KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

COLLEGE OF HEALTH SCIENCES

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

USE OF SURROGATE REFERENCE STANDARDS IN QUANTITATIVE HPLC

SUBMITTED BY

ERNEST OPPONG-DANQUAH

MAY, 2010

USE OF SURROGATE REFERENCE STANDARDS IN QUANTITATIVE HPLC

THESIS SUBMITTED IN PARTIAL

FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

PHARMACEUTICAL ANALYSIS AND QUALITY CONTROL

In the

Department of Pharmaceutical Chemistry,

Faculty of Pharmacy and Pharmaceutical Sciences

By

ERNEST OPPONG-DANQUAH

KWAME NKRUMAH UNIVERSITY OF SCIENCE & TECHNOLOGY, KUMASI

MAY, 2010

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.

…… ……

Prof. J. K. Kwakye Dr. R. K. Adosraku

(SUPERVISOR) (Head of Department)

……..

Ernest Oppong-Danquah

(STUDENT)

DEDICATION

This work is dedicated to my father, Mr. J. Oppong-Danquah.

ACKNOWLEDGEMENT

*What shall we then say to these things? If God be for **us**, who can be **against us**? Rom 8:31*

I acknowledge with esteemed gratitude and honour, the Almighty God, for His abundant grace and mercies bestowed upon me throughout this period of studies. It is only by His loving kindness and providence that I have gotten this far.

I also express my thanks to my supervisor Prof. J. K. Kwakye for the immense help and support given me.

I want to appreciate my dearest and loving friend, Amma Gyapomah for the diverse help and affection she gave me.

My gratitude also goes to all my colleagues, the teaching and non-teaching staff of the department of pharmaceutical chemistry at the Kwame Nkrumah University of Science and Technology (KNUST).

The joint family support and affection demonstrated by my siblings cannot be left out. I duly acknowledge Amos, Frank, Georgina and Jennifer for their prayers and support throughout the challenging times. God bless you all.

ABSTRACT

In quantitative HPLC, pure reference standards of analytes are used either to draw calibration curves or for one point assays. These reference standards are sometimes very scarce to come by and if available may be very expensive. The use of readily available drugs to replace the reference standards of the analytes was therefore investigated. A simple and sensitive HPLC method was developed for the assay of **Indometacin** and **Diazepam**. The assay method made use of surrogate reference standards being run together with the pure sample of the analyte to obtain a constant of proportionality, 'K' that relates the concentration and peak areas of the two. The K values obtained were then used to assay the analyte.

The assay of Indometacin employed a microbore column packed with a C18 reversed-phase material (5µm HICHROM ODS column) with an isocratic mixture of methanol and phosphate buffer, pH 5.8±0.02 (60:40, v/v) as the mobile phase. The chromatographic separation was monitored by a UV detector at a wavelength of 254 nm. When Naproxen was used as surrogate standard the K value obtained was 1.6735±0.021 with percentage content of 99.20%±1.98, 95.09%±1.85 and 96.98%±1.70 for the three brands of Indometacin capsule used. With Benzoic acid as surrogate standard, the K value obtained was 3.426±0.073 with percentage content of 99.02%±1.81, 94.89±1.47 and 98.52%±1.63 for the three brands. When Diazepam was used as surrogate reference standard the K value was 3.0955±0.19 with assays of 99.65%±0.59, 92.99%±1.28 and 98.14%±0.81 respectively for the three brands. Comparatively, the standard method (BP method) of assay showed percentage content of 98.04%±0.21, 92.51%±0.77 and 99.15%±0.77 respectively for the three brands. The methods were statistically comparable at the 99% confidence interval.

The assay of Diazepam also employed a microbore column packed with a C18 reversed-phase material (5µm HICHROM ODS column) with an isocratic mixture of methanol and phosphate buffer, pH 5.8±0.02 (75:25, v/v) as the mobile phase. The chromatographic separation was monitored by a UV detector at a wavelength of 300 nm. When Piroxicam was

used as surrogate, the K value obtained was 0.20418±0.0015 with percentage content of 95.00%±1.98, 103.01%±1.01 and 95.67%±1.05 for the three brands of Diazepam tablet used. With Metronidazole as surrogate standard, the K value obtained was 0.135271±0.009 with percentage content of 94.84%±0.55, 103.94±1.44 and 98.10%±0.82 for the three brands. When Indometacin was used as surrogate reference standard the K value was 0.3230±0.018 with assays of 95.10%±1.25, 97.57%±1.27 and 93.11%±1.30 respectively for the three brands of Diazepam tablet. The standard method from the BP also showed results of 94.38%±0.74, 98.07%±0.48 and 99.05%±0.38 respectively for the three brands of Diazepam tablets. The methods were statistically comparable to the Standard BP method at the 99% confidence interval except for two brands which showed slight systematic errors with Piroxicam as surrogate reference standard.

All the assays for the Diazepam and Indometacin with the developed methods however fell within the permissible range of the British Pharmacopoeia with detection limit of 0.37µg/ml for Indometacin and 0.67µg/ml for Diazepam.

Naproxen, Benzoic acid and Diazepam can be used as surrogate reference standards for the assay of Indometacin using the K values obtained. Piroxicam, Metronidazole and Indometacin can be used as surrogate reference standard for the assay of Diazepam.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ED – Diazepam Tablets from Ernest Chemist Pharmaceuticals

GD – Diazepam Tablets from Golden Tower Limited

PD – Diazepam Tablets from Pharmanova Limited

IL – Indometacin Capsules from Letap Pharmaceuticals Limited

IE – Indometacin Capsules from Ernest Chemist Limited

IM- Indometacin Capsules from M & G Pharmaceuticals

Piro – Piroxicam

Metro – Metronidazole

Indo – Indometacin

Napro – Naproxen

HPLC –High Performance Liquid Chromatography

UV – Ultra Violet

BPCRS – British Pharmacopoeia Chemical Reference Standard

Rf – Retardation Factor

IUPAC – International Union of Pure and Applied Chemistry

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The number of drugs introduced onto the market is increasing every year. These drugs may either be totally new or partial structural modifications of the existing ones. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for the analysis of these drugs may not be available in the pharmacopoeias. Already existing analytical procedures for certain drugs may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable. In such situations, the analytical scientist's burden of profiling drugs becomes very challenging. It becomes necessary therefore, to develop newer analytical methods for such drugs. Such method development can be time and effort consuming, hence a tremendous need to employ a comprehensive method development strategy to meet the challenges of high throughput and rapid turn-round time.

Chromatography, now the most widely used separation technique, is defined as the process of separating the components of mixtures that are distributed between a stationary phase and a flowing mobile phase according to the rate at which they are transported through the stationary phase [1]. Chromatography has now developed into a number of related but quite different forms that enable the components of complex mixtures to be separated and quantified.

High-performance liquid chromatography (HPLC) is today widely used for separation and analysis. HPLC offers a combination of speed, reproducibility, and sensitivity. HPLC's popularity and wide usage lies in its versatility. It can be used to separate and analyze

compounds with a wide polarity range in a single run. Almost anything that can be dissolved can be separated on some type of HPLC column [2]. Aqueous samples can be run directly after a simple filtration. It can be used to analyze thermally labile compounds and even volatile compounds. [2] It can also be used to separate compounds of very wide molecular weight differences. Amounts of material to be detected can vary from picograms and nanograms (analytical scale) to micrograms and milligrams (semi-preparative scale) to multigrams (preparative scale) [2].

High Performance Liquid Chromatography (HPLC) analytical method development is a critical process in pharmaceutical analysis. Methods need to separate the desired components satisfactorily, generate the required results, and they must be reproducible and robust so that they can be used from time to time without problems.

Method validation which includes robustness, reproducibility among others is a prerequisite for acceptance of any newly developed method. Good Manufacturing Practices (GMP) outlined by the World Health Organization (WHO) requires that every non-compendia analytical method (or modified compendia method) must be validated and the validation results documented [10]. Significant tests should also be done to ensure that there is no significant difference between the outcome of the developed method and the standard method. [17]

The HPLC separation and analysis of samples is achieved by injecting the sample dissolved in a solvent into a stream of mobile phase being pumped onto a column packed with a solid separating material. The interaction is a liquid-solid separation. It occurs when a mixture of compounds dissolved in a solvent can either stay in the solvent or adhere to the packing material in the column.

The detector measures the concentration of sample bands as they leave the column and pass through the detector flow cell. When no band is passing through the detector, a constant signal is recorded called the Baseline of the chromatogram or detector. When a sample band reaches

the detector, the detector responds to the difference in the mobile phase properties caused by the presence of the sample compound, giving rise to a change in detector signal, seen as a Peak. The result is that components with the highest affinity for the column packing stick the longest and wash out last. This differential washout or elution of compounds is the basis for the HPLC separation. This peak data can be used either to quantitate, with standard calibration, the amounts of each material present or to control the collection of purified material in a fraction collector.

1.2 JUSTIFICATION

There are two approaches to the application of UV spectroscopy to analyze pharmaceuticals. One approach is to make a calibration curve for the reference standard, determine the absorbance for the test sample and deduce the concentration of the test solution from the graph. Such calibration curves are drawn based on the Beer-Lambert Law that absorption is proportional to concentration [10].

The second approach is to determine the specific absorbance, A (1%, 1cm), of the substance using a reference standard sample. The absorbance of the test sample is measured and its percentage content calculated. It is obvious the use of the A (1%, 1cm) decreases the volume of data and consequently the time required to analyze a sample.

Likewise in HPLC analysis of most drugs, a reference standard of the test sample is obtained and a calibration curve is drawn to ascertain the concentration of the test sample. This approach of HPLC analysis is time consuming. Sometimes the reference standards of the samples to be analyzed are scarce and even if available, are very expensive. In such cases acquisition of these reference standards for use becomes a problem. Industries and regulatory bodies who need to assure the quality of these drugs are therefore not able to do so. This makes apparent the need to develop surrogate standards which are more readily available.

Reference standards are solely for use in the tests and assays of the BP in which a BPCRS is required. They are issued only for chemical analysis and may not be suitable for any other purposes. They are not intended for administration to humans or animals [5].

Importing these reference standards into Ghana however is expensive. The cost of Diazepam and Indometacin reference standards are given in the table below [20].

Table 1.1 Cost of Reference Standard

Catalog Number	**Product Description**	**Current Lot**	**Previous Lot**	**Unit Price**
1185008	Diazepam CIV (100 mg)	I2G270	I1C364 (03/09)	$263.00 EACH
1341001	Indometacin (200 mg)	J1G345	J0B165 (10/08)	$199.00 EACH

There is therefore a tremendous need to employ a comprehensive method development strategy to meet the challenges of high throughput and rapid turn-around time and also to minimize cost by excluding the reference standards of the analytes. To circumvent this problem, readily available drug substances are used as surrogate reference standards replacing the pure reference standards of the analytes. This research to analyze pharmaceutical products without using its reference standard has been started for some years now in the Department of Pharmaceutical Chemistry of KNUST. Instead of the reference standard of the analyte, a surrogate reference standard is employed and a constant 'K" synonymous to A(1%,1cm) in UV spectroscopy is determined with other conditions.

Work on Paracetamol, Aspirin and Diclofenac has already been done [29]. The project seeks to extend the search by working on Diazepam and Indometacin as another case study.

1.3 MAIN OBJECTIVE

This project was to investigate the possibility of using various compounds as surrogate reference standards for the analysis of **Diazepam tablets** and **Indometacin capsules** using HPLC.

1.4 SPECIFIC OBJECTIVES

The specific objectives of this research included;

1. To establish the conditions for an HPLC assay procedure for Diazepam tablets and Indometacin capsules using surrogate reference compounds.
2. To elute the analyte together with a surrogate reference standard at different times.
3. To determine the limit of detection (LOD), limit of quantitation (LOQ) and the retention times of Diazepam and Indometacin as well as their surrogate reference compounds.
4. To determine a constant K that can effectively be used for quantitative analysis.
5. To determine the percentage content of Diazepam and Indometacin in various tablets and capsules using the method developed.
6. To compare results obtained from the method developed for Diazepam with a standard method (for the analysis of Diazepam tablets) in the British Pharmacopoeia.
7. To compare results obtained from the method developed for Indometacin with a standard method (for the analysis of indometacin capsules) in the British Pharmacopoeia.
8. To determine the precision, accuracy, repeatability and reproducibility of the method developed.

1.5 HYPOTHESIS OF STUDY

The UV detectors used in HPLC generate electrical signals whose magnitude is determined by the concentration of the analyte. Consequently the area under a peak 'A' is proportional to the concentration 'C' of analyte injected.

Implies $A \alpha C$

Introducing a constant, Y

$$A = YC$$

$$Y = A/C$$

For similar compounds, the constant remains the same

$$\frac{\text{A analyte}}{\text{C analyte}} = \frac{\text{A standard}}{\text{C standard}},$$

the standard is the pure sample of the analyte.

However, using surrogate compounds as standard,

$$\frac{\text{A analyte}}{\text{C analyte}} \neq \frac{\text{A standard}}{\text{C standard}},$$

Introducing a constant, K

$$\frac{\text{A analyte}}{\text{C analyte}} = K\frac{\text{A standard}}{\text{C standard}}$$

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

$A_{analyte}$ is the peak area of the analyte, $A_{standard}$ is the peak area of the standard.

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

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

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

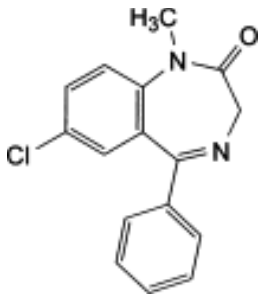
$C_{analyte} = (A_{analyte} \times C_{standard}) / (A_{standard} \times K)$

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

1.6 LITERATURE REVIEW

1.6.1 PROFILE OF ANALYTES AND SURROGATE REFERENCE STANDARDS

1.6.1.1 DIAZEPAM



$C_{16}H_{13}ClN_2O$ 284.7

Fig. 1.1 Chemical structure of diazepam

Diazepam was first marketed as *Valium* by Hoffman-La Roche Limited, a Swiss global health-care pharmaceutical and diagnostic company that operates worldwide. [25]

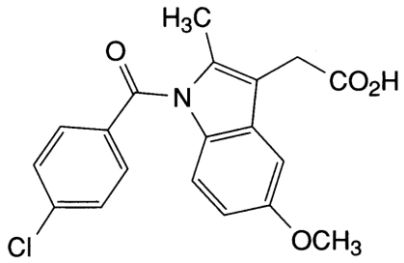
Diazepam is a **benzodiazepine** derivative drug which is a white or almost white crystalline powder. It is very slightly soluble in water and has a solubility profile of 1 in 25 of ethanol, 1 in 2 of chloroform and 1 in 39 of ether. It has a melting point of between 131 to 135^{o}C. [7]

It contains not less than 99.0 per cent and not more than the equivalent of 101 per cent of *7-chloro-1-methyl-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one*, calculated with reference to the dried substance.[5]

In a mixture of sulphuric acid and methanol, diazepam exhibits maximum absorption at two wavelengths of 242nm and 285nm with the specific absorbance at the maximum of 242nm as 1020 [5].

It is indicated in status epilepticus, febrile convulsions and convulsions due to poisoning.[6]

1.6.1.2 INDOMETACIN



$C_{16}H_{14}O_3$ 254.3

Fig.1.2 Chemical structure of Indometacin

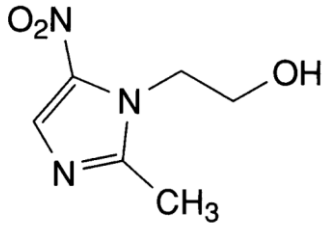
Indometacin is an example of the arylacetic and arylpropionic acid derivatives of the Non-Steroidal Anti-inflammatory drugs (NSAIDs). [11] Indometacin is a white to yellow-tan, crystalline powder with a melting point of between 158 to 162^o. It exhibits polymorphism. It has a solubility profile of 1 in 50 of ethanol, 1 in 30 of chloroform, 1 in about 40 of ether, soluble in acetone and practically insoluble in water. [7]

Indometacin contains not less than 98.5 per cent and not more than the equivalent of 100.5 per cent of [1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl]acetic acid, calculated with reference to the dried substance.[5]

In a methanolic HCl, Indometacin shows a maximum UV absorption at a wavelength of 318nm with a specific absorbance of between 170 and 190.

Indometacin is an NSAID (Non-Steroidal Anti-Inflammatory Drug) and has more of anti-inflammatory and analgesic effect. It is indicated in rheumatic diseases and other acute musculoskeletal disorders; acute gout; dysmenorrhoea and closure of ductus arteriosus.[6]

1.6.1.3 METRONIDAZOLE



$C_6H_9N_3O_3$ 171.2

Fig. 1.3 Chemical structure of Metronidazole

Metronidazole is white to pale yellow crystalline powder or crystal which darkens on exposure to light. It has melting point of between 159 and 163^{o}C.

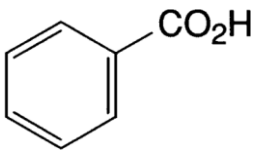
It has a solubility profile of 1 in 100 of water, 1 in 200 of ethanol, 1 in 250 of chloroform and slightly soluble in ether [7].

It has the IUPAC name, *2-(2-Methyl-5-nitro-1H-imidazol-1-yl) ethanol* and has a percentage content of between 99 and 101% with reference to the dried sample. Metronidazole in a 0.1M

HCl will exhibit a UV absorption maximum at 277nm with specific absorbance between 365 and 395. [5]

Metronidazole is an antibacterial with activity against anaerobic organisms including dental infections. It also has some anti- protozoal activity [6].

