
		2205642*	1913747*	167775*	227045*	230709*	89205*	227115*	44130*
Day4	1	2152030	1912349	165689	230112	235398	91238	230435	42348
	2	2178099	1882090	161745	231006	238080	90105	231012	47450
		2165065*	1897220*	163717*	230559*	236739*	90672*	230724*	44899*
Day5	1	2163459	1875640	169884	222898	235640	91911	233204	45201
	2	2193456	1924058	170102	225858	231434	94501	234098	44802
		2178458*	1899849*	169993*	224378*	233537*	93206*	233651*	45002*

* = Mean peak area for each day

App.7 K Constant Data and Calculation

App.7.1 Naproxen (0.001% w/v) vs. Benzoic acid (0.0005% w/v)

Run	Peak area Naproxen	Peak area Benzoic acid
1	5368314	4536499
2	5402356	4559098
3	5394578	4443877
4	5428098	4521045
5	5413385	4560047
6	5374777	4514398

App.7.2 Naproxen vs. Benzoic acid; multiple concentration levels

Conc. Naproxen	Conc. Benzoic acid	Peak area Naproxen	Peak area Benzoic acid
0.004	0.002	62224683	53144198
0.002	0.001	22118272	20019579
0.001	0.0005	20758624	19439780
0.0008	0.0004	11676781	10755489
0.0004	0.0002	6495755	5506166
0.0002	0.0001	3813536	3015276
0.00004	0.00002	426052	339073

App.7.3 Naproxen (0.001% w/v) vs. Paracetamol (0.0005% w/v)

Run	Peak area Naproxen	Peak area Paracetamol
1	303190	274856
2	320863	280201
3	312450	277885
4	304893	275900
5	315488	273688
6	311876	278235

App.7.4 Naproxen vs. Paracetamol; multiple concentration levels

Conc. Naproxen	Conc. Paracetamol	Peak area Naproxen	Peak area Paracetamol
0.004	0.002	1043119	940387
0.002	0.001	498326	435654
0.001	0.0005	303190	274856
0.0008	0.0004	243117	226571
0.0004	0.0002	125390	111428
0.0002	0.0001	71749	66558
0.00004	0.00002	19349	16512

App.7.5 Naproxen (0.001% w/v) vs. Prednisolone (0.0005% w/v)

Run	Peak area Naproxen	Peak area Prednisolone
1	1027608	576466
2	1064589	580453
3	1082058	578901
4	1054465	577300
5	1038011	576998
6	1027840	577199

App.7.6 Naproxen vs. Prednisolone; multiple concentration levels

Conc. Naproxen	Conc. Prednisolone	Peak area Naproxen	Peak area Prednisolone
0.004	0.002	2845197	1600356
0.002	0.001	1525178	848990
0.001	0.0005	837193	456886
0.0008	0.0004	577033	323523
0.0004	0.0002	300607	165357
0.0002	0.0001	166782	92172
0.00004	0.00002	32089	17979

App.7.7 Glibenclamide (0.0005% w/v) vs. Salicylic acid (0.00025% w/v)

Run	Peak area Glibenclamide	Peak area Salicylic acid
1	459628	179762
2	460202	180102
3	459102	179460
4	459242	179768
5	459280	180100
6	460802	180305

App.7.8 Glibenclamide vs. Salicylic acid; multiple concentration levels

Conc. Glibenclamide	Conc. Salicylic acid	Peak area Glibenclamide	Peak area Salicylic acid
0.001	0.0005	922256	362524
0.0005	0.00025	465628	182762
0.0003	0.00015	286354	111866
0.00015	0.000075	142031	56076
0.00006	0.00003	57214	22287
0.00002	0.00001	18486	7256
0.001	0.0005	922256	362524

App.7.9 Glibenclamide (0.0005% w/v) vs. Indomethacin (0.00025% w/v)

Run	Peak area Glibenclamide	Peak area Indomethacin
1	423071	273162
2	430987	270586
3	432403	272604
4	421228	273982
5	435602	275983
6	425568	271876

