KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

COLLEGE OF HEALTH SCIENCES

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

THE USE OF SURROGATE REFERENCE STANDARDS IN QUANTITATIVE HPLC

SUBMITTED BY

JOSEPH EMIL AMEGADZIE

MAY, 2010

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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.

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(Student)

DEDICATION

To God Almighty, through His grace, all things become possible.

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ABSTRACT

The possibilities of using surrogate reference standards in quantitative high performance liquid chromatography (HPLC) have been explored. A quantitative reverse phase-High Performance Liquid Chromatography method has been developed to determine a constant, K with a surrogate reference that makes it possible to analyse prednisolone in a formulation in the absence of pure prednisolone as reference. Indometacin, Naproxen and Piroxicam were considered as surrogate reference standards. The chromatographic apparatus consisted of Shimadzu LC-6A Liquid Chromatograph-pump, Hichrom H5ODS column, Applied Biosystems 783 programmable Absorbance UV detector at 254nm and Shimadzu CR501 Chromatopac-Integrator as a recorder. Elution was isocratic with a mobile phase consisting of methanol and phosphate buffer (pH=5.8) in a ratio 1:1. The mean retention times for both drug and surrogates were; **prednisolone**: (4.30 ± 0.7 min), **indometacin**: (9.79 ± 0.3 min), **naproxen**: (2.25 ± 0.6 min) and **piroxicam**: (1.26 ± 0.3 min). The average K constant obtained for the surrogate reference standards were, **Indometacin**: 1.8323, **Naproxen**: 1.6925 and **Piroxicam**: 1.3550. These constants were used in the analysis of four samples of prednisolone tablets. The percentage content obtained for each sample was then compared with that obtained using the BP 2007 method. The results showed that the surrogate reference standard can be used for the analysis of prednisolone without the use of pure prednisolone powder as reference.

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CHAPTER ONE

1.0 INTRODUCTION

The quality of a drug is determined, after establishing its authenticity, by testing its purity and the quantity of the pure substance in the drug using various analytical methods. The determination of all these indices forms the basics of pharmaceutical analysis, and the results for each drug must strictly correspond to the requirements of a Pharmacopoeia. However, in most quantitative pharmaceutical analysis of drugs using HPLC, a pure reference standard of the analyte is needed. Sometimes buying and importing these reference standards are very costly to the drug manufacturer, pharmaceutical laboratories and regulatory bodies. Another factor that also comes to play is time as resource in procuring these standards. These phenomena have necessitated the need for the use of surrogate reference standards as a substitute for these reference standards most importantly in quantitative HPLC. Therefore there is the need to develop analytical methods that will make it possible to analyse pharmaceutical products in the absence of the reference standards using its surrogate standard.

Nevertheless, method developing using High Performance Liquid Chromatography (HPLC) can become cumbersome and time consuming if adequate and responsible steps are not taken. Firstly, one must define the method and the separation goals, enough and adequate information must be obtained about the analyte and conclusively, this should lead to validation of the method developed. Upon embarking on method development, one must consider certain parameters. Such parameters are; detector selection, selection of chromatographic mode and selection of the HPLC column. [1]

Validation of a method is documented evidence that provides a high degree of assurance that an analytical method demonstrates that it is adequate for its intended purpose. This also implies that the method is reproducible, accurate and robust within the specified analyte range. According to World Health Organization (WHO), Good Manufacturing Practices (GMP) of every non-compendia analytical method must be validated and the validation method and result must be documented. [2]

The validation of an analytical method must demonstrate that it fulfils all the requirements of the analytical applications, ensuring the reliability of the results. Various validation parameters are Precision, Accuracy, Limit of detection (LOD), Limit of Quantification (LOQ), Linearity and Range, Ruggedness, Reproducibility, Selectivity and Sensitivity. However, not all parameters are applicable to every developed method as much is depended upon the objectives on which the method is developed. Nevertheless, the accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and value found. [6, 7]

High pressure liquid chromatography or High performance liquid chromatography, popularly known as HPLC, is one mode of chromatography; the most widely used analytical method for the analysis of pharmaceutical products. It is used in every stage of drug development as a quality control tool to monitor the purity of both drugs and excipients in the course of drug manufacturing. [3]

HPLC utilizes a liquid mobile phase to separate the components of a mixture. These components (or analytes) are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture is resolved into its components. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC requires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures. [18, 19]

The simplest HPLC system is made up of a high-pressure solvent pump, an injector, a column, a detector, and a data recorder. Solvent (mobile phase) from a solvent reservoir is pulled up the solvent inlet line into the pump head through a one-way check valve. Pressurized in the pump head, the mobile phase is driven by the pump against the column back-pressure through a second check valve into the line leading to the sample injector. The pressurized mobile phase passes through the injector and into the column, where it equilibrates with the stationary phase and then exits to the detector flow cell and out to the waste collector. The sample, dissolved in mobile phase or a similar solvent, is first loaded into the sample loop and then injected by turning a handle swinging the sample loop into the pressurized mobile phase stream. Fresh solvent pumped through the injector sample loop

washes the sample onto the column head and down the column. The separated bands in the effluent from the column pass through the column exit line into the detector flow cell. The detector reads concentration changes as changes in signal voltage. This change in voltage with time passed out to the recorder or computer over the signal cable and is traced on paper as a chromatogram, allowing fractions to be detected as rising and falling peaks [3].

There are many ways to classify liquid column chromatography. If the classification is based on the nature of the stationary phase and the separation process, three modes can be specified. In adsorption chromatography the stationary phase is an adsorbent (like silica gel or any other silica based packings) and the separation is based on repeated adsorption and desorption steps.

In ion-exchange chromatography the stationary bed has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time. In size exclusion chromatography the column is filled with material having precisely controlled pore sizes, and the sample is simply screened or filtered according to its solvated molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the porous of the packing particles and elute later. Mainly for historical reasons, this technique is also called gel filtration or gel permeation chromatography although, today, the stationary phase is not restricted to a "gel". Concerning the first type, two modes are defined depending on the relative polarity of the two phases: normal and reversed-phase chromatography. [6]

In normal-phase chromatography, the stationary bed is strongly polar in nature (e.g., silica gel), and the mobile phase is nonpolar (such as n-hexane or tetrahydrofuran). Polar samples are thus retained on the polar surface of the column packing longer than less polar materials. Reversed-phase chromatography is the inverse of this. The stationary bed is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. Here the more nonpolar the material is, the longer it will be retained. [6]

Each mode of HPLC has its own requirements. For normal phase mode solvents are mainly nonpolar, for reversed-phase eluents are usually a mixture of water with some polar organic

solvent such as acetonitrile. Size-exclusion HPLC has special requirements, SEC eluents has to dissolve polymers, but the most important is that SEC eluent has to suppress all possible interactions of the sample molecule with the surface of the packing material. Pharmaceutical analysis based on calibration curve with an external standard is one of several quantitative methods in HPLC. In this method of analysis, calibration curves are drawn by the injection of several known concentrations. An electrical signal is generated by a detector out of the several injections which magnitude is determined by the concentration of the analyte. The response from the detector is plotted against the concentration of the analyte which is depicted in a straight line graphical representation. The accuracy of the standard preparations is shown by the linearity of the plotted values with linearity dependent on the response output of the detector. Invariably, the concentration of the diluted sample extract is determined from the calibration curve by substituting the area of its chromatographic peak into the equation for the calibration curve drawn [4].

1.1 JUSTIFICATION

In UV analysis for quantitative determinations, the use of the specific absorbance A (1%, 1cm) has been developed. This value however is dependent on specific location as the value varies from place to place and this is so because of certain factors such as the state of the purity of the substance, the conditions of the solvent that was originally used to establish the data and the extent to which they correspond to those of particular test laboratory. Upon this development, it became advisable to establish periodically a "local" value of the specific absorbance that can be used in calculating sample concentrations and this is done only when a pure form of the sample is present. [5, 20]

Moreover, in UV spectroscopy analyses of pharmaceutical products, calibration curves are drawn based upon the Beer-Lambert Law that absorption is proportional to concentration. However, it has been observed that beyond certain concentrations of the test sample, there is no linearity between the concentration and absorbance, making quantitative work impossible. Also, the use of A (1%, 1cm) to determine the specific absorbance of substances using reference standard sample leads to decrease in volume of working data.

Nevertheless, UV absorbance detection is probably the most common detection mode used. The detector used in the lab is a variable wavelength detector which allows the operator to choose the wavelength to monitor. [36]

In most quantitative pharmaceutical analysis of drugs using HPLC, a pure reference standard of the analyte is needed. *In industry and pharmaceutical laboratories, there are times when such standards become unavailable and very costly*. In such dilemma, quality control monitoring of medicines during pre-manufacturing and post-manufacturing becomes cumbersome for laboratories both in industries and regulatory purposes in defining the safety of the ultimate interest of the consumer.

Pharmaceutical reference standards from the United States of America which are established and released with critical characteristics of each lot of specimen determined independently in two or more laboratories. The America's Food and Drugs Association(FDA) laboratories and the United States Pharmacopoeia, USP, and other laboratories both academia and industrial reference standards laboratory are mostly involved in the testing of all new standards and their possible replacements of some existing standards [6].

In Ghana, sometimes buying and importing these reference standards into our country however is very expensive. The relative cost of Prednisolone reference standards as on the 25th February, 2010 is detailed in Table below;

Table 1.1 ***COST OF REFERENCE STANDARD***

Catalog #	**Product Description**	**Current Lot**	**Previous Lot**	**Unit Price**
1555005	Prednisolone (200mg)	N0D212	M (02/06)	$199.00 EACH

Also, the availability of such reference standards taking time factor into consideration also comes to play. Therefore it has thus become imperative to develop research that evolve a mechanism that makes it possible to assess the quality of pharmaceuticals in the absence of the pure sample as a reference standard, thus the research topic "Surrogate Standards", which also simply means 'a substitute for a reference standards'.

A baseline study in this regard for Paracetamol, Aspirin and Diclofenac Sodium products has successfully been completed at the Department of Pharmaceutical Chemistry. My project therefore seeks to extend the search by using the analysis of Prednisolone as another case study.

1.2 MAIN OBJECTIVES

This project seeks to investigate the possibility of using surrogate reference standards for the analyses of Prednisolone tablet in quantitative HPLC.

1.3 SPECIFIC OBJECTIVES

The specific objectives of this research are;

1. To develop an HPLC assay procedure for Prednisolone tablet using surrogate reference compounds.
2. To elute the analyte together with three (3) surrogate reference standards at different times.
3. To validate the method developed by using validation parameters such as Specificity and Selectivity, Linearity, precision, accuracy, Limit of detection (LOD), Limit of quantification (LOQ).
4. To determine a constant, K that can effectively be used for quantitative analysis.
5. To determine the percentage content of Prednisolone for various brand of tablets using the method developed.
6. To compare the results obtained from the method developed with a standard method in the British Pharmacopoeia.

1.4 HYPOTHESIS OF STUDY

In HPLC quantitative analysis,

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

where; ‘A’ is the area under a peak and ‘C’ is the concentration of the sample:
where the standard is the pure sample of the analyte.

However, using surrogate compounds as standard,

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

It is rather;

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

where;

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

$\mathbf{A}_{analyte}$ is the peak area of the analyte.

$\mathbf{A}_{standard}$ is the peak area of the surrogate standard.

$\mathbf{C}_{standard}$ is the concentration of the surrogate standard.

$\mathbf{C}_{analyte}$ is the concentration of the analyte.

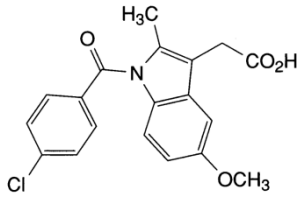
Once K, $A_{analyte}$ and $C_{standard}$ are known for a particular system, $C_{analyte}$ can be calculated.

Thus;

Percentage Content = (Actual concentration / Nominal concentration) × 100%

1.5 INTRODUCTION TO ANALYTES AND SURROGATE REFERENCE STANDARDS

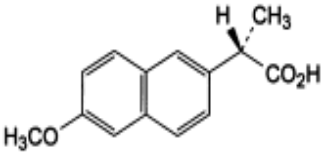
1.5.1 Indometacin



*The chemical structure of **Indometacin*** [7].

Indometacin has the following proprietary names; **artracin**, **indocid**, **indocin**, **indoflex**, **rheumacin**. It is a crystalline powder with a white to yellow-tan colour. It is used as an analgesic to relieve minor aches and pains, and as anti-inflammatory medication.[9] Its IUPAC name is **[1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl] acetic acid**, its formula is $C_{19}H_{16}ClNO_4$; has a molar mass of 357.8g/mol and a melting point range of 158^0C to 162^0C. It is practically insoluble in water, solubility of 1g in 50ml of ethanol, 1g in 30ml chloroform, 1g in about 40ml ether and insoluble in acetone.[8] Indometacin has a side effect of frequent gastro-intestinal disturbances (including diarrhoea), headache, dizziness, and light-headedness. [7, 21]

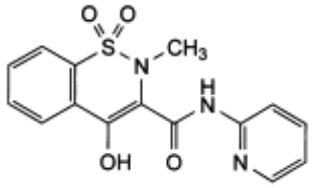
1.5.2 Naproxen



*The chemical structure of **Naproxen***[7].

Naproxen has the following as its proprietary names; **equiproxen**, **floginax**. It is a white crystalline powder. It is used as an analgesic activity comparable to paracetamol but paracetamol is preferred particularly in the elderly to relieve pains and aches and also as an anti-inflammatory [9]. Its IUPAC name is **(2*S*)-2-(6-Methoxynaphthalen-2-yl) propanoic acid**. Its formula is $C_{14}H_{14}O_3$; has a molar mass of 230.3g/mol; a melting point range of 154^oC to 158^oC. It is practically insoluble in water, 96% soluble in ethanol, solubility of 1g in 15ml of chloroform and 1g in 40ml of ether.[8] Naproxen has side effects such as gastro-intestinal discomfort, nausea, diarrhoea and occasionally, bleeding and ulceration may occur [7, 22].

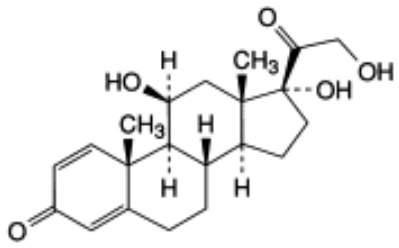
1.5.3 Piroxicam



*The chemical structure of **Piroxicam**.* [7]

Piroxicam has felden as its proprietary name. It is a white or yellow crystalline powder. It has both analgesic and anti-inflammatory properties. Piroxicam is as effective as Naproxen and has a long duration of action which permits once-daily administration. However, it has more gastro-intestinal side-effects than most other anti-inflammatory drugs, and is associated with more frequent serious skin reactions [9]. Its IUPAC name is 4-hydroxy-2-methyl-*N*-(pyridin-2-yl)-2*H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide, a molecular formula of $C_{15}H_{13}N_3O_4S$ and a molar mass of 331.4g/mol with a melting range of 198^{o}C to 200^{o}C. It is practically insoluble in water, insoluble in methylene chloride and slightly soluble in ethanol. Piroxicam shows polymorphism. [23]

1.5.4 Prednisolone



*The chemical structure of **Prednisolone*** [7].

Prednisolone has the following proprietary names; codelcortone, delta-phoricol, delta-cortef and deltastab. It is a white crystalline powder and hygroscopic in nature. Prednisolone is a corticosteroid that is used for the suppression of inflammatory and allergic disorders [9]. It has an IUPAC name of 11b,17,21-trihydroxypregna-1,4-diene-3,20-dione and a molecular formula of $C_{21}H_{28}O_5$ with a molar mass of 360.4g/mol. It has a melting point range of 230^{o}C to 235^{o}C with decomposition. It is soluble in 1g in 1300ml of water, 1g in 30ml of ethanol, 1g in 27ml of dehydrated alcohol and 1g in 180ml chloroform. [7]

Prednisolone is soluble in dioxin and methanol. It has several side effects and this includes gastro-intestinal effects including dyspepsia, peptic ulceration (with perforation), abdominal distension, acute pancreatitis, oesophageal ulceration and candidiasis; musculoskeletal effects including proximal myopathy, osteoporosis, vertebral and long bone fractures, avascular

osteonecrosis, tendon rupture; endocrine effects include adrenal suppression, menstrual irregularities and amenorrhoea. The side-effects are minimised by using lowest effective dose for minimum period as possible. [7, 24]

1.6 LITERATURE REVIEW

1.6.1 Theory and Instrumentation of Various Analytical Methods

1.6.1.1 *UV-Visible Spectrophotoscopy*

The fundamental application of ultraviolet and visible spectrophotometry is in quantitative analysis. It can be used for assay of single, mixture and bulk drugs, dissolution tests for tablets and capsules, limit tests for impurities (colorimetry methods). Also, it can be used for measurements such as pK_a or velocity constants in enzymatic reactions. [10]

Absorption in the ultraviolet and visible regions of the electromagnetic spectrum corresponds to transitions between electronic energy levels and provides useful analytical information for both inorganic and organic samples. Molecules may possess several excited states. After excitation by absorption of radiation, rapid transitions can occur to lower energy excited states, which then revert to the ground state, emitting electromagnetic radiation at a lower energy and by lower processes referred to as photoluminescence. The components of ultraviolet and visible spectrometers include a source of radiation, a means of dispersion and a detector specific to this spectral region. [11]

The ultraviolet (UV) and visible region of the electromagnetic spectrum covers the wavelength range from about 200nm to about 800nm. The vacuum ultraviolet region, which has the shortest wavelengths and highest energies (100-200nm), is difficult to make measurements in and is little used in analytical procedures. Most analytical measurements are in the UV region are made between 200 and 400nm. The visible region occurs between 400 and 800nm. The absorbance of a sample is proportional to the number of absorbing molecules in the spectrophotometer light beam. It has therefore become necessary to correct absorbance value for this and other operational factors if the spectra of different compounds are to be compared. The corrected absorption value is the specific absorbance.

Specific absorbance, A (1%, 1cm) is used for quantitative determinations in samples, using the absorbance measured in the specified solvent.

A (1%, 1cm) is defined as the 'absorbance of a 1%w/v solution in a cell of 1cm path length'.