1.6.1.4 BENZOIC ACID



$C_7H_6O_2$ 122.1

Fig. 1.4 Chemical structure of Benzoic acid

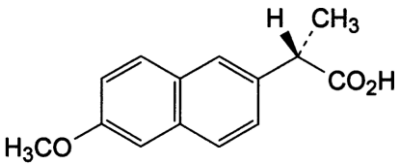
Benzoic acid is also called benzenecarboxylic acid or phenylformic acid. It is a colourless, light feathery crystals or white scales of powder with melting point of between 121 to 124^oC and sublimes on heating. It has a solubility of 1 in about 350 of water, 1 in 3 of ethanol, 1 in 20 of boiling water, 1 in 5 of chloroform, 1 in 3 of ether and freely soluble in acetone.[7]

It has a percentage content of not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of benzenecarboxylic acid. [5]

Benzoic acid's UV spectrum in aqueous acid shows absorbances at 230nm and 273nm with specific absorbances of 923 and 85 respectively. In methanol, shows absorbances at 227nm and 280nm with specific absorbances of 895 and 61 respectively. [7]

Benzoic acid is used as a preservative in a concentration of 0.1%. The salt sodium benzoate is given orally to test for liver function [7].

1.6.1.5 NAPROXEN



$C_{14}H_{14}O_3$ 230.3

Fig. 1.5 Chemical structure of Naproxen

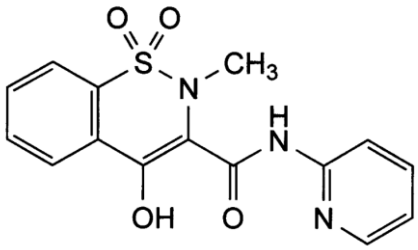
Naproxen is a white crystalline powder with melting point 156°. It is practically insoluble in water, 1 in 25 of ethanol, 1 in 15 of chloroform and 1 in 40 of ether. [7]

The IUPAC name for Naproxen is (2*S*)-2-(6-Methoxynaphthalen-2-yl) propanoic acid with content of 99.0 per cent to 101.0 per cent (dried substance). [5]

Naproxen in methanol exhibits 4 absorption maxima, at 262 nm, 271 nm, 316 nm and 331 nm. The specific absorbances at the absorption maxima are 216 to 238, 219 to 241, 61 to 69 and 79 to 87, respectively.[5]

Naproxen is also an NSAID with anti-inflammatory, analgesic and anti-pyretic effect. It is indicated in pain and inflammation in rheumatic disease (including juvenile idiopathic arthritis) and other musculoskeletal disorders, dysmenorrhoea and acute gout. [6]

1.6.1.6 PIROXICAM



$C_{15}H_{13}N_3O_4S$ 331.4

Fig. 1.6 Chemical structure of Piroxicam

Piroxicam contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of *4-hydroxy-2-methyl-N-(pyridin-2-yl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide*, calculated with reference to the dried substance [5]. It has a melting point of 198 to 200°C [7].

It is a white or slightly yellow, crystalline powder, practically insoluble in water, soluble in methylene chloride, slightly soluble in ethanol. It shows polymorphism [5].

Piroxicam is also an NSAID with anti-inflammatory, analgesic and anti-pyretic effect. It is indicated in pain and inflammation in rheumatic disease (including juvenile idiopathic arthritis) and other musculoskeletal disorders, dysmenorrhoea and acute gout [6].

1.6.2 INSTRUMENTATION OF ANALYTICAL METHODS

1.6.2.1 ULTRA- VIIOLET VISIBLE SPECTROSCOPY

Analytical spectroscopy is the science of determining how much of a substance is present in a sample by accurately measuring how much light is absorbed or emitted by atoms or

molecules within it. [8] Different types of spectroscopy are available, depending on the type or wavelength of electromagnetic radiation absorbed or emitted by the atom or molecule. The region in the electromagnetic spectrum with wavelengths ranging from 4×10^{-7} to 1×10^{-8} m is called the UV region but only a small part of the UV spectrum is used to characterize organic compounds. [9] This region is usually expressed as 200 to 400 nm. The energy of electromagnetic radiation in this region ranges from 300 to 600 kJ/mol (70 to 140 kcal/mol). This is sufficient energy to promote electrons into higher energy levels (excited states). [9]

It is a technique mostly used for quantitative trace analysis. It is also used as an adjunct to other spectrometric techniques in the identification and structural analysis of organic materials. [12] Commercial instruments for U.V usually cover the visible region therefore have two light sources; a deuterium or hydrogen discharge tube for U.V region and a tungsten filament lamp for the visible region.[4] The lower limit of measurement in normally 190nm owing to the fact that oxygen absorbs radiation below that. Below this region, quatz becomes less transparent and measurements below this region require the use of diffraction gratings and special vacuum techniques. [4]

The use of the UV-visible spectrometer for quantitative work follows the **Beer-Lambert law**. It states that the proportion of light absorbed by a solute in a transparent solvent is independent of the intensity of the incident light and is proportional to the number of absorbing molecules in the light path[4]: $\log_{10} (I_o/I) = A = \varepsilon cl$

Where; I_o = intensity of incident light

I = intensity of transmitted light

ε = molar absorptivity or molar extinction coefficient

c = concentration of solute in moles / litre

l = cell path length (cm)

A = absorbance

Sometimes ε is defined as specific absorbance when 'c' is %w/v concentration. Assay of drugs can easily be computed with the specific absorbance and absorbance of the sample. Pharmaceutical products formulated with more than one drug, typically referred to as combination products, are intended to meet previously unmet patients need by combining the therapeutic effects of two or more drugs in one product. These combination products can present daunting challenges to the analytical chemist. However the U.V spectrometer can be employed. The basis of all the spectrophotometric techniques for multicomponent samples is the property that at all wavelengths:

- the absorbance of a solution is the sum of absorbance of the individual components or
- the measured absorbance is the difference between the total absorbance of the solution in the sample cell and that of the solution in the reference cell.

There are various spectrophotometric techniques which can be used for the analysis of combination samples;

-Simultaneous equation method

-Derivative spectrophotometric method

-Absorbance ratio method (Q-Absorbance method)

-Difference spectrophotometry

-Solvent extraction method [14]

1.6.2.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

All chromatographic techniques depend upon the same basic principle, that is, variation in the rate at which different components of a mixture migrate through a stationary phase under the influence of a mobile phase. Rates of migration vary because of differences in distribution ratios. This chromatography method gives a more efficient separation than does column chromatography and hence the name high performance liquid chromatography. [9]

An HPLC instrument has at least the following components: solvent reservoir, transfer line with frit, high-pressure pump, sample injection device, column, detector, and data recorder. It is quite common to work with more than one solvent, thus a mixer and controller are needed. If the data acquisition is done by a computer it can also be used for the control of the whole system [13].

1.6.2.2.1 Liquid Chromatographic Separation Modes

Adsorption Chromatography [13]

This principle of adsorption chromatography is known from classical column and thin-layer chromatography. A relatively polar material with a high specific surface area is used as the stationary phase, silica being the most popular, but alumina and magnesium oxide are also often used. The mobile phase is relatively non-polar (heptane to tetrahydrofuran). The different extents to which the various types of molecules in the mixture are adsorbed on the stationary phase provide the separation effect. A non-polar solvent such as hexane elutes more slowly than a medium-polar solvent such as ether. Polar compounds are eluted later than non-polar compounds.

Reversed-Phase Chromatography

The reverse of the above applies:

(i) The stationary phase is very non-polar.

(ii) The mobile phase is relatively polar (water to tetrahydrofuran).

(iii) A polar solvent such as water elutes more slowly than a less polar solvent such as acetonitrile.

Non-polar compounds are eluted later than polar compounds.

Ion-Exchange Chromatography

The stationary phase contains ionic groups (e.g. NR_3 þ or SO_3_) which interact with the ionic groups of the sample molecules. The method is suitable for separating, e.g. amino acids, ionic metabolic products and organic ions.

Ion-Pair Chromatography

Ion-pair chromatography may also be used for the separation of ionic compounds and overcomes certain problems inherent in the ion-exchange method. Ionic sample molecules are ‘masked’ by a suitable counter ion. The main advantages are, firstly, that the widely available reversed-phase system can be used, so no ion exchanger is needed, and, secondly, acids, bases and neutral products can be analyzed simultaneously.

Ion Chromatography

Ion chromatography was developed as a means of separating the ions of strong acids and bases (e.g. Cl^-, NO_3^-). It is a special case of ion-exchange chromatography but the equipment used is different.

Size-Exclusion Chromatography

This mode can be subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solutions). Size-exclusion chromatography separates molecules by size, i.e. according to molecular mass. The largest molecules are eluted first and the smallest molecules are eluted last. This is the best method to choose when a mixture contains compounds with a molecular mass difference of at least 10%.

Affinity Chromatography

In this case, highly specific biochemical reversible interactions provide the means of separation. The stationary phase contains specific groups of molecules which can only adsorb the sample if certain charge-related conditions are satisfied. It is the only technique that enables the purification of a biomolecule on the basis of its biological function [27].

1.6.2.2.2 Mobile Phase Reservoirs

They contain the mobile phase and are mostly made of glass or stainless steel. They have degassers for removing dissolved gases (O_2, N_2) and also a means of filtering off dust from the solvent.

1.6.2.2.3 Mobile Phase

The solvent strength is a measure of its ability to pull analytes from the column. It is generally controlled by the concentration of the solvent with the highest strength; for example, in reverse phase HPLC with aqueous mobile phases, the strong solvent would be the organic modifier; in normal phase HPLC, it would be the most polar one. The aim is to find the correct concentration of the strong solvent. With many samples, there will be a range of solvent strengths that can be used within the aforementioned capacity limits. Other factors (such as pH and the presence of ion pairing reagents) may also affect the overall retention of analyte. [15]

Solvents used for the preparation of mobile phases in HPLC should ideally have high solubility and purity, must be low in cost, non-corrosive to HPLC system components and be UV transparent [9].

In reversed phase HPLC, the retention of analytes is related to their hydrophobicity. The more hydrophobic the analyte, the longer it is retained. When an analyte is ionized, it becomes less hydrophobic and, therefore, its retention decreases. Acids lose a proton and become ionized when pH increases and bases gain a proton and become ionized when pH decreases. Therefore, when separating mixtures containing acids and/or bases by reversed phase HPLC, it is necessary to control the pH of the mobile phase using an appropriate buffer in order to achieve reproducible results.

Table 1.2 *Common buffers used in HPLC*

Buffer	**pKa**	**Buffer Range**	**UV Cutoff (nm)**
Phosphate	2.1	1.1-3.1	200
	7.2	6.2-8.2	
	12.3	11.3-13.3	
Formic acid*	3.8	2.8-4.8	210

Acetic acid*	4.8	3.8-5.8	210
Citrate	3.1	2.1-4.1	230
	4.7	3.7-5.7	
	5.4	4.4-6.4	
Tris	8.3	7.3-9.3	205
Triethylamine*	11.0	10.0-12.0	200
Pyrrolidine	11.3	10.3-12.3	200
* Volatile buffers			

It is a good practice to prepare only as much mobile phase as will be used within a short time. The shelf-life of aqueous solutions without an organic solvent is very low if rigorous quality standards are not followed [13]. The following are shelf lives of some solvents often used.

Water from water purification system	3 days
Aqueous solutions (without buffer)	3 days
Buffer solutions	3 days
Aqueous solutions with < 15% organic solvent	1 month
Aqueous solutions with > 15% organic solvent	3 months
Organic solvents	3 months

1.6.2.2.4 Pumping System

This forces the mobile phase to the column and then through it. The pump normally contains corrosive-resistant components to avoid rusting. The pump must be able to provide pressure of up to 6000psi, pulse free output, flow rate ranging from 0.1 – 10ml/min, flow control and flow reproducibility. [10] Common pumps normally used are the reciprocating, displacement and the pneumatic or constant pressure pump.

1.6.2.2.5 Injector

Sample feed is one of the critical aspects of HPLC. Even the best column produces a poor separation result if injection is not carried out carefully. In theory, an infinitely small volume of sample mixture should be placed in the centre of the column head, care being taken to prevent any air from entering at the same time. Sample injections can be done; with syringe and septum injector; with a loop valve or with an automated injection system (autosampler)

A disposable guard column is sometimes positioned between the injector and the analytical column. [1] Overloading of the column must be avoided to prevent band broadening.[10]

1.6.2.2.6 Column

The column is where the separation process occurs and it is therefore the central component of high performance liquid chromatograph. Although the column is the most important part, it is usually the smallest one. There are two types of HPLC columns:, conventional and microbore. [1] Microbore columns have three principal advantages over conventional columns;

1) Solvent consumption is about 80% less because of much lower mobile phase flow rate (10-100µl/min)

2) The low volume flow rate makes them ideal for interfacing with a mass spectrometer

3) Sensitivity is increased because solutes are more concentrated, which is especially useful if sample size is limited.

Columns are usually made of stainless steel containing the packing material. Columns can also be classified as bonded-phase (reverse) columns and normal phase columns. The surface of silica (normal phase) is polar and slightly acidic due to the presence of silanol (Si-OH) groups. The reverse phase columns are however non-polar, because the silanol groups have

been replaced with other functional groups which are non-.polar. The resulting bonded phases, which are heterolitically stable through the formation of siloxane (Si-O-Si-C) bonds, have different chromatographic characteristics and selectivities to unbonded silica [1]. The choice of column will therefore depend on the physical and chemical characteristics of the mixture to be separated [10].

Table 1.3 *Polarity trend for columns and solvents* [2]

Column	C18	0	C8	C4	CN	Diol	NH_2	Si
Solvents	Hexane	Benzene	CH_2Cl_2	$CHCl_3$	THF	AN	MeOH	H_2O

Table 1.3 *Increasing polarity from C18 (bonded silica) to silica: C18 (ods)octadecyl silica, phenyl, C8, cyano, C3, diol, amino, and silica. Under that, their solvents in opposite order of polarity from hexane under C18 to water under silica: hexane, benzene, methylene chloride, chloroform, THF, acetonitrile, MeOH, and water respectively.*

Column efficiency [10]

This is dependent on the degree of band broadening relative to the time taken to elute. An efficient column must be able to maintain sharp narrow peaks on the function of retention time. A quantitative measurement of column efficiency is the number of theoretical plates (N) calculated from the chromatogram by:

$N = 16 (Rt/W)2$

Where Rt = retention time, W = peak width at base

The larger the N, the more efficient a column is. A value of 10,000 to 20,000 plates is ideal for a 25cm 5µl column.

Another useful parameter for column efficiency if the height equivalent to theoretical plate (HETP) or the H-value that is the length of a column required to generate one plate.

H = L/N, where L is length of column and N is number of theoretical plate.

A column of low H-value is better than one with a high H-value.

Column Ageing and Healing [2]

There are five basic types of "killers" of column efficiency:

1. Effects that remove bonded phase

2. Effects that dissolve the column surface or the packing itself (End void)

3. Materials that bind to the column.

4. Things that cause pressure increase

5. Column channeling

Some of these effects cause permanent damage to the columns and need to be replaced. Some of the effects however can be avoided by simply working within the pH range of 2.5–7.5 at ambient temperature. Buffers can be used to avoid pH below 2. These end voids can be repaired by refilling the column with fresh packing materials that has been worked into a paste with mobile phase. Column channeling can also be healed by rapping the column at both ends against a counter, hooking it up backwards and running it at high flow rate for a minute or two.

1.6.2.2.7 Detector

The function of the detector is to monitor the mobile phase as it comes out. A good HPLC detector should have a low dead volume in order to reduce zone broadening. It should be highly sensitive and of good stability [10]. It should give signals directly proportional to concentrations over a wide range.[1] It should also have rapid and reproducible response to solute.

Three types of detectors are available:

A. Bulk property detector which detects differences in bulk property between a pure mobile phase and a mobile phase containing the dissolved solute, examples, Conductivity and refractive index.

B. Solute property detector which detects based on the unique chemical properties of solutes, like UV-Visible absorption characteristic, fluorescence and redox behavior.

C. Desolution detector which utilizes some property of the analyte after the mobile phase has been removed, example, Mass spectrometer.

The most widely used detector is the UV and visible absorption detector because of its high sensitivity, reproducibility and ease of operation. Fluorescence detector is highly sensitive and selective for florescent compounds or derivatized florescent compounds. Electrochemical detectors (include coulometric, amperometric, potentiometric or polarographic detectors) are also selective for bioactive compounds which are electroactive and can be oxidized or reduced. Refractive index detector is also universally used because nearly all dissolved solutes alter the refractive index of the mobile phase. Other detectors include the infrared absorption and mass spectrometer detectors.

1.6.2.2.8 Recorder

This could be a simple chart recorder or an elaborate interface with a computer to provide us with a hard copy of the separation profile.

1.6.2.3 THIN LAYER CHROMATOGRAPHY

Thin layer chromatography is a technique where the components of a mixture separate by differential migration through a planar bed of a stationary phase with the mobile phase flowing by virtue of capillary forces. The solutes are detected in-situ on the surface of the thin layer plate by visualizing reagents after the chromatography has been completed.[1] Finely divided particulate sorbents are used as stationary phase. These may include; silica gel, cellulose powder, kieselguhr and magnesium silicate. They normally contain binding agents like calcium sulphate (15%) to ensure adherence to the plate.

The basic chromatographic measurement of a substance in TLC is the Rf value, defined as

$$Rf = \frac{\text{distance the substance travels from origin}}{\text{distance the solvent travels from origin.}}$$

Thin layer chromatography primarily is used as qualitative analytical technique for the identification of organic and inorganic solutes by the comparison of samples with standard chromatogram simultaneously. [1]

1.6.2.4 TITRIMETRIC AND CHEMICAL ANALYSIS

There are various classes of titrimetry. Examples include:

Complexometric titrations, which are used for the estimation of metal salts. It basically involves the use of Ethylenediamine tetracetic acid (EDTA) as the titrant with an indicator dye being used to determine the endpoint in the metal solution. This form of titration is used for the

assay of magnesium hydroxide, magnesium trisilicate, calcium acetate, calcium chloride, zinc undecanoate and zinc chloride.

