KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI.

COLLEGE OF SCIENCE

DEPARTMENT OF CHEMISTRY

DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD WITH PDA DETECTION FOR THE SIMULTANEOUS ESTIMATION OF ACETYLSALICYLIC ACID, PARACETAMOL AND CAFFEINE IN FIXED DOSE COMBINATION TABLETS

BY

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Bsc. CHEMISTRY

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of

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DECLARATION

The research work reported herein was carried out at the Department of Chemistry, Faculty of Physical Sciences, College of Science, Kwame Nkrumah University of Science and Technology, Kumasi. This work has not been submitted for any other degree. Any assistance obtained therein has been duly acknowledged.

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ABSTRACT

Acetylsalicylic acid and paracetamol belong to a class of drugs called analgesics and are often formulated together in combination with Caffeine, an adjuvant for the relief of pain, fever and inflammations. A RP-HPLC method has been developed and validated for the simultaneous estimation of acetylsalicylic acid, paracetamol and caffeine in multi-component drug formulation. The developed method made use of a Brownlee Analytical column C8, 5 µm, 150 x 4.6 mm as the stationary phase and acidic water-methanol mixture (60:40 $^{v}/_{v}$) ratio as the mobile phase. All the three components were eluted within 5.5 minutes with mean retention times of 2.05 ± 0.0062 for paracetamol, 2.45 ± 0.0030 for caffeine and 5.03 ± 0.0140 for acetylsalicylic acid. The method was validated based on validation parameters such as accuracy, linearity, precision, robustness, specificity, limit of detection (LOD) and limit of quantification (LOQ). The mean recoveries were 99.39 ± 1.58 %, 99.69 ± 1.45 % and 100.56 ± 1.60 % for acetylsalicylic acid, paracetamol and caffeine respectively. Linearity of the developed method was in the concentration range of 20-100 ppm with R^2 value of 0.9933 for acetylsalicylic acid, 2.5-20 ppm with R^2 value of 0.9978 for paracetamol and 1.25-10 ppm with R^2 value of 0.9991 for caffeine. The % RSD for inter and intra- days precisions were 0.408; 0.143, 0.056; 0.091, and 0.470; 0.207 for acetylsalicylic acid, paracetamol and caffeine respectively. The LOD and LOQ for acetylsalicylic acid, paracetamol and caffeine were $1.078x10^{-5}$; $3.267x10^{-5}$, 0.00; 0.00 and $1.237x10^{-7}$; $2.193x10^{-6}$ ppm respectively. Accurate results obtained for the assay of both tablet samples (A and B) also confirmed the validity of the developed method. The developed method is therefore, accurate, linear, precise, specific, robust, sensitive and cost effective and can be used in routine quality control analysis of multi-component drug formulations containing these three active ingredients.

DEDICATION

This work is dedicated to my parents, Mr. John Kofi Mensah and madam Comfort Nyamekye, my wife, Mrs. Beatrice Addai-Arhin, my son, Kweku Twumasi Addai, my brothers and sisters and my family friend Pee.

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LIST OF ABBREVIATIONS

ASA	Acetylsalicylic Acid
APIs	Active Pharmaceutical Ingredients
BNF	British National Formulary
BP	British Pharmacopoeia
CDER	Centre for Drug Evaluation and Research
CNS	Central Nervous System
COX	Cyclooxygenase
HPLC	High Performance Liquid Chromatography
ICH	International Conference on Harmonization
ISO	International Standard Organization
LALLS	Low Angle Laser Light Scattering
LOD	Limit of Detection
LOQ	Limit of Quantification
MALLS	Multiple Angle Laser Light Scattering
NQA	Nano Quantity Aerosol
ODS	Octadecylsilane
OS	Octylsilane
PDA	Photodiode Array
PGES	Prostaglandins Endoperoxide Synthase
RP-HPLC	Reversed Phase – High Performance Liquid Chromatography

RSD	Relative Standard Deviation
SD	Standard Deviation
TLC	Thin Layer Chromatography
UV	Ultra-Violet

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

A drug is any chemical substance that produces or exerts a biological effect on humans or animals or affect body functions of living organisms especially humans or animals (Fulcher *et al*., 2009; BNF, 2012). This biological effect or effect on body functions may be physiological, performance enhancing or an intoxicating effect (Fulcher *et al*., 2009). Physiological effect of drugs may be achieved through cure, diagnosis, treatment, prevention and/or management of diseases. Drugs meant for any of these purpose(s) are pharmacologically termed as medicines (Fulcher *et al*., 2009; BNF, 2012).