A (1%, 1cm) = **A / c l**

where;

A is the absorbance,

c is the concentration in g/100ml,

l is the light path through the sample in cm. [12]

Single component analyses

For samples within which only one component absorbs significantly, a wavelength must be chosen and this wavelength must be the wavelength of maximum absorption in the spectrum in order to minimize wavelength-setting errors. Stray light errors may occur if wavelength at the extreme ends of the ultraviolet and visible ranges is used. The specific absorbance can be used for the calculation of sample concentration. [13]

Multicomponet analyses

An overlap of the absorption spectra of two or more drugs of interest may occur. There may also be some interference from impurities in manufacturing, decomposition products and formulation excipients. These irrelevant absorptions, if not removed can cause systematic error to the assay of the sample component. The basis of all the spectrophotometric techniques for multicomponent sample is the property that at all wavelengths:

a. the absorbance of a solution is the sum of absorbances of the individual components; or;

b. 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 (blank) cell. [10]

1.6.1.2 *Nuclear Magnetic Resonance*

NMR spectroscopy is one of the most important analytical methods available today. The reasons are manifold: it is applied by chemists and physicists to gases, liquids, liquid crystals and solids (including polymers). Biochemists use it routinely for determining the structures of peptides and proteins, and it is also widely used in medicine (**MRI- Magnetic Resonance**

Imaging). With the advent of spectrometers operating at very high magnetic fields (up to 21.1 T, i.e. 900 MHz proton resonance frequency) it has become an extremely sensitive technique, so that it is now standard practice to couple NMR with high pressure liquid chromatography (HPLC). [11, 14]

The wide range of nuclei which are magnetically active makes NMR attractive not only to the organic chemist but also to the organometallic and inorganic chemist. The latter in particular often has the choice between working with liquid or solid samples; the combination of high resolution and magic angle spinning (HR/MAS) of solid samples provides a wealth of structural information which is complementary to that obtained by X-ray crystallography. The same suite of techniques, slightly adapted, is now available to those working in the field of combinatorial chemistry. This is only a selection of the possibilities afforded by NMR, and the list of methods and applications continues to multiply. One of the applications of NMR Spectroscopy in quantitative analysis is the analysis of multicomponent assays such as sulphatriad. [14]

1.6.1.3 *Infra red Spectroscopy*

Infra red spectroscopy operates on the principle of vibrational transitions in molecules which causes absorption in the infrared region of the electromagnetic spectrum. They may also be studied and applied using the technique of Raman spectrometry, where they scatter exciting radiation with an accompanying shift in its wavelength. The vibrational spectra give information about the functional groups in molecules, and the observed group frequencies are affected by molecular interactions such as hydrogen bonding. Basically, Infrared instruments include a radiation source, a means of analyzing the radiation and detection and data processing system. Additionally, sampling methods to deal with gases, liquids, solids, micro samples and mixtures are available. [11, 15]

1.6.1.4 *Mass Spectrometry*

Mass spectrometry (MS) is a technique whereby materials are ionized and dissociated into fragments characteristic of the molecule(s) or elements present in the sample. Mass spectrometry has come a long way from its role in the discovery of isotopes for many of the chemical elements. In just a few decades, difficulties with introducing large, highly polar molecules such as proteins into a mass spectrometer have been overcome and the mass spectrometer, in its many guises, stands as a central technology for the analysis of pharmaceutical products. Perhaps even more astounding, given its construct, is the increasing

role that mass spectrometry now plays in the study of medicines and other macromolecular interactions. A mass spectrometer which operates under high vacuum incorporates a sample inlet and ion source, a mass analyzer, an ion detector and a data processing system. [11, 16]

1.6.1.5 *Thin Layer Chromatography*

Thin-layer chromatography (TLC) is without doubt one of the most versatile and widely used separation methods in chromatography. Commercially, many sorbents on a variety of backings are now available. Most stages of the technique are now automated (can now be operated instrumentally) and modern HPTLC (High performance thin-layer chromatography) allows the handling of a large number of samples in one chromatographic run. Speed of separation, high sensitivity and good reproducibility all result from the higher quality of chromatographic layers and the continual improvement in instrumentation. [11]

In addition TLC has remained relatively inexpensive and one can easily see why it is still popular today. It has found a use in a wide range of application areas as the concept of TLC is so simple and samples usually require only minimal pretreatment. It is often thought of only in terms of its use in pharmaceutical analysis and production and in clinical analysis, but many standard methods in industrial pharmacy, drug toxicology, food chemistry and herbal analysis rely upon TLC as the preferred approach. In its simplest form, TLC costs little, but even including the more sophisticated instrumentation, it still remains less expensive per sample analysis than, for example HPLC. [37]

TLC includes a mobile phase which basically by a capillary action moves across a stationary phase which may include of the following adsorbents; alumina, cellulose, magnesium silicate and silica gel. These adsorbents may contain a binder, usually calcium sulphate and a fluorescence indicator such as fluorescein, to allow detection of compounds which quench the fluorescence when the plate is observed in UV light of 254nm wavelength. The stationary phase is bonded to a suitable plate made of glass, aluminium foil, or plastic. [17]

The basic chromatographic measurement of a substance in TLC is the Retardation factor, R_f value which is defined as;

$$R_f = \frac{\text{Distance the substance travels from the origin}}{\text{Distance the solvent front travels from the origin}}$$

1.6.1.6 *Titrimetric and Chemical Analysis*

Titrimetry is an analytical method that is widely used in the pharmaceutical industry because of its robustness, capability for high precision and accuracy and also for its cheapness. Titrations usually involve the addition of controlled volumes of a standard solution, whose concentration is known accurately, to a solution of reactant of unknown concentration. The theoretical amount of solution that must be added until the reaction is just complete is the equivalence point and the end point in a titration is the point at which change is detected accurately. In an ideal case, these points should be the same. There are however, various classes of titrimetry. [25]

Aqueous Acid-Base Titrations

Proton transfer reactions in aqueous solutions are quite fast. Aqueous acid-base titrations are thus suitable for the analysis of any Bronsted acid or base. Practically, the PK_a or PK_b of the analyte should be less than 10 (i.e., PK_a or $PK_b < 10$) for a complete reaction between analyte and titrant. In order for the titration reaction to go to completion, a strong acid or a strong base is the usual choice for a titrant in acid-base titrations. The levelling effect in aqueous solutions should be kept in mind, however: the strongest acid that can exist at a substantial concentration is the hydronium ion, H_3O^+, since any strong acid HA will react completely with water. [25]

Thus, titrating with any strong acid is equivalent to titrating the analyte with hydronium ion. Similarly, the strongest base that can exist in water is the hydroxide ion, OH^-. For the analysis of bases, the most common aqueous titrant is HCl; sometimes H_2SO_4 or $HClO_4$ are also used. Any of these may be standardized by tris (hydroxymethyl) aminomethane, $(HOCH_2)_3CNH_2$, which is sometimes referred to simply as tris. sodium carbonate, Na_2CO_3, can also serve as a primary standard, but it is less desirable than tris due to its lower equivalent weight. Titrations of bases are sometimes called alkalimetric titrations. [26]

Nonaqueous Acid-Base Titrations

Sometimes acid-base titrations are performed using a solvent other than water. There are several reasons why nonaqueous acid-base titrations may be used instead of aqueous titrations:

1. The sample is insoluble in water.
2. Sample and or titrant react with water in undesirable ways.

3. For the analysis of very weak acids or bases. As mentioned previously, an aqueous acidimetric titration is limited to bases with PK_b less than about 10. Otherwise, the reaction between titrant (i.e., H_3O^+) and analyte will be incomplete. One solution to this problem for weak bases would be to use a stronger titrant; an impossibility in aqueous solutions. However, using glacial acetic acid as the solvent would solve that problem, since the strongest possible acid is H_2OAc^+ (a strong acid indeed). Most strong acids do not completely dissociate in acetic acid. Thus, perchloric acid in acetic acid is a much stronger titrant than the same acid in water. Similar considerations apply to alkalimetric titrations.
4. Selectivity is sometimes enhanced in nonaqueous solutions (analysis of analytes with similar dissociation constants). In aqueous solutions, a difference of 2 pK units is necessary to observe distinct endpoints. However, careful choice of solvent can sometimes allow the observation of distinct endpoints that cannot be measured in aqueous solution. [25]

Redox Titrations

Redox reactions are the most diverse of the four main classes of inorganic aqueous reactions (acid-base, complexation and redox). In principle, redox titrations can be used to analyze any oxidizing or reducing agent. However, many redox reactions are either too slow or have inconsistent stiochiometry. The stability of titrant and analyte solutions can also be a problem. Nevertheless, a wide variety of analytes can be conveniently determined by redox titrations. [25]

Complexometric Titrations

Complexometric titrations are based on the reaction between Lewis acids (usually metal cations) and Lewis bases. Lewis acids and bases react to form a complex. The base donates two electrons to form a bond with the acid. Since the proton, H^+, is a good Lewis acid, by definition any Bronsted base will be a Lewis base. Lewis bases will possess at least a single lone pair of electrons that it will donate to the Lewis acid. Lewis bases are also sometimes called ligands, and the atoms containing the lone pair is the ligand binding site. A special subset of ligands are those that contain more than one binding site on the molecule; these are called chelating agents. Chelating agents form particularly strong complexes called chelates with Lewis acids. [25]

By far the most common complexometric titrant is ethylenediaminetetraacetic acid, EDTA. This is a hexadentate chelating ligand, meaning that there are six ligand binding sites on

EDTA molecule. EDTA titrations are very versatile: they can be used for the analysis of all the metal cations except the alkali metals, and can even be used (through back-titration and similar methods) for the analysis of many anions. EDTA titrations are also fairly sensitive, capable of detecting concentrations of some metals at levels of approximately 10 ppm (i.e., 10 mg/L). [27]

1.6.1.7 *High Performance Liquid Chromatography*

The ability to separate and analyse complex samples is integral to the pharmaceutical and medical sciences. Classic column chromatography has evolved over the years, with chromatographic innovations introduced at roughly decade intervals. These techniques offered major improvements in speed, resolving power, detection, quantification, convenience and applicability to new sample types. The most notable of these modifications was high performance liquid chromatography (HPLC). [8]

The systems used in chromatography are often described as belonging to one of four mechanistic types: adsorption, partition, ion exchange and size exclusion. Adsorption chromatography arises from interactions between solutes and the surface of the solid stationary phase. Generally, the eluents used for adsorption chromatography are less polar than the stationary phases and such systems are described as 'normal phase'. Partition chromatography involves a liquid stationary phase that is immiscible with the eluent and coated on an inert support. Partition systems can be normal phase (stationary phase more polar than eluent) or reversed–phase chromatography, referred to as RPC (stationary phase less polar than eluent). Ion–exchange chromatography involves a solid stationary phase with anionic or cationic groups on the surface to which solute molecules of opposite charge are attracted. Size–exclusion chromatography involves a solid stationary phase with controlled pore size. Solutes are separated according to their molecular size, with the large molecules unable to enter the pores elute first. However, this concept of four separation modes is an over simplification. In reality, there are no distinct boundaries and several different mechanisms often operate simultaneously. [28, 36]

1.6.1.7.1 Mobile Phase

The mobile phase is the solvent that moves the analyte through the column. In HPLC, type and composition of the mobile phase (eluent) is one of the variable influencing the separation. Despite the large variety of solvents used in HPLC, there are several common

properties. These properties include; purity, detector compatibility, solubility of the sample, low viscosity, chemical inertness and reasonable price. Each mode of HPLC has its own requirements. For normal phase mode solvents are mainly nonpolar, for reversed-phase eluents are usually a mixture of water with some polar organic solvent such as acetonitrile. [8]

Table 1.2 Common HPLC solvents and their properties

Solvent	Polarity Index	Viscosity (cp) at 20°C	Boiling Point (°C) at 1 atm	Miscibility Number	Refractive Index	UV Cut Off (nm)
Acetic Acid	6.2	1.26	117.9	14	1.372	230*
Acetone	5.4	0.32	56.3	15, 17	1.359	330
Acetonitrile	6.2	0.37	81.6	11, 17	1.344	190
Benzene	3.0	0.65	80.1	21	1.501	280
Chloroform	3.4	0.57	61.2	19	1.443	245
Cyclohexane	0.0	0.98	80.7	28	1.427	200
Dimethyl Sulphoxide	6.5	2.24	189.0	9	1.477	268
p-Dioxane	4.8	1.54	101.3	17	1.422	215
Ethanol	5.2	1.20	78.3	14	1.361	210
Ethyl Acetate	4.3	0.47	77.1	19	1.370	256
Formamide	7.3	3.76	210.5	3	1.446	≈260
Hexane	0.0	0.31	68.7	29	1.372	195
Methanol	6.6	0.60	64.7	12	1.329	205
Methyl Ethyl Ketone	4.5	0.43	80.0	17	1.381	330
1-Propanol	4.3	2.30	97.2	15	1.380	210
2-Propanol	4.3	2.35	117.7	15	1.380	205
i-Propyl Ether	2.2	0.33	68.3	26	1.368	220
Tetrahydrofuran	4.2	0.55	66.0	17	1.408	230
Toluene	2.3	0.59	101.6	23	1.496	285
Water	9.0	1.00	100.0	–	1.330	190
p-Xylene	2.4	0.70	138.0	24	≈1.50	290

** Value refers to a 1% solution in water*

Buffer Solutions

A buffer solution is an aqueous solution consisting of a mixture of a weak acid and its conjugate base or a weak base and its conjugate acid. Buffers are basically solutions prepared to resist changes in pH due to external influences some of which are temperature, pressure, volume, redox potential or acidity. [30] It has the property that the pH of the solution changes very little when a small amount of strong acid or base is added to it. Buffer solutions

are used as a means of keeping pH at a nearly constant value in a wide variety of chemical applications. [29]

The following table list a series of commonly used HPLC buffers, their alternative name, where applicable, and their pKa values at 20°C.

Table 1.3 Common Buffers Used in HPLC

Buffer	Buffer Range	pK_a at 200C
ACES/N-(2-Acetamido)-2-aminoethanesulfonic Acid	6.4 – 7.4	6.9
Acetamidoglycine/N-(2-Acetamido) Glycine		7.72
Acetic Acid/Ammonium (K- & Na-) Acetate	3.8 – 5.8	4.8
ADA/N-(2-Acetamido)-iminodiacetic Acid	6.4 – 7.4	6.6
Mono- & Di- Ammonium (K- & Na-) Carbonate	5.4 – 7.4 9.3 – 11.3	6.4 10.3
Ammonium Hydroxide (Chloride)/Ammonia	8.2 – 10.2	9.2
BES /N,N-Bis(2-hydroxyethyl)-2-aminoethane-sulfonic Acid	6.6 – 7.6	7.15
Bicine/N,N-Bis(hydroxyethyl) glycine	7.8 – 8.8	8.35
BIS-TRIS Propane/1,3-Bis[tris (hydroxymethyl)methylamino]propane	5.8 – 7.8	6.8
Borate	8.2 – 10.2	9.24
Cholamine Chloride/(2-Aminoethyl) trimethylammonium Chloride Hydrochloride	–	7.1
Citric Acid/Tri potassium citrate	2.1 – 6.4	3.1 4.7 5.4
Diethylamine Hydrochloride/Diethylamine	9.5 – 11.5	10.5
Formic Acid/Ammonium (K- & Na-) Formate	2.8 – 4.8	3.8
Glycinamide/Glycinamide Hydrochloride	–	8.2
Glycine Hydrochloride/Glycine	8.8 – 10.8	9.8
Glycylglycine	–	8.4
HEPES/N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid	7.0 – 8.0	7.55
MES/2-(N-Morpholino)-ethanesulfonic Acid	5.8 – 6.5	6.15
1-Methylpiperidine Hydrochloride/1-Methylpiperidine	9.1 – 11.1	10.1
Phosphoric Acid; Mono & Di potassium phosphate	<3.1 6.2 – 8.2 11.3 – 13.3	2.1 7.2 12.3
PIPES/Piperazine-N,N'-Bis (2-ethanesulphonic Acid)	6.4 – 7.2	6.8
TAPS/N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic Acid	7.7 – 9.1	8.4
TES/N-Tris(hydroxymethyl)methyl-2-amino-ethanesulfonic Acid	7.0 – 8.0	7.5
Trifluoroacetic Acid	1.5 – 2.5	>>2
Tricine/N-Tris(hydroxymethyl)methylglycine	7.6 – 8.8	8.15
Triethylamine Hydrochloride/Triethylamine	10.0 – 12.0	11.0
TRIS/Tris(hydroxymethyl)aminomethane	7.3 – 9.3	8.3

The dissociation constant pK_a of the analyte is vital for a correct buffer pH to be chosen. Usually, for good and refined peak shape, a buffer 1.5 pH units above or below the pK_a is recommended. A buffer concentration of 0.025M – 0.05M is adequate for most reverse phase applications. [31]

All pH adjustments are made in the aqueous buffer alone before mixing it with any organic solvents. Between 3 to 10 days, a buffered mobile phase can be used after which absorption of carbon dioxide leads to bacteria growth. [32]

1.6.1.7.2 Pumps

High–pressure pumps are needed to force solvents through packed stationary phase beds. Smaller bed particles (e.g. 3 μm) require higher pressures. There are many advantages to using smaller particles, but they may not be essential for all separations. The most important advantages are higher resolution, faster analyses and increased sample load capacity. However, only the most demanding separations require these advances in significant amounts. Many separation problems can be resolved with larger particle packings (e.g. 5 μm) that require less pressure. Flow–rate stability is another important pump feature that distinguishes pumps. Constant–flow systems are generally of two basic types: reciprocating piston and positive displacement (syringe) pumps. The basic advantages of both systems are their ability to repeat elution volume and peak area, regardless of viscosity changes or column blockage, up to the pressure limit of the pump. [8]

Although syringe–type pumps have a pressure capability of up to 540 000 kPa (78 000 psi), they have a limited ability to form gradients. Reciprocating piston pumps can maintain a liquid flow for an indefinite length of time, while a syringe pump needs to be refilled after the syringe volume has been displaced. Dual–headed reciprocating piston pumps provide more reproducible and pulse–free delivery of solvent, which reduces detector noise and enables more reliable integration of peak area. Reciprocating pumps now dominate the HPLC market and are even useful for micro-HPLC applications, as they can maintain a constant flow at flow rates in μL/min ranges. An additional pump feature found on the more elaborate pumps is external electronic control. Although it adds to the expense of the pump, external electronic control is a very desirable feature when automation or electronically controlled gradients are to be run. Alternatively, this becomes unnecessary when using isocratic methods. The degree of flow control also varies with pump expense. More expensive pumps include such state of

the art technology as electronic feedback and multiheaded configurations. Modern pumps have the following parameters:

• Flow–rate range, 0.01 to 10 mL/min.

• Flow–rate stability, not more than 1% (short term).

• For size exclusion chromatography (SEC), flow–rate stability should be <0.2%.