App.7.10 Glibenclamide vs. Indomethacin; multiple concentration levels

Conc. Glibenclamide	Conc. Indomethacin	Peak area Glibenclamide	Peak area Indomethacin
0.001	0.0005	838142	532324
0.0005	0.00025	423071	273162
0.0003	0.00015	220786	140581
0.00015	0.000075	131203	83786
0.00006	0.00003	66601	42893
0.00002	0.00001	29640	17157
0.001	0.0005	838142	532324

App.7.11 Glibenclamide (0.0005% w/v) vs. Chloramphenicol (0.00025% w/v)

Run	Peak area Glibenclamide	Peak area Chloramphenicol
1	64939	85610
2	65022	86222
3	64846	88102
4	64940	84820
5	65010	85668
6	64982	83865

App.7.12 Glibenclamide vs. Chloramphenicol; multiple concentration levels

Conc. Glibenclamide	Conc. Chloramphenicol	Peak area Glibenclamide	Peak area Chloramphenicol
0.001	0.0005	124878	165220
0.0005	0.00025	64939	85610
0.0003	0.00015	35290	48015
0.00015	0.000075	17145	22907
0.00006	0.00003	7855	10206
0.00002	0.00001	2701	3618
0.001	0.0005	124878	165220

App.8 Assay Of Tablets Using The Developed methods

App.8.1 Naprosyn EC tablets vs. surrogate standards

Run	Peak area Benzoic acid	Peak area Paracetamol	Peak area Prednisolone
1	4536499	282550	389825
2	4563456	284230	439355
3	4719387	289128	451345
4	4318101	279985	399148
5	4291297	295908	434834
6	4557321	292333	386204

App.8.2 Naprox ECL tablets vs. naproxen surrogate standards

Run	Peak area Benzoic acid	Peak area Paracetamol	Peak area Prednisolone
1	4456499	478873	421789
2	4428429	490945	435897
3	4494002	488340	419876
4	4722011	465349	406285
5	4582390	488871	398206
6	4351293	467925	384345

App.8.3 Naproxen tablets peak areas for each surrogate standard

Brand	Surrogate Standard	Runs					
		1	2	3	4	5	6
Naprosyn EC	Prednisolone	768234	798345	768678	745987	734111	702349
	Paracetamol	316571	320345	324653	314784	334111	328344
	Benzoic acid	5458314	5503468	5634582	5201289	5148093	5432811
Naprox ECL	Prednisolone	768234	798345	768678	745987	734111	702349
	Paracetamol	545893	560291	554901	532823	549341	540812
	Benzoic acid	5183391	5204582	5341039	5452910	5382398	5201684

App.8.4 Glibenclamide tablets peak areas for each surrogate standard

Brand	Surrogate Standard	Runs					
		1	2	3	4	5	6
Daonil	Salicylic acid	460235	460222	459824	460808	460448	459806
	Indomethacin	434172	452345	421390	444231	431238	450045
	Chloramphenicol	64939	68294	62893	66291	65222	64883
Clamide	Salicylic acid	455061	478549	458857	478056	470222	478024
	Indomethacin	476129	481230	468455	480349	478201	490456
	Chloramphenicol	71234	70190	68223	72349	69451	71291
Glibenil	Salicylic acid	455855	450402	450800	455800	451244	456780
	Indomethacin	420491	423402	423762	419349	425719	418901
	Chloramphenicol	74453	71290	78529	76391	74911	75882

App.8.5 Daonil tablets vs. surrogate standards

Run	Peak area Salicylic acid	Peak area Indomethacin	Peak area Chloramphenicol
1	179762	273162	84810
2	180102	288124	88832
3	179460	265793	83410
4	179768	282238	87671
5	180100	272894	86573
6	180305	287345	85672

App.8.6 Clamide tablets vs. surrogate standards

Run	Peak area Salicylic acid	Peak area Indomethacin	Peak area Chloramphenicol
1	175112	298345	91234
2	182387	301231	90333
3	175340	295410	88237
4	184573	302131	93139
5	181201	294341	88818
6	180482	304982	92888

App.8.7 Clamide tablets vs. surrogate standards

Run	Peak area Salicylic acid	Peak area Indomethacin	Peak area Chloramphenicol
1	178232	276814	94456
2	182562	275134	92459
3	176412	276912	99634
4	183768	271239	96720
5	184199	277123	95529
6	181565	265919	96222