There are many different types of drugs but these types are grouped under several classes depending on the nature (chemistry), action (uses) and the mechanisms of action of the drugs (Fulcher *et al*., 2009; BNF, 2012). For example, Amoxycillin, Cefuroxime, Ciprofloxacin etc belong to the antibacterial (antibiotic) class, hence meant for the treatment of bacterial infections. Paracetamol, acetylsalicylic acid, diclofenac etc are also meant to relieve pain, hence are called analgesics. Metronidazole, tinidazole and furanzolidone belong to the antifungal class, hence meant for the treatment of fungal infections. (BNF, 2012). It must be noted that several classes exist with several drug types falling under these classes (BNF, 2012).

Before the advent of multi-component drug formulations, drugs and even currently some drugs are still available as single component drug formulations containing only one active ingredient which was/is responsible for the therapeutic activity of the drug (Li *et al*., 2010). Most of the single component drugs that are meant to treat infections are easily susceptible to resistance by microorganisms (Li *et al*., 2010). This has led to the development of multicomponent drug formulations containing two or more active ingredients which are responsible for a combined

therapeutic activity of the drug (Li *et al*., 2010). ‘’The concept of multi-component drug formulations is beneficial when the selected agents possess differing mechanisms of actions that provide additive or synergistic efficacy’’ (Li *et al*., 2010).

Examples of multi-component drug formulations include amoxycillin and clavulanic acid for the treatment of bacterial infections, artemeter and lumenfantrine for the treatment of malaria, artesunate and amodiaquine for the treatment of malaria (BNF, 2012). Besides synergistic efficacy, other advantages of multi-component drug formulations include lesser treatment failure rate, slower development of resistance, reduced case fatality ratios and less drug development cost (Li *et al*., 2010).

The increased efficacy of multi-component drug formulations, increased resistance of microorganisms to single component drug formulations and dependence and/or tolerance of single component drug formulations have resulted in the increased production of multicomponent drug formulations which has further led to increased drug counterfeiting and adulteration (Mackey and Liang, 2011). Drug counterfeiting and adulteration are a major problem worldwide and has contributed significantly to global health and patient safety including drug resistance and death of patients (Mackey and Liang, 2011). Statistically, there are about thousand counterfeit incidents per year globally and this includes all types of therapeutic classes (Mackey and Liang, 2011). Drug counterfeiting and adulteration has in turn affected the purity, strength and subsequent potency of multi-component drug formulations, hence the need to carry out laboratory analysis to check the purity and strength of bulk drug formulations.

Drug analysis forms an integral part of several stages of drug development such as stability studies, quality control and formulation (Santos *et al*., 2009; Shrivastava and Gupta, 2011).

The concept of drug quality in terms of its purity, identity and strength is very important because it gives a measure of the safety of the drug and its subsequent therapeutic potency. Safety of drugs is not only limited to purity and strength, but it is also determined by other inherent factors of the drug material such as side effects (Gorog, 2007). Of these, purity is of much prominence because by the identification and quantitative determination of impurities and degradation products, its contribution to the side effects of the drug material as whole can be minimized (Gorog, 2007). It must be noted that drug purity is also very important because it can be influenced by the analyst and the type of analytical method used (Gorog, 2007). The purity of pharmaceutical formulations can only be determined through laboratory testing of the intermediate and finished drug product (bulk drug product). This means that the certainty of purity and/or strength of drug formulations largely depend on the analytical method(s) employed in the analysis of such drug formulations (Gorog, 2007). Hence, the need to develop analytical methods that can be used in the analysis of these multi-component drug formulations in order to ascertain their purity and/or strength (Vichare *et al*., 2010). Again the Pharmacopoeias such as United States Pharmacopoeia (USP), British Pharmacopoeia (BP), European Pharmacopoeia (EP) etc mostly have standardised methods for the analysis of single component drug formulations, hence the need to develop methods which aim at accurate and precise analysis of these multi-component drug formulations (BP, 2009).

Many analytical methods are frequently being developed especially in the area of chromatography and spectrophotometry (Suresh *et al*., 2010). Some examples of developed analytical methods especially in the area of High Performance Liquid Chromatography (HPLC) for the analysis of multi-component drug formulations include method for the simultaneous determination of paracetamol and caffeine in tablet formulation (Tsvetkova *et al*., 2012), method for the simultaneous estimation of paracetamol, caffeine, cetirizine and phenylephrine in tablet dosage form (Malakar *et al*., 2013), method for the analysis of acetylsalicylic acid in

the presence of its degradation products (Suresh *et al*., 2010), method for simultaneous quantitation of aspirin, salicylic acid and caffeine in effervescent tablets and others (Sawyer and Kumar, 2003).