• Maximum pressure, up to 34 500 kPa (5000 psi). [8, 28]

1.6.1.7.3 Injectors

An injector for an HPLC system should provide injection of the liquid sample within the range of 0.1 to 100 mL of volume with high reproducibility and under high pressure (up to 27 600 kPa). The injector should also minimise disturbances to the flow of the mobile phase and produce minimum band broadening. Sample introduction can be accomplished in various ways. The injection valve has, in most cases, replaced syringe injection. Valve injection offers rapid, reproducible and essentially operator–independent delivery of a wide range of sample volumes. The most common valve is a six–port Rheodyne valve in which the sample fills an external stainless steel loop. A clockwise turn of the valve rotor places the sample filled loop into the mobile–phase stream, which deposits the sample onto the top of the column. These valves can be operated manually or actuated via computer–automated systems. One minor disadvantage of valve injection is that the sample loop must be changed to obtain various sample volumes. However, this is a simple procedure that requires a few minutes only. In more sophisticated HPLC systems, automatic sampling devices are incorporated. [28, 36]

These autosamplers have a piston–metering syringe–type pump to suck the preset sample volume into a line and transfer it to a sample loop of adequate size in a standard six–port valve. Most autosamplers are computer controlled and can serve as the master controller for the whole system. In HPLC, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. The solvent need not be the mobile phase, but frequently it is wise to choose the mobile phase to avoid detector interference, column component interference, loss in efficiency or all of these. It is always best to remove particles from the sample by filtration or centrifugation, since continuous injections of particulate material eventually cause blockage of injection devices or columns. Sample sizes may vary widely. The availability of highly sensitive detectors frequently allows the use of small samples that yield the highest column performance. [28]

1.6.1.7.4 Detectors

Today, optical detectors are used most frequently in HPLC systems. These detectors pass a beam of light through the flowing column effluent as it passes through a flow–cell. Flow–cells are available in preparative, analytical and micro–analytical sizes. The variations in light intensity, caused by ultraviolet (UV) absorption, fluorescence emission or change in refractive index (depending on the type of detector used) from the sample components that pass through the cell, are monitored as changes in the output voltage. These voltage changes are recorded on a strip–chart recorder and frequently are fed into an integrator or computer to provide retention time and peak–area data. Most applications in drug analysis use detectors that respond to the absorption of UV radiation (or visible light) by the solute as it passes through the flow–cell. [8]

Absorption changes are proportional to concentration, following the Beer–Lambert Law. Flow–cells generally have path–lengths of 5 to 10 mm with volumes between 5 and 10 μL. These detectors give good sensitivities with many compounds, are not affected by slight fluctuations in flow rate and temperature, and are non–destructive, which allows solutes to be collected and further analysed if desired. The simplest detectors are of the fixed–wavelength type and usually contain low–pressure mercury lamps that have an intense emission line at 254 nm. Some instruments offer conversion kits that allow the energy at 254 nm to excite a suitable phosphor to give a new detection wavelength (e.g. 280 nm).

Variable–wavelength detectors have a deuterium lamp with a continuous emission from 180 to 400 nm and use a manually operated diffraction grating to select the required wavelength. Tungsten lamps (400 to 700 nm) are used for the visible region. Many organic compounds absorb at 254 nm and hence a fixed–wavelength detector has many uses. However, a variable–wavelength detector can be invaluable to increase the sensitivity of detection by using the wavelength of maximum absorption. This is particularly useful when analysing proteins that absorb at 280 nm, or peptides that are detected commonly at 215 nm. Using a variable–wavelength detector can also increase the selectivity of detection by enhancing the peak of interest relative to interfering peaks. [37]

Eluents must have sufficient transparency at the selected detection wavelength. Buffer salts can also limit transparency. The spectra of some drugs change with pH and the sensitivity and selectivity of an assay can sometimes be controlled by changing the eluent pH. The influence

of such changes on the chromatography must also be considered. Other detectors commonly used include diode array, refractive index (RI), fluorescence (FL), electrochemical (EC) and mass spectrometry (MS). Infra–red (IR) and nuclear magnetic resonance (NMR) spectrometers may also be used as detectors. [8, 33]

1.6.1.7.5 Columns

Typical HPLC columns are 10, 15 and 25 cm in length and are fitted with extremely small diameter (3, 5 or 10 μm) particles. The columns may be made of stainless steel, glass–lined stainless steel or polyetheretherketone (PEEK). The internal diameter of the columns is usually 4.0 or 4.6 mm for traditional detection systems such as UV. This is considered the best compromise between sample capacity, mobile phase consumption, speed and resolution. However, if pure substances are to be collected, larger diameter columns may be needed. Smaller diameter columns (2.1 mm or less) are often used when HPLC is coupled with Mass Spectrometer. The smaller diameter columns also have the advantage of consuming less solvent because of their lower optimal flow rates. HPLC systems sold today can often be plumbed with narrower tubing diameters to take advantage of the benefits of these smaller column diameters. [8]

Packed capillary microcolumns are also gaining wider use when interfacing the HPLC to a mass spectrometer and extremely low flow rates (mL/min) are needed to maximise sensitivity for the analysis of proteins and peptides. Packing of the column tubing with small diameter particles requires high skill and specialised equipment. For this reason, it is generally recommended that all but the most experienced chromatographers purchase pre–packed columns, since it is difficult to match the high performance of professionally packed HPLC columns without a large investment in time and equipment. In general, HPLC columns are fairly durable and one can expect a long service life unless they are used in some manner that is intrinsically destructive, such as with highly acidic or basic eluents, or with continual injections of ‘dirty’ biological or crude samples. It is wise to inject some test mixture into a column when new and to retain the chromatogram. If questionable results are obtained later the test mixture can be injected again under specified conditions. [8, 33]

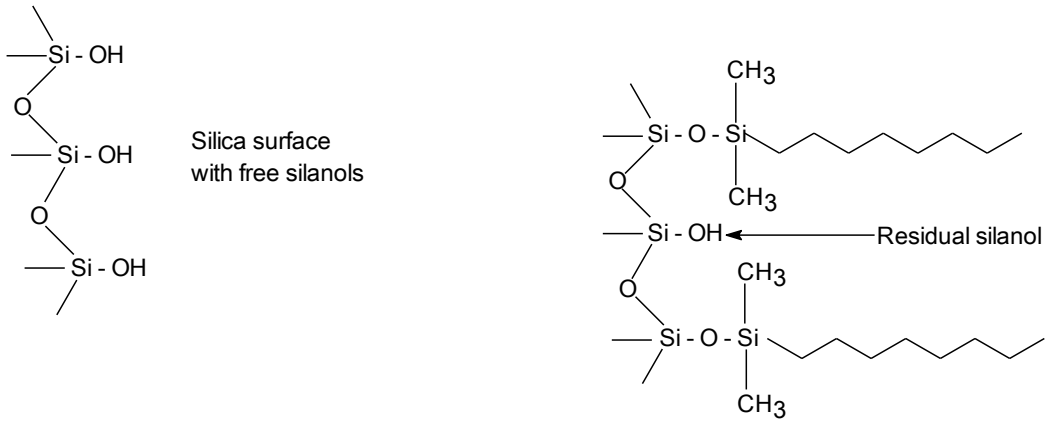
Packing characteristics

Silica–based packing materials

Silica (SiO_2, xH_2O) is the most widely used substance for the manufacture of packing materials. It consists of a network of siloxane linkages (Si–O–Si) in a rigid three–dimensional structure that contains interconnecting pores. The size of the pores and the concentration of silanol groups (Si–OH), which line the pores, can be controlled in the manufacturing process. Thus, a wide range of commercial products is available with surface areas that range from 100 to 800 m^2/g and average pore sizes from 4 to 33 nm. Spherical packing materials are now the only types being introduced for analytical HPLC. Irregular shaped materials are still being used to pack preparative columns. The silanol groups on the surface of silica give it a polar character, which is exploited in adsorption chromatography using organic eluents. Silanol groups are also slightly acidic and hence basic compounds are adsorbed particularly strongly. Unmodified silica can thus be used with aqueous eluents for the chromatography of basic drugs. [37]

Silica can be altered drastically by reaction with organochlorosilanes or organoalkoxysilanes to give Si–O–Si–R linkages with the surface. The attachment of hydrocarbon chains to silica produces a non–polar surface suitable for RPC in which mixtures of water and organic solvents are used as eluents. The most popular material is octadecylsilica (ODS), which contains C18 chains, but materials with C1, C2, C4, C6, C8 and C22 chains are also available. The latest silica–based bonded phase to be introduced is a long C30 phase, which has 24% carbon coverage to make it one of the most retentive phases available. [36]

During manufacture, such materials may be reacted with a small monofunctional silane (e.g. trimethylchlorosilane) to reduce further the number of silanol groups that remain on the surface (endcapping). Recent advances in column technology include multiple reactant endcapping, use of Type B (high purity, low trace metal, low acidity) silica and encapsulating the surface with a polymeric phase. These silicas are often referred to as ‘base–deactivated’ and are especially useful in RPC in the pH range of 4 to 8 when many basic compounds are partially ionised. Variations in elution order on different commercial packing materials of the same type (e.g. ODS) are often attributed to differences in surface coverage and the presence of residual silanol groups. For this reason it must not be assumed that a method developed with one manufacturer’s ODS column can be transferred easily to another manufacturer’s ODS column. [8, 34]



Speciality silicas

A vast range of materials have intermediate surface polarities that arise from the bonding to silica of organic compounds that contain groups such as phenyl, cyano, nitro, amino, fluoro, sulfono and diols. There are also miscellaneous chemical moieties bound to silica, as well as polymeric packings, designed to purify specific compounds. [8, 37]

Phenyl

Propylphenylsilane ligands attached to the silica gel show weak dipole–induced dipole interactions with polar analytes. Usually this type of bonded phase is used for group separations of complex mixtures. Newer phases have phenyl backbones that allow π–π (stacking) interactions. These are recommended for peptide mapping applications. Amino–compounds show some specific interactions with phenyl–modified adsorbents. [8]

Cyano

A cyano–modified surface is very slightly polar. Columns with this phase are useful for fast separations of mixtures that consist of very different components. These mixtures may show a very broad range of retention times on the usual columns. Cyano–columns can be used on both normal- and reversed–phase modes of HPLC. [8]

Amino

Amino–phases are weak anion–exchangers. This type of column is mainly used in normal phase mode, especially for protein separation and also the selective retention of aromatic compounds. [8, 37]

Fluoro

A newer type of silica packing has fluorinated surfaces. This phase is generally more hydrophilic than phases with hydrocarbons of similar chain length. It has increased retention and unique selectivity for halogenated organic compounds and lipophilic compounds. [8]

Sulfono

Sulfonic functional groups separate compounds on the basis of hydrophobic interactions. These packing materials allow the isocratic separation of mixtures that normally require gradient elution. [8, 37]

Diols

Diols are slightly polar adsorbents for normal–phase separations. These are useful to separate complex mixtures of compounds with different polarities that usually have a strong retention on unmodified silica. [8, 37]

Miscellaneous

Cyclodextrins, amylose, avidin, ristocetin, nitrophenylethyl, carbamate, ester and diphenylethyldiamine functional groups are all bound to silica packing material to enable enantiomeric separations. These columns are often referred to as chiral columns. Strong ion exchangers are also available, in which sulfonic acid groups or quaternary ammonium groups are bonded to silica. These packing materials are useful to separate proteins. There are also proprietary functional groups added to silica packing materials for a variety of uses. These include petrochemical analysis, environmental analysis; detection of deoxyribose nucleic acid (DNA) adducts, purification of double stranded DNA,
separation of cationic polymers and separation of nitro–aromatic explosives. [8, 34]

CHAPTER TWO

2.0 EXPERIMENTAL METHODS, MATERIALS AND REAGENTS

2.1 Materials / Reagents

Perchloric acid, 70% A.C.S (Aldrich), Methanol (BDH Analar grade), Potassium dihydrogen orthophosphate (BDH), Hydrochloric acid, 32% (BDH), Sodium hydroxide pellets, 99% (BDH), Phenol red, Ethanol (BDH Analar grade), Chloroform, Acetone, Methylene Chloride (BDH Analar grade), Sulphamic acid (BDH Analar grade), Sulphuric acid, 98.0% (BDH), Acetone (BDH), Acetic anhydride (BDH) and Anhydrous acetic acid (BDH) were provided by the Department of Pharmaceutical Chemistry, KNUST-Kumasi, Ghana. Alugram Sil-G fur dieDC (Macherey-Nagel-Germany) for TLC was obtained from the Chemistry Department, College of Science, KNUST-Kumasi, Ghana.

The following pure samples were obtained from Ernest Chemist in Tema.

Table 2.1 Pure Samples Used

Name	Batch No.	Date of Manu.	Date of Expiry	Assay (%)
Indometacin	0923002	Jun, 2009	Jun, 2011	99.50
Naproxen	0903201	Mar, 2009	Mar, 2012	99.40
Piroxicam	20061101	Nov, 2006	Nov, 2010	98.80
Prednisolone	K04A20081217	Dec, 2008	Nov, 2013	100.40

Also, pure sample of dexamethasone as internal standard for HPLC assay of prednisolone tablets was obtained from Letap Pharmaceuticals Limited, Accra.

Table 2.2 Pure Sample

Name	Batch No.	Date of Manu.	Date of Expiry	Assay (%)
Dexamethasone	X_2-091008	Oct., 2009	Oct., 2014	100.02

Prednisolone tablets manufactured by Letap Pharmaceuticals, M & G Pharmaceuticals, EFAH Pharmaceuticals Limited and Ernest Chemist Ltd all in Ghana were bought from pharmacy shops at Ayeduase, Tech junction and Adum all in Kumasi.

2.2 Profile of Samples

PREDNISOLONE SAMPLE	MANUFACTURING COMPANY	BATCH NUMBER	MANU. DATE	EXPIRY DATE
LE (5mg)	Letap Pharmaceuticals Ltd., Ghana	0050099	Oct, 2009	Oct, 2011
MG (5mg)	M & G Pharmaceuticals Ltd., Ghana	PD324M	Aug, 2010	Aug, 2012
EF (5mg)	EFAH Pharmaceutical Ltd., Ghana	708004	Jan, 2010	Jan, 2012
EC (5mg)	Ernest Chemist Ltd., Ghana	0108J	Aug, 2009	Aug, 2013

2.3 Instrumentation

Table 2.3 Instrumentation

Number	Instrumentation
1	EUTECH Instruments Cyberscan pH Meter
2	Cecil CE 2041 2000 Series-UV Spectrophotometer
3	Shimadzu LC-6A Liquid Chromatograph-pump
4	Hichrom H5ODS, 15cm × 4.6mm column
5	Applied Biosystems 783 programmable Absorbance Detector
6	Stuart Melting Point SMP 10 Apparatus
7	Shimadzu CR 501 Chromatopac-Integrator
8	Büchi rotary evaporator
9	Whatman Filter Paper
10	FS 28H Fisher Scientific Sonicator
11	AE ADAM PW124 Analytical balance
12	PG Instruments Ltd. T90+ UV-VIS Spectrometer

2.4 Identification Tests and Assays for Pure Samples

2.4.1 Indometacin

Identification; UV Spectrophotometry

A concentration of 0.0025%w/v of indometacin in a mixture of 1 volume of 1 M HCl and 9 volumes of methanol was prepared. An absorption maximum at 318 nm was observed. Refer to section 3.1.1

Assay

Indometacin (0.3009g) was dissolved in 75ml of acetone, through which carbon dioxide free nitrogen has been passed for 15 min. A constant stream of nitrogen through the solution was maintained. 0.1ml of phenolphthalein solution was added and titrated with 0.1M sodium hydroxide. A blank titration was then carried out.

Each ml of 0.1M sodium hydroxide is equivalent to 35.78mg of $C_{19}H_{16}ClNO_4$. Refer to section 3.1.1

2.4.2 Naproxen

Identification Test; UV Spectrophotometry

40.0mg of pure naproxen was dissolved in methanol and diluted to 100.0ml with the same solvent. 10.0ml of the solution was diluted to 100.0ml with methanol.

This was examined between 230nm and 350nm. The solution shows 4 absorption maxima at 262nm, 271nm, 316nm and 331nm. Refer to section 3.1.2

Assay

Naproxen (0.2007g) was dissolved in a mixture of 25 ml of water and 75mL of methanol.

It was then titrated with 0.1M sodium hydroxide, using 1mL of phenolphthalein solution as an indicator.

Each ml of 0.1M sodium hydroxide is equivalent to 23.03mg of $C_{14}H_{14}O_3$. Refer to section 3.1.2

2.4.3 Piroxicam

Assay

Piroxicam (0.2507g) was dissolved in 60 ml of a mixture of equal volumes of acetic anhydride and anhydrous acetic acid. It was then titrated with 0.1 M perchloric acid and the end-point was determined potentiometrically.

Each ml of 0.1M perchloric acid is equivalent to 33.14 mg of $C_{15}H_{13}N_3O_4S$. Refer to section 3.1.3

2.4.4 Prednisolone

Assay

Prednisolone (0.1008g) was dissolved in alcohol and dilute to 100.0 ml with the same solvent. 2.0 ml of the solution was diluted to 100.0 ml with alcohol. The absorbance was measured at the maximum at 243.5 nm.

Serial dilutions of various concentrations were prepared and their respective absorbances were taken and a calibration curve was plotted. Refer to section 3.1.4

Thin Layer Chromatography

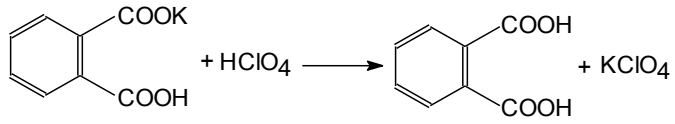
The chromatography was carried out using pre-coated plates (layer = 0.20mm silica gel 60). Significant amount of the prednisolone pure sample and the powder of the prednisolone tablet were dissolved in a mixture of 10mL volume of methanol and 9mL volumes of methylene chloride and dilute to 10mL with the same mixture of solvents. 10mg of pure prednisolone was also prepared and used as a reference. Each solution was spotted on the plate prepared and put in a mobile phase tank which was prepared by adding a mixture of 1.2 volumes of water and 8 volumes of methanol to a mixture of 15 volumes of ether and 77 volumes of methylene chloride. The plate was allowed to dry in air and examined in ultraviolet light at 254nm. The plates were sprayed with alcoholic solution of sulphuric acid, and allowed to dry until the spots appeared. It was allowed to cool and examined in ultraviolet light at 365nm. The principal spot in the chromatogram obtained with the test solution was similar in position and size to the principal spot in the chromatogram obtained with that of the reference. Refer to Fig 2.1

2.5 Melting Point Determination

The dry pure powder of each of the reference standards and analyte were introduced into separate capillary tubes sealed at one end. The solid was shaken down the tube by tapping the sealed end on a hard surface so as to form a tightly packed column from 3 to 5 mm in height. These were then placed in a melting point determination apparatus and their various melting points determined. Refer to section 3.1 for results

2.6 Standardization of Solutions

0.1M Perchloric Acid



Potassium hydrogen phthalate (0.5006g) was weighed into a 100ml conical flask. Glacial acetic acid (25ml) was added. It was then warmed for the dissolution of the salt. Subsequently, it was cooled and titrated with Perchloric acid (0.1M). Oracet blue was used as the indicator. [35] Refer to Section 3.2

0.1M Sodium Hydroxide

Sulphamic acid (0.9679g) was weighed and dissolved in a volumetric flask (100ml). 20ml of the resulting solution was delivered into a conical flask and titrated against a NaOH (0.5M) using methyl orange as the indicator. Refer to Section 3.2

2.7 Uniformity of Weight

Twenty tablets each of all the four brands of prednisolone, LE, MG, EP and EC were weighed individually. 20 tablets for the individual brands were then weighed together to obtain total weight for 20 tablets. An average tablet weight for each of the four brands was then computed and percentage deviations from average weights were deduced for each brand.