Redox titrations, which involve the transfer of electrons between the titrant and the analyte. In Potentiometric Redox titrations, standard Ag/AgCl or Hg/Hg_2Cl_2 electrodes are used as a reference in conjunction with an inert redox electrode, e.g. platinum. Hydrogen peroxide, ferrous salts, sodium perborate and benzoyl peroxide are all assayed using $KMnO_4$ as the titrant. [19s]

Direct acid/base titrations in the aqueous phase have various forms. Strong acid/strong base titrations are used for the assay of thiamine hydrochloride, sulphuric acid, hydrochloric acid and perchloric acid. Weak acid/strong base titrations are used for the assay of nicotinic acid tablets, mustine injection, chlorambucil injection, benzoic acid and undecanoic acid. Non-aqueous titration of acidic drugs is used for the assay of barbiturates, some sulphonamides and phenols. Non-aqueous titration of weakly basic drugs is used for the assay of adrenaline, chlordiazepoxide and chloroquine phosphate. [18]

Chapter 2

MATERIALS, REAGENTS AND METHODS

2.1 MATERIALS/ REAGENTS

Methanol (Analar Grade), glacial acetic acid (BDH), Acetic anhydride (BDH), Benzoic acid, Sodium hydroxide(BDH), Hydrochloric acid (BDH), Ethanol (BDH), Sulphuric acid (BDH), Perchloric acid(BDH), Phenolphthalein, Acetone, Potassium dihydrogen orthophosphate, Phenol red, sulphamic acid.

Pure samples were obtained from Ernest Chemist in Tema.

Table 2.1 Profiles of Pure samples used

PURE DRUG	BATCH NUMBER	MAN. DATE	EXPIRY DATE	ASSAY%
Diazepam	20080204	Feb-08	Feb-11	100.1
Indometacin	X061210	Dec-07	Nov-10	99.1
Metronidazole	081001801	Oct-08	Oct-12	99.6
Naproxen	0903201	Mar-09	Mar-12	99.8
Piroxicam	20061101	Nov-07	Nov-10	98.6

Diazepam tablets, manufactured by Pharmanova Ltd, Ernest Chemist and Golden Tower Limited, and Indometacin capsules, manufactured by Letap Pharmaceuticals Limited, M&G Pharmaceuticals and Ernest Chemist Limited, were purchased from retail pharmacies in and around the Kwame Nkrumah University of Science and Technology.

Table 2.2 Profile of Diazepam tablet samples

Name	Strength(mg)	Company	Batch no.	Exp. Date
GD	5	Golden tower Ltd	DZ5015	Nov-11
PD	5	Pharmanova Ltd	8008	Mar-12
ED	10	Ernest Chemist	0110H	Oct-12

Table 2.3 Profile of Indometacin capsule samples

Name	Strength(mg)	Company	Batch no.	Exp. Date
IL	25	Letap pharmaceuticals Ltd.	019046	July-11
IE	25	Ernest Chemist	**	**
IM	25	M&G Pharmaceutical	**	**

** not available

2.2 INSTRUMENTATION

Hanna instruments pH 211 microprocessor pH meter

Cecil CE 2041 2000 Series-UV Spectrophotometer

Shimadzu LC-6A Liquid Chromatograph-pump

Applied Biosystems 783 programmable Absorbance Detector

Shimadzu CR 501 Chromatopac-Integrator

HICHROM ODS column

FS 28H Fisher Scientific Sonicator

Adam-analytical weighing balance, WA 210 ; 210/0.0001g

Büchi rotary evaporator

2.3 STANDARDIZATION OF SOLUTIONS

2.3.1 STANDARDIZATION OF 0.1M SODIUM HYDROXIDE

Sulphamic acid solution (25ml) was pippetted into a conical flask and titrated against NaOH (0.1M) using methyl orange as the indicator. A triplicate determination was done.

2.3.2 STANDARDIZATION OF (0.1 M) PERCHLORIC ACID

Potassium hydrogen phthalate (0.4998g) was weighed into a conical flask. Glacial acetic acid (25ml) was added. The solution was then warmed to ensure dissolution of the salt. It was allowed to cool and titrated with Perchloric acid (0.1M). Oracet blue was used as the indicator.

2.4 IDENTIFICATION TESTS AND ASSAY FOR SURROGATE REFERENCE SAMPLES

2.4.1 BENZOIC ACID

Colour test

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.

Assay

Benzoic acid (0.200 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. The titre value was recorded and repeated.

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

2.4.2 NAPROXEN

Identification

0.004%w/v of naproxen was prepared in methanol. It was then examined between 230 nm and 350 nm to observe 4 absorption maxima, at 262 nm, 271 nm, 316 nm and 331 nm.

Assay

Naproxen (0.200 g) was accurately weighed into a conical flask containing a mixture of 25 ml of water and 75 ml of methanol. It was then titrated with standardized 0.1 M sodium hydroxide, using phenolphthalein solution as indicator.

1 ml of *0.1 M sodium hydroxide* is equivalent to 23.03 mg of $C_{14}H_{14}O_3$.

2.4.3 INDOMETACIN

Identification

Indometacin (0.0025%w/v) in a mixture of 1 volume of 1 M HCl and 9 volumes of methanol was prepared. It was then examined between 300 nm and 350 nm to observe an absorption maximum at 318 nm.

Assay

Indometacin (0.300 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 sodium hydroxide* is equivalent to 35.78 mg of $C_{19}H_{16}ClNO_4$.

2.4.4 PIROXICAM

Assay

Piroxicam (0.250g) was accurately weighed into a conical flask. 60 ml of a mixture of equal volumes of acetic anhydride and anhydrous acetic acid was added. It was titrated with previously standardized 0.1M perchloric acid with oracet blue as indicator.

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

2.4.5 DIAZEPAM

Identification test

About 0.0100g of diazepam was dissolved in 3 ml of sulphuric acid. The solution was examined under ultraviolet light at 365 nm.

Assay

Diazepam (0.500g) was accurately weighed into a 250 ml conical flask. Acetic anhydride (50ml) was added with few drops of oracet blue solution as indicator. The resulting solution was titrated with standardized 0.1 M perchloric acid.

1 ml of 0.1 M perchloric acid is equivalent to 28.47 mg of $C_{16}H_{13}ClN_2O$.

2.4.6 METRONIDAZOLE

Identification

Metronidazole (0.002%w/v) was prepared with 0.1M HCl as solvent. The solution was then examined with the UV spectrometer to observe an absorption maximum at 277 nm and a minimum at 240 nm.

Assay

Metronidazole (0.150g) was weighed accurately into a conical flask containing 50 ml of anhydrous acetic acid. It was then titrated with already standardized 0.1 M perchloric acid, determining the end-point with oracet blue as indicator..

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

2.5 IDENTIFICATION TEST FOR DRUG SAMPLES

2.5.1 DIAZEPAM TABLETS

Test solutions of all the diazepam tablets were prepared by shaking a quantity of the powdered tablets with sufficient methanol to produce solutions containing 0.5%w/v of diazepam. It was allowed to cool and then decanted to obtain a clear solution. A reference solution 0.5%w/v was also prepared in methanol with the pure diazepam reference standard.

A thin layer chromatography was carried out using silica gel as the coating substance and a mixture of 100 volumes of chloroform and 10 volumes of methanol as the mobile phase. About 2 µl of each of the solutions was applied separately to the plate and kept in the mobile phase. After removal of the plate, it was sprayed with a 10% v/v solution of sulphuric acid in absolute ethanol. It was then heated at 105°C for 10 minutes and examined under ultraviolet light (365 nm).

2.5.2 INDOMETACIN CAPSULES

A quantity of the contents of the capsules containing 25 mg of Indometacin was mixed with 2ml of water. 2 ml of 2M sodium hydroxide was then added. The colour was observed.

2.6 MELTING POINT DETERMINATION

The dry surrogate standards and pure samples of analyte were introduced into separate capillary tubes with ends sealed to contain samples. Samples were pushed down the capillary tubes with a clean and tiny metallic rod to ensure good and tight packing (about 2mm in height).

The capillaries containing the samples were each fed into the melting point determination apparatus to determine the melting points. The melting range for each sample was then determined and recorded accordingly.

2.7 HPLC METHOD DEVELOPMENT

2.7.1 PREPARATION OF SOLVENT SYSTEM

2.7.1.1 Preparation Of 0.2m Potassium Dihydrogen Orthophosphate (KH_2PO_4)

Potassium dihydrogen orthophosphate (5.4440g) was accurately weighed and dissolved in about 100ml of water in a 200ml volumetric flask. It was sonicated for uniform dissolution and topped up to the mark with distilled water to produce a 200ml of 0.2M KH_2PO_4

2.7.1.2 Preparation of The Buffer pH 5.8

0.2M NaOH (4.5ml) was pippeted into a 250ml volumetric flask. 0.2M KH_2PO_4 (62.5ml) was measured and added. Enough distilled water was added to the mark and shaken for uniform mixing. The buffer was allowed to stand for about ten minutes then the pH was recorded.

2.7.1.3 Preparation of The Mobile Phase And Solvent

Diazepam

The mobile phase used was a mixture of methanol and the prepared buffer in the ratio of 75 to 25 respectively. The required volume was prepared and filtered with a number 1 sintered glass funnel.

Indometacin

The mobile phase used was a mixture of methanol and the prepared buffer in the ratio of 60 to 40 respectively. The required volume was prepared and filtered with a number 1 sintered glass funnel.

2.7.2 DETERMINATION OF WAVELENGTH OF MAXIMUM ABSORPTION

Different concentrations of each sample (pure drugs of analyte and surrogates references) were prepared with the mobile phase. The UV absorption spectrum for each sample was observed between wavelengths of 250nm to 400nm.

2.7.3 LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ)

A concentration of 0.1% w/v of pure samples of all the surrogate standards and analytes were accurately prepared with the mobile phase. They were serially diluted to produce different concentrations. Twenty micro-litres (20 µl) of the resultant solutions were injected onto the column one after the other and ran. Chromatograms were recorded and peak areas measured. LODs and LOQs for each sample was calculated from the calibration curves. That is LOD = 3.3 x σ/s and LOQ = 10 x σ/s

Where ‘σ’ is the residual standard deviation of calibration curve and ‘s’ is the slope of the curve.

Chromatographic conditions

Column: HICHROM ODS column

Chromatograph settings: attenuation = 1; chart speed = 5 inches per minutes

Flow rate: 1.5ml/min (Indometacin) and 1ml/min (Diazepam)

Detector: UV-visible detector; 300nm (Diazepam) and 254nm (Indometacin)

Solvent system: 75ml methanol:25ml buffer pH 5.8 for Diazepam

60ml methanol: 40ml buffer pH 5.8 for Indometacin

Injector: 20µl

2.7.4 DETERMINATION OF K USING THE SURROGATE REFERENCE STANDARDS

The surrogate references used for diazepam were Indometacin, Piroxicam and Metronidazole. Stock solutions of the pure sample of anlyte and surrogate references were prepared with the mobile phase. Just like with the use of internal standards in HPLC, every run was to contain the analyte pure sample and surrogate reference standard. 1ml each of diazepam stock solution (analyte) and Piroxicam stock solution (surrogate) were pipetted into a 10ml volumetric flask, mixed and made to the mark with the mobile phase producing certain concentrations of the analyte and surrogate standard (Piroxicam). Twenty micro-litres (20µl) of the resultant solution was injected onto the column and eluted isocratically. Chromatograms were recorded and peak areas computed. The constant K was determined. The concentration of the diazepam and Piroxicam were varied in the mixture to determine whether these variations would affect the constant K.

The whole process was repeated using Indometacin and Metronidazole as surrogate standards for diazepam and the 'K' values consequently determined.

The same procedure was followed for Indometacin as the analyte and benzoic acid and naproxen as the surrogate standards. 'K' was to be determined in each case.

2.7.5 ANALYSIS OF COMMERCIAL SAMPLES

2.7.5.1 Diazepam Tablets Using the Surrogate Reference Standards

An amount of ground diazepam tablets equivalent to 0.02g of diazepam was accurately weighed and dissolved in an amount of the mobile phase in a 100ml volumetric flask. Sufficient mobile phase was added to produce 100ml which was then filtered. 1ml each of the analyte and prepared surrogate standard were pippeted into a 10ml volumetric flask mixed and toped up with the mobile phase. Twenty microlitres (20µl) of the resultant solution was injected onto the column and eluted. The major peaks corresponding to diazepam and the surrogate standard were measured and areas computed. This was done for the analyte against all the three surrogate standards (Metronidazole, Piroxicam and Indometacin). The content of the diazepam tablets determined with the knowledge of the 'K' values. Each surrogate was also used against all three brands of diazepam tablets.

2.7.5.2 Indometacin Capsules Using the Surrogate Reference Standards

An amount of mixed content of 20capsules equivalent to 0.05g of Indometacin was accurately weighed and dissolved in an amount of the mobile phase in a 100ml volumetric flask. Sufficient mobile phase was added to produce 100ml which was then filtered. 1ml each of the analyte and prepared surrogate standard were pippeted into a 10ml volumetric flask mixed and toped up with the mobile phase. Twenty microlitres (20µl) of the resultant solution was injected onto the column and eluted. The major peaks corresponding to Indometacin and the

surrogate standard were measured and areas computed. This was done for the analyte against all the three surrogate standards (Diazepam, Benzoic acid and Naproxen). The content of the Indometacin capsule was determined with the knowledge of the 'K' values. Each surrogate was also used against all three brands of Indometacin capsules.

2.8 BP. METHOD OF ASSAY FOR COMMERCIAL SAMPLES

2.8.1 UNIFORMITY OF WEIGHT TEST

Diazepam tablets

Twenty tablets each of all the three brands of diazepam were weighed individually. The 20 tablets for the individual brands were then weighed together to obtain total weight for 20 tablets. The average tablet weight for the three brands was then computed. Percentage deviations from average weights were also deduced.

Indometacin capsules

Weight of intact capsule was obtained. The capsule was then opened without losing any part of the shell and completely voided of its content. The empty shell was then weighed to compute weight of content per capsule. The procedure was repeated for 19 other capsules of the same brand. The average weight of powder per capsule was obtained.

The process was repeated for 20 capsules each of the two other brands.

2.8.2 DETERMINATION OF PERCENTAGE CONTENT OF DIAZEPAM TABLETS (B.P)

To a quantity of the powder containing 10 mg of Diazepam 5 ml of water was added, mixed and allowed to stand for 15 minutes. To this 70 ml of 0.5% w/v solution of sulphuric acid in methanol was added and shaken for 15 minutes. Enough of the methanolic sulphuric acid was added to produce 100 ml and filtered.

10 ml of the filtrate was measured into a 50ml volumetric flask and made up to volume with the solvent. The absorbance of the resulting solution at the maximum at 284 nm was measured. The percentage content of diazepam was computed taking 450 as the value of A(1%, 1 cm).

2.8.3 DETERMINATION OF PERCENTAGE CONTENT OF INDOMETACIN CAPSULES (B.P)

To a quantity of the mixed contents of 20 capsules containing 50 mg of Indometacin, 10ml of water was added and allowed to stand for 10 minutes with occasional swirling. 75ml of methanol was added and shaken very well. Sufficient methanol was added to produce 100 ml and filtered.

5 ml of the filtrate was measured and a mixture of equal volumes of methanol and phosphate buffer pH 7.2 was added to produce 100 ml. The absorbance of the resulting solution was measured at the maximum at 320 nm, the content was calculated taking 193 as the value of A(1%, 1 cm).

2.9 ANALYTICAL PERFORMANCE PARAMETERS (VALIDATION PARAMETERS)

2.9.1 LINEARITY

Stock solutions of all the surrogate reference standards and reference samples for analyte were prepared and serially diluted to different concentrations. Twenty micro-litres (20µl) of the resultant solutions were injected onto the column. The peak areas were measured and plotted against their respective concentrations.

2.9.2 ACCURACY, PRECISION AND REPRODUCIBILITY

Three concentrations were each prepared from the reference standards based on the calibration curve. Five injections for each concentration were made to test for their accuracy, precision and reproducibility. The peak areas were measured. The Relative Standard Deviation (RSD) for the individual concentrations was determined.