Recently, most over-the-counter (OTC) analgesics are available as multi-component drugs. Acetylsalicylic acid (ASA), paracetamol and caffeine are usually put together in a tablet formulation and used as analgesic, antipyretic (fever reducing) and anti-inflammatory. It is therefore very important that the quantities of these active drugs in a single tablet formulation are estimated or quantified using very reliable, sensitive, precise and accurate analytical techniques.

1.2 PROBLEM STATEMENT

Tolerance to single component drugs, increased resistance by microorganisms to single component drugs and increased efficacy of multi-component drugs have led to increased production of multi-component drugs. Most of these multi-component drugs are available as over-the-counter (OTC) analgesics with acetylsalicylic acid, paracetamol and caffeine as main ingredients of these multi-component OTC analgesics. This means that most of these multi-component OTC analgesics contain at least two of these three active ingredients. However, standard analytical methods that are less time consuming and can aid in accurate and precise analysis of these multi-component analgesics containing acetylsalicylic acid, paracetamol and caffeine are not readily available especially in the pharmacopoeias.

1.3 MAIN OBJECTIVE

To develop and validate a reliable, less time consuming, precise, accurate, sensitive and a cost effective RP-HPLC method for the simultaneous estimation of acetylsalicylic acid, paracetamol and caffeine in fixed dose combination tablets.

1.3.1 SPECIFIC OBJECTIVES

- To develop a RP-HPLC method for simultaneous estimation of acetylsalicylic acid, paracetamol and caffeine in fixed dose combination tablets.
- To validate the developed method based on ICH guidelines.
- To determine the suitability of RP-HPLC method in assessing the quality of fixed dose combination tablets containing acetylsalicylic acid, paracetamol and caffeine.

1.4 JUSTIFICATION OF THE STUDY

The development and/or emergence of multi-component drugs has led to the development and validation of many analytical methods for the analysis of these multi-component drugs. For accurate quantification of individual components in a multi-component drug, a very highly sensitive analytical technique which can aid the separation of multi-component drug into its respective component drugs is required. This is because the amount of active drug goes a long way to affect the bioavailability and subsequent potency of the drug. Hence, the need to develop a highly sensitive, accurate, precise, cost effective and a reliable HPLC method for simultaneous determination of ASA, paracetamol and caffeine in fixed dose combination tablets.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 ACETYLSALICYLIC ACID

Acetylsalicylic acid (ASA), chemically known as 2-(acetyloxy)benzoic acid is an acetyl derivative of salicylic acid. It is a white crystalline, weakly acidic compound having a melting point of about 136 ºC and a boiling point of 140 ºC. The acid dissociation constant (Ka) of ASA is 3.162 x 10^{-4} at room temperature (25 ºC) (Seshasai *et al*., 2012).

Acetylsalicylic acid decomposes rapidly in solutions of ammonium acetate or of the acetates, carbonates, citrates or hydroxides. Though stable in dry air, it can be hydrolysed into acetic acid and salicylic acid upon contact with moisture. Alkalis speed up the hydrolysis of ASA into acetates and salicylates (Seshasai *et al*., 2012).

Acetylsalicylic acid (ASA) belongs to a group or class of analgesic drugs known as NonSteroidal Anti-Inflammatory Drugs (NSAIDs) (Burke *et al*., 2006). ASA is a salicylate drug which is used to relieve mild to moderate pain, to reduce fever (antipyretic) and to treat and manage inflammations (Brayfield, 2014). ASA can also prevent blood clot (antiplatelet effect) by inhibiting the production of thromboxanes which have the ability to bind platelet molecules leading to the formation of clot within blood vessels. As a result, ASA is employed at low doses to prevent and manage cardiac related diseases such as stroke, hypertension, congestive cardiac failure (CCF) etc (Seshasai *et al*., 2012).

The basic mechanism of action of ASA is to irreversibly inactivate Cyclooxygenase (COX) or Prostaglandins Endoperoxide Synthase (PTGS) enzyme, which is known for the synthesis of prostaglandins and thromboxane. This is achieved when ASA acts as an acetylating agent by covalently bonding an acetyl group to a serine residue in the active site of the PTGS enzyme (Seshasai *et al*., 2012).

ASA irreversibly inhibits COX-1 and changes enzymatic activity of COX-2 which is responsible for the formation or production of prostanoids which are mostly proinflammatory. ASA modified COX-2 produce lipoxins which are mostly anti-inflammatory (Seshasai *et al*., 2012).

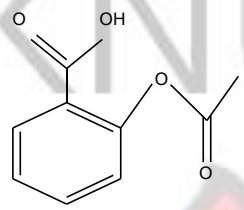

Figure 2.1: Chemical Structure of ASA

Source: (BP, 2009).