2.8 Determination of Percentage content of Prednisolone Tablets (5mg) using Standard Method from the British Pharmacopoeia

Twenty (20) tablets were weighed and powdered. A solution containing 0.005% w/v of prednisolone and 0.0075% w/v of dexamethasone (internal standard) in a mixture of 58 volumes of methanol and 42 volumes of water was prepared.

A second solution was also prepare by adding 58ml of methanol to a quantity of the powder containing 5mg of Prednisolone, shook for 10 minutes and a sufficient water was added to produce 100ml which was mixed and filtered.

A third solution was also prepared in the same manner as the second solution but 10ml of a 0.075% w/v solution of dexamethasone in methanol and 48 ml of methanol was added in place of the 58ml of methanol.

An isocratic chromatographic procedure was carried out using a stainless steel column (20 cm × 4.6 mm) packed with stationary phase Column of 5µm Spherisorb ODS, using a mobile

phase of a mixture of 42 volumes of water and 58 volumes of methanol with a flow rate of about 1ml per minute and a detection wavelength of 254nm. Refer to A.12 for results

2.9 HPLC ANALYSIS

2.9.1 Preparation of Mobile Phase

For effective pH control of the mobile phase, a phosphate buffer was settled upon. For prednisolone and its surrogate standards, a buffer of pH 5.8 and methanol in a ratio of 1:1 was found to give good resolutions in between peaks with a temperature reading of 27.1^{o}C. The phosphate buffer was prepared by weighing 13.6100g of potassium dihydrogen orthophosphate into 500mL volumetric flask. It was then dissolved with distilled water in a 500mL volumetric flask. 50mL of the buffer was placed in 200mL volumetric flask and 3.6mL of 0.2M NaOH was added and topped up with water to volume to obtain a pH of 5.8.

2.9.2 Detection of Wavelength of Maximum Absorption

0.0500g of each sample was weighed and dissolved with methanol into a 50ml volumetric flask. A quantity of each sample was poured into a cuvette and it was scanned within a wavelength range of 225 to 350nm using a T90+ UV-VIS Spectrometer from PG Instruments. Refer to Table 3.2 and Fig. 3.2, 3.3, 3.4 and 3.5 for results

2.9.3 Chromatographic Conditions

- Column: Hichrom 5ODS 4.6mm x 15cm; surface area of 300m^{2}g; pore size of 70A; manufactured by Agilent Company, USA.
- Chromatograph settings: attenuation = 2; chart speed = 5 inches per minutes
- Flow rate: 2.3ml/min
- Detector: UV-Visible detector; 254nm
- Solvent system: methanol: phosphate buffer of ratio 1:1
- Injector: 20µl

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

A stock solution of 0.05% w/v of all the surrogate reference standards were prepared and serially diluted to different concentrations. Twenty micro-litres (20µl) of the resultant

solutions were injected into the column. The peak areas were then determined. The Limit of Detection (LOD) and the Limit of Quantification (LOQ) were determined using the following formulae;

LOD = 3.3 × STD / slope; and **LOQ** = 10 × STD / slope

Where;

STD is the residual standard deviation and;

Slope is the gradient/slope of the calibration curve drawn. Refer to table A.14 for results

2.9.5 Validation Parameters

Linearity

A stock solution of 0.05% w/v of all the pure samples were prepared and serially diluted to different concentrations. Twenty micro-litres (20µl) of the resultant solutions were injected into the column. The peak areas were measured and calculated and plotted against their respective concentrations. Refer to Table A.15 for results.

Specificity and Selectivity

The Specificity and Selectivity describe the capacity of the analytical method to measure the drug in the presence of impurities or excipients. [18, 19] A quantity of 0.0010%w/v of the pure sample of prednisolone was prepared and 20µl is injected and its retention time noted. Prednisolone tablet powder of 0.0010%w/v was also prepared and 20µl is injected and its retention time was noted on the chromatogram. Refer to table A.16 for result.

Precision (Repeatability)

The precision was determined by repeatability (intra-day). The intra-day precision was calculated as the relative standard deviation (RSD) of three (3) different concentrations for six (6) replicates during the same day. Four (4) sample solutions of the pure samples (0.0500%w/v) were prepared and serially diluted and the standard deviation (SD) and RSD were calculated. Refer to table A.17, A.18, A.19 and A.20 for results

Accuracy

According to ICH, 2003; the accuracy of an analytical method can be inferred if the method developed satisfies three criteria; these are Linearity, Specificity and Precision. [18]

Determination of the Constant K using surrogate reference standards

The surrogate reference standards used were indometacin, naproxen and piroxicam. All the stock solutions of both the analyte and surrogate standards were prepared using phosphate buffer of pH 5.8 and methanol in a ratio of 1:1. Just like with the use of internal standards in normal quantitative HPLC, every successive run must contain the pure sample of the analyte and its surrogate reference standard. Stock solutions each of prednisolone (analyte) and indometacin (surrogate standard) were prepared. 1ml of each stock solution was pipetted into a 10ml volumetric flask which was thoroughly mixed by shaking or sonicating and finally made to the mark with the mobile phase thereby producing a particular concentration of both the analyte and the surrogate standard. 20µl of the resultant solution was injected via an injector into the column system and eluted isocratically with its resultant chromatograms recorded and their various peak areas calculated. Varied concentrations of both the analyte (prednisolone) and surrogate standard (indometacin) were prepared and analysed and the constant K determined for each concentration analysed. Finally, an average constant, K was determined.

The same process was repeated for the other surrogate standards of naproxen and piroxicam and their respective average constant, K determined.

2.9.6 Analysis of Commercial Samples using Surrogate Reference Standards

Prednisolone tablets of brand LE

Twenty tablets of Prednisolone of brand LE were grinded. 0.1009g of the Prednisolone powder was dissolved in 100ml of the solvent system (50ml methanol : 50ml phosphate buffer of pH=5.8). 1.92ml of the analyte and 1ml of the already prepared surrogate standard solution were subsequently dissolved in 10ml of the same solvent system which was subsequently mixed by shaking and sonicating. 20µl of the resulting solution was injected and their corresponding peak areas were recorded from the chromatograph. This procedure was repeated for all the surrogate reference standards of indometacin, naproxen and piroxicam. The content of prednisolone tablets of brand LE was thus determined with the knowledge of the average 'K' value of each surrogate standard. Refer to Table A.24, A.25 and A.26 for results

Prednisolone tablets of brand MG

Twenty tablets of Prednisolone of brand MG were grinded. 0.1000g of the Prednisolone powder was dissolved in 100ml of the solvent system (50ml methanol : 50ml phosphate buffer). 9.03ml of the analyte and 1ml of the already prepared surrogate standard solution were subsequently dissolved in 10ml of the same solvent system which was subsequently mixed by shaking and sonicating. 20µl of the resulting solution was injected and their corresponding peak areas were recorded from the chromatograph. This procedure was repeated for all the surrogate reference standards of indometacin, naproxen and piroxicam. The content of prednisolone tablets of brand MG was determined with the knowledge of the average 'K' value of each surrogate standard. Refer to Table A.27, A.28 and A.29 for result

Prednisolone tablets of brand EP

Twenty tablets of Prednisolone brand EP were grinded. 0.0506g of the prednisolone powder was dissolved in 50ml of the solvent system (50ml methanol: 50ml phosphate buffer). 6.56ml of the analyte and 1ml of the already prepared surrogate standard solution were subsequently dissolved in 10ml of the same solvent system which was subsequently mixed by shaking and sonicating. 20µl of the resulting solution was injected and their corresponding peak areas were recorded from the chromatograph. This procedure was repeated for all the surrogate reference standards of indometacin, naproxen and piroxicam. The content of prednisolone tablets of brand EP was determined with the knowledge of the average 'K' value of each surrogate standard. Refer to Table A.30, A.31 and A.32 for results.

Prednisolone tablets of brand EC

Twenty tablets of Prednisolone brand EC were grinded. 0.1033g of the prednisolone powder was dissolved in 50ml of the solvent system (50ml methanol: 50ml phosphate buffer). 1.93ml of the analyte and 1ml of the already prepared surrogate standard solution were subsequently dissolved in 10ml of the same solvent system which was subsequently mixed by shaking and sonicating. 20µl of the resulting solution was injected and their corresponding peak areas were recorded from the chromatograph. This procedure was repeated for all the surrogate reference standards of indometacin, naproxen and piroxicam. The content of prednisolone tablets of brand EC were determined with the knowledge of the average 'K' value of each surrogate standard. Refer to Table A.33, A.34 and A.35 for results.

CHAPTER THREE

3.0 RESULTS AND CALCULATIONS

3.1 Identification Tests and Assays for Pure Samples

3.1.1 Indometacin

Melting Point Determination

LITERATURE VALUE (^{o}C)	EXPERIMENTAL RANGE (^{o}C)
158 ℃ to 162 ℃	159 ℃ to 162 ℃

UV Spectrophotometry

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

= 8.45mL

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 × 8.45ml

= 0.3025g

Thus the percentage purity (%) = (0.3025/0.3035) × 100%

= 99.67%

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

Average purity is;

(99.67 + 98.69) / 2

= 99.17%

3.1.2 Naproxen

Melting Point Determination

LITERATURE VALUE (^{o}C)	EXPERIMENTAL RANGE (^{o}C)
154 ℃ to 158 ℃	154 ℃ to 157 ℃

UV Spectrophotometry

A spectrum of a methanolic solution 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}) = 0.9607 × 9.3 × 0.02303

= 0.2058g

Percentage 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%

3.1.3 Piroxicam

Melting Point Determination

LITERATURE VALUE (^{o}C)	EXPERIMENTAL RANGE (^{o}C)
198 ℃ to 200 ℃	199 ℃ to 200 ℃

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

= 0.2475g

Percentage purity of Piroxicam = (0.2475g/0.25) × 100%

= 98.98%

The second Percentage purity of Piroxicam is 98.78%

Therefore, Average Purity = (98.98 + 98.78) / 2 = 98.88%

3.1.4 Prednisolone

Melting Point Determination

LITERATURE VALUE (^{o}C)	EXPERIMENTAL RANGE (^{o}C)
230 ℃ to 235 ℃	230 ℃ to 235 ℃

Thin Layer Chromatography

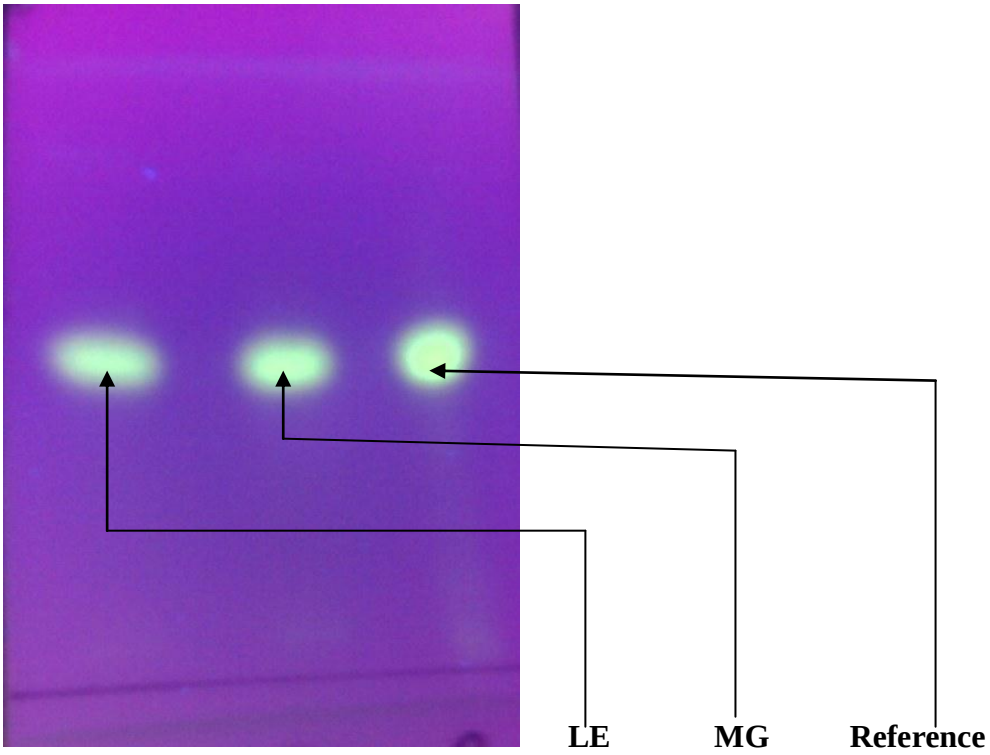


Fig 2.1 Thin Layer Chromatograph

R_f value of Tablet powder = (Distance the substance travels from the origin) / (Distance the solvent front travels from the origin)

For tablet of brand LE:

= 1.4 / 2.7

= 0.52

For tablet of brand MG:

= 1.4 / 2.7

= 0.52

For the Reference:

= 1.4 / 2.7

= 0.52

Assay

C_1 = 0.1005%, V_1 = 2mL and V_2 = 100mL

Therefore, C_1 = (2mL × 0.1005) / 100mL

= 0.00201%

From the equation of line drawn from the calibration curve;

y = 412.0x – 0.001

at y = 0.821

x = 0.001995

therefore; Percentage Purity = (Actual / Nominal) × 100%

= (0.001995 / 0.00201) × 100%

= 99.3%

3.1.5 Dexamethasone

Melting Point Determination

LITERATURE VALUE (^oC)	**EXPERIMENTAL RANGE (^oC)**
150 – 153	151 – 153

Assay

C_1 = 0.1007%, V_1 = 2mL and V_2 = 100mL

Therefore, C_1 = (2mL × 0.1007) / 100mL

= 0.002014%

From the equation of line drawn from the calibration curve;

y = 433.1x – 0.002

at y = 0.8650

x = 0.002002

therefore; Percentage Purity = (Actual / Nominal) × 100%

= (0.002002 / 0.002014) × 100%

= 99.40%

3.2 Standardization of Solutions

Standardization of 0.1M NaOH using Sulphamic acid

Factor (H_2NSO_3) = Actual weight / Nominal weight

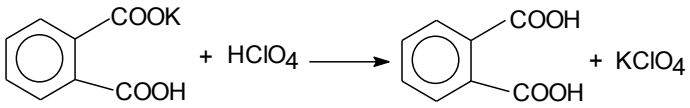
= 2.2024g / 2.4518g

= 0.8983

Factor (NaOH) = [Factor (H_2NSO_3) × Volume (H_2SO_3)] / Volume (NaOH)

Factor (NaOH) using Methyl Orange as the indicator = [0.8983 × 20] / 18.70 = 0.9607

Standardization of 0.1M Perchloric acid



mole ratio = 1:1

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

Average titre = 25.20ml

Expected endpoint ⇒ 2.0422g = 100ml of 0.1M Perchloric acid, therefore 0.5g = 24.48ml

99% = 24.48ml

Therefore 100% = 24.50; Factor = 25.20ml / 24.50ml = 0.9469

3.3 Determination of Percentage Content of Prednisolone in Prednisolone tablets (5mg) using a Standard Method from the British Pharmacopoeia.

Product brand: LE

In HPLC quantitative analysis, for a formulated drug;

Percentage Content = $(A_t / A_p) \times 100\%$

where;

A_t = Peak Area ratio of the formulated tablet

A_p = Peak Area ratio of pure sample

Also where;

Peak Area ratio of formulated drug = Area of formulated drug / Area of internal standard

Peak Area ratio of pure sample = Area of pure sample / Area of internal standard

Area = ½ base × height

Area (pure sample) = ½ (1.5mm) × 94mm = $70.5mm^2$

Area (internal standard) = ½ (1.5mm) × 122mm = $91.5mm^2$

therefore "peak area ratio" = area of pure sample / area of internal standard (dexamethasone)

= $70.5mm^2$ / $91.5mm^2$

= 0.7705

Area (prednisolone tablet) = ½ (1.5mm) × 107mm = $80.25mm^2$

Area (internal standard) = ½ (1.5mm) × 134mm = $100.5mm^2$

therefore "peak area ratio" = area of pred. tablet / area of internal standard (dexamethasone)

= $80.25mm^2$ / $100.5mm^2$

= 0.7985

Therefore; Percentage Content(%) = $(A_t / A_p) \times 100\%$

= (0.7985 / 0.7705) × 100%

= 103.64%

Table 3.1 Results obtained from the analyses

Prednisolone Sample	Batch Number	Percentage Content (%)				
		Sample 1	*Sample 2*	*Sample 3*	*Sample 4*	*Sample 5*
LE	0050099	103.64	108.92	107.23	104.63	104.62
MG	PD324M	103.33	102.65	102.77	102.02	104.01
EP	708004	117.02	116.01	115.23	119.15	119.67
EC	0108J	107.38	108.03	105.65	106.98	106.44

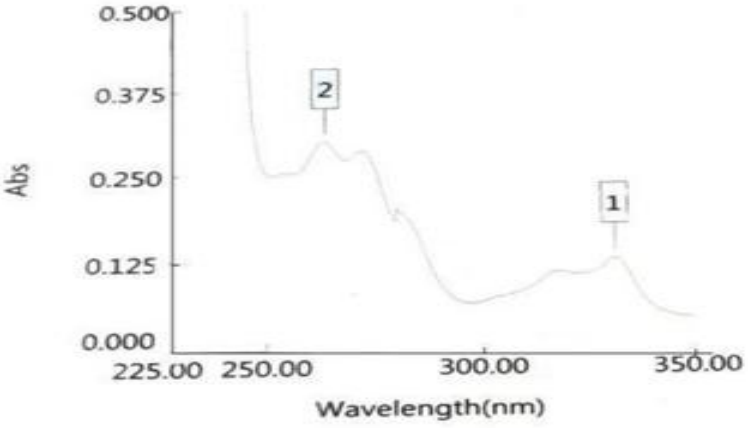
3.4 Method Development

3.4.1 UV Spectrum of samples

Table 3.2 Spectra analysis of pure samples

Sample	Wavelength range (nm) of Absorbance	Wavelength of Maximum absorbance
Prednisolone	225.00 – 350.00	243.5
Indometacin	225.00 – 350.00	320.0
Naproxen	225.00 – 350.00	331.0
Piroxicam	225.00 – 350.00	242.0

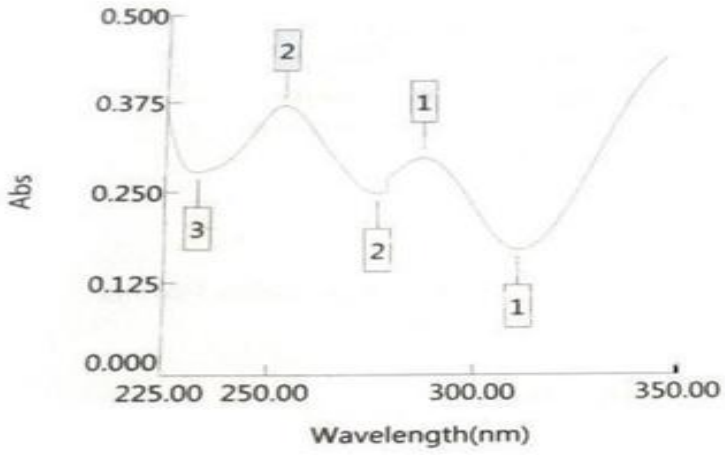
Fig 3.2 A UV spectrum of pure sample of Naproxen