Chapter 3

RESULTS AND CALCULATIONS

3.1 STANDARDIZATION OF SOLUTIONS

3.1.1 STANDARDIZATION OF 0.1M NaOH WITH H_2NSO_3H

$H_2NSO_3H + NaOH \longrightarrow H_2NSO_3Na + H_2O$

Mole ratio 1 : 1

Nominal wt = **0.9807g** of H_2NSO_3H

Amount of H_2NSO_3H weighed = 0.9800g

Factor of H_2NSO_3H = Actual weight/ nominal weight

= 0.9800/0.9807

$F_{(H_2NSO_3H)}$ = 0.9992

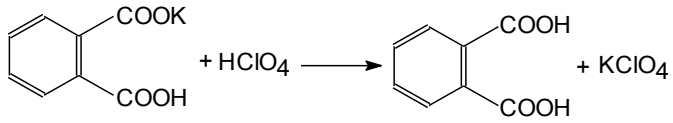
$Factor_{(H_2NSO_3H)} \times Volume_{(H_2NSO_3H)} = Factor_{(NaOH)} \times Volume_{(NaOH)}$

$F_{(NaOH)} = [F_{(H_2NSO_3H)} \times V_{(H_2NSO_3H)}]/V_{(NaOH)}$

$F_{(NaOH)} = (0.9992 \times 25)/26$

$F_{(NaOH)}$ = **0.9607**

3.1.2 STANDARDIZATION OF PERCHLORIC ACID ($HClO_4$) WITH POTASSIUM HYDROGEN PHTHALATE



Mole ratio 1 : 1

204.2g of $C_8H_5O_4K$ in 1000ml ≡ 1M $HClO_4$

0.2042g of $C_8H_5O_4K$ in 1ml ≡ 0.1M $HClO_4$

Factor $C_8H_5O_4K$ = actual wt/nominal wt

= 0.4998/0.5g

= 0.9996

0.020422g $C_8O_4H_5K$ ≡ 1ml of 0.1M $HClO_4$

Average titre = 25.20ml- Blank titre

= 25.20-0.1 = 25.10ml

$F_{HClO_4} = F_{C_8O_4H_5K}$* $Vol_{C_8O_4H_5K}/Vol_{HClO_4}$

= 0.9996*25ml/25.01ml

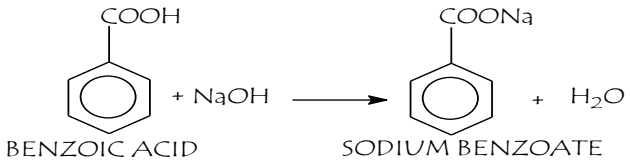
Factor of $HClO_4$ = **0.9956**

3.2 IDENTIFICATION AND ASSAY OF SURROGATE AND ANALYTE REFERENCE STANDARDS

3.2.1 BENZOIC ACID

A solution of benzoic acid gave a dull-yellow precipitate which was soluble in ether.

Assay



Factor of NaOH = 0.9607

Actual volume of NaOH for 1st determination = titre × F(NaOH)

= 17.2 ×0.9607

= 16.52ml

From milliequivalent

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

∴ Actual amount = 16.52 ×0.01221

= 0.2017g

%purity = (actual wt/nominal wt) ×100%

= (0.2017/0.2019) ×100%

= 99.9%

Same was done for 2nd determination to obtain 99.4%

Average %purity = (99.9+99.4)/2

= **99.65%**

3.2.2 NAPROXEN

Identification

A spectrum of a methanolic solution of Naproxen showed 4 absorption maxima, at 262 nm, 271 nm, 316 nm and 331 nm.

Assay

$F_{(NaOH)}$ = 0.9607

1 ml of 0.1 M sodium hydroxide is equivalent to 23.03 mg of $C_{14}H_{14}O_3$.

Actual amount (1^{st} det) = 0.9607×9.3×0.02303

= 0.2058g

%purity = (0.2058/0.2090) ×100%

= 98.5%

Same was done for 2^{nd} determination to obtain a purity of 99.8%

Average purity = (98.5+99.8)/2 = **99.15%**

3.2.3 INDOMETACIN

Identification

An absorption maximum at 318nm was observed on the UV spectrum.

Assay

Titre value = 9.6ml-blank titre(0.8) = 8.8ml

Factor of NaOH = 0.9607

Actual titre = 8.8 * 0.9607 = 8.45416ml

1 ml of 0.1 M sodium hydroxide is equivalent to 0.03578 g of $C_{19}H_{16}ClNO_4$.

Actual amount of indometacin = 0.03578 * 8.45416ml

= 0.3024898g

% purity = (0.3024898/0.3045) * 100% = 99.33%

Similar calculation was done for other weighed sample to obtain 98.69%

Average purity is (99.33+98.69)/2 = **99.01%**

3.2.4 PIROXICAM

Assay

Titre = 8ml-0.5(blank) =7.5ml

Actual titre = 7.5 * 0.9956 = 7.467ml

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

Actual amount of piroxicam = 7.467ml * 0.03314 = 0.247456g

% purity of piroxicam = (0.247456g/0.25) * 100%

= 98.98%

The second determination gave purity of 98.78% giving an average of **98.88%**

3.2.5 DIAZEPAM

Identification

The solution shows greenish-yellow fluorescence in ultraviolet light at 365 nm.

Assay

9.30ml (titre) – 0.5ml (blank) = 8.8ml

Actual titre = 8.8ml * 0.9956(factor of $HClO_4$) = 8.76ml

From milliequivalent

1 ml of 0.1 M perchloric acid is equivalent to 0.02847g of $C_{16}H_{13}ClN_2O$.

Amount of diazepam = 8.76 * 0.02847 = 0.24943g

% purity = (0.24943/0.2501) * 100% = 99.73%

2^{nd} determination gave a purity of 100.01%

Average % purity = **99.87%**

3.2.6 METRONIDAZOLE

Identification

The solution showed an absorption maximum at 277 nm and a minimum at 240 nm.

Assay

Titre (0.1504g) = 8.80mml and Factor of $HClO_4$ = 0.9956

Actual titre = 8.80ml * Factor of $HClO_4$

= 8.80 *0.9956 = 8.7612ml

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

Actual amount of Metronidazole = 0.01712*8.7612ml = 0.14999g

% purity of Metronidazole = (0.14999/0.1504)*100%

= 99.7%

Similar calculation was done for other samples to obtain 99.8% to obtain an average purity of **99.75%**

3.3 MELTING POINT

Table 3. *Melting point determination*

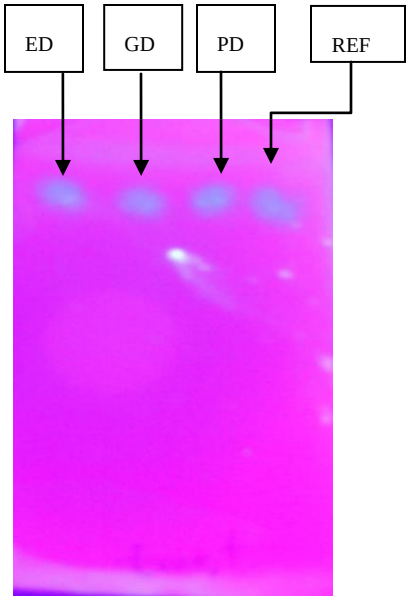
Sample	Experimental range (^oC)	Literature range(^oC)
Benzoic acid	122-124	121-124
Metronidazole	160-162	159-163
Naproxen	154-156	154-158
Indometacin	158-160	158-162
Piroxicam	192-194	200-204
Diazepam	132-134	131-135

3.4 IDENTIFICATION OF DRUG SAMPLES

A mixture of the content of all the brands of **Indometacin** capsules and sodium hydroxide produce a bright yellow colouration which faded rapidly hence Indometacin present.

A thin layer chromatography was done for all the brands of **diazepam** with the reference sample. There was no significant difference in the shape of their spots and also in their retention times.

Fig TLC.1



f value (GD) = Distance moved from the origin / Distance moved by solvent front

= 40mm / 50mm = 0.80

Rf value of Pure sample = 40mm / 50mm = 0.80

3.5 3.5 HPLC METHOD DEVELOPMENT

UV spectra of all the samples

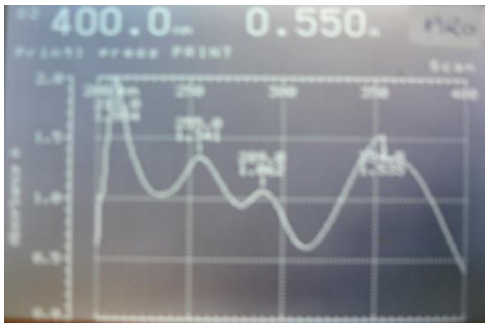


Fig. 3.1 UV spectrum of Piroxicam

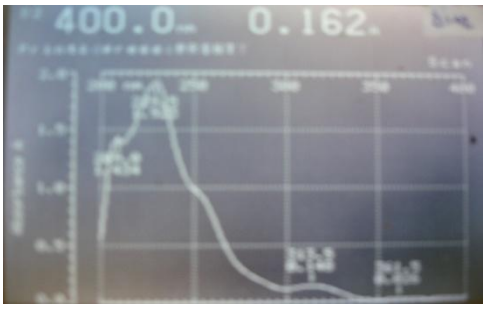


Fig.3.2 UV spectrum of Diazepam

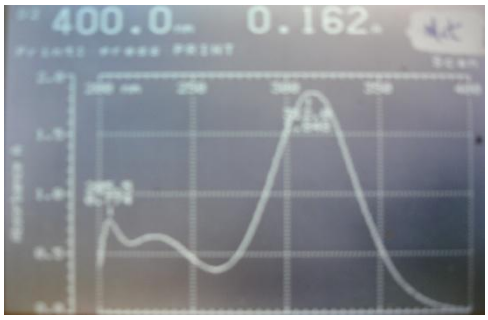


Fig. 3.3 UV spectrum of Metronidazole

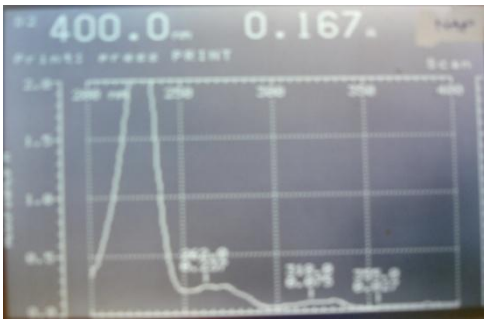


Fig. 3.4 UV spectrum for Naproxen

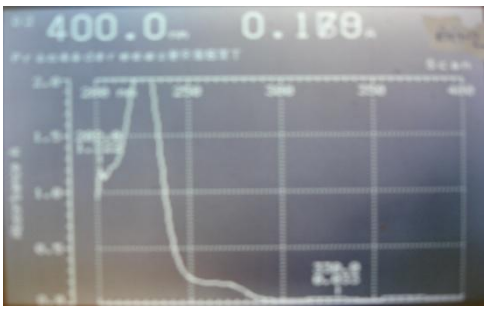


Fig. 3.5 UV spectrum for Benzoic acid

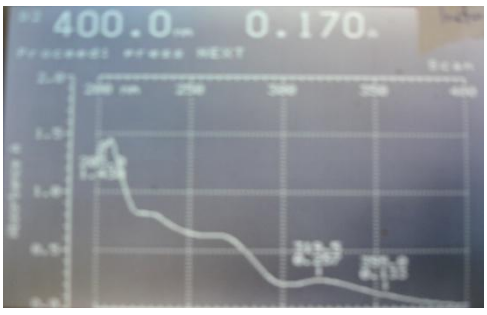


Fig. 3.6 UV spectrum for Indometacin

From the UV spectra, it was realized that Benzoic acid and Naproxen could not absorb at 300nm. Work on them as surrogate standards were therefore done at a wavelength of 254nm while the others were done at 300nm

Retention times

Table M.1 Mean Retention Times (min) for Diazepam and its surrogate reference Standards

Mobile Phase System Phosphate Buffer (pH 5.80 ± 0.02) and Methanol	**Mean Retention Time/min**			
	Diazepam	**Indometacin**	**Piroxicam**	**Metronidazole**
Composition = 25:75	6.67±0.21	2.92±0.11	2.72±0.07	2.21±0.15

Table M.2 Mean Retention Times (min) for Indometacin and its surrogate reference Standards

Mobile Phase System Phosphate Buffer (pH 5.80 ± 0.02) and Methanol	**Mean Retention Time/min**			
	Indometacin	**Naproxen**	**Benzoic acid**	**Diazepam** *Mobile ph.25:75*
Composition = 40:60	5.35±0.12	2.58±0.03	1.72±0.05	6.76±0.21

3.6 CALCULATION OF LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ)

LOD = 3.3 x σ/s; LOQ = 10 x σ/s; where'σ' is the residual standard deviation; 's' is the number of sample injections.

Sample calculation for Piroxicam at 300nm.

Concentrations (%w/v) used for piroxicam; 0.00003125, 0.0000625, 0.000125, 0.00025, 0.0004, 0.0005; s =640199 and σ = 4.800024761

Therefore LOD = (3.3 x 4.800024761)/640199 = 2.47424E-05% w/v;