2.2 PARACETAMOL

Paracetamol, also known as acetaminophen and chemically known as N-(4-hydroxyphenyl)acetamide is a widely used over-the-counter (OTC) analgesic and antipyretic employed in the management and treatment of mild to moderate pain such as headaches (Graham *et al*., 2013). The analgesic properties of acetaminophen are similar to that of ASA but its anti-inflammatory activity is very weak. For example acetaminophen can relieve pain in mild arthritis but has no effect on the underlying inflammation which causes redness and swelling of the joints (Graham *et al*., 2013). Paracetamol is better tolerated than ASA in patients who experience gastric irritations upon administration of ASA (Graham *et al*., 2013). This has made paracetamol a more common drug in various homes (Graham *et al*., 2013). It is believed that weak opioids such as codeine potentiates paracetamol in approximately 50 % of patients but with an increased number of patients experiencing adverse effects (Murnion, 2010). However, drugs containing paracetamol and strong opioids such as morphine reduce the amount of opioid needed and improve analgesia (Murnion, 2010).

The mechanism of action of paracetamol is not completely understood but it is believed that paracetamol has inhibitory activity against cyclooxygenase (COX) particularly the COX-1 (Hinz *et al*., 2008). Due to its high affinity for COX-1, it has little or no anti-inflammatory activity (Hinz *et al*., 2008).

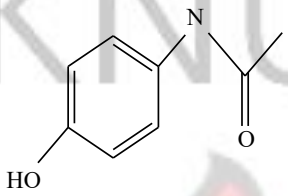

Figure 2.2: Chemical Structure of paracetamol

Source: (BP, 2009).

2.3 CAFFEINE

Caffeine which is chemically known as 1,3,7-trimethylxanthine is a bitter, white crystalline xanthine alkaloid present in the seeds, leaves and fruit of some plants such as the coffee and tea leaves (Tavallali and Sheikhaei, 2009). Caffeine is a stimulant drug, hence acts as a central nervous stimulant to prevent sleep (Lovett, 2005). It is considered as the world's most widely consumed psychoactive drug since it is available in most beverages containing caffeine such as tea, soft drinks and energy drinks (Lovett, 2005).

Continuous use of caffeine leads to physical dependence or addiction and also tolerance which results from a decreased in autonomic effects over a period of time (Juliano and Griffiths, 2004).

The basic mechanism of action of caffeine is to inhibit or antagonise adenosine receptors in the brain since adenosine functions as an inhibitory neurotransmitter that suppresses activity in the central nervous system (CNS) (Fisone *et al*., 2004; Latini and Pedata, 2010). Caffeine also inhibits phosphodiesterase and activates leukotriene synthesis leading to reduction in inflammation. As a result, caffeine is employed in the management and treatment of pain

resulting from inflammation (Peters-Golden *et al*., 2005). It is believed to potentiate or enhance the analgesic effect of paracetamol and/or ASA, hence included in most paracetamol and/or ASA containing preparations meant for analgesia (Derry *et al*., 2012; Liu *et al*., 2011).

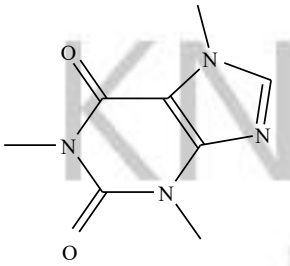

Figure 2.3: Chemical Structure of Caffeine

Source: (Tavallali and Sheikhaei, 2009; BP, 2009).

2.4 HIGH PERFORMANCE/PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

This is a type of chromatographic technique or method in which the mobile phase is pumped under high pressure through a more efficient column which contains the stationary phase (Ahuja, 2003; Kenkel, 2003). HPLC makes use of columns that have frits at both ends in order to contain the stationary phase. These columns can be reused, i.e. several individual separations can be performed on a particular column. This makes it possible to even use a high expensive column packing in order to achieve the best results (Ahuja, 2003).

HPLC is considered as the most widely used chromatographic technique basically due to its high performance nature and its several advantages compared to older, non-instrumental open column chromatographic technique (Kenkel, 2003). In HPLC, separation and quantitation procedures can be done within the shortest period, i.e. HPLC ensures short analysis time. Modern column technology and gradient solvent elution system have also contributed to the best separation results achieved with the use of HPLC (Kenkel, 2003). HPLC can be used for both qualitative and quantitative analysis of several compounds, probably due to the different

types of detectors that can be attached to the LC component of the HPLC set up (Ahuja, 2003).”The greater reproducibility and continuous quantitative detection in HPLC allows more reliable qualitative analysis or more accurate and precise quantitative analysis than classical or open column chromatography’’ (Ahuja, 2003).