Comment :

No.	P/V	Wavelength(nm)	Abs	Comment
1	Peak	331.15	0.130	
2	Peak	262.30	0.303	
3	Peak	231.50	4.175	

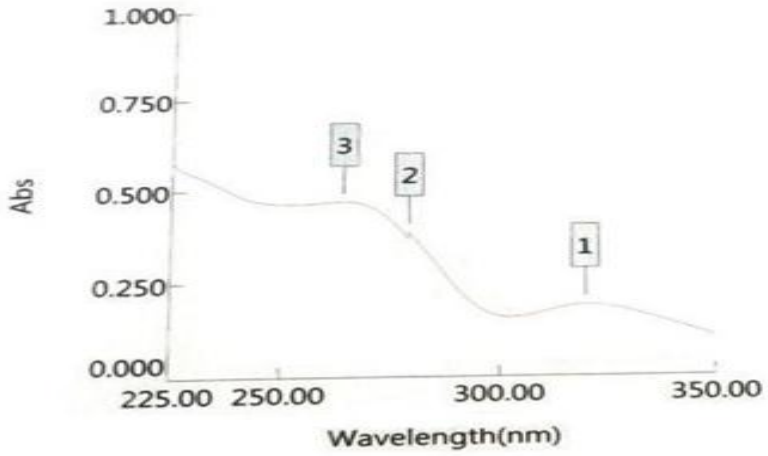
Fig 3.3 A UV spectrum of pure sample of Piroxicam



Comment

No.	P/V	Wavelength(nm)	Abs	Comment
1	Peak	288.45	0.300	
2	Peak	254.10	0.373	
1	Valley	311.25	0.172	

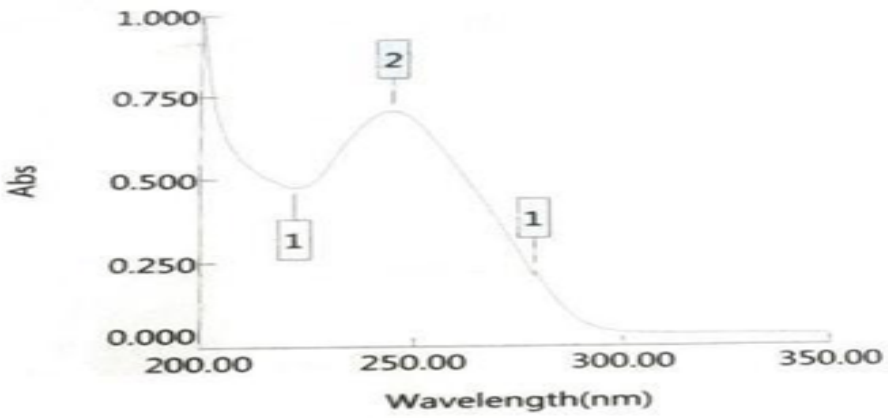
Fig 3.4 A UV spectrum of pure sample of Indometacin



Comment :

No.	P/V	Wavelength(nm)	Abs
1	Peak	320.25	0.194
2	Peak	279.40	0.392
3	Peak	264.30	0.475

Fig 3.5 A UV spectrum of Prednisolone pure sample



Comment :

No.	P/V	Wavelength(nm)	Abs	Comment
1	Peak	279.40	0.227	
2	Peak	246.20	0.710	
1	Valley	222.30	0.478	

3.4.2 Retention times

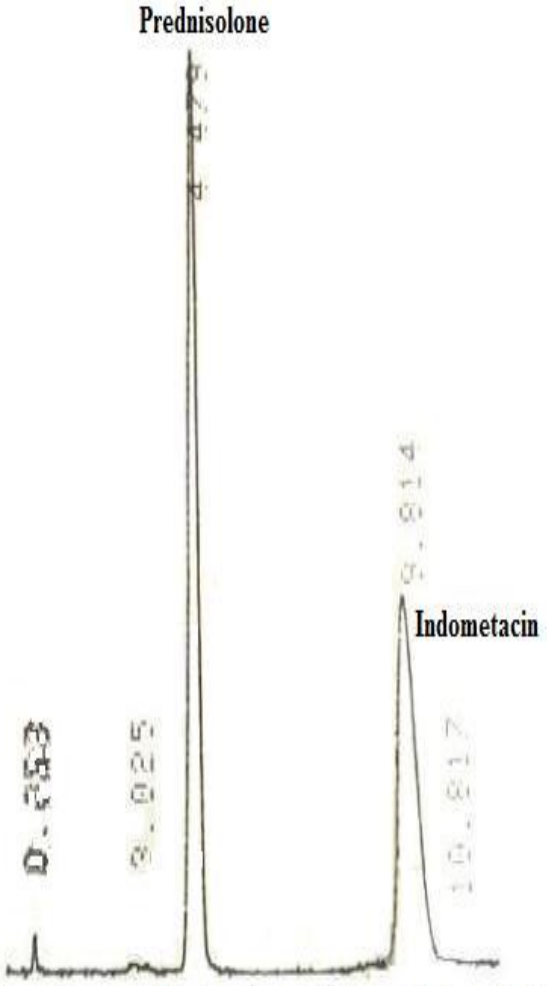
Table 3.3 Mean Retention Time for both drug and surrogates

Samples	Mean Retention Time (min)
Prednisolone	4.30 ± 0.7
Indometacin	9.79 ± 0.3
Naproxen	2.25 ± 0.6
Piroxicam	1.26 ± 0.3

3.4.3 Chromatograms

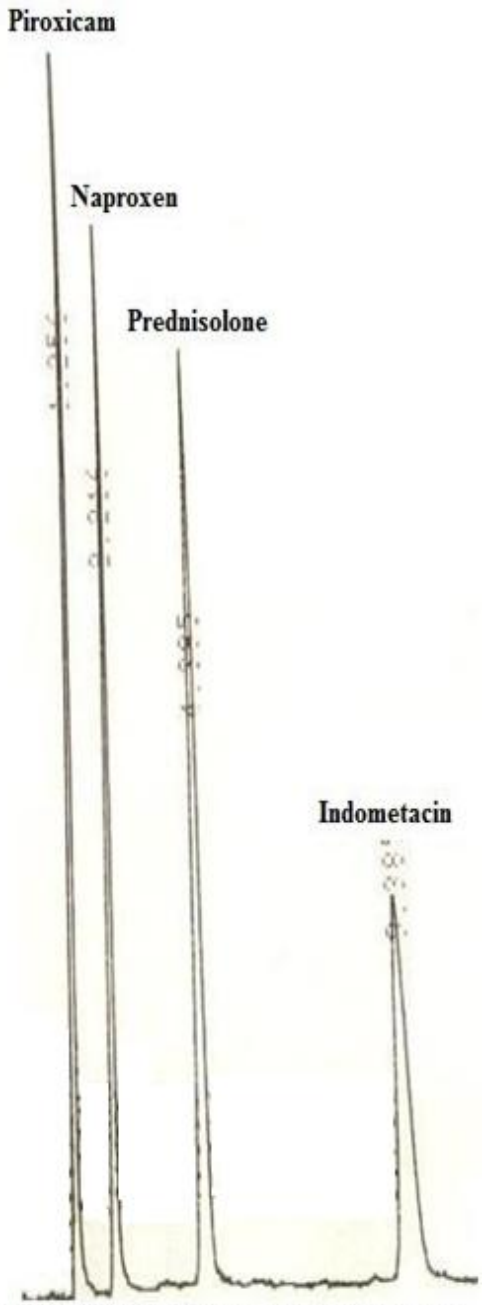
A chromatogram of Prednisolone tablet maufactured by LE Pharmaceuticals Ltd.

A chromatogram of Prednisolone tablet manufactured by LE Pharmaceuticals and Naproxen(surrogate standard)

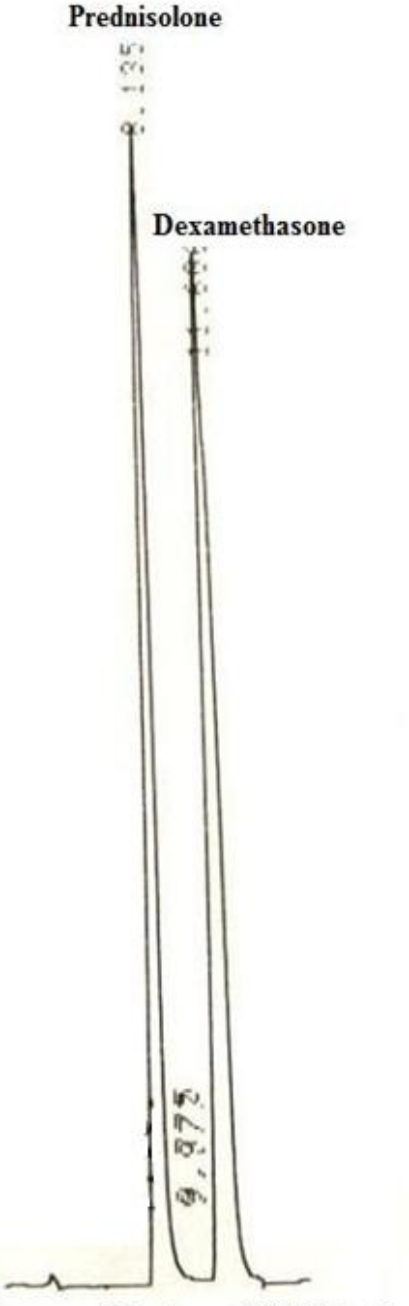


A chromatogram of Prednisolone tablet manufactured by LE Pharmarceuticals and Indometacin(surrogate standard)

A chomatogram of Prednisolone tablet manufactured by MG Pharmaceuticals Ltd and Piroxicam(surrogate standard)



A chromatogram of Prednisolone tablet manufactured by LE Pharm. Ltd and all 3 surrogate standards



A chromatogram of the Assay (BP 2007) of Prednisolone tablet from LE Pharmaceuticals and Dexamethasone(internal standard).

3.5 Statistical Analysis

3.5.1 Calculation of Limit of Detection (LOD) and Limit of Quantification (LOQ)

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

where;

σ = residual standard deviation ie. $\boldsymbol{\sigma_{res}} = \{\Sigma(Y - Y_{est}) / n\text{-}1\}^{1/2}$, also where: Y = y values (Area) from a calibration curve and Y_{est} = y values calculated using the equation of line

$y = mx + c$

S = the slope of the equation of line from the calibration curve drawn.

For example; the following concentrations (%w/v) of Prednisolone were injected (0.0012, 0.0010, 0.009, 0.0011, 0.0007, 0.0006, and 0.0004) and their corresponding peak Areas (cm^2) were noted as (1.1109, 0.9290, 0.8207, 1.0178, 0.6450, 0.5637 and 0.3740).

The values for Y_{est} (ie. Y estimated) were calculated using the equation of line;

$y = 918.6x + 0.005$.

For Y = 1.1109 and x = 0.0012; Y_{est} = 1.10732: and so on and so forth

Hence, σ_{res} (for the sum of $Y - Y_{est}$) = 0.006218

From the equation of line, $y = 918.6x + 0.005$

S (slope) = 918.6

Thus, LOD = $(3.3 \times 0.006218) / 918.6$

= 0.00002234% w/v

And, LOQ = $(10 \times 0.006218) / 918.6$

= 0.00006769% w/v

3.5.2 Validation Parameters

Linearity

Concentrations were prepared for Prednisolone pure samples between the ranges of 0.0004 – 0.0012 and their corresponding areas in cm^2 noted. This was then used to plot a calibration graph with an equation of line; $y = 924.5x + 0.004$ and R^2 of 0.9990. Refer to Fig A.4

Specificity and Selectivity

These parameters were determined by comparing the chromatograms of the prednisolone pure sample with that of prednisolone tablet with references to their mean retention times with a mean retention time of 4.32min and 4.31min respectively. Refer to Table A.16

Precision (Repeatability)

Relative Standard Deviation, RSD (%) = (Sd × 100%) / x

where; Sd = Standard deviation of the Area of the pure drug

x = the mean of the Area of the pure drug

For a concentration of 0.0012%w/v of prednisolone in six replicates, its corresponding areas (cm^2) are 1.340; 1.326; 1.340; 1.340; 1.326 and 1.330.

The mean, x = (1.340 + 1.326 + 1.340 + 1.340 + 1.326 + 1.330) / 6

= 1.3337

$Sd = \sqrt{\{(x - x)^2 / N-1\}}$

= 0.0071

Thus, RSD = (S_d × 100%) / x

= (0.0071 × 100%) / 1.3337

= 0.53%

Accuracy

Since the analytical method developed is able to satisfy Linearity, Selectivity and Specificity and Precision; Accuracy can thus be inferred. [18]

3.6 Sample Calculation of Percentage Content of Prednisolone in Prednisolone Tablets from brand LE

0.1009g of Prednisolone powder was weighed and dissolved in 100mL of the solvent system (50mL of phosphate buffer and 50mL of methanol). 2.1mL of the resulting solution was dissolved in 10mL of the same solvent system. 20µL was then injected.

Average weight of tablet = 0.0977g.

0.0977g ≡ 5mg of Prednisolone;

$$0.1009g \equiv (0.005g \times 0.1009g) / 0.0977g$$
$$\equiv 0.0052g$$

Hence, 0.1009g will contain 52mg of pure Prednisolone.

Therefore, 0.0052g of prednisolone was actually dissolved and serially diluted to make a concentration of 0.0011%w/v and injected to give a peak Area of 1.47cm^2.

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

Surrogate standard = Indometacin; Analyte = Prednisolone.

Area of analyte, Aa = 1.2802; Concentration of analyte, Ca = 0.0010%w/v

Area of standard, As = 0.8980; Concentration of standard, Cs = 0.0013%w/v

K value = (1.2802cm^2 × 0.0013%w/v) / (0.8980cm^2 × 0.0010%w/v)

= 1.8533.

Four different concentrations for both analyte were prepared and their respective peak areas in cm^2 computed. Refer to table A.21, A.22 and A.23 for results.

Thus the Average K value (Indometacin) = 1.8323

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

» (1.47cm^2 × 0.0010%w/v) / (1.8323 × 0.6978cm^2)

» 0.001470 / 1.278579

= 0.001150% w/v

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

= (0.001150 / 0.001100) × 100%

= 104.55%

3.7 Comparison of the Method Developed with Standard Method (BP 2007) using T-Test.

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

where;

X_d = the mean difference between paired values,

S_d = the estimated standard deviation of the differences and

N is the number of values within the sets (5).

Calculating for T_{exp};

The percentage contents (%) for pure prednisolone in prednisolone tablet from LE prednisolone brand, using a standard method in BP 2007 and developed method (with indometacin as a surrogate standard) were 103.64%; 108.92%; 107.23%; 104.63%; 104.62% and 104.18%; 104.55%; 105.55%; 105.27% 103.82% respectively.

$X_d = \{(-0.54) + 4.37 + 1.68 + (-0.64) + 0.80\} / 5$

$= 1.134$

$S_d = 2.051$

$N = 5$

Therefore, $t_{exp} = (X_d / S_d) \times \sqrt{N}$

$t_{exp} = (1.134 / 2.051) \times \sqrt{5}$

$= 1.24$

Hence t_{exp} for Indometacin from prednisolone sample LE is 1.24. Table A.36, A.37, A.38 and A.39

CHAPTER FOUR

4.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

4.1 DISCUSSION

4.1.1 Identification tests and Assays

Identification tests and assays serve to identify and determine the percentage purity of pharmaceutical starting materials, intermediates and finished materials to ensure compliance with appropriate standards and specifications. Hence identification tests were done to ascertain the identity of the samples and to ensure that all samples have been labelled correctly. [38]

Indometacin:

The percentage purity of indometacin when it was analyzed was 99.67%; 98.69% with an average of 99.17%. The British Pharmacopoeia states that, its percentage purity ought to be between 98.5% and 102.5%. Indometacin has an experimental melting point range of 159^0C to 162^0C. Its literature value is 158^0C to 162^0C. The sample analysed is surely Indometacin.

Naproxen

The percentage purity of naproxen when it was analyzed was 98.50%; 99.80% with an average of 99.15%. The British Pharmacopoeia states that, its percentage purity ought to be between 99.00% and 101.0%. Naproxen has an experimental melting point range of 154^0C to 157^0C. Its literature value is 154^0C to 158^0C. The sample analysed is definitely Naproxen.

Piroxicam

The British Pharmacopoeia states that, its percentage purity ought to be between 98.5% and 100.5%. The percentage purity of Piroxicam when it was analyzed was 98.98%; 98.78% with an average of 98.88%. Piroxicam has an experimental melting point range of 199^0C to 200^0C. Its literature value is 198^0C to 200^0C. The sample analysed is definitely Piroxicam.

Prednisolone

The percentage purity of prednisolone when it was analyzed was 99.30%. The British Pharmacopoeia states that, its percentage purity ought to be between 97.0% and 103.0%.

Prednisolone has an experimental melting point range of 230^0C to 235^0C. Its literature value is 230^0C to 235^0C. The R_f values of the powdered tablets from both prednisolone tablets LE and MG were 0.52 compared with that of the reference which also gave 0.52. The sample analysed is therefore Prednisolone.

Dexamethasone

The percentage purity of dexamethasone when it was analyzed was 99.40%. The British Pharmacopoeia states that, its percentage purity ought to be between 97.0% and 103.0%. Dexamethasone has an experimental melting point range of 150 - 153^0C. Its literature value ranges from 151-153^0C with subsequent decomposition. The sample analysed is definitely Dexamethasone.

4.1.2 Uniformity of Weight

Table 4.1 Uniformity of weight of tablets

Average weight of tablet	Percentage deviation permissible
80mg or less	± 10
More than 80mg and less than 250mg	± 7.5
250mg or more	± 5

The average weight of a tablet is determined by weighing 20 tablets selected at random, and not more than two tablets should deviate from the average weight by a greater percentage than that shown in **Table 4.1** and none should deviate by more than twice that percentage. [7]

The average weight obtained when uniformity test was carried out on prednisolone tablets LE was 97.7mg; prednisolone tablets MG was 180.6mg; prednisolone tablets EP was 206.74mg; and prednisolone tablets EC was 99.7mg.

A critical look at **Table A.8** for average weight of 80mg or less shows that none of the tablets deviated. For **Table A.9**, two tablets deviated having a percentage deviation of 10.4651 and 10.4097. None of the tablets in both **Table A.10** and **A.11** deviated. The batch of Prednisolone tablets which were taken through the uniformity test are therefore within the control limits of pharmacopoeial standards.

4.1.3 Determination of Percentage Content of Prednisolone in Prednisolone tablets using Standard Method from the British Pharmacopoeia (2007).