LOQ = (10 x 4.800024761)/640199 = 7.49771E-05% w/v

3.7 CHROMATOGRAMS

Indometacin

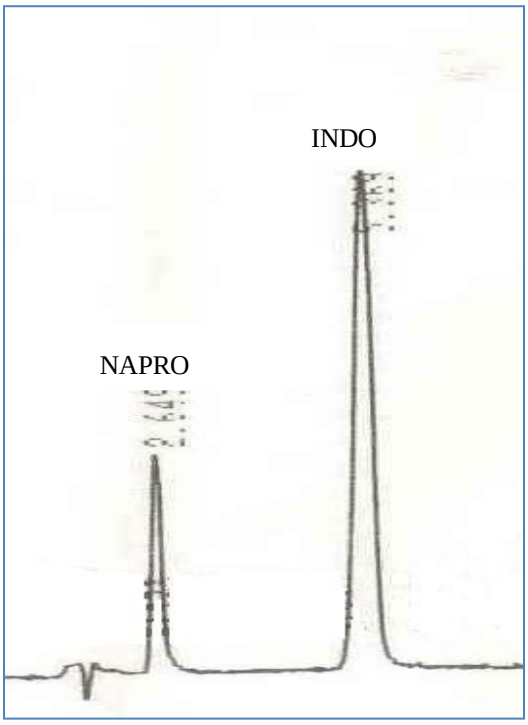


Fig CI.1 Napro. & Indo

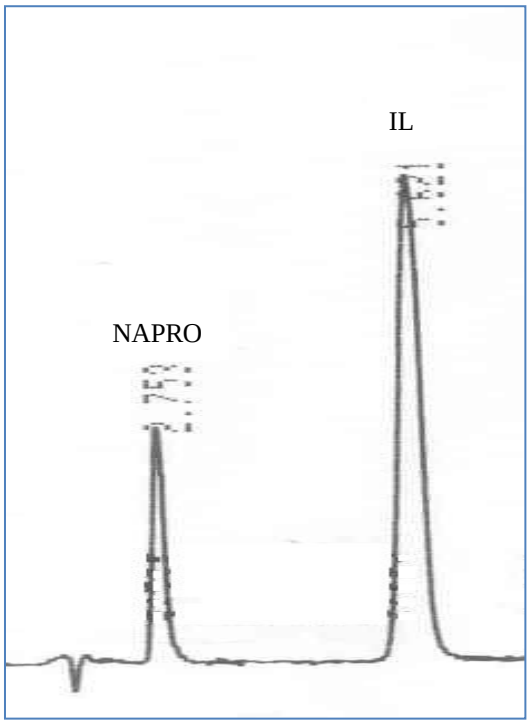


Fig CI.2 Napro & IL

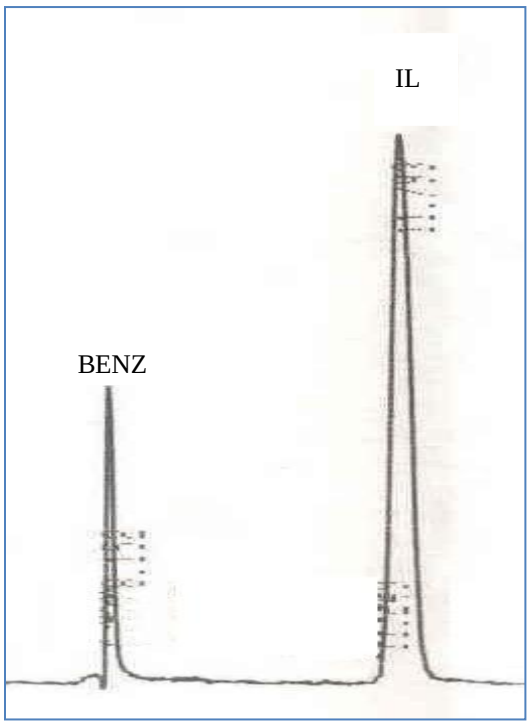


Fig CI.3 Benzoic acid & IL

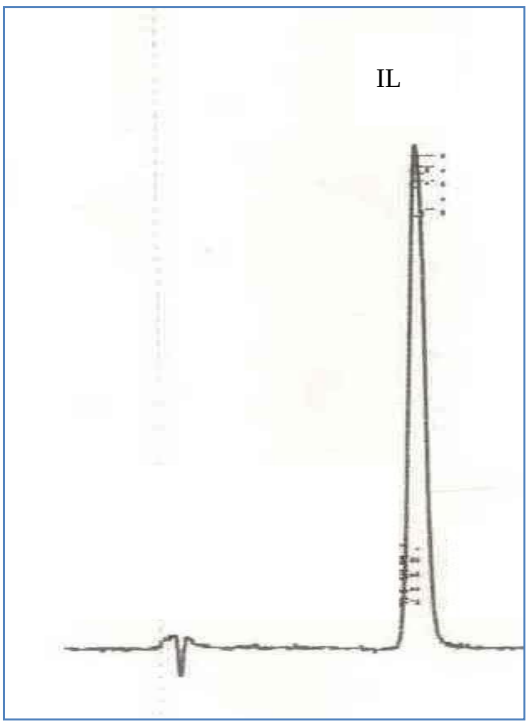


Fig CI.4 IL

Diazepam

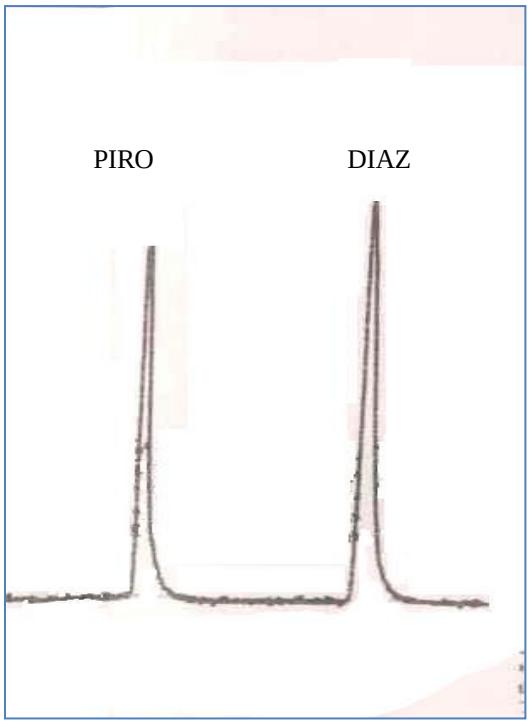


Fig CD.1 Piroxicam & diazepam

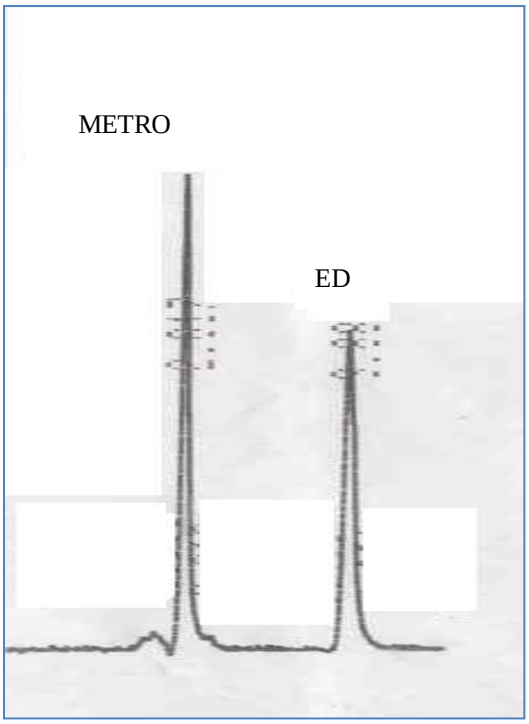


Fig CD.2 Metronidazole & ED

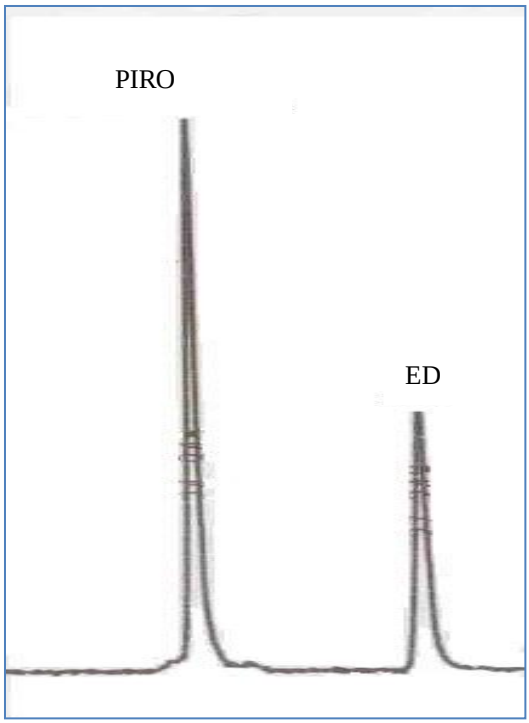


Fig CD.3 Piro & ED

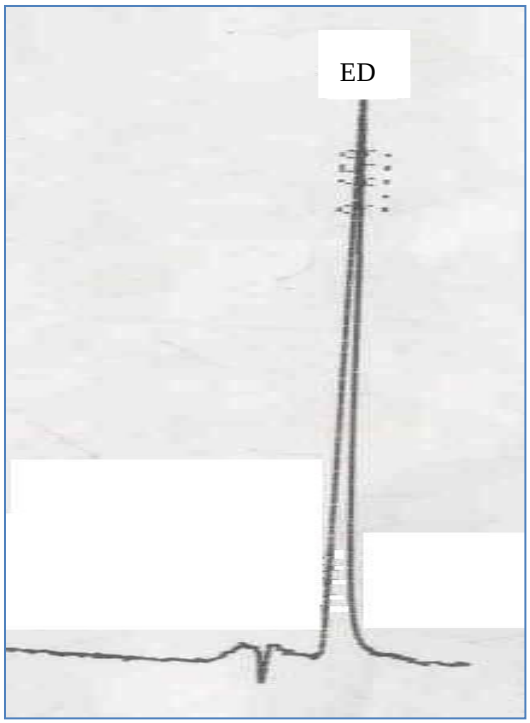


Fig CD.4 ED

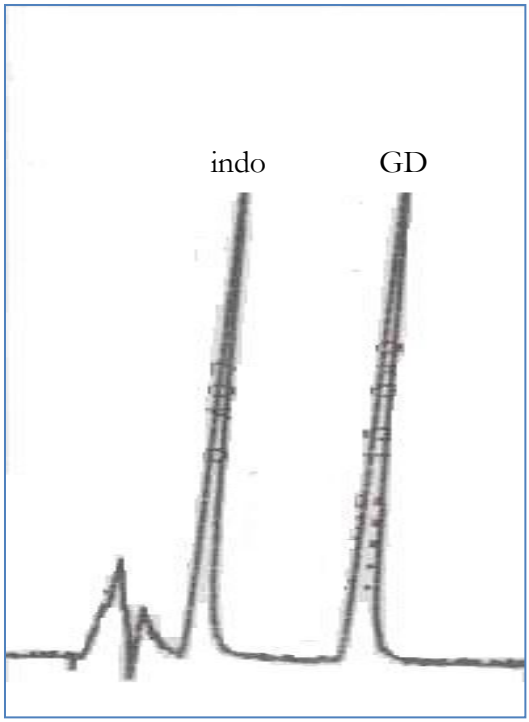


Fig CD.5 Indo & GD

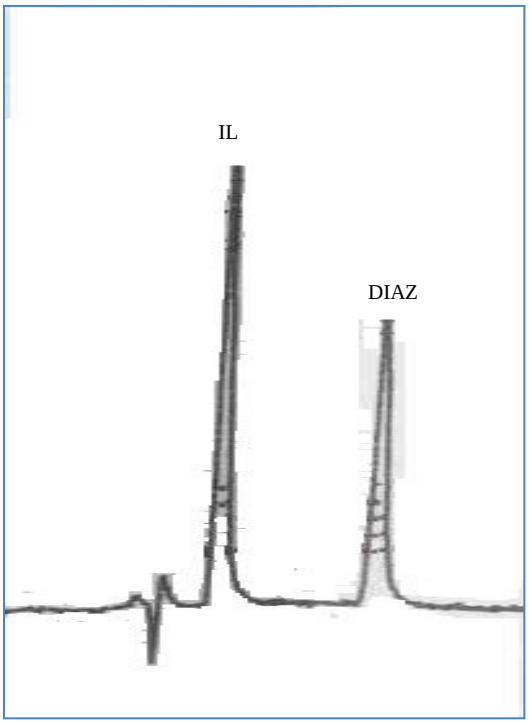


Fig CD.6 Diazepam & IL

3.8 SAMPLE CALCULATION FOR 'K' OF DIAZEPAM

One surrogate standard for diazepam was Metronidazole. A prepared mixture of the two containing diazepam (0.0004%) and Metronidazole (0.000205%) was injected to produce peak areas of 19mm^2 and 74mm^2 respectively.

K value = (Area of analyte × Concentration of standard) / (Concentration of analyte × Area of standard)

Analyte is diazepam and surrogate is Metronidazole.

Substituting the parameters into the formula

K = (19 ×0.000205)/(0.0004×74)

K = 0.131588

Calculating for K at different concentrations gave an average K= **0.135271**

Similar calculations were done for all the different concentration pairs and the other surrogates to find their corresponding K values

3.9 SAMPLE CALCULATION FOR PERCENTAGE CONTENT OF DIAZEPAM TABLETS ED WITH K VALUE OF METRONIDAZOLE

0.1010g of diazepam(ED) powdered tablets was weighed and dissolved in 50ml of the solvent system (75 methanol and 25 buffer of pH 5.8) and filtered. 1ml of the resulting solution and 0.5ml of pure Metronidazole solution (0.082%w/v) were mixed and more solvent added to produce 10ml. 20µl was then injected.

Average weight of Dizepam ED tablet = 0.1946g containing 0.01g (10mg) pure diazepam

Therefore actual diazepam contained in weighed powder = (0.01×0.1010)/0.1946

= **0.00519g** pure diazepam

0.00519g of pure Diazepam was actually dissolved in 50ml to obtain 0.0104%w/v.

Diluting 1ml to 10ml produces **0.00104%w/v** while 0.5ml of pure Metronidazole(0.082%) solution to 10ml produces 0.0041%

Peak area for diazepam ED = 50mm^2; peak area for Metronidazole = 74mm^2

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

Substituting the parameters;

= (50 x 0.000205) / (0.135271 x 74);

= 0.001024%w/v

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

= (0.001024 / 0.00104) x 100 = **98.46%**

Similar calculations were done for all other brands and the assay for Indometacin at 254nm

3.10 DETERMINATION OF PERCENTAGE CONTENT OF DIAZEPAM TABLETS (BP)

From Beer-Lambert's law A =abc

Where A = absorbance' a = specific absorbance, b = path length, c = concentration (%w/v)

Sample calculation with PD brand of diazepam tablets

Specific absorbance for diazepam 'a' = 450 and 'b' = 1cm

For absorbance 'A' of 0.795

c = A/ab

c = 0.795/(1×450)

c = 0.001767%

Amount weighed = 0.3428g

From uniformity of weight, 0.1901g of powdered tablets is equivalent to 0.005g diazepam.

∴ 0.3428g will contain (0.005×0.3428)/0.1901 = 0.009016g of diazepam in 100ml of solution

10ml of this solution was diluted to 50ml to produce a concentration of

= (0.009016×10)/50

= 0.0018%w/v

%content = (actual amount/expected amount)×100%

%content = (0.001767/0.0018) ×100%

= **98.16%**

Table 3.2 *Table of result for calculated %contents of different brands of diazepam tablets*

Brands of diazepam	Percentage content(%w/v)				
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
GD(5mg)	93.1	94.5	94.5	94.9	94.9
PD(5mg)	98.08	98.16	97.65	97.65	98.82
ED(10mg)	99.18	99.50	99.20	98.49	98.90

3.11 DETERMINATION OF PERCENTAGE CONTENT OF INDOMETACIN CAPSULES (BP)

Sample calculation with IL brand of Indometacin capsules

From Beer-Lambert's law A =abc

c = A/ab

Specific absorbance for Indometacin 'a' = 193 and 'b' = 1cm

For absorbance 'A' of 0.728

c = 0.728/(1×193)

c = 0.003772%

Amount of powder weighed = 0.3103g

From uniformity of weight, 0.10027g of powdered tablets is equivalent to 0.025g Indometacin.

∴ 0.3103g will contain (0.025×0.3103)/0.10027 = 0.0774g of Indometacin in 100ml solution

5ml of this solution was diluted to 100ml to produce a concentration of

= (0.0774×5)/100

= 0.00387%w/v

%content = (actual amount/expected amount)×100%

%content = (0.003772/0.00387) ×100%

= **97.47%**

Table 3.3 *Table of result for calculated %contents of different brands of Indometacin capsules*

Brands of Indometacin	Percentage content(%w/v)				
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
IL(25mg)	97.47	97.40	97.20	98.00	100.14
IE(25mg)	91.40	93.01	93.20	91.98	92.94
IM(25mg)	98.7	98.4	99.80	100.15	98.7

Chapter 4

DISCUSSION, CONCLUSSION AND RECOMMENDATION

4.1 DISCUSSION

4.1.1 IDENTIFICATION AND ASSAY

Drug reference standards play a very prominent role in all drug quality assurance procedures. In many cases, the absence of a drug reference standard is the quality-limiting factor in the analysis of the drug sample. It is therefore imperative to identify the reference standards and also ascertain their purities. This helps surmount the problems of wrong or poor labeling on the part of the supplier.

Benzoic acid showed a percentage purity of 99.65%. The British pharmacopoeia states that Benzoic acid contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of benzene carboxylic acid. The result obtained indicates that its purity falls within the literature range hence, a pure standard. Further testing was done to identify it as stipulated by the BP. An alcoholic solution of benzoic acid produced a dull-yellow precipitate upon addition of ferric chloride solution which dissolved in ether. Its melting point was determined and showed a range of 122-124^{0}C as compared to the BP range of 121 – 124^{o}C. These were good markers to confirm that the sample under investigation was truly Benzoic acid.

Naproxen was one of the pure samples used as surrogate references. It was assayed and a purity of 99.15% was obtained which fell within the permissible range stated by the BP. The British pharmacopoeia states that the purity of naproxen should be between 99.0% and 101.0%. The naproxen sample is therefore considered pure since its experimental purity fell within BP's range of purity. The UV spectrum of naproxen (refer to fig 3.4 page 49) produced four different absorption maxima just like the literature (BP) stipulated. The experimental melting point range was 154 – 156^{o}C. The literature value is 154 – 158^{o}C. The sample was therefore confirmed to be Naproxen.

Indometacin pure reference standard was also assayed and identified. The percentage content of Indometacin was calculated to be 99.01% which fell within the BP specification of between 98.5-100.5%. An absorption maximum at 318nm was observed on the UV spectrum obtained for identification. (Refer to fig.3.6 page 49) The experimental melting point was 158-160^{o}C compared to the literature range of 158 °C to 162 °C.

Piroxicam was assayed to obtain a purity of 99.98% and a melting point range of 192^{o}C to 194^{o}C. The BP states that piroxicam contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent, calculated with reference to the dried substance. It is inferred that the piroxicam pure reference used was of good purity.

Diazepam pure reference sample was assayed titrimetrically to obtain a purity of 99.87%. The percentage content as stated by the BP says diazepam should contain not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of diazepam, calculated with reference to the dried substance. It can be seen that the purity of diazepam falls within BP specifications. The pure powder was also identified by determining the melting point range, 132^{o}C to 134^{o}C, which is comparable to BP reference range of 131 °C to 135 °C. A solution of diazepam in sulfuric acid showed a greenish-yellow fluorescence in ultraviolet light at 365 nm which is peculiar to diazepam.

Lastly, Metronidazole pure reference sample was assayed and identified like all others. A hydrochloric acid solution of it showed the desired absorption maximum at 277nm and a minimum at 240nm on the UV spectrum obtained. A melting point range of 160°C to 162°C was obtained. This range lies comfortably within the required melting point range of 159 °C to 163 °C for Metronidazole. A percentage purity of 99.75% was estimated which fell within the BP specification of between 99% and 101%. The sample was therefore considered to be pure.

Commercial diazepam tablets and Indometacin capsules procured for the research were also identified to defeat the fear of total counterfeiting of drugs. Thin layer chromatography was done for all the brands of diazepam. The spots obtained were comparable to the spot corresponding to the pure reference sample with 0.8 as the retardation factor (Rf) for all.

Indometacin capsules obtained also produced the recommended bright yellow colour with sodium hydroxide. The samples used were therefore identified as the right samples.

4.1.2 UNIFORMITY OF WEIGHT

In the uniformity of weight test, not more than 2 of the individual masses should deviate from the average mass by more than the percentage deviation shown in Table 4.1 and none should deviate by more than twice that percentage.

Table 4.1 Uniformity of weight

Pharmaceutical form	Average mass	Percentage deviation
Tablets (uncoated and film coated)	80mg or less	10
	More than 80mg and less	7.5
	250mg or more	5
Capsules	Less than 300mg	10
	300mg or more	7.5

The diazepam GD brand had a strength of 5mg which is less than 80mg. This implies from the reference table above that not more than two of the masses should deviate by more than 10%. From the table of deviation of GD, only one tablet deviated by 12.37% with the rest falling within the permissible range. The diazepam GD brand can therefore be said to have passed the uniformity of weight with an average weight of 0.1706g per tablet.

The diazepam PD brand had a strength of 5mg which is also less than 80mg. According to the British Pharmacopoeia, tablets of 5mg strength should not have any two masses deviating by more than 10%. After the uniformity of weight was done on the PD tablets, none of the individual tablets deviated by more than 10%. The diazepam PD tablets therefore passed the uniformity of weight test with an average tablet weight of 0.1901g

The average weight obtained when the uniformity of weight test was carried out on diazepam tablets ED (10mg) was 0.1946g with none of the individual masses deviating by more than 10%. The ED tablets also passed the uniformity of weight test.

Uniformity of weight was also carried out on the three brands of Indometacin capsules. Indometacin capsules IM and IE showed deviations falling within the permissible range of the BP with average weights of 0.1602g and 0.1824g respectively. The Indometacin IL capsules had 6 of the individual masses deviating by more than 10%. This brand thus failed the uniformity of weight test. It can be inferred that either the capsule shells had varying weights or that capsule filling was not uniform. The average content per capsule was however found to be 0.10027g. Refer to pages 76-81 for uniformity of wt. of tablets

4.1.3 DETERMINATION OF PERCENTAGE CONTENT OF DIAZEPAM TABLETS (B.P)

The three different brands of Diazepam tablets were assayed using the standard method from the British pharmacopoeia. Diazepam tablets GD gave an average percentage content of 94.38%. Diazepam tablets PD gave an average percentage content of 98.07%. The average percentage content obtained for diazepam ED was 99.05%. The British pharmacopoeia states that diazepam tablets should have 92.5 to 107.5% of the stated amount. From the results, (Refer to pages 56, Table 3.2) it is observed that all three brands of diazepam assayed passed the test.