Figure 2.4 is a schematic diagram of an HPLC system. The HPLC is made of two major components namely, the LC component and the detector component. The LC component comprises a solvent reservoir which contains the mobile phase, a high pressure pump for pumping the mobile phase through the column, a specially designed injector for introduction of the sample and column for separating the sample mixture into its respective components (Kenkel, 2003). The detector component is responsible for sensing or detecting the eluted or separated components of the sample mixture and a data processing unit which receives signals from the detector and displays the signals in the form of results (chromatogram) (Kenkel, 2003).

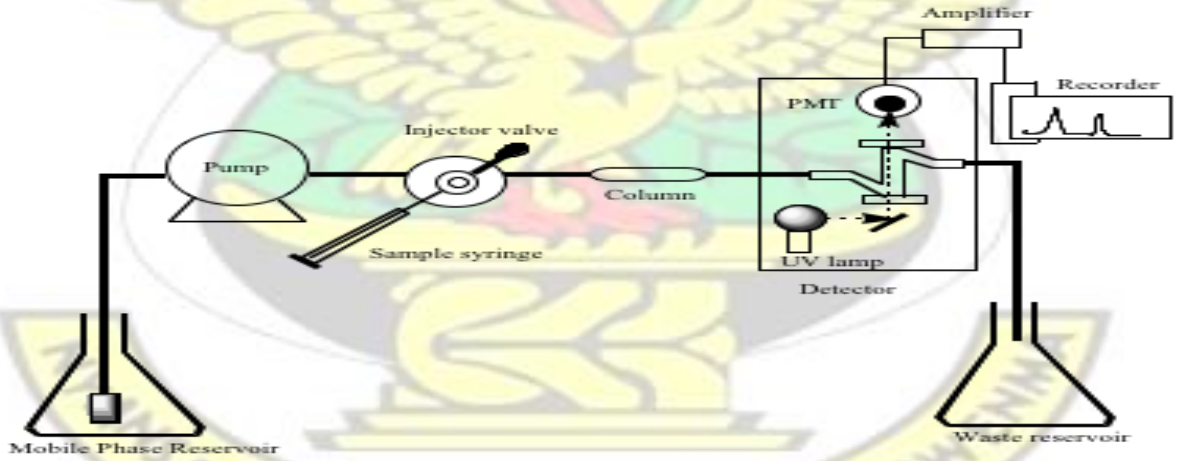

Figure 2.4: Schematic diagram of an HPLC system

Source: (Mac-mod analytical, 2008).

2.4.1 Mobile Phase in HPLC

The mobile phase, sometimes called the eluent, is usually a mixture of two or more solvents having different polarities, buffered to a suitable pH and pumped under high pressure (about

5000 psi) through the chromatographic column (Ahuja, 2003; Kenkel, 2003). This solution is contained in an inert glass material called the reservoir into which a tube fitted with a coarse metal filter is immersed. The tube is connected to a pump which draws the mobile phase through the tube into the chromatographic column. The mouth of the reservoir is also covered with a cap which prevents particulate matter from entering the mobile phase (Kenkel, 2003). In order to ensure that there are no interferences from dissolved gases as a result of the pump drawing the mobile phase into the column through vacuum action, the HPLC system is connected to a device called the degasser which removes dissolved gases from the flow stream to ensure better performance of the pump and the detector (Kenkel, 2003).

The mobile phase is one of the most important factors to be considered in HPLC method development especially when a reverse phase HPLC is to be employed. The type of mobile phase used may have a considerable effect on the retention of the components of a sample, promote or hinder ionization of the component of a sample or even shield an accessible residual silanol or any other active adsorption centres on the surface of the adsorbent (stationary phase) (Skoog *et al*., 2004). Proper selection of the mobile phase is considered the second most important factor after adsorbent type in HPLC method development. The main requirement of a mobile phase is that it should be able to dissolve the components of the sample mixture at concentrations suitable for their detection (Kenkel, 2003).

For a mobile phase to exhibit effective elution properties, the polarity of the mobile phase in relation to the polarities of the components of the sample mixture must be considered. A more polar eluent retains the more polar components of the sample mixture and vice-versa (Ahuja, 2003; Kenkel, 2003). In other words the polarity of the eluent (mobile phase) is one of the two factors that determine whether or not a chromatographic method is a normal phase (non-polar mobile phase and polar stationary phase) or reverse phase (polar mobile phase and non-polar stationary phase) (Ahuja, 2003; Kenkel, 2003). For instance RP-HPLC is the most

widely used chromatographic method in drug analysis. This means that mobile phases for drug analysis are polar since most drugs are polar compounds. This ensures adequate interaction of the drug components with the mobile phase leading to better separation (Ahuja, 2003; Kenkel, 2003). Table 2.1 shows the most important parameters of some common solvents used as mobile phases in normal and RP-HPLC.