Various brands of prednisolone LE, MG, EP and EC from four (4) different pharmaceutical companies in Ghana were analysed using standard methods from the British Pharmacopoeia. Prednisolone tablets of brand LE gave an average percentage content of 105.81%. Prednisolone tablets of brand MG gave an average percentage content of 102.96%. Prednisolone tablets of brand EP gave an average percentage content of 117.42%. Prednisolone tablets of brand EC gave an average percentage content of 106.90%.
The British pharmacopoeia states that Prednisolone tablets should have 90.0% to 110.0% of Prednisolone. This implies that, apart from the Prednisolone tablets EP which deviated from the acceptable range, all the Prednisolone tablets analysed passed the test.

4.1.4 Method Development

Virtually, all organic compounds can be analysed by UV-Visible detectors. It has been published that almost 70% of HPLC analyses have been performed with UV-Visible detectors since its operation is quite easy hence its world wide use in liquid chromatography as a detector. UV-Visible spectra of all the pure samples were recorded so as to determine their maximum wavelength of absorption. Eventually, a wavelength of 254nm was chosen.

The solubility properties of the compounds were important because HPLC deals solely with liquid, that is, every sample has to be in the liquid state before the analysis can be made possible. There was therefore the need to select a solvent in which the selected compounds could easily dissolve. Moreover, the compounds used in this project have ionizable functional groups which are vastly influenced by pH changes of the mobile phase. Consequently, phosphate buffer was settled upon with pH ranges of **0.6 – 3.60; 5.70 – 8.70; 10.8 – 13.80** were used. The pH values of all the samples were within the range of 5.20 – 8.25 therefore, all the samples would give a good peak within these pH values.

Emphasis was placed on shorter run time, resolution of key components, optimizing selectivity, and understanding potential interferences by reaction components. The chosen method was highly selective for all the surrogate reference standards.

Eventually, a pH of 5.8 ± 0.1 was chosen as the combination with methanol in a ratio of 1:1 as mobile phase was able to give well resolved peaks coupled with virtually no interference at a wavelength of 254nm.

4.1.5 Chromatogram

Both Naproxen and Indometacin have carboxylic acid functional groups. Generally, all carboxylic acids are polar substances and therefore form very strong hydrogen bonds with each other and water. Acids generally show increase in retention times which decreases down to the basic region as the pH is reduced. This phenomenon explains the high retention time of both naproxen and indometacin with mean retention times of 2.25 ± 0.6(min) and 9.79 ± 0.3(min) respectively. Piroxicam is a basic drug and that accounts for its retention time of 1.26 ± 0.4(min) and Prednisolone which is more of a neutral compound with a mean retention time of 4.30 ± 0.4(min).

4.1.6 Determination of the Constant K

The respective values for the analytes and the surrogate reference standards were inserted into the formula for the determination of K values. The various K values for the analytes were determined. Thus, it was observed for each of the surrogate reference standards that, a change in concentration or other wise of the analyte has no significant effect on the K value.

Table A.21, A.22 and A.23

4.1.7 Determination of Percentage Content

The percentage content of Prednisolone in prednisolone tablets was determined using the K values. The British pharmacopoeia gives a limit of 90 to 110.0% of the stated amount for Prednisolone tablets. A critical look at **Tables A.24** – **A.26**, **A.27** – **A.29**, **A.30** – **A.32** and **A.33** – **A.35** revealed that, all samples analysed fell within the control limits in the British pharmacopoeia except for the brand of tablets in **Table A.30 – A.32** which fell outside the acceptable range and this also failed the standard method from BP 2007.

4.1.8 Statistical Analysis (Validation)

Linearity

The analytical curve for pure prednisolone was constructed by plotting the area under the curve (AUC) of the main peak versus drug concentration. It was found to be linear over a wide concentration range (0.0004 – 0.0012%w/v) with a correlation coefficient of 0.9990. The straight line equations obtained from the experimental results were found to be;

$y = 924.5x + 0.004$;

$y = 923.1x + 0.003$; and

$y = 918.6x + 0.005$;

The RSD of the slope and of the intercept of the three lines were 0.53% and 0.38%, and 0.70% respectively. Thus, this HPLC method can be considered to show adequate linearity in the concentration range (0.0004 – 0.0012%w/v) for quantitative analysis of prednisolone under the experimental conditions described. The detailed Linearity data are shown at **Table A.15.**

Selectivity and Specificity

The specificity and selectivity describe the capacity of the analytical method to measure the drug in the presence of impurities, excipients, degradation products or matrix components (ICH 2003; USP 2004). These parameters were determined by comparing the chromatograms of the prednisolone pure standard with that of the prednisolone tablet.

The chromatogram of the prednisolone pure standard presented a peak in mean time retention of 4.32 ± 0.1 (min). The chromatogram peaks are well resolved, indicating the high specificity of the method. The retention time of 4.32 ± 0.1 (min) is a good value for routine procedures in quality control. In fact, compared to values obtained from BP2007 with a retention time of 8.135 min, the method developed proved advantageous, with a shorter retention time.

Precision

The precision refers to the variability of the results in repeated analyses of the sample under identical experimental conditions. The method was validated by evaluating the intra-day precision. The precision was calculated from nine determinations of four homogeneous samples.[19] The intra-day precisions (prednisolone) were expressed as relative standard

deviation (RSD) 0.38, indicating that the method presents a good precision. [18] The detailed precision data are shown at **Table A.17 to A.20.**

Accuracy

Since parameters such as Linearity, Selectivity and Specificity and Precision met the accepted criteria of analytical method that was developed, Accuracy can thus be inferred. [18] Therefore the method developed can best be described to show a high degree of accuracy.

Limit of Detection

The Limit of Detection (%w/v) was determined to be 0.00041; 0.000040; 0.000049; and 0.000022 for Indometacin, Naproxen, Piroxicam and Prednisolone respectively.

Limit of Quantification

The Limit of Quantification (%w/v) was determined to be 0.000121; 0.000120; 0.000150; and 0.000068 for Indometacin, Naproxen, Piroxicam and Prednisolone respectively.

Comparison of Developed Method to that of Standard Method using T-test

The T-test is used to compare the experimental means of two sets of data or to compare the experimental mean of one set of data with a known or reference value. A statistic, t, is thus defined as; $t = (X_d \times \sqrt{N}) / S_d$. [11]

For four (4) degrees of freedom, the critical values of "t" at the 99% (P = 0.01) levels for a two-tailed test is 4.60.

The t_{exp} of Prednisolone tablets from brand LE were 1.24, 1.83 and 1.72 for Indometacin, Naproxen and Piroxicam respectively.

Prednisolone tablets MG had the following t_{exp} values; 0.53, 1.46 and 0.05 for Indometacin, Naproxen and Piroxicam respectively.

Prednisolone tablets from EP had the following t_{exp} values; 0.87, 2.40 and 2.53 for Indometacin, Naproxen and Piroxicam respectively.

Also, the t_{exp} of Prednisolone tablet from brand EC were, 1.39, 0.75 and 0.58 for Indometacin, Naproxen and Piroxicam respectively.

From the above values, as t_{exp} is less than t_{tab} at 99% probability levels for all tablets, there was no significant difference between the means of the standard method (BP 2007) and the method developed.

5.0 CONCLUSION

The major objective for this project was to find the possibility of using surrogate reference standards for the analysis of **Prednisolone** tablets. Thus, the method developed can effectively replace the conventional and traditional methods available in the pharmacopoeias.

A mobile phase system of phosphate buffer of pH 5.8 ± 0.1 and methanol in the ratio of 1:1 was found to give well resolved chromatograms between the peaks of prednisolone and its surrogate reference standards. The retention time of 4.31 ± 0.1min for prednisolone tablet is a good value for routine procedures in quality control which also proved advantageous with a shorter retention time than the standard method in BP 2007.

The **K** values for **Indometacin**, **Naproxen**, and **Piroxicam** were 1.8323, 1.6907 and 1.3550 respectively.

The results show that the HPLC method presented here can be considered very suitable owing to its high selectivity and specificity, linearity in the concentration range and high precision and adequate accuracy at the concentrations studied.

6.0 RECOMMENDATION

Surrogate reference standards must be found for the analyses of other pharmaceutical preparations to ease the burden on manufacturing companies, research institutions, educational and regulatory bodies especially in developing countries like Ghana as reference standards are becoming expensive.

7.0 REFERENCES

1. Meyer V.R Practical HPLC. Fourth Edition. Wiley Publications. Pp 1 - 23
2. Ajibola A. Olaniyi, Principles of Drug Quality Assurance and Pharmaceutical Analysis, 2000; Mosuro Publishers, Ibadan, pp 116 – 129, 453 - 456.
3. http://www.pharminfo.net/reviews/introduction to analytical method development in pharmaceutical formulations
4. Miller J.C, Statistics for Analytical Chemistry. 1993, Croper Street: Eliss Horwood Limited, Market Cross House, pp. 30 - 48.
5. Jackson J. V., Moss M. S., Widdop B., Clarkes's Isolation and Identification of Drugs. Second ed. 1986; The pharmaceutical press, London; pp 201 – 213; 221 – 236.
6. United States Pharmacopoeia and NF, 2004
7. British Pharmacopoeia, 2007
8. Clarks; Introduction to HPLC. pdf file
9. British National Formulae, 2007
10. Beckett A. H., Stenlake J.B., Practical Pharmaceutical Chemistry Part Two. Fourth in ed. 1988: Printed in England at the University Press, Cambridge, pp 281 – 282
11. Kealey D., P.J. Haines., Instant Notes on Analytical Chemistry. Printed by BIOS Scientific Publishers Limited, 2002 pp 26 – 53.
12. Jackson J. V, Moss M. S., Widdop B., Clarkes's Isolation and Identification of Drugs. Second ed. 1986; The pharmaceutical press, London; pp 201 – 213; 221 – 236.
13. Kemp, W., Organic Spectroscopy. Third Ed; printed in china; pp 30 - 34
14. NMR – From Spectra to Structures An Experimental Approach Second Revised and Expanded Edition. Pages 1-3
15. http://en.wikipedia.org/wiki/ Infrared spec
16. Kevin M. Downnard, Dominic M. Desiderio Wiley Interscience Series on Mass Spectrometry Pages 1-3
17. Braithwaite F. J. Smith Department of Physical Sciences Trent Polytechnic, N., Chromatographic Methods, Great Britain: Chapman and Hall, pp 253 - 256.
18. International Conference on Harmonization (ICH); validation of analytical procedures: Methodology, Q2B (CPMP/ICH/281/03), 2003.
19. United States Pharmacopoeia and National Formulae, 2004
20. Melentyeva G. Antonova L. Pharmaceutical Chemistry.

Mir Publishers, Moscow. Pp 21 – 59.

21. Smyth JM, Collier PS, Darwish M. Indometacin and Aspirin; www.wikipedia.com
22. Peter JH, Eric L. "Twenty Years of Naproxen Technology"; www.wikipedia.com
23. Mammen L, Schmidt CP "Piroxicam"; www.wikipedia.com
24. Davis M, Williams R, Chakraborty J; "Prednisolone" www.wikipedia.com
25. Introduction to Titrimetry and Chemical Analyses Methods. pdf file
26. Shulamit L. M. Introduction to HPLC Modes. pdf file
27. Complexometric titration on EDTA. www.answers.com
28. Alexander E. Introduction to HPLC. L5. pdf file
29. Armelle V. Good Laboratory Practice HPLC. pdf file
30. Henry R. A., Design of Volatile Buffer Systems for LC Applications: Keystone Scientific, Inc. Bellefonte, PA. Pp 23 - 27
31. http://teachline.Is.huji.ac.il/72682/Booklets/Amersham RPC Manual. pdf
32. Neue, U.D., Separations Solutions: Mobile Phase pH. March 1999: American Laboratory. page 60.
33. Dong M. W., Mordern HPLC for Practicing Scientist. 2006, Hoboken, New Jersey: John Wiley & Sons, Inc., pp 87 – 96, 195 – 219.
34. http://www.inertsil.com/Technical-Data/Bonding.pdf
35. Ajibola A. O., Ogungbamila F.O., Experimental Pharmaceutical Chemistry. 1998; Shaneson C. I. Limited, Ibadan; page 34.
36. StandardBase Techniques: High Performance Liquid Chromatography Drenthe College, The Netherlands pdf file pages 1-8
37. Chrom-Ed Book Series Raymond P. W. Scott Liquid Chromatography.pdf file page 2-8
38. 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.

APPENDIX

P.1.0 Preparation of Solutions

P.1.1 Preparation of 0.1M Sodium Hydroxide

1g NaOH in 250ml ≡ 0.1M NaOH

98% = 1.0g therefore 100% = 1.0204g x 1.0107 = 1.0313g; Actual weight = **1.0311g**

1.0311g of sodium hydroxide was weighed into a 25ml beaker. It was then dissolved in the beaker and transferred into a 250ml volumetric flask using a funnel. It was stoppered and swirled for thorough mixing. The 250ml volumetric flask was finally filled to the mark with distilled water and stoppered.

P.1.2 Preparation of 0.1M Perchloric Acid

In order to avoid the explosive acetyl perchlorate from being formed, 0.82ml of Perchloric acid was diluted with 90ml of Glacial acetic acid and 3.2ml Acetic anhydride was subsequently added. The mixture was cooled to room temperature and finally topped up to the 100ml mark with Glacial acetic acid. It was then allowed to stand for 24 hours before use (to compete the reaction between the acetic anhydride and the water).

P.1.3 Preparation of Sulphamic acid

$H_2NSO_3 + NaOH \rightarrow H_2NSO_3Na + H_2O$

97.09g H_2NSO_3 in 1000ml ≡ 1M NaOH

4.8545g H_2NSO_3 in 100ml ≡ 0.5M NaOH

Actual weight = 4.8397g

4.8397g of Sulphamic acid was weighed and dissolved in 100ml distilled water.

Titration Tables

Table A.1 Assay of Pure Indometacin

Burette Readings	**1st (0.3035g)**	**2nd (0.3030g)**	**Blank**
Final reading, mL	9.60	19.50	0.80
Initial reading, mL	0.00	10.00	0.00
Titre value, mL	9.60	9.50	0.80

Table A.2 Assay of Pure Naproxen

Burette Readings	**1st (0.2090g)**	**2nd (0.2039)**
Final reading, mL	9.40	18.60
Initial reading, mL	0.10	9.50
Titre Value, mL	9.30	9.10

Table A.3 Assay of Piroxicam

Burette 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 A.4 Standardization of 0.1M Perchloric acid

Burette reading(ml)	**1st determination**	**2nd determination**	**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

Table A.5 Standardization of 0.1M NaOH

Burette Reading	**1st**	**2nd**	**3rd**
Final Reading, mL	18.7	38.4	18.7
Initial Reading, mL	0.00	19.7	0.00
Titre Value, mL	18.7	18.7	18.7

Table A.6 Assay of Pure Prednisolone by UV Spectrophotometry

Concentration, %w/v	**Absorbance**
0.0020	0.8210
0.0010	0.4120

0.0005	0.2000
0.0015	0.6210
0.0013	0.5300
0.0003	0.1040

Table A.7 Assay of Pure Dexamethasone by UV Spectrophotometry

Concentration, %^{w}/v	**Absorbance**
0.0020	0.8650
0.0010	0.4350
0.0005	0.2130
0.0015	0.6450
0.0013	0.5610
0.0003	0.1280

Table A.8 Uniformity of Weight for Prednisolone Tablets LE

Prednisolone Tablets, 5mg			
Prednisolone sample : LE			
Batch Number : 0050099			
Weight of 20 Tablets = 1.9548g			
Average Weight = 0.0977g			
Number	**Individual wt (g)**	**Deviation**	**% Deviation**
1	0.1002	0.0025	2.5589
2	0.0911	-0.0066	-6.7554
3	0.0987	0.0010	1.0235
4	0.0942	-0.0035	-3.5824
5	0.0968	-0.0009	-0.9212
6	0.0989	0.0012	1.2283
7	0.0972	-0.0005	-0.5118
8	0.0965	-0.0012	-1.2283
9	0.0952	-0.0025	-2.5589
10	0.0991	0.0014	1.4330
11	0.0993	0.0016	1.6377
12	0.0973	-0.0004	-0.4094
13	0.0937	-0.0040	-4.0942
14	0.0880	-0.0097	-9.9284
15	0.0980	0.0003	0.3071
16	0.0972	-0.0005	-0.5118
17	0.0980	0.0003	0.3071
18	0.1003	0.0026	2.6612
19	0.0986	0.0009	0.9212
20	0.1027	0.0050	5.1177

Table A.9 Uniformity of weight for Prednisolone Tablets MG

Prednisolone Tablets, 5mg			
Prednisolone sample : MG			
Batch Number : PD324M			
Weight of 20 Tablets = 3.6118			
Average Weight = 0.18059g			
Number	**Individual wt (g)**	**Deviation**	**% Deviation**
1	0.1856	0.0050	2.7686
2	0.1896	0.0090	4.9334
3	0.1796	-0.0010	-0.5537
4	0.1840	0.0034	1.8826
5	0.1940	0.0134	7.4197
6	0.1770	-0.0036	-1.9934
7	0.1617	-0.0189	-10.4651
8	0.1764	-0.0042	-2.3256
9	0.1842	0.0036	1.9934
10	0.1734	-0.0072	-3.9867
11	0.1941	0.0135	7.4751
12	0.1839	0.0033	1.8272
13	0.1797	-0.0009	-0.4983
14	0.1895	0.0089	4.9280
15	0.1857	0.0051	2.8239
16	0.1733	-0.0073	-4.0421
17	0.1843	0.0037	2.0487
18	0.1763	-0.0043	-2.3810
19	0.1618	-0.0188	-10.4097
20	0.1769	-0.0037	-2.0487

Table A.10 Uniformity of Weight for Prednisolone Tablets EP

Prednisolone Tablets, 5mg			
Prednisolone sample : EP			
Batch Number : 708004			
Weight of 20 Tablets = 4.1347g			
Average Weight = 0.2067g			
Number	**Individual wt (g)**	**Deviation**	**% Deviation**
1	0.2114	0.0047	2.2565
2	0.2034	-0.0033	-1.6132
3	0.2044	-0.0023	-1.1295
4	0.2011	-0.0056	-2.7257
5	0.2031	-0.0036	-1.7583
6	0.2020	-0.0047	-2.2904
7	0.2011	-0.0056	-2.7257
8	0.2201	0.0134	6.4648
9	0.2052	-0.0015	-0.7425
10	0.2015	-0.0052	-2.5322
11	0.2104	0.0037	1.7728
12	0.1966	-0.0101	-4.9024
13	0.2039	-0.0028	-1.3713
14	0.2167	0.0100	4.8202
15	0.2128	0.0061	2.9337
16	0.1993	-0.0074	-3.5964
17	0.2144	-0.0077	-3.7077
18	0.2111	0.0044	2.1114
19	0.2084	0.0017	0.8054
20	0.2040	-0.0027	-1.3229