4.1.4 DETERMINATION OF PERCENTAGE CONTENT OF INDOMETACIN CAPSULES (B.P)

The percentage content of the various brands of Indometacin capsules were investigated using standard method from the BP. This was a UV spectroscopic method with a stated specific absorptivity of Indometacin. A one point UV assay was done on all the brands after extracting the pure Indometacin from the formulation with methanol. Indometacin IL had an average percentage content of 98.04%, Indometacin IM had an average percentage

content of 99.15% and IE had a content of 92.51%. The British pharmacopoeia states that Indometacin capsules should have 90.0 to 110.0% of the stated amount. This implies that, the Indometacin capsules analyzed passed the test. Refer to page 58, Table 3.3

4.1.5 METHOD DEVELOPMENT

Methods for analyzing drugs in multicomponent dosage forms or even single formulations with other excipients can be developed provided one has knowledge about the nature of the sample, mostly its molecular weight, polarity, ionic character and the solubility parameters. An exact recipe for HPLC, however, cannot be provided because method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase. In general one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with many polar groups and are water soluble. In such situations a polar solvent system is employed. Changing the polarity of mobile phase can alter elution of drug molecules. The elution strength of a mobile phase depends upon its polarity; the stronger the polarity, the higher the elution strength.

In the method for Indometacin and Diazepam, a reverse phase and a polar solvent system were used since they are polar compounds. A mixture of water and methanol was used which was able to elute the drugs but gave delayed peaks and slight tailing. Since an adjustment of the organic phase composition of the mobile phase is required to shorten or lengthen the retention time and avoid tailing, different compositions of the mobile phase system were tried. A lot of trials were done to give good peaks but upon the introduction of the surrogates, resolution was very poor.

For weakly acidic or weakly basic solutes, the role of pH is crucial. Changes in ionization states of solutes affects affinity of stationary phase consequently irregular peaks. In order therefore to control the pH of the mobile phase, a buffer was employed. Use was made of the different buffers like a combination of equimolar concentrations of mono and dibasic sodium phosphates, acetic acids and phosphates adjusted with phosphoric acid, some of which produced tailing peaks and others splitting peaks. Finally, a pH of 5.8 obtained from

Potassium phosphate monobasic adjusted with sodium hydroxide was used with the organic solvent, methanol. 25 parts buffer and 75 parts methanol was chosen for the analysis of diazepam while 40 parts buffer and 60 parts methanol was chosen for indometacin.

The UV spectrometer was chosen based on the analyte, its selectivity, sensitivity and availability. The analytes have chromophores to enable UV detection. In UV detection, good analytical results are obtained only when the wavelength is selected carefully. This requires knowledge of the UV spectra of the individual components present in the sample. All the samples were run and their spectra carefully observed. A wavelength of 300nm was chosen for the analysis of diazepam with its surrogate standards (indometacin, metronidazole and piroxicam) while 254nm was chosen for the analysis of indometacin and its surrogate standards (benzoic acid, naproxen and diazepam)

4.1.6 CHROMATOGRAM

A chromatogram of indometacin, benzoic acid and naproxen showed that indometacin had the highest retention time followed by naproxen then benzoic acid. All three samples have carboxylic acid functional groups making them polar. Very polar molecules would have very little interaction with the non-polar stationary phase and hence come out early. The bulky indole ring and benzene ring in indometacin introduces some degree of non polarity increasing interaction with stationary phase and consequently a higher retention time of around 6 minutes. Naproxen also has a naphthalene ring which introduces some level of non-polarity but not as much as that of indometacin. The polarity of the three therefore increases from benzoic acid to naproxen then indomethacin and hence the increasing retention time from benzoic acid to indometacin. Refer to page50, Tables M1and M2. The retention times of all the samples were basically affected by the relative degrees of polarities and the mobile phase employed for the elution. Diazepam has the least of polarities hence the highest retention time among the samples with a mobile phase composition of 25 parts buffer and 75 parts methanol. Tailing in these peaks were avoided with larger amounts of the organic composition in the mobile phase.

4.1.7 DETERMINATION OF THE CONSTANT K

From the hypothesis of this research, K is a constant that relates the concentration and corresponding peak area of a sample to another. The constant K for any two samples does not depend on the concentration of one since a change in concentration will be compensated for by the corresponding change in the peak area. Ideally, the calculated K for every two samples at different concentrations should be the same. However, the slight difference observed in the table of results, Refer to pages 84- 85 for tables was due to random errors as the statistical data infers. The concentrations and corresponding peak areas for the analytes and the surrogate reference standards were substituted into the formula for the determination of K. The various K values for the analytes with the surrogate samples were determined.

4.1.8 DETERMINATION OF PERCENTAGE CONTENT

In method development pure reference samples are used to develop the conditions and then the conditions are employed for the analysis of the actual formulation. Formulations normally have excipients in addition to the active ingredients like diazepam in the case of diazepam tablets. Diazepam tablets have lactose, corn starch, pregelatinized starch and calcium stearate, methyl and propyl parabens with particular dyes depending on the strength [23]. Some of these excipients also absorb UV light therefore interfering with the established conditions with the pure samples. A good analytical method developed should exclude interferences from the excipients used in the formulation of interest. According to the British Pharmacopoeia, the content of diazepam in the tablet should be between 92.5 to 107.5% while the content of Indometacin in the capsules should be between 90% and 110%. A look at the table of results for the K estimated contents, (Refer to pages 86 – 93 for tables) reveals that all the estimated percentage contents fell within the limits of the British Pharmacopoeia.

Pages 93 and 94, Table C. 1 and Table C. 2 also compare the percentage content obtained with the BP standard method of assay and the developed method of assay for Indometacin and Diazepam respectively.

4.1.9 STATISTICAL ANALYSIS

4.1.9.1 T-Test

This is a significance test that compares experimental means with known values. T test checks the truth about the null hypothesis which says that there is no difference between the values of the developed method and that of the standard method other than that which can be attributed to random variation. For 8 degrees of freedom, the critical value of 't' at the 99% probability is 3.36. The estimated critical value should always be less than the tabulated critical value for the null hypothesis to be true.

The t_{exp}(T calculated) for Indometacin IL were 1.116, 1.003 and 0.9301 for Naproxen, Benzoic acid and Diazepam surrogate reference standards respectively when compared to the standard method. The t_{exp} for Indometacin IM were 0.7756, 2.593 and 2.013 for Benzoic acid, Naproxen and Diazepam surrogate reference standards respectively. The t_{exp} for Indometacin IE were 2.883, 3.476 and 0.7239 for Naproxen, Benzoic acid and Diazepam surrogate reference standards upon comparing with the standard method of assay. Inferring from the values obtained the null hypothesis is retained showing no evidence of systematic error except in the case of IE with benzoic acid as surrogate reference.

The t_{exp} for diazepam ED were 2.334, 9.792 and 6.73 for Metronidazole, Indometacin and Piroxicam reference standards respectively when compared with the standard method of assay for Diazepam. The t_{exp} for GD were 1.116, 1.104 and 0.655 for Metronidazole, Indometacin and Piroxicam surrogate reference standards respectively. The t_{exp} for PD were 9.867, 8.617 and 0.8232 for Piroxicam, Metronidazole and Indometacin surrogate reference standards respectively. Although ED with Indometacin surrogate standard, ED with Piroxicam standard, PD with Piroxicam and Metronidazole surrogate standards gave percentage yields within BP specifications, their null hypothesis is rejected since their t_{exp} are greater than the critical value. Their means are said to be different from the mean of the standard method.

4.1.9.2 Specificity

Based on ICH guidelines, an investigation of specificity should be conducted during the validation of identification tests, the determination of impurities, and the assay. The procedure was shown to be specific for the analyte showing distinctly the component of interest from excipients with no interference. (Refer to fig.CD.6 page 53)

4.1.9.3 Linearity

A linear relationship should be evaluated across a range of concentrations. The coefficient of regression (r^2) should be 0.995 to 1.0. [26] From the tables of linearity, (refer to tables L1-L7, pages 96-97) all the regression coefficients fell within the ICH stipulated range. The procedure was therefore linear for all the samples.

4.1.9.4 Precision (Repeatability)

The relative standard deviations (RSD) of all samples were calculated for their reproducibility and repeatability. The ICH guidelines state that, the RSD should not be more than 2% [28]. RSD of all samples was below 2%. Refer to pages 94-95 for table of results

4.1.9.5 Limit of Detection and Quantitation

The LOD and LOQ were calculated for all the drug samples worked with. (Refer to tables LOD.1 and LOD.2 page 82 for computed limits)

4.2 CONCLUSIONS

A mobile phase system of pH 5.8 ± 0.02 phosphate buffer and methanol (40: 60) was found to resolve the peaks of indometacin and its surrogate reference standards. For the K values

associated with indometacin, naproxen gave a value of 1.6735±0.021; benzoic acid gave a value of 3.426±0.073 and diazepam gave 3.0955±0.19

A mobile phase system of pH 5.80 ± 0.02 phosphate buffer and methanol (25: 75) was found to effectively resolve the peaks of diazepam and its reference standards. Piroxicam gave a K value of 0.20418±0.0015; metronidazole gave 0.135271±0.009 and indometacin gave 0.3230±0.018.

The results presented in the study suggest that the surrogate reference standards that were used in this research can be used for the analysis of Indometacin and diazepam without the use of their pure reference standards. This method is simple, fast and cheaper than the existing methods. The developed method can therefore effectively replace the standard method.

4.3 RECOMMENDATION

Surrogate reference standards must be found for other drugs to ease the burden on our academic institutions searching for pure reference standards for analysis.

Some work should also be done on the use of surrogates for quantitation of plant extracts since the quest for pure samples for analysis can be tedious.

APPENDIX

2.3 Preparation of solutions

2.3.1. Preparation of 0.1 M NaOH

Molecular mass = 40g/mol Assay = 98%

40g of NaOH in 1000ml ≡ 1M NaOH

4g of NaOH in 1000ml ≡ 0.1M NaOH

0.4g of NaOH in 100ml ≡ 0.1M NaOH

From assay 98% ≡ 0.4g

100% ≡ (0.4g / 98) × 100 = **0.4081g** of NaOH pellets

Analar sodium hydroxide (0.4081g) was weighed into a beaker. Some amount of distilled water was added and stirred to ensure complete dissolution. The solution was allowed to stand to cool. It was then transferred quantitatively into a 100ml volumetric flask using a cleaned and dried funnel. The solution was shaken to ensure proper mixing and then topped up to the mark to produce a 100ml of 0.1M sodium hydroxide.

2.3.2. Preparation of 0.2M Sodium hydroxide (NaOH)

0.4g of NaOH in 100ml ≡ 0.1M NaOH

0.4g of NaOH in 50ml ≡ 0.2M NaOH

From assay 98% ≡ 0.4g

100% ≡ (0.4g / 98) × 100 = **0.4081g** of NaOH pellets

Sodium hydroxide (0.40816g) was accurately weighed into a beaker. About 20ml of water was added to dissolve it with stirring. The solution was allowed to cool and transferred

quantitatively into a 50ml volumetric flask. More water was added to the mark to obtain 0.2M NaOH solution.

2.3.3. Preparation of Sulphamic acid

$H_2NSO_3H + NaOH \longrightarrow H_2NSO_3Na + H_2O$

Mole ratio 1 : 1

$\therefore$ 97.09g of H_2NSO_3H in 1000ml ≡ 1M NaOH

0.9709g of H_2NSO_3H in 100ml ≡ 0.1M NaOH

0.009709g of H_2NSO_3H in 1ml ≡ 0.1M NaOH

Assay of H_2NSO_3H = 99%

$\therefore$ (0.9709/99) × 100 = **0.9807g** of H_2NSO_3H

Sulphamic acid (0.9807g) was weighed and dissolved in about 50ml of water in a beaker. It was transferred quantitatively into a clean dried 100 ml volumetric flask using a funnel. It was then topped up to the mark and shaken to ensure uniform dissolution.

2.3.4. Preparation of 0.1M Perchloric Acid

Glacial acetic acid (450 ml) was measured and transferred into a 500ml volumetric flask. Perchloric acid (5.1 ml of 70%) was added gently with continuous swirling. 15 ml of acetic anhydride was added slowly with continuous and efficient mixing to prevent the formation of acetyl perchlorate. The volume was adjusted to 500ml with glacial acetic acid. The solution was labeled and allowed to stand for 24 hrs before it was used.

3.1.2 Preparation of 1M Hydrochloric acid (HCl)

Molecular mass = 36.5g/mol Assay = 36% Specific gravity=1.18g/ml

36.5g of HCl in 1000ml ≡ 1M HCl

3.65g of HCl in 100ml ≡ 1M HCl

From assay 36% ≡ 3.65g

100% ≡ (3.65g/36) × 100 = 10.1389 g of HCl

Volume= mass/gravity

= 10.1389/1.18 = **8.6ml** of HCl

HCl (8.6ml) was measured into a beaker containing about 40ml of disilled water. The solution was allowed to cool and transferred quantitatively into a cleaned and dried 100ml volumetric flask. It was then topped up to the mark, stoppered and shaken to ensure uniformity of the solution.

Table of results

Titration tables

Table T.T.1 Standardization of NaOH with H_2NSO_3H

Burrete reading(ml)	1st determination	2nd determination	3rd determination
Final reading	26.00	25.90	26.00
Initial reading	0.00	0.00	0.00
Titre value	26.00	25.90	26.00

Average titre = (26.00+26.00)/2

= 26.00ml

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

Burrete reading(ml)	1st determination	2nd determinaation	Blank determination
Final reading	26.70	35.20	0.10
Initial reading	1.50	10.00	0.00

Titre value	25.20	25.20	0.10

Average titre = (25.2+25.2)/2 =25.2

Titre =25.2-blank = 25.10ml

Table T.T.3 Assay of Benzoic acid

Burrete reading(ml)	1st determination (0.2019g)	2nd determination (0.2041g)
Final reading	21.40	38.70
Initial reading	4.20	21.4
Titre value	17.20	17.30

Table T.T.4 Assay of Naproxen

Burrete reading(ml)	1st determination (0.2090g)	2nd determination (0.2039)
Final reading	9.40	18.60
Initial reading	0.10	9.50
Titre value	9.30	9.10

Table T.T.5 Assay of Metronidazole

Burrete reading(ml)	1st det.(0.1504g)	2nd det. (0.1503g)	Blank determination
Final reading	9.30	18.60	0.50
Initial reading	0.00	9.30	0.00
Titre value	9.30	9.30	0.50

Table T.T.6 Assay of Indometacin

Burrete reading(ml)	1st det.(0.3045g)	2nd det. (0.3030g)	Blank determination
Final reading	9.60	19.50	0.80
Initial reading	0.00	10.00	0.00
Titre value	9.60	9.50	0.80

Table T.T.7 Assay of Piroxicam

Burrete reading(ml)	1st det.(0.2500g)	2nd det. (0.2505g)	Blank determination
Final reading	8.00	16.00	0.50
Initial reading	0.00	8.00	0.00
Titre value	800	8.00	0.50

Table T.T.8 Assay of Diazepam

Burrete reading(ml)	1st det.(0.2501g)	2nd det. (0.2494g)	Blank determination
Final reading	9.30	18.60	0.50
Initial reading	0.00	9.30	0.00
Titre value	9.30	9.30	0.50

Uniformity of weight

Table UW.1 **Diazepam GD(5mg)**

Weight of 20 tablets = 3.4125g			
Average wt/tablet = 0.1706g			
Number	Individual wt(g)	Deviation(g)	% Deviation
1	0.1583	-0.0123	-7.2098

2	0.1684	-0.0022	-1.2895
3	0.1728	0.0022	1.2895
4	0.1687	-0.0019	-1.1137
5	0.1759	0.0053	3.1066
6	0.1593	-0.0113	-6.6236
7	0.1767	0.0061	3.5756
8	0.1867	0.0161	9.4372
9	0.1692	-0.0014	-0.8206
10	0.1602	-0.0104	-6.0961
11	0.1749	0.0043	2.5205
12	0.1710	0.0004	0.2344
13	0.1495	-0.0211	-12.3681
14	0.1875	0.0169	9.9062
15	0.1751	0.0045	2.6377
16	0.1637	-0.0069	-4.0445
17	0.1807	0.0101	5.9202
18	0.1595	-0.0111	-6.5064
19	0.1823	0.0117	6.8581
20	0.1720	0.0014	0.8206

Table UW.2 **Diazepam PD(5mg)**

Weight of 20 tablets = 3.8015g			
Average wt/tablet = 0.1901g			
Number	Individual wt(g)	Deviation	%Deviation
1	0.1880	-0.0021	-1.1047
2	0.1947	0.0046	2.4197
3	0.1920	0.0019	0.9995

4	0.1884	-0.0017	-0.8943
5	0.1851	-0.0050	-2.6301
6	0.1860	-0.0041	-2.1567
7	0.1938	0.0037	1.9463
8	0.1885	-0.0016	-0.8416
9	0.1868	-0.0033	-1.7359
10	0.1858	-0.0043	-2.2619
11	0.1944	0.0043	2.2619
12	0.1903	0.0002	0.1052
13	0.1921	0.0020	1.0521
14	0.1960	0.0059	3.1036
15	0.1886	-0.0015	-0.7890
16	0.1883	-0.0018	-0.9468
17	0.1930	0.0029	1.5255
18	0.1919	0.0018	0.9468
19	0.1951	0.0050	2.6301
20	0.1875	-0.0026	-1.3677

Table UW.3 **Diazepam ED(10mg)**

Weight of 20 tablets = 3.8918g			
Average wt/tablet = 0.1946g			
Number	Individual wt(g)	Deviation	%Deviation
1	0.1881	-0.0065	-3.3401
2	0.1916	-0.0030	-1.5416
3	0.1961	0.0015	0.7708
4	0.1989	0.0043	2.2096
5	0.1895	-0.0051	-2.6207