Table 2.1: Important parameters of some commonly used solvents as mobile phase in Normal and RP-HPLC.

Solvent	UV Absorption Maxima (nm)	Polarity Index	Suitable HPLC Method
Acetonitrile	190	5.8	RP
Dioxane	215	4.8	RP
Ethanol	210	4.3	RP
Methanol	210	4.2	RP
Isopropanol	205	4.3	RP
Tetrahydrofuran	220	4.0	RP
Water	185	10.2	RP
Ethyl acetate	255	4.4	RP
Cyclohexane	210	0.04	Normal
n-hexane	210	0.1	Normal
Isopropyl ether	220	2.4	Normal
Toluene	286	2.4	Normal
Diethyl ether	218	2.8	Normal
CCl_4	265	1.6	Normal

Source: (Harvey, 2000).

2.4.1.1 Elution Types/ Methods (Isocratic and Gradient) in HPLC

The composition of a mobile phase is extremely important in any liquid chromatographic method. The mobile phase composition is one of the factors that determine the elution rate,

hence, the retention times and better separation of the components of the sample mixture (Kenkel, 2003). As a result, one of the two elution methods, i.e. isocratic or gradient elution method can be employed in RP-HPLC or liquid chromatography in general (Kenkel, 2003).

Isocratic and gradient elution are the two elution methods of the mobile phase employed for the elution of components of a sample mixture from the stationary phase (Kenkel, 2003; Skoog *et al*., 2004). In isocratic elution, the composition of the mobile phase is kept constant throughout the elution or separation process. Although a new or different mobile phase composition can be used, this can only be achieved when the flow is stopped, mobile phase reservoirs changed and the flow restarted (Kenkel, 2003).

In gradient elution there is a changeable mixing ratio of the composition of the mobile phase during the elution or separation process. Gradient elution method helps in achieving a more better separation (Kenkel, 2003). This can be done by using a gradient programmer (part of the HPLC system software) which is able to draw at least two different solvents from two mobile phase reservoirs at same time and gradually mix them according to the mobile phase composition set up or programmed by the operator (Kenkel, 2003).

2.4.2 Stationary Phase/Columns in HPLC

In HPLC, stationary phase consists of active solid particles (packing materials) such as silica particles usually about 5 µm in size packed into a cylindrically shaped metal tube called the chromatographic column (Smith and Marton, 1997). The term packing material refers to a loose, usually particulate material intended for chromatographic use before packing into a column (Smith and Marton, 1997). The column has specific dimensions although these dimensions differ from column to column (Kenkel, 2003). The stationary phase is basically defined as the immobilized phase upon which the separation of components of a mixture is

effected (Kenkel, 2003) or ‘’that part of the chromatographic system responsible for the retention of components of a sample mixture which are being carried through by the mobile phase’’ (Smith and Marton, 1997). This means that for a better separation of components of the sample mixture to be achieved, the physical and chemical nature of the stationary phase must be considered, hence proper selection of the stationary phase is considered the first most important factor in HPLC method development (Ahuja, 2007; Kenkel, 2003; Skoog *et al*., 2004).

The stationary phase can also be a gel or liquid (as in partition HPLC) that is chemically bonded to a solid support (bonded phase) or immobilized onto it (immobilized phase). ‘’The solid support may or may not contribute to the separation process’’ (Smith and Marton, 1997). There are several stationary phases for HPLC but these stationary phases are classified based on the type of liquid chromatography i.e. either adsorption, ion-exchange, size exclusion or partition chromatography (Kenkel, 2003).

In drug analysis using RP-HPLC, the most common type of stationary phase employed is the non-polar type particularly the C18 columns such as Hypersil HyPurity C18, µ-Bondapak C18, Hypersil BDS C18, Kromasil C18 etc although C8 columns can also be used (Kenkel, 2003). Since these stationary phases are less polar, their use ensures that the drug molecules (mostly polar molecules) are retained for longer period by the polar mobile phase, hence resulting in fast elution and better separation of the drug molecules. Table 2.2 shows specifications of some C18 stationary phases used in RP-HPLC;

Table 2.2: Specifications of some stationary phases/Columns used in RP-HPLC.