Table A.11 Uniformity of weight for Prednisolone tablets EC

Prednisolone Tablets, 5mg			
Prednisolone sample : EC			
Batch Number : 0108J			
Weight of 20 Tablets = 1.8945g			
Average Weight = 0.0997g			
Number	**Individual wt (g)**	**Deviation**	**% Deviation**
1	0.1057	0.0060	6.0181
2	0.0971	-0.0026	-2.6078
3	0.0981	-0.0016	-1.6048
4	0.0953	-0.0044	-4.4132
5	0.1012	0.0015	1.5045
6	0.0941	-0.0056	-5.6169
7	0.0994	-0.0003	-0.3009
8	0.0976	-0.0021	-2.1063
9	0.1037	0.0040	4.0120
10	0.0990	-0.0007	-0.7021
12	0.1006	0.0009	0.9027
13	0.0967	-0.0030	-3.0090
14	0.0987	-0.0010	-1.0030
15	0.1024	0.0027	2.7081
16	0.0964	-0.0033	-3.3099
17	0.1066	0.0069	6.9208
18	0.1009	0.0012	1.2036
19	0.1022	0.0025	2.5075
20	0.0991	-0.0006	-0.6018

TABLE OF RESULT FOR PERCENTAGE CONTENT OF PREDNISOLONE TABLETS USING STANDARD METHOD, BP 2007

Table A.12 Percentage Content of Prednisolone Using BP 2007

Prednisolone Brand	**Batch Number**	**Percentage Content (%)**				
		Sample 1	***Sample 2***	***Sample 3***	***Sample 4***	***Sample 5***
LE	0050099	103.64	108.92	107.23	104.63	104.62
MG	PD324M	103.33	102.65	102.77	102.02	104.01
EP	708004	117.02	116.01	115.23	119.15	119.67
EC	0108J	107.38	108.03	105.65	106.98	106.44

Table A.13 Retention times for both drug (prednisolone) and surrogates

Samples	**Mean Retention Time (min)**
Prednisolone	4.30±0.4
Indometacin	9.79±0.3
Naproxen	2.25±0.6
Piroxicam	1.26±0.4

Table A.14 LIMIT OF DETECTION AND QUANTIFICATION

Samples	**LOD (%w/v)**	**LOQ (%w/v)**
Indometacin	0.000041	0.000121
Naproxen	0.000040	0.000120
Piroxicam	0.000049	0.000150
Prednisolone	0.000022	0.000068

LINEARITY

Table A.15 Linearity for Pure Drugs

Pure Drugs	Straight Line Equation	R^2	Range of Concentration, % w/v
Prednisolone	1. y = 924.5x + 0.004 2. y = 923.1x + 0.003 3. y = 918.6x + 0.005	1. 0.9990 2. 0.9990 3. 0.9990	0.0004 – 0.0012 0.0004 – 0.0012 0.0004 – 0.0012
Indometacin	1. y = 719.6x + 0.010 2. y = 718.9x + 0.009 3. y = 720.0x + 0.009	1. 0.9980 2. 0.9980 3. 0.9990	0.0003 – 0.0013 0.0002 – 0.0012 0.0002 – 0.0013
Piroxicam	1. y = 843.2x + 0.001 2. y = 840.7x + 0.002 3. y = 835.5x + 0.004	1. 0.9980 2. 0.9990 3. 0.9990	0.0003 – 0.0012 0.0003 - 0.0012 0.0002 – 0.0012
Naproxen	1. y = 603.6x – 0.008 2. y = 604.1x – 0.007 3. y = 603.5x – 0.006	1. 0.9980 2. 0.9980 3. 0.9990	0.0002 – 0.0011 0.0002 – 0.0012 0.0002 – 0.0012

SPECIFICITY AND SELECTIVITY

Table A.16 Specificity and Selectivity

Samples	Retention Time (min)
Prednisolone Pure Sample	4.32±0.02
Prednisolone Tablet	4.31±0.02

PRECISION (Repeatability) For Intra-day

Table A.17 Relative Standard Deviation for pure Indometacin

Concentration (% w/v)	Number of Injections	Standard Deviation	Relative Standard Deviation, RSD (%)
0.00120	6	0.004472	0.53
0.00100	6	0.001255	0.17
0.00080	6	0.005009	0.94

Table A.18 Relative Standard Deviation for pure Naproxen

Concentration (% w/v)	Number of Injections	Standard Deviation	Relative Standard Deviation, RSD (%)
0.0010	6	0.001936	0.26
0.0012	6	0.002739	0.25
0.0007	6	0.001940	0.39

Table A.19 Relative Standard Deviation for pure Piroxicam

Concentration (% w/v)	Number of Injections	Standard Deviation	Relative Standard Deviation, RSD (%)
0.0010	6	0.002076	0.28
0.0011	6	0.005999	0.66
0.0007	6	0.005178	0.98

Table A.20 Relative Standard Deviation for pure Prednisolone

Concentration (% w/v)	Number of Injections	Standard Deviation	Relative Standard Deviation, RSD (%)
0.0012	6	0.007090	0.53
0.0010	6	0.004893	0.38
0.0007	6	0.005925	0.70

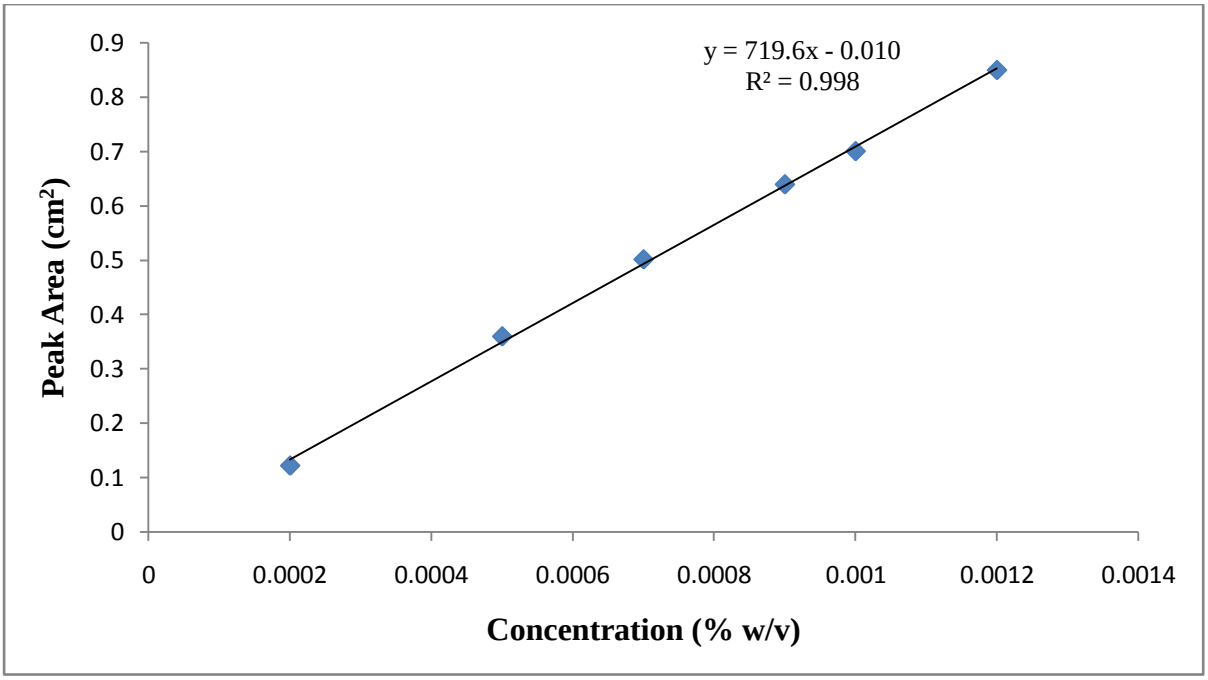


Fig. A.1 Calibration curve for Pure Indometacin.

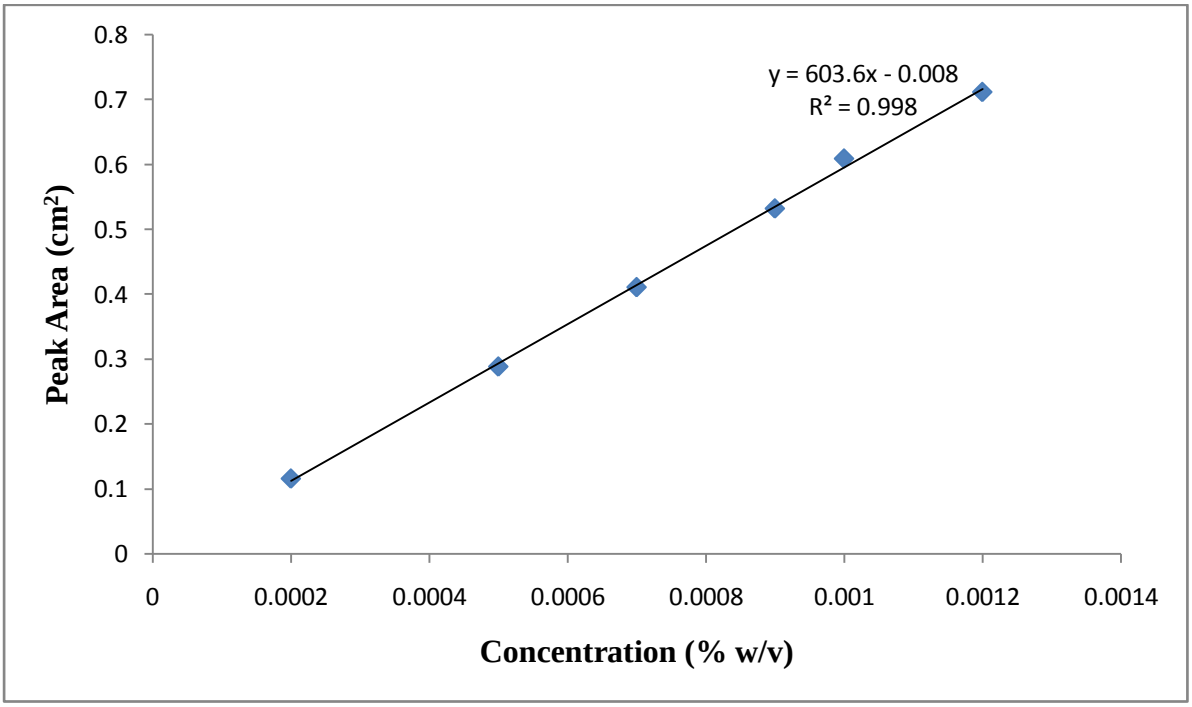


Fig. A.2 Calibration curve for Pure Naproxen.

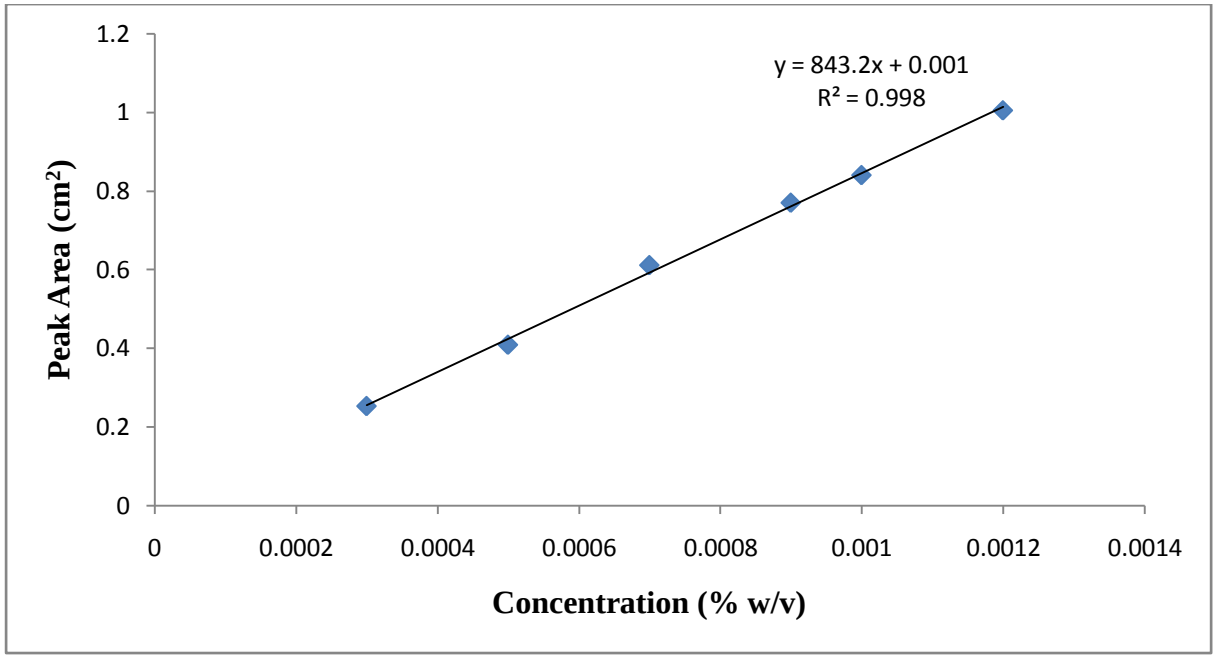


Fig. A.3 Calibration curve for Pure Piroxicam.

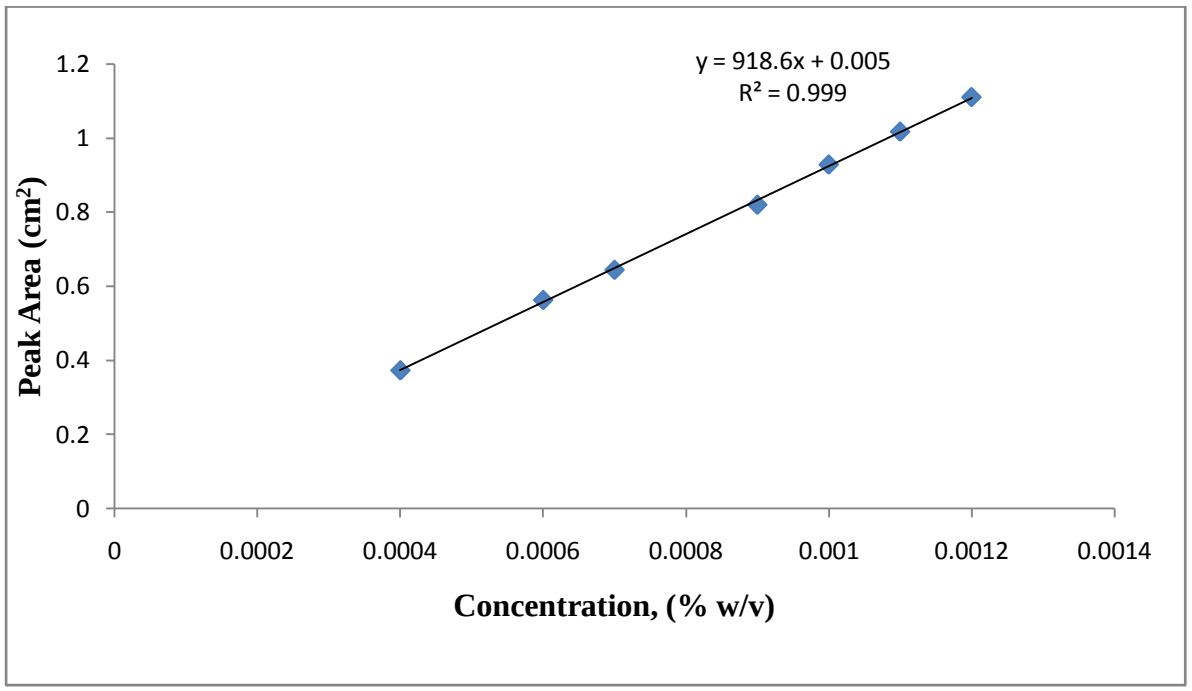


Fig. A.4 Calibration curve for Pure Prednisolone.