6	0.1949	0.0003	0.1542
7	0.1935	-0.0011	-0.5652
8	0.1943	-0.0003	-0.1542
9	0.1989	0.0043	2.2096
10	0.2043	0.0097	4.9845
11	0.1983	0.0037	1.9013
12	0.1904	-0.0042	-2.1582
13	0.2006	0.0060	3.0832
14	0.1956	0.0010	0.5138
15	0.1969	0.0023	1.1819
16	0.1865	-0.0081	-4.1624
17	0.1968	0.0022	1.1305
18	0.1909	-0.0037	-1.9013
19	0.1976	0.0030	1.5416
20	0.1922	-0.0024	-1.2333

Table UW.4 **Indometacin IL(25mg)**

Total weight of powder in 20 capsules = 2.0054g					
Average wt of powder/capsule = 0.10027					
Number	Wt of capsule(g)	Wt of empty shell(g)	Wt of powder(g)	Deviation(g)	%Deviation
1	0.1251	0.0409	0.0842	-0.0161	-16.0518
2	0.1279	0.0406	0.0873	-0.0129	-12.8614
3	0.1329	0.0434	0.0895	-0.0107	-10.6679
4	0.1418	0.0433	0.0985	-0.0017	-1.6949
5	0.1361	0.0384	0.0977	-0.0026	-2.5922
6	0.1408	0.0417	0.0991	-0.0012	-1.1941

7	0.1394	0.0414	0.0980	-0.0023	-2.2931
8	0.1423	0.0413	0.1010	0.0007	0.6979
9	0.1631	0.0425	0.1206	0.0203	20.2392
10	0.1377	0.0400	0.0977	-0.0026	-2.5922
11	0.1566	0.0419	0.1147	0.0144	14.3569
12	0.1509	0.0411	0.1098	0.0095	9.4715
13	0.1403	0.0443	0.0960	-0.0043	-4.2871
14	0.1323	0.0417	0.0906	-0.0097	-9.6709
15	0.1533	0.0429	0.1104	0.0101	10.0697
16	0.1334	0.0437	0.0897	-0.0106	-10.5682
17	0.1603	0.0462	0.1141	0.0138	13.7587
18	0.1522	0.0429	0.1093	0.0090	8.9730
19	0.1373	0.0366	0.1007	0.0004	0.3988
20	0.1406	0.0441	0.0965	-0.0038	-3.7886

Table UW. 5 **Indometacin IE(25mg)**

Total weight of powder in 20 capsules = 3.6489g					
Average wt of powder/capsule = 0.1824g					
Number	Wt of capsule(g)	Wt of empty shell(g)	Wt of powder(g)	Deviation(g)	%Deviation
1	0.2232	0.0394	0.1838	0.0014	0.7675
2	0.2203	0.0402	0.1801	-0.0023	-1.2609
3	0.2263	0.0403	0.1860	0.0036	1.9736
4	0.2197	0.0391	0.1806	-0.0018	-0.9868
5	0.2194	0.0390	0.1804	-0.0020	-1.0965
6	0.2205	0.0397	0.1808	-0.0016	-0.8772
7	0.2222	0.0408	0.1814	-0.0010	-0.5482

8	0.2263	0.0386	0.1877	0.0053	2.9057
9	0.2254	0.0399	0.1855	0.0031	1.6995
10	0.2230	0.0395	0.1835	0.0011	0.6031
11	0.2175	0.0392	0.1783	-0.0041	-2.2478
12	0.2277	0.0403	0.1874	0.0050	2.7412
13	0.2131	0.0402	0.1731	-0.0093	-5.0986
14	0.2222	0.0391	0.1831	0.0007	0.3838
15	0.2127	0.0402	0.1725	-0.0099	-5.4276
16	0.2263	0.0398	0.1865	0.0041	2.2478
17	0.2228	0.0395	0.1833	0.0009	0.4934
18	0.2267	0.0409	0.1858	0.0034	1.8640
19	0.2245	0.0405	0.1840	0.0016	0.8772
20	0.2244	0.0393	0.1851	0.0027	1.4802

Table UW.6 **Indometacin IM(25mg)**

Total weight of powder in 20 capsules = 3.2035g					
Average wt of powder/capsule = 0.1602g					
Number	Wt of capsule(g)	Wt of empty shell(g)	Wt of powder(g)	Deviation(g)	%Deviation
1	0.2054	0.0499	0.1555	-0.0047	-2.9338
2	0.2095	0.0480	0.1615	0.0013	0.8114
3	0.2092	0.0478	0.1614	0.0012	0.7491
4	0.2000	0.0478	0.1522	-0.0080	-4.9937
5	0.2088	0.0492	0.1596	-0.0006	-0.3745
6	0.2166	0.0490	0.1676	0.0074	4.6192
7	0.2106	0.0489	0.1617	0.0015	0.9363
8	0.2112	0.0499	0.1613	0.0011	0.6866

9	0.2145	0.0508	0.1637	0.0035	2.1847
10	0.2048	0.0488	0.1560	-0.0042	-2.6217
11	0.2126	0.0487	0.1639	0.0037	2.3096
12	0.2074	0.0487	0.1587	-0.0015	0.9363
13	0.2172	0.0491	0.1681	0.0079	4.9313
14	0.2082	0.0493	0.1589	-0.0013	-0.8114
15	0.2073	0.0498	0.1573	-0.0029	-1.8102
16	0.2032	0.0487	0.1545	-0.0057	3.5580
17	0.2096	0.0499	0.1597	-0.0005	-0.3121
18	0.2083	0.0479	0.1604	0.0002	0.1248
19	0.2082	0.0493	0.1589	-0.0013	-0.8115
20	0.2121	0.0495	0.1626	0.0024	1.4981

Table LOD.1 Limit of detection and quantitation of pure samples at 300nm

Sample	LOD(%w/v)	LOQ(%w/v)
Piroxicam	0.0000247424	0.0000749771
Indometacin	0.0000182782	0.0000553886
Diazepam	0.0000677529	0.000205312
Metronidazole	0.0000166095	0.000050332

Table LOD.2 Limit of detection and quantitation of pure samples at 254nm

Sample	LOD(%w/v)	LOQ(%w/v)
Indometacin	0.0000373776	0.000113265
Naproxen	0.000029306	0.0000888059
Benzoic acid	0.000074559	0.000225936

Calibration curves

Fig CC.1 Diazepam at 300nm

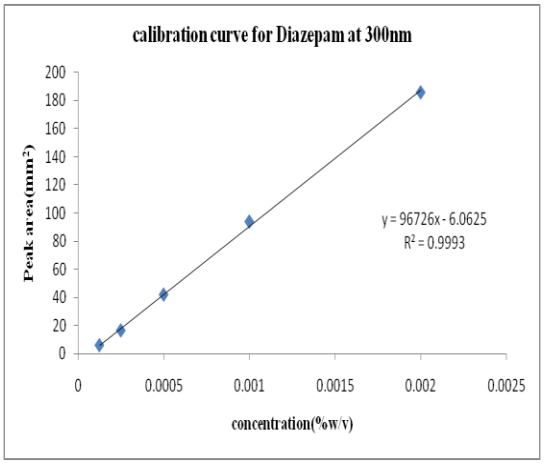


Fig CC.2 Piroxicam at 300nm

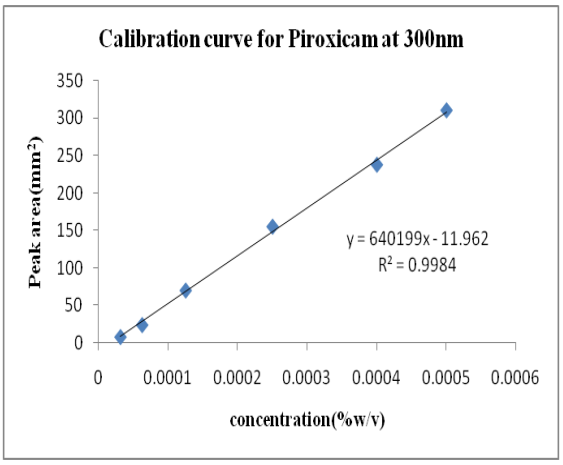


Fig CC.3 Metronidazole at 300nm

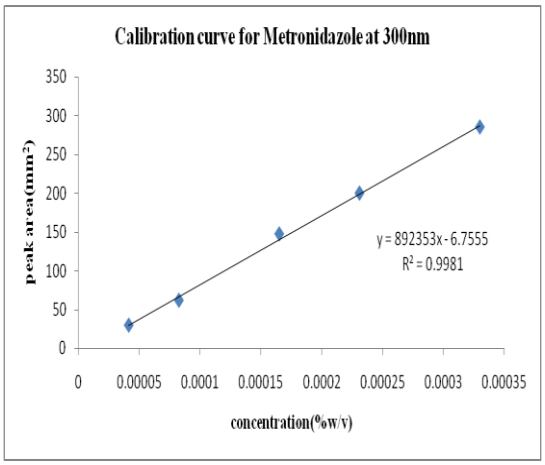


Fig CC.4 Indometacin at 300nm

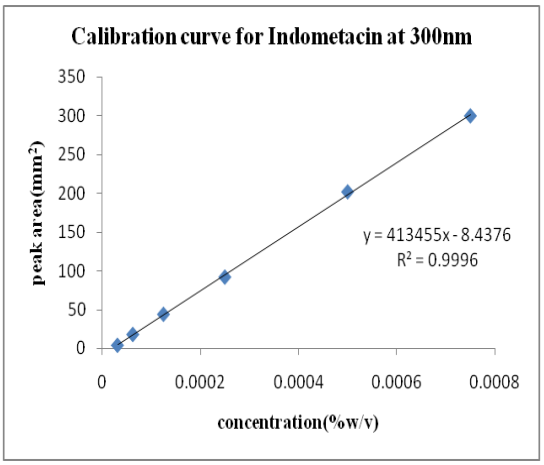


Fig. CC.5 Benzoic Acid at 254nm

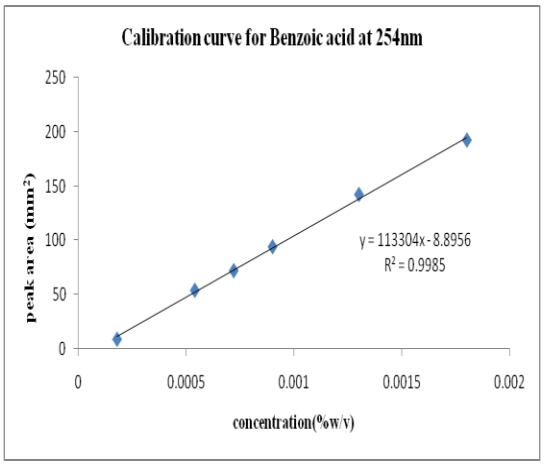


Fig. CC.6 Naproxen at 254nm

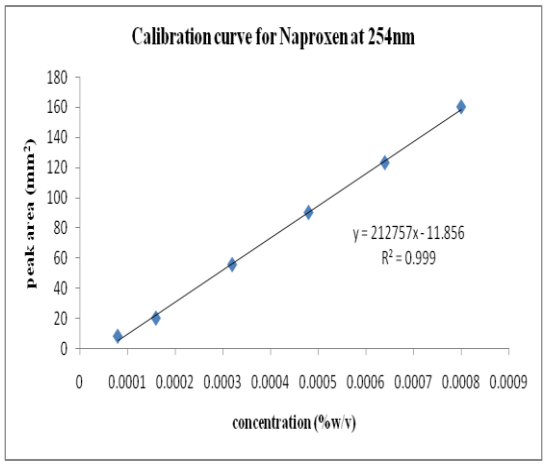
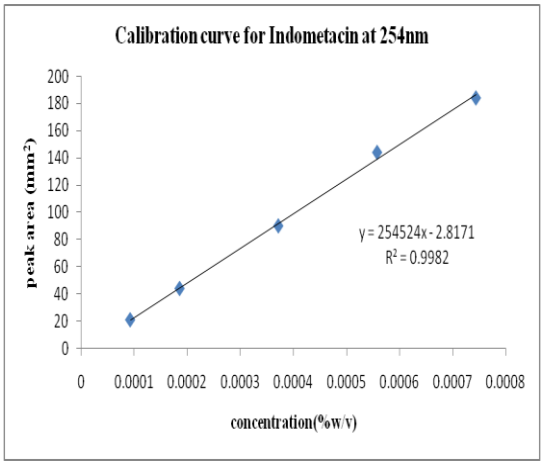


Fig. CC. 7 Indometacin at 254nm



Determination of the constant K values for Diazepam as analyte

Table K.1. *K values using Metronidazole as surrogate reference standard at 300nm*

Conc. 0f Metro (%w/v)	Peak area of Metro (mm^2)	Conc. of diazepam(%w/v)	Peak area of diazepam(mm^2)	K value
0.000205	74.0	0.0004	19.00	0.131588

0.000205	75.0	0.0004	19.00	0.129833
0.000205	74.0	0.0004	22.00	0.152336
0.000205	74.0	0.0012	56.25	0.129856
0.000205	74.0	0.0012	57.50	0.132742

Average K = 0.135271

Table K.2 *K values using Indometacin as surrogate reference standard at 300nm*

Conc. Indometacin (%w/v)	Peak area of Indometacin (mm^2)	Conc. of diazepam(%w/v)	Peak area of diazepam(mm^2)	K value
0.0005	144	0.001	94.00	0.3264
0.0005	142	0.002	194.00	0.3415
0.0005	166	0.002	218.25	0.3287
0.0005	166	0.002	194.00	0.2922
0.0003	87	0.001	96.00	0.3310

Average K = 0.3230

Table K .3 *K values using Piroxicam as surrogate reference standard at 300nm*

Conc. 0f Piroxicam (%w/v)	Peak area of Piroxicam (mm^2)	Conc. of diazepam(%w/v)	Peak area of diazepam(mm^2)	K value
0.0002	96	0.001	98	0.20417
0.0002	96	0.001	98	0.20417
0.0002	98	0.002	196	0.20417
0.0004	190	0.002	196	0.20631
0.0004	190	0.001	94	0.20211

Average K = 0.20418

Determination of the constant K values for Indometacin as analyte

Table K.4 *K values using Naproxen as surrogate reference standard at 254nm*

Conc. of Naproxen. (%w/v)	Peak area of Naproxen (mm^2)	Conc. of Indometacin(%w/v)	Peak area of Indometacin(mm^2)	K value
0.00032	38	0.000372	75.0	1.6978
0.00032	38	0.000372	73.5	1.6638
0.00048	56	0.000372	72.0	1.6590

Average K = 1.6735

Table K.5. *K values using Benzoic acid as surrogate reference standard at 254nm*

Conc. 0f Metro (%w/v)	Peak area of Metro (mm^2)	Conc. of Indometacin(%w/v)	Peak area of Indometacin(mm^2)	K value
0.0009	52	0.000372	75	3.4894
0.0009	54	0.000372	75	3.3602
0.0009	52	0.000558	108	3.3499
0.0009	51	0.000558	108	3.4156
0.0009	53	0.000186	38.5	3.5149

Average K = 3.4260

Table K.6 *K values using Diazepam as surrogate reference standard at 300nm*

Conc. of Diazepam (%w/v)	Peak area of Diazepam (mm^2)	Conc. of Indometacin(%w/v)	Peak area of Indometacin(mm^2)	K value
0.001	94	0.0005	144	3.0638
0.002	194	0.0005	142	2.9278
0.002	218.25	0.0005	166	3.0424
0.002	194	0.0005	166	3.4227
0.001	96	0.0003	87	3.0208

Average K = 3.0955

Determination of Percentage content of Diazepam in Diazepam tablets using the 'K'

Table PC.1. *% content of diazepam ED using **Metronidazole** as surrogate*

K = 0.135271					
Concentration of ED tablet(Ca = 0.00104%w/v); corresponding peak Area(Aa = 50mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	Percentage
0.000205	74.0	0.01025	10.01005	0.001024	**98.12**
0.000205	75.0	0.01025	10.14532	0.001012	**97.15**
0.000165	60.0	0.00825	8.11626	0.001017	**97.74**
0.000165	60.0	0.00825	8.11626	0.001017	**97.74**
0.000205	74.0	0.01025	10.01005	0.001024	**98.12**

***Cs** is the concentration of surrogate and **As** is the area of peak corresponding to concentration of surrogate*

Table PC.2 *% content of diazepam GD using **Metronidazole** as surrogate*

K = 0.135271					
Concentration of GD tablet(Ca = 0.00145%w/v); corresponding peak Area(Aa = 67.5mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	% Content
0.000205	74.0	0.01384	10.01005	0.001383	**95.38**
0.000205	75.0	0.01384	10.14532	0.001364	**94.07**
0.000165	60	0.01114	8.11625	0.001373	**94.69**
0.000165	60	0.01114	8.11625	0.001373	**94.69**
0.000205	74.0	0.01384	10.01005	0.001383	**95.38**

Table PC.3 *% content of diazepam PD using **Metronidazole** as surrogate*

K = 0.135271					
Concentration of PD tablet(Ca = 0.002709%w/v); corresponding peak Area(Aa = 141.25mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	%Content
0.000205	76.0	0.02895	10.2828	0.002815	**103.91**
0.000205	75.0	0.02895	10.1475	0.002853	**105.31**

0.000165	62	0.02330	8.3886	0.002777	**102.51**
0.000165	62	0.01918	8.3886	0.002286	**105.49**[¥]
0.000165	62	0.02330	8.3886	0.002777	**102.51**

[¥] *Aa=116.25 Ca=0.002167*

Table PC.4 *% content of diazepam ED using* ***Indometacin*** *as surrogate*

K = 0.3230					
Concentration of ED tablet(Ca = 0.001%w/v); corresponding peak Area(Aa = 52mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	%Content
0.0003	51	0.0156	16.47	0.000947	**94.72**
0.0003	51.5	0.0156	16.63	0.000938	**93.80**
0.0003	52	0.0156	16.79	0.000929	**92.91**
0.0003	53	0.0156	17.11	0.000912	**91.20**
0.0003	52	0.0156	16.79	0.000929	**92.91**

Table PC.5 *% content of diazepam GD using* ***Indometacin*** *as surrogate*

K = 0.3230					
Concentration of GD tablet(Ca = 0.001465%w/v); corresponding peak Area(Aa = 81 mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	% Content
0.0003	54	0.0243	17.442	0.001393	**95.09**
0.0003	53	0.0243	17.119	0.001419	**96.89**
0.0003	54	0.0477	17.442	0.002735	**93.33**[¥]
0.0003	53	0.0477	17.119	0.002786	**95.09**
0.0003	54	0.0243	17.442	0.001393	**95.09**

[¥] *Aa=159 Ca=0.00293*

Table PC.6 *% content of diazepam PD using* ***Indometacin*** *as surrogate*

K = 0.3230					
Concentration of PD tablet(Ca = 0.002167%w/v); corresponding peak Area(Aa = 117.5mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	%Content
0.0003	51	0.03525	16.473	0.002139	**98.75**
0.0003	52	0.03525	16.796	0.002098	**96.85**
0.0003	50	0.03525	16.150	0.002647	**97.71**[¥]
0.0003	51	0.03525	16.473	0.002595	**95.80**[¥]
0.0003	51	0.03525	16.473	0.002139	**98.75**

[¥]*Aa=142.5mm^2 Ca=0.002709%*

Table PC.7 *% content of diazepam ED using* ***Piroxicam*** *as surrogate*

K = 0.20418					
Concentration of ED tablet(Ca = 0.00104%w/v); corresponding peak Area(Aa =52 mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	% Content
0.0004	102	0.0208	20.8263	0.0009987	**96.03**
0.0004	103	0.0208	21.0305	0.0009890	**95.10**
0.0004	104	0.0208	21.2347	0.0009795	**94.19**
0.0004	102	0.0208	20.8263	0.0009987	**96.03**
0.0004	101	0.0208	20.6222	0.0010086	**96.98**

Table PC.8 *% content of diazepam GD using* ***Piroxicam*** *as surrogate*

K = 0.20418					
Concentration of GD tablet(Ca = 0.00293%w/v); corresponding peak Area(Aa = 159mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	%Content
0.0004	109	0.0636	22.2556	0.002857	**97.53**

0.0004	111	0.0636	22.6640	0.002806	**95.77**
0.0004	101	0.0278	20.6221	0.001348	**92.97**¥
0.0004	101	0.0278	20.6221	0.001348	**92.97**¥
0.0004	111	0.0636	22.6640	0.002806	**95.77**

¥ *Aa=69.5mm2 Ca=0.00145%*

Table PC.9 *% content of diazepam PD using* ***Piroxicam*** *as surrogate*

K = 0.20418					
Concentration of PD tablet(Ca = 0.002167%w/v); corresponding peak Area(Aa = 116.25mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	% Content
0.0004	102	0.0465	20.8263	0.00223	**102.91**
0.0004	103	0.0465	21.0305	0.00221	**102.03**
0.0004	101	0.0465	20.6222	0.00225	**104.05**
0.0004	101	0.0465	20.6222	0.00225	**104.05**
0.0004	103	0.0465	21.0305	0.00221	**102.03**

Table PC.10 *% content of Indometacin IL using* ***Naproxen*** *as surrogate*

K = 1.6735					
Concentration of IL capsules (Ca = 0.000678%w/v); corresponding peak Area(Aa =148.75 mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	%Content
0.00032	41	0.0476	68.614	0.000693	**102.32**
0.00032	43	0.0476	71.9605	0.0006615	**97.56**
0.00032	42	0.0476	70.287	0.000677	**99.88**
0.00016	20	0.0112	33.47	0.0003346	**98.70**
0.00032	43	0.0476	71.9605	0.0006615	**97.56**

Table PC.11 *% content of Indometacin IE using* ***Naproxen*** *as surrogate*

K = 1.6735					
Concentration of IE capsules(Ca = 0.0005098%w/v); corresponding peak Area(Aa = 109.5mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	% Content
0.00032	42	0.03504	70.2870	0.0004985	**97.78**
0.00032	43	0.03504	71.9605	0.0004869	**95.51**
0.00032	43	0.03504	71.9605	0.0004869	**95.51**
0.00016	22	0.01752	36.817	0.0004758	**93.34**
0.00016	22	0.01752	36.817	0.0004758	**93.34**

Table PC.12 *% content of Indometacin IM using* ***Naproxen*** *as surrogate*

K = 1.6735					
Concentration of IM capsules(Ca = 0.0006616%w/v); corresponding peak Area(Aa = 141mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	%Content
0.00032	42	0.0451	70.287	0.0006417	**96.99**
0.00032	41	0.0451	68.614	0.0006573	**99.35**
0.00016	21	0.0225	35.143	0.0006419	**97.03**
0.00016	22	0.0225	35.980	0.0006253	**94.52**
0.00032	42	0.0451	70.287	0.0006417	**96.99**

Table PC.13 *% content of Indometacin IL using* ***Benzoic acid*** *as surrogate*

K = 3.4260					
Concentration of IL capsules(Ca = 0.000678%w/v); corresponding peak Area(Aa =148.75 mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	%Content
0.0009	56	0.1338	191.860	0.00069	**101.77**
0.0009	58	0.1338	198.708	0.000673	**99.31**
0.00063	39	0.0441	133.614	0.00033	**97.36**[¥]

0.00063	39	0.0441	133.614	0.00033	**97.36**¥
0.0009	58	0.1338	198.708	0.000673	**99.31**

¥ *Aa = 70mm^2 Ca = 0.000339%*

Table PC.14 *% content of Indometacin IE using* ***Benzoic acid*** *as surrogate*

K = 3.4260					
Concentration of IE capsules(Ca = 0.0005098%w/v); corresponding peak Area(Aa =108.75 mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	%Content
0.0009	59	0.09787	202.134	0.000484	**94.97**
0.0009	58	0.09787	198.700	0.000492	**96.61**
0.0009	58	0.09585	198.700	0.000482	**94.62**¥
0.0009	59	0.09585	196.995	0.000474	**93.01**¥
0.0009	59	0.09787	202.134	0.000484	**94.97**

¥ *Aa = 106.5 %*

Table PC.15 *% content of Indometacin IM using* ***Benzoic acid*** *as surrogate*

K = 3.4260					
Concentration of IM(Ca = 0.0006616%w/v); corresponding peak Area(Aa =136.5 mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	%Content
0.0009	55	0.12285	188.43	0.000652	**98.55**
0.0009	56	0.12285	193.76	0.000634	**95.83**
0.00063	38	0.0860	130.188	0.000660	**99.84**
0.00063	38	0.0860	130.188	0.000660	**99.84**
0.0009	55	0.12285	188.43	0.000652	**98.55**

Table PC.16 *% content of Indometacin IL using* ***diazepam*** *as surrogate*

K = 3.0955					
Concentration of IL capsules(Ca = 0.0005%w/v); corresponding peak Area(Aa = 124.25mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	% Content
0.0012	96	0.1491	297.168	0.000501	100.34
0.0012	97	0.1491	200.264	0.000496	99.31
0.0004	22.5	0.0168	69.648	0.000241	96.48¥
0.0004	22	0.0168	68.101	0.000247	98.68¥
0.0012	97	0.1491	200.264	0.000496	99.31

¥ *Aa =42mm2; Ca= 0.00025%*

Table PC.17 *% content of Indometacin IE using* ***diazepam*** *as surrogate*

K = 3.0955					
Concentration of IE capsules(Ca = 0.0005756%w/v); corresponding peak Area(Aa = 151.5mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	% Content
0.0014	130	0.2121	402.415	0.000527	**91.56**
0.0014	128	0.2121	396.224	0.000535	**93.00**
0.0007	36	0.0609	111.438	0.000547	**95.03**¥
0.0007	37	0.0609	114.533	0.000532	**92.37**¥
0.0014	128	0.2121	396.224	0.000535	**93.00**

¥ *Aa=87mm^2 ; Ca=0.0002818%*

Table PC.18 *% content of Indometacin IM using* ***diazepam*** *as surrogate*

K = 3.0955					
Concentration of IM capsules(Ca = 0.0004215%w/v); corresponding peak Area(Aa =117.5 mm^2)					
Cs	As	Aa * Cs	K* As	Aa*Cs/ K*As	% Content
0.0014	128	0.1645	396.224	0.000414	**98.29**
0.0014	127	0.1645	393.1285	0.000418	**99.27**

0.0004	21	0.0268	65.0055	0.000412	**97.81**
0.0014	128	0.1645	396.224	0.000414	**98.29**
0.0014	130	0.1645	402.415	0.000409	**97.03**

Comparing the %contents of drugs obtained from standard method and developed methods

Table C.1 %content of Indometacin for both methods

STANDARD METHOD			DEVELOPED METHOD					
Brand	Average %content	S.D	Naproxen		Benzoic acid		Diazepam	
			Average %content	S.D	Average %content	S.D	Average %content	S.D
IL	**98.04**	0.21	**99.20**	1.98	**99.02**	1.81	**99.65**	0.59
IE	**92.51**	0.77	**95.09**	1.85	**94.89**	1.47	**92.99**	1.28
IM	**99.15**	0.77	**96.98**	1.70	**98.52**	1.63	**98.14**	0.81

Table C.2 %content of Diazepam for both methods

STANDARD METHOD			DEVELOPED METHOD					
Brand	Average %content	S.D	Metronidazole		Indometacin		Piroxicam	
			Average %content	S.D	Average %content	S.D	Average %content	S.D
GD	**94.38**	0.74	**94.84**	0.55	**95.10**	1.25	**95.00**	1.98
PD	**98.07**	0.48	**103.94**	1.44	**97.57**	1.27	**103.01**	1.01
ED	**99.05**	0.38	**98.10**	0.82	**93.11**	1.30	**95.67**	1.05

Relative standard deviations (RSD)

Table RSD.1 Relative standard deviation of pure diazepam

Concentration (% w/v)	Number of Injections	S.D	RSD (%)
0.0004	5	0.273861	1.26
0.0005	5	0.83666	1.98
0.0012	5	0.83666	0.90

Table RSD.2 Relative standard deviation of pure Indometacin

Concentration (% w/v)	Number of Injections	S.D	RSD (%)
0.00025	5	1.254990	1.98
0.0003	5	0.836660	1.55
0.0006	5	1.677051	0.88

Table RSD.3 Relative standard deviation of pure Metronidazole

Concentration (% w/v)	Number of Injections	S.D	RSD (%)
0.0000825	5	1.045825	1.57
0.000165	5	1.25	0.84
0.000231	5	1.045825	0.53

Table RSD.4 Relative standard deviation of pure benzoic acid

Concentration (% w/v)	Number of Injections	S.D	RSD (%)
0.00054	5	0.41833	1.58
0.00072	5	0.67082	1.95
0.0009	5	0.83666	1.56

Table RSD.5 Relative standard deviation of pure naproxen

Concentration (% w/v)	Number of Injections	S.D	RSD (%)
0.00032	5	0.74162	1.81
0.00048	5	1	1.78
0.00064	5	1.25499	1.01

Table RSD.6 Relative standard deviation of pure piroxicam

Concentration (% w/v)	Number of Injections	S.D	RSD (%)
0.00025	5	1.25	0.8
0.0004	5	1.25	0.53
0.0005	5	1.045825	0.36

Table L.1

LINEARITY OF INDOMETACIN; Concentration (%w/v) range = 0.00075-0.00003125	
Equation of Line	**Correlation coefficient, R^2**
Y=413455x-8.4376	0.9996

Table L.2

LINEARITY OF DIAZEPAM; Concentration (%w/v) range = 0.002-0.000125	
Equation of Line	**Correlation coefficient, R^2**
Y=96726x-6.0625	0.9993

Table L.3

LINEARITY OF PIROXICAM; Concentration (%w/v) range = 0.0005-0.00003125	
Equation of Line	**Correlation coefficient, R^2**
Y=640199x-11.962	0.9984

Table L.4

LINEARITY OF METRONIDAZOLE; Concentration (%w/v) range = 0.00033-0.00004125	
Equation of Line	**Correlation coefficient, R^2**
Y=892353x-6.7555	0.9981

Table L.5

LINEARITY OF INDOMETACIN; Concentration (%w/v) range = 0.000744-0.000093	
Equation of Line	**Correlation coefficient, R^2**
Y=254524x-2.8171	0.9982

Table L.6

LINEARITY OF BENZOIC ACID; Concentration (%w/v) range = 0.00018-0.0018	
Equation of Line	**Correlation coefficient, R^2**
Y=113304x-8.8956	0.9985

Table L.7

LINEARITY OF NAPROXEN; Concentration (%w/v) range = 0.0008-0.00008	
Equation of Line	**Correlation coefficient, R^2**
Y=212757x-11.856	0.999

REFERENCES

[1] Kealey D. and Haines P. J. (2002), Analytical Chemistry
Bios scientific publishers, pages 119- 129.

[2] McMaster M. C. (2007), A Practical User's Guide, Second Edition.
John Wiley & Sons Inc, pages 2-3

[3] Snyder L. R. and Dolan J. W. (2007),High Performance Gradient Elution.
John Wiley & Sons Inc, page 15

[4] Furniss B. S., Hannaford A. J. etal. Vogel's Textbook of Practical Organic Chemistry, Fifth Edition. John Wiley & Sons Inc 605 third avenue, New York. Pagesm 384

[5] British Pharmacopoeia 2007 CD ROM, Incorporating the requirements of the 5th Edition of the European Pharmacopoeia 2004 as amended by Supplements 5.1 and 5.5, Version 110.

[6] BNF 58, September 2009, BMS Group and RPS Publishing. Tavistock Square, Lndon WCH 9JP, UK.

[7] Clarke's Isolation and Identification of Drugs, (1986) Second edition. The pharmaceutical press London.

[8] Carins D.(2008), Essentaials of Pharmaceutical Chemistry ,Third Edition. The Pharmaceutical Press, London, Chicago. Pages 176-200.

[9] Bloch D. R. (2006), Organic Chemistry Demystified, A Self Teaching Guide.
McGraw Hill Company, London. Pages 221-230

[10] Olaniyi A. A., Principles of Drug Quality Assurance and Pharmaceutical Analysis, 2000; Mosuro Publishers, Ibadan, pp 116 – 129, 453 - 456.

[11] Olaniyi A. A.(2005), Essential Medicinal Chemistry, Third Edition.

Hope Publications, GAAF Building, Nigeria. Pages 202-209

[12] Fifield F. W.(2000) and Kealey D. Principles and Practice of Analytical Chemistry,Fifth Edition. University Press , Cambridge, U.K. Page 426

[13] Meyer V. R. (2004), Practical High Performance Liquid Chromatography, Fourth Edition. John Wiley & Sons Inc

[14] Rashmin B. (07/22/2008), An Introduction To Analytical Method Development For Pharmaceutical Formulations, Vol 6, Issue 4.

[15] Shabir G. A.(March 1, 2004), HPLC Method Development and Validation for Pharmaceutical Analysis. Pharmaceutical Technology Europe.

[16] Dong Micheal W, Mordern HPLC for Practicing Scientist. 2006, Hoboken, New Jersey: John Wiley & Sons, Inc., pp 87 – 96, 195 – 219.

[17] Miller J. C. and Miller J. N.(1993), Statistics for Analytical Chemistry, Third Edition. Ellis Horwood PTR Prentice Hall, New York. Pages54-71

[18] Olaniyi A. A. and Ogumgbamila F. O, Experimental Pharmaceutical Chemistry. Shanson C.I. limited,1A Adebo ojo street, Obokun,Akintola avenue. Pages 8-50

[19] David, W.G., Pharmaceutical Analysis, A text book for pharmacy students and pharmaceutical chemists. 1999: Churchill Livingstone, imprint of Harcourt Publishers Limited, pp 49 – 71.

[20] United States Pharmacopoeia Daily Reference Standard Catalogue; pdf. Last updated on the 25th February, 2010.

[21] Beckett A. H., Stenlake J.B., Practical Pharmaceutical Chemistry Part One. Fourth in two parts ed. 1988: Printed in England at the University Press, Cambridge, pp 16.

[22] United State Pharmacopoeia 30 NF 25 The official compendia of Standard Asian.

Edition 2007

[23] Kohli D.P.S (1991), Drug Formulation Manual, First edition. Eastern Publishers.

[24] Beckett A. H., Stenlake J.B., Practical Pharmaceutical Chemistry Part Two. Fourth in two parts ed. 1988: Printed in England at the University Press, Cambridge, pp 281 – 282.

[25] Wikipedia, The Free Encyclopedia (Internet)
http://en.wikipedia.org/wiki/Diazepam.

[26] Text on validation of Analytical Procedures, International conference on Harmonization (ICH) of Technical requirement for registration of pharmaceuticals for Human use, ICH steering committee,1994

[27] Handbook from Amersham Biosciences. Affinity Chromatography, Principles and Methods. Edition AD. Page 7.

[28] Baghel U.S., Singhal M. et al. Analytical method validation for tablet of phenoxymethyl penicillin potassium by RP-HPLC method. Journal of Chemical and Pharmaceutical Research, 2009, 1(1): 271-275. Page 273

[29] Tuani T. (2010). Use of Surrogate Reference Compounds In Quantitative HPLC. Assay of Acetylsalicylic acid and Diclofenac. Printed in KNUST Printing Press, Kumasi.