Stationary Phase	Particle Size (μm)	Pore Size (Å)	Surface Area (m^2/g)	Carbon Load (%)
Zorbax Extend C18	5	80	180	12.5
Zorbax ODS	5	70	330	20
Zorbax Rx-C18	5	80	180	12
Zorbax SB-C18	5	80	180	10
Zorbax XDB-C18	5	80	180	10
Kromasil C18	5	100	340	19
Hypersil BDS C18	5	130	170	11
Novapak C18	5	60	120	7.3
μ-Bondapak C18	10	125	330	10
Symmetry C18	5	100	335	19

Source:(Mac-Mod Analytical, 2008)

2.4.3 Normal and Reverse Phase Chromatographic Methods

The choice for a normal phase or reverse phase chromatographic method basically depends on the nature (polarity) of the components of the sample mixture (Kenkel, 2003). The nature of the sample components in turn determine the type or nature (polarities) of both the stationary and mobile phases required for better resolution or separation of such components (Kenkel, 2003).

In normal phase chromatography, the mobile phase is non-polar while the stationary phase is polar. This means that polar components are more retained or strongly adsorbed by the stationary phase, hence increasing retention time for each component in the elution process

(Kenkel, 2003). On the other hand if non-polar components are used, retention time of each component is decreased since the components are more retained by the eluent (Kenkel, 2003). Typical examples of stationary phases used in normal phase chromatography contain silica, Si-O bonding sites attached to either a cyano (-CN) group, amino ($-NH_2$) group or a diol (-CHOH-CH_2OH) group. Typical examples of mobile phases also include hexane, carbon tetrachloride, toluene, chloroform, cyclohexane and benzene (Kenkel, 2003).

Reverse phase chromatography which is the most widely used HPLC method and mostly used in drug analysis makes use of a polar mobile phase and non-polar stationary phase (Kenkel, 2003). Retention time is always shorter in reverse phase chromatography since polar components of a sample mixture are retained more by the mobile phase, hence eluted rapidly (Kenkel, 2003). Non-polar stationary phases in reverse phase are chemically bonded to silica particles and the degree of non-polarity is determined by the number of carbon atoms that make up the stationary phase. The greater the number of carbon atoms the greater the nonpolarity and vice versa (Kenkel, 2003). Typical examples include stationary phases containing C8 or C18 carbon chain length. Commonly used mobile phases include acetonitrile, methanol, water or acetic acid buffered solutions (Kenkel, 2003). It must be noted that normal phase and reverse phase chromatographic methods are both types of partition HPLC, which is also the most common type of HPLC (Kenkel, 2003).

2.4.4 HPLC Detectors

The basic function of the detector is to detect the components of a sample mixture that are eluted from the column and display an electronic or electrical signal in the form of peaks on the readout (Arti *et al*., 2011; Kenkel, 2003). The displayed peaks represent the results (chromatogram) and each peak is directly proportional to the concentration of a particular component (analyte) in the sample mixture (Arti *et al*., 2011; Kenkel, 2003). Although several

HPLC detectors are available but the two major classes of HPLC detectors are the solute property detectors and bulk property detectors (Arti *et al*., 2011; Kenkel, 2003). Solute property detectors sense or detect changes in the physical and chemical properties of the eluates (components of the sample mixture) while bulk property detectors measure changes in the properties of the eluent and eluates (Arti *et al*., 2011; Kenkel, 2003). The use of a particular detector is dependent on the nature of the analytes or components of the sample mixture under study (Kenkel, 2003; Skoog *et al*., 2004). An ideal HPLC detector should characteristically have an excellent linear response as a function of concentration of the analytes, wide linear dynamic range, high signal-to-noise ratio and sensitivity within the range of 0.01 to 100 µg of the eluting components (Arti *et al*., 2011).

2.4.4.1 Ultraviolet-Visible (UV-Visible) Detectors

The UV-Visible detectors are the most commonly used HPLC detectors and are basically a spectrophotometer or a photometer consisting of a light source, wavelength selector and a phototube (Arti *et al*., 2011; Kenkel, 2003). Most organic compounds absorb radiation within UV region (200-400 nm) and visible region (400-800 nm) of the electromagnetic spectrum, hence these detectors are mostly suitable for the determination of drug compounds (Arti *et al*., 2011). Fixed wavelength detectors, variable wavelength detectors and multi wavelength detectors are the most common detectors used for detection in these regions. Since these detectors are spectrophotometers, they operate on the principle of Beer-Lambert's law and sensitivity of the detector is dependent on the specific absorbance of the analyte (Arti *et al*., 2011). Effluent from the column flows into the cuvette which holds the sample. When only the mobile phase flows into the cuvette, a zero absorbance is detected when the eluted components of a sample mixture flow into the cuvette, the components absorb UV radiation of the selected

wavelength. This causes a change in the absorbance from zero and a peak is observed which is recorded as the chromatogram (Kenkel, 2003).

2.4.4.1.1 Fixed Wavelength UV-Visible Detector

This is a type of UV-Visisble detector which operates at a constant wavelength of 254 nm or 280 nm in the UV region with mercury lamp as the source of radiation (Arti *et al*., 2011).

2.4.4.1.2 Variable Wavelength UV-Visible Detector

This is also another type of UV-Visible detector that can operate within the full uv-visible region of the electromagnetic spectrum. The most suitable source of radiation for this type of uv-visible detector is the xenon arc lamp (Arti *et al*., 2011).

2.4.4.1.3 Multi Wavelength UV-Visible Detector

This detector operates by selecting a single or narrow band of wavelength. The two types are dispersion detectors which monitor mobile phase at one wavelength and can be used for the detection of some carboxylic acids and series of common fatty acids and diode array detectors (DAD) that monitor absorption of radiation by analytes simultaneously over a range of wavelengths (Arti *et al*., 2011). DAD can be used for the detection and identification of poison in herbal medicines (Arti *et al*., 2011). An ideal multi wavelength detector combines dispersive and DAD systems of operation (Arti *et al*., 2011).

2.4.4.2 Fluorescence Detector

The design, theory and principle of operation of the HPLC fluorescence detector are the same as in fluorescence spectrophotometer or the fluorometer. Like the fluorometer, the HPLC fluorescence detector consists of a radiation source, first monochromator or filter for isolating and selecting the excitation wavelength, sample compartment, a second monochromator with phototubes for isolating and selecting the emission or fluorescence wavelength (Kenkel, 2003). The second monochromator or filter and the phototube are arranged at right angles to the incident radiation. This is to prevent the incident radiation from reaching the phototube so that only the fluorescence wavelength can be detected and measured (Kenkel, 2003). This means that the fluorescence detector only measures the fluorescence intensities of individual components in the sample mixture as they exit the column (Kenkel, 2003). This makes the HPLC fluorescence detector highly sensitive (10 to 1000 times higher than the uv-visible detector) (Arti *et al*., 2011; Kenkel, 2003). As the phototube detects the components individually, the electronic or electric signal produced by the phototube is recorded and the chromatogram is displayed. The HPLC fluorescence detector is only suitable for compounds that fluoresce such as proteins, nucleic acids, aflatoxins, fluorescein, toxic compounds such as cyanide etc (Arti *et al*., 2011; Kenkel, 2003). The three types of HPLC fluorescence detectors are single wavelength fluorescence detector which uses low pressure mercury lamp as an excitation source and has a sensitivity of 1 ng/ml, laser induced fluorescence detector and multi wavelength fluorescence detector (Arti *et al*., 2011).

2.4.4.3 Refractive Index Detector

Refractive index (n) of a compound is defined as the ratio of a speed of light in a vacuum or air (C_{air}) to the speed of light in a liquid (C_{liquid}) (Kenkel, 2003). It is expressed mathematically as;

$$n = C_{(air)}/C_{(liquid)}$$

Refractive index (n) of a material is always greater than one because $C_{(liquid)}$ or speed of light in any material medium is always less than the speed of light in a vacuum or air (Kenkel, 2003).

‘’Refractive index is a bulk property of column eluent’’, hence detection by this detector is dependent on solute modifying the total refractive index of the mobile phase (Arti *et al*., 2011). HPLC refractive index detectors include Christianssen effect detector, interferometer detector, thermal lens detector, dielectric constant detector but the most commonly used one is the refractometer which can give both qualitative and quantitative data on the analyte(s) of interest (Arti *et al*., 2011; Kenkel, 2003).

The refractometer measures the extent of refraction of light rays moving through a thin film of liquid. The refraction occurs as the rays move from a more dense medium (vacuum or air) to a less dense medium (liquid or any other material), hence the more dense medium is always used as the reference point (Kenkel, 2003).

The HPLC refractive index detector is a modification of the basic refractometer. The most popular design of the HPLC refractive index detector consists of both column effluent and pure mobile phase (which act as reference) passing through adjacent cells within the detector (Kenkel, 2003). Light rays which pass through both cells are also directed onto a photosensitive surface and the position of the light rays when the two cells contain the mobile phase is used as the reference point with the recorder pen being set to zero (Kenkel, 2003).

When components of a sample mixture exit the column, the refractive index in one cell changes, beam of light is refracted and directed onto a different position on the photosensitive surface. This causes the recorder pen to deflect and trace the peak which is recorded as the chromatogram (Kenkel, 2003). The main advantage of this detector is that it can be used for almost all compounds since every compound has its characteristic refractive index. The disadvantages include limited sensitivity, recorder output sensitive to temperature effects and

difficulty in using the detector for gradient elution method due to its sensitivity to the composition of the eluent (Arti *et al*., 2011; Kenkel, 2003). The HPLC refractive index detector is very suitable for