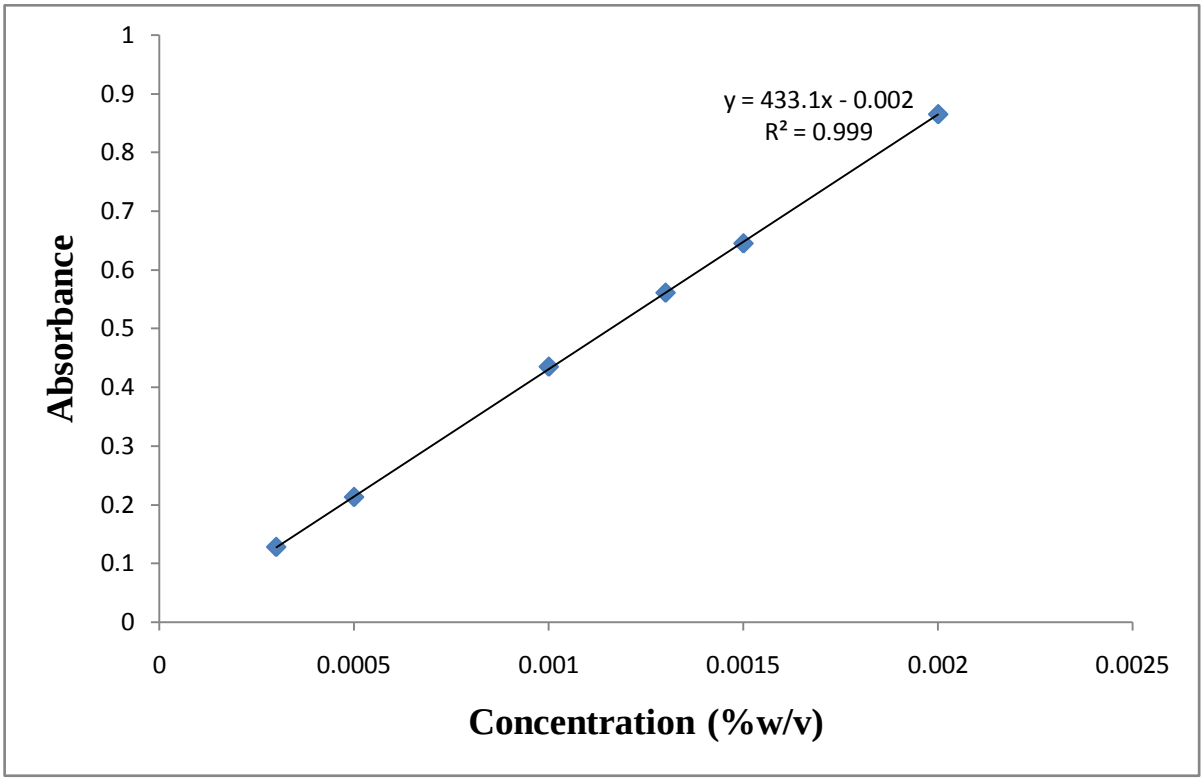


Fig A.5 Calibration curve for the assay of pure dexamethasone

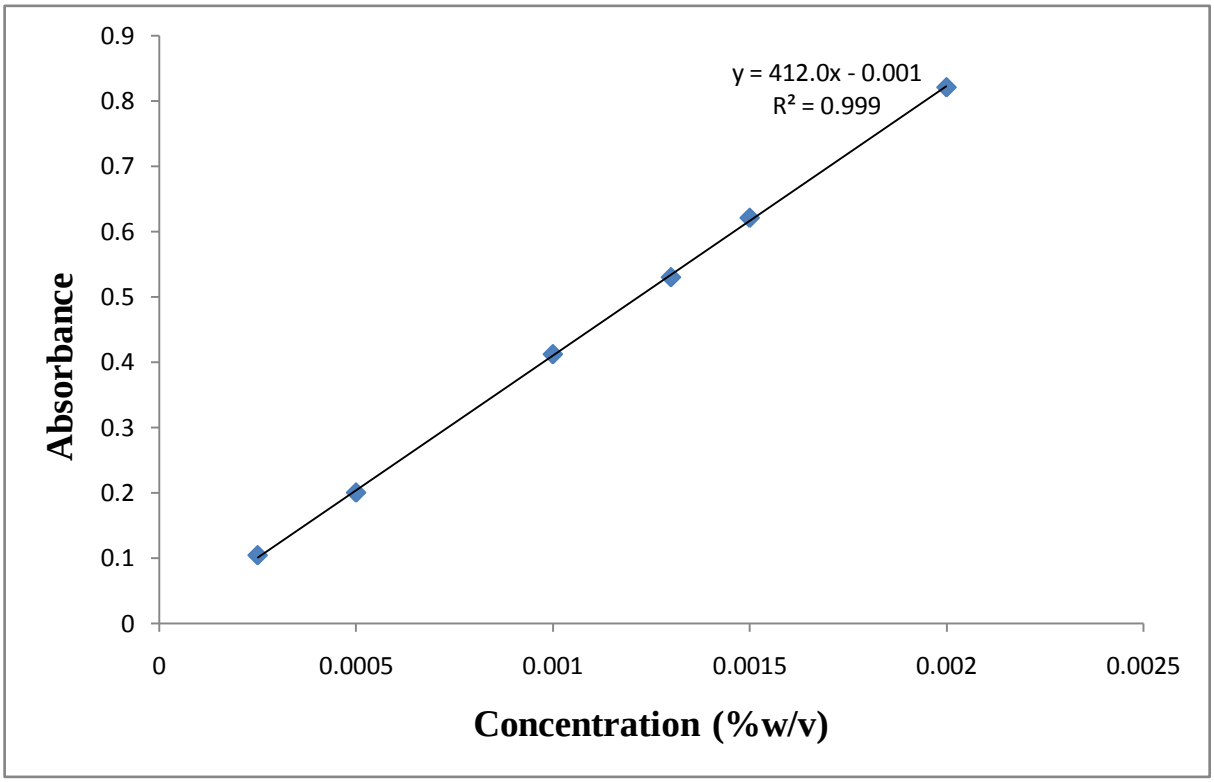


Fig A.6 Calibration curve for the assay of pure prednisolone

Determination of the constant K, for Prednisolone

Table A.21 K Values for Prednisolone using Indometacin as a surrogate standard

Determination of K values for Prednisolone at a concentration of 0.0012% w/v using Indometacin as the standard. Percentage Purity of Indometacin = 99.80%				
Peak Area of Indometacin, As	**Concentration of Indometacin, Cs**	**Peak Area of Prednisolone, Aa**	**Concentration of Prednisolone, Ca**	**K**
0.8400	0.0012	1.2800	0.0010	1.8286
0.6978	0.0010	1.5402	0.0012	1.8394
0.8980	0.0013	1.2802	0.0010	1.8533
0.9010	0.0013	1.0101	0.0008	1.8218
0.8431	0.0012	1.5329	0.0012	1.8182

Average K value = 1.8323

Table A.22 K values for Prednisolone using Naproxen as a surrogate standard

Determination of K values for Prednisolone at a concentration of 0.0012% w/v using Naproxen as the standard. Percentage Purity of Naproxen = 99.30%				
Peak Area of Naproxen, As	**Concentration of Naproxen, Cs**	**Peak Area of Prednisolone, Aa**	**Concentration of Prednisolone, Ca**	**K**
0.7875	0.0012	1.3400	0.0012	1.7000
0.7901	0.0012	1.1010	0.0010	1.6722
0.7290	0.0011	1.3640	0.0012	1.7151
0.5881	0.0009	1.0404	0.0009	1.7691
0.6001	0.0009	1.1043	0.0010	1.6562

Average K value = 1.6925

Table A.23 K values for Prednisolone using Piroxicam as a surrogate standard

Determination of K values for Prednisolone at a concentration of 0.0012%w/v using Piroxicam as the standard. Percentage Purity of Piroxicam = 99.80%				
Peak Area of Piroxicam, As	**Concentration of Piroxicam, Cs**	**Peak Area of Prednisolone, Aa**	**Concentration of Prednisolone, Ca**	**K**

0.9050	0.0011	1.4300	0.0013	1.3370
0.8388	0.0010	1.1130	0.0010	1.3269
0.5720	0.0007	1.3580	0.0012	1.3849
0.5722	0.0007	1.3578	0.0012	1.3842
0.9121	0.0011	1.1128	0.0010	1.3421

Average K value = 1.3550

Determination of Percentage Content of Prednisolone

Table A.24 Percentage content of tablets LE using Indometacin as a surrogate standard

Percentage content of Prednisolone in Prednisolone tablet using Indometacin as the standard.					
Tablet = Prednisolone, 5mg; Average weight = 0.0977g					
Prednisolone sample LE; Batch No. = 0050099					
Concentration of Prednisolone tablet,Ca=0.0011%w/v;Peak area of Prednisolone,Aa= 1.47					
Peak Area of Indometacin,**As**	Concentration(%w/v) of Indometacin, **Cs**	**Aa** × **Cs**	**K** × **As**	(**Aa** × **Cs**) / (**K** × **As**)	Percentage Content(%)
0.8400	0.0012	0.001764	1.539132	0.001146	104.18
0.6978	0.0010	0.001470	1.278579	0.001150	104.55
0.8980	0.0013	0.001911	1.645405	0.001161	105.55
0.9010	0.0013	0.001911	1.650902	0.001158	105.27
0.8431	0.0012	0.001764	1.544812	0.001142	103.82

Table A.25 Percentage content of tablets LE using Naproxen as a surrogate standard

Percentage content of Prednisolone in Prednisolone tablet using Naproxen as the standard.					
Tablet = Prednisolone, 5mg; Average weight = 0.0977g					
Prednisolone sample LE; Batch No. = 0050099					
Concentration of Prednisolone tablet,Ca=0.0012%w/v Peak area of Prednisolone, Aa= 1.478					
Peak Area of Naproxen, **As**	Concentration(%w/v) of Naproxen, **Cs**	**Aa** × **Cs**	**K** × **As**	(**Aa** × **Cs**) / (**K** × **As**)	Percentage Content(%)

0.7490	0.0011	0.001626	1.267683	0.001282	106.83
0.6081	0.0009	0.001330	1.029209	0.001292	107.67
0.6078	0.0009	0.001330	1.028702	0.001293	107.75
0.6078	0.0009	0.001330	1.028702	0.001293	107.75
0.7490	0.0011	0.001626	1.267683	0.001282	106.83

Table A.26 Percentage content of tablets LE using Piroxicam as a surrogate standard

Percentage content of Prednisolone in Prednisolone tablet using Piroxicam as the standard.					
Tablet = Prednisolone, 5mg; Average weight = 0.0977g					
Prednisolone sample LE; Batch No. = 0050099					
Concentration of Prednisolone tablet,Ca=0.0012%w/v Peak area of Prednisolone,Aa= 1.4775					
Peak Area of Piroxicam, **As**	Concentration(%w/v) of Piroxicam, **Cs**	**Aa** × **Cs**	**K** × **As**	(**Aa** × **Cs**) / (**K** × **As**)	Percentage Content(%)
0.9125	0.0011	0.001625	1.236438	0.001314	109.50
0.8482	0.0010	0.001478	1.149311	0.001286	107.17
0.5902	0.0007	0.001034	0.799721	0.001293	107.75
0.5912	0.0007	0.001034	0.801076	0.001291	107.58
0.9221	0.0011	0.001625	1.249446	0.001301	108.42

Table A.27 Percentage content of MG using Indometacin as a surrogate standard

Percentage content of Prednisolone in Prednisolone tablet using Indometacin as the standard.					
Tablet = Prednisolone, 5mg; Average weight = 0.18059g					
Prednisolone sample MG; Batch No. = PD324M					
Concentration of Prednisolone tablet,Ca=0.0010%w/v Peak area of Prednisolone, Aa = 1.21					
Peak Area of Indometacin, **As**	Concentration(%w/v) of Indometacin, **Cs**	**Aa** × **Cs**	**K** × **As**	(**Aa** × **Cs**) / (**K** × **As**)	Percentage Content(%)
0.7732	0.0012	0.001452	1.416754	0.001025	102.50

0.6456	0.0010	0.001210	1.182933	0.001023	102.30
0.8308	0.0013	0.001573	1.522275	0.001033	103.30
0.8312	0.0013	0.001573	1.523008	0.001033	103.30
0.7787	0.0012	0.001452	1.426812	0.001018	101.80

Table A.28 Percentage content of MG using Naproxen as a surrogate standard

Percentage content of Prednisolone in Prednisolone tablet using Naproxen as the standard.					
Tablet = Prednisolone, 5mg; Average weight = 0.18059g					
Prednisolone sample MG; Batch No. = PD324M					
Concentration of Prednisolone tablet,Ca=0.0010%w/v Peak area of Prednisolone,Aa= 1.16					
Peak Area of Naproxen, **As**	Concentration(%w/v) of Naproxen, **Cs**	**Aa** × **Cs**	**K** × **As**	(**Aa** × **Cs**) / (**K** × **As**)	Percentage Content(%)
0.7875	0.0012	0.001392	1.332844	0.001044	104.40
0.7901	0.0012	0.001392	1.337244	0.001041	104.10
0.7290	0.0011	0.001276	1.233833	0.001034	103.40
0.5881	0.0009	0.001044	0.995359	0.001049	104.90
0.6001	0.0009	0.001044	1.015669	0.001028	102.80

Table A.29 Percentage content of MG using Piroxicam as a surrogate standard

Percentage content of Prednisolone in Prednisolone tablet using Piroxicam as the standard.					
Tablet = Prednisolone, 5mg; Average weight = 0.18059g					
Prednisolone sample MG; Batch No. = PD324M					
Concentration of Prednisolone tablet, Ca= 0.0009%w/v Peak area of Prednisolone, Aa = 1.04					
Peak Area of Piroxicam, **As**	Concentration(%w/v) of Piroxicam, **Cs**	**Aa** × **Cs**	**K** × **As**	(**Aa** × **Cs**) / (**K** × **As**)	Percentage Content(%)
0.9050	0.0011	0.001122	1.226275	0.000933	103.67
0.8388	0.0010	0.001020	1.136574	0.000915	101.67

0.5720	0.0007	0.000714	0.775060	0.000939	104.33
0.5722	0.0007	0.000714	0.775331	0.000921	102.33
0.9121	0.0011	0.001122	1.235896	0.000926	102.89

Table A.30 Percentage content of EP using Indometacin as a surrogate standard

Percentage content of Prednisolone in Prednisolone tablet using Indometacin as the standard.					
Tablet = Prednisolone, 5mg; Average weight = 0.206735g					
Prednisolone sample EP; Batch No. = 708004					
Concentration of Prednisolone tablet,Ca=0.0008%w/v: Peak area of Prednisolone,Aa= 1.089					
Peak Area of Indometacin, **As**	Concentration(%w/v) of Indometacin, **Cs**	**Aa × Cs**	**K × As**	**(Aa × Cs) / (K × As)**	Percentage Content(%)
0.7804	0.0012	0.001307	1.429927	0.000914	114.25
0.6303	0.0010	0.001089	1.154899	0.000943	117.88
0.8200	0.0013	0.001416	1.502486	0.000942	117.75
0.8302	0.0013	0.001416	1.521175	0.000931	116.38
0.7802	0.0012	0.001307	1.429560	0.000914	114.25

Table A.31 Percentage content of EP using Naproxen as a surrogate standard

Percentage content of Prednisolone in Prednisolone tablet using Naproxen as the standard.					
Tablet = Prednisolone, 5mg; Average weight = 0.206735g					
Prednisolone sample EP; Batch No. = 708004					
Concentration of Prednisolone tablet,Ca=0.0008%w/v: Peak area of Prednisolone,Aa= 1.027					
Peak Area of Naproxen, **As**	Concentration(%w/v) of Naproxen, **Cs**	**Aa × Cs**	**K × As**	**(Aa × Cs) / (K × As)**	Percentage Content(%)
0.7875	0.0012	0.001232	1.332844	0.000925	115.63
0.7901	0.0012	0.001232	1.337244	0.000922	115.25
0.7290	0.0011	0.001130	1.233833	0.000916	114.50

0.5881	0.0009	0.000924	0.995359	0.000929	116.13
0.6001	0.0009	0.000924	1.015669	0.000910	113.75

Table A.32 Percentage content of EP using Piroxicam as a surrogate standard

Percentage content of Prednisolone in Prednisolone tablet using Piroxicam as the standard.					
Tablet = Prednisolone, 5mg; Average weight = 0.206735g					
Prednisolone sample EP; Batch No. = 708004					
Concentration of Prednisolone tablet,Ca=0.0008%w/v: Peak area of Prednisolone,Aa= 1.073					
Peak Area of Piroxicam, **As**	Concentration(%w/v) of Piroxicam, **Cs**	**Aa × Cs**	**K × As**	(**Aa × Cs**) / (**K × As**)	Percentage Content(%)
0.9050	0.0011	0.001183	1.226275	0.000963	120.38
0.8388	0.0010	0.001073	1.136574	0.000944	118.00
0.5720	0.0007	0.000751	0.775060	0.000970	121.25
0.5722	0.0007	0.000751	0.775331	0.000969	121.13
0.9121	0.0011	0.001183	1.235896	0.000955	119.38

Table A.33 Percentage content of EC using Indometacin as a surrogate standard

Percentage content of Prednisolone in Prednisolone tablet using Indometacin as the standard.					
Tablet = Prednisolone, 5mg; Average weight = 0.0997g					
Prednisolone sample EC; Batch No. = 0108J					
Concentration of Prednisolone tablet, Ca=0.0008%w/v;Peak area of Prednisolone, Aa= 1.089					
Peak Area of Indometacin,**As**	Concentration(%w/v) of Indometacin, **Cs**	**Aa × Cs**	**K × As**	(**Aa × Cs**) / (**K × As**)	Percentage Content(%)
0.8400	0.0012	0.001321	1.539132	0.000858	107.25
0.6978	0.0010	0.001101	1.278579	0.000861	107.63
0.8980	0.0013	0.001431	1.645405	0.000870	108.75
0.9010	0.0013	0.001431	1.650902	0.000867	108.38
0.8431	0.0012	0.001321	1.544812	0.000855	106.88

Table A.34 Percentage content of EC using Naproxen as a surrogate standard

Percentage content of Prednisolone in Prednisolone tablet using Naproxen as the standard.					
Tablet = Prednisolone, 5mg; Average weight = 0.18059g					
Prednisolone sample EC; Batch No. = 0108J					
Concentration of Prednisolone tablet,Ca=0.0010%w/v Peak area of Prednisolone,Aa= 1.1901					
Peak Area of Naproxen, **As**	Concentration(%w/v) of Naproxen, **Cs**	**Aa** × **Cs**	**K** × **As**	(**Aa** × **Cs**) / (**K** × **As**)	Percentage Content(%)
0.7875	0.0012	0.001428	1.332844	0.001071	107.10
0.7901	0.0012	0.001428	1.337244	0.001068	106.80
0.7290	0.0011	0.001309	1.233833	0.001061	106.10
0.5881	0.0009	0.001071	0.995359	0.001076	107.60
0.6001	0.0009	0.001071	1.015669	0.001055	105.50

Table A.35 Percentage content of EC using Piroxicam as a surrogate standard

Percentage content of Prednisolone in Prednisolone tablet using Piroxicam as the standard.					
Tablet = Prednisolone, 5mg; Average weight = 0.0997g					
Prednisolone sample EC; Batch No. = 0108J					
Concentration of Prednisolone tablet,Ca=0.0012%w/v Peak area of Prednisolone,Aa= 1.4564					
Peak Area of Piroxicam, **As**	Concentration(%w/v) of Piroxicam, **Cs**	**Aa** × **Cs**	**K** × **As**	(**Aa** × **Cs**) / (**K** × **As**)	Percentage Content(%)
0.9125	0.0011	0.001602	1.236438	0.001296	108.00
0.8482	0.0010	0.001478	1.149311	0.001267	105.58
0.5902	0.0007	0.001034	0.799721	0.001275	106.25
0.5912	0.0007	0.001034	0.801076	0.001273	106.08
0.9221	0.0011	0.001625	1.249446	0.001282	106.83

T-tests

Table A.36 T-Test for Tablets LE

Tablet = Prednisolone, 5mg; Average weight = 0.0977g			
Prednisolone brand: LE Batch Number: 0050099			
Standard Method (B.P., 2007)	**New Method (Indometacin)**	**New Method (Naproxen)**	**New Method (Piroxicam)**
103.64	104.18	106.83	109.50
108.92	104.55	107.67	107.17
107.23	105.55	107.75	107.75
104.63	105.27	107.75	107.58
104.62	103.82	106.83	108.42
Standard Deviation 2.19	0.73	0.49	0.91
t_{exp}	1.23	1.83	1.72

Table A.37 T-Test for Tablets MG

Tablet = Prednisolone, 5mg; Average weight = 0.18059g			
Prednisolone brand: MG Batch Number: PD324M			
Standard Method (B.P., 2007)	**New Method (Indometacin)**	**New Method (Naproxen)**	**New Method (Piroxicam)**
103.33	102.50	104.40	103.67
102.65	102.30	104.10	101.67
102.77	103.30	103.40	104.33
102.02	103.30	104.90	102.33
104.01	101.80	102.80	102.89
Standard Deviation 0.75	0.65	0.83	1.05
t_{exp}	0.53	1.46	0.05

Table A.39 E-Test for sample EP

Tablet = Prednisolone, 5mg; Average weight = 0.206735g			
Prednisolone brand: EP Batch Number: 708004			
Standard Method (B.P., 2007)	**New Method (Indometacin)**	**New Method (Naproxen)**	**New Method (Piroxicam)**
117.02	114.25	115.63	120.38
116.01	117.88	115.25	118.00
115.23	117.75	114.50	121.25
119.15	116.38	116.13	121.13
119.67	114.25	113.75	119.38
Standard Deviation 1.94	1.78	0.94	1.36
$\mathbf{t_{exp}}$	0.86	2.40	2.53

Table A.38 T-Test for sample EC

Tablet = Prednisolone, 5mg; Average weight = 0.206735g			
Prednisolone brand: EC Batch Number: 0108J			
Standard Method (B.P., 2007)	**New Method (Indometacin)**	**New Method (Naproxen)**	**New Method (Piroxicam)**
107.38	107.25	107.10	108.00
108.03	107.63	106.80	105.58
105.65	108.75	106.10	106.25
106.98	108.38	107.60	106.08
106.44	106.88	105.50	106.83
Standard Deviation 0.91	0.78	0.83	0.93
$\mathbf{t_{exp}}$	1.39	0.75	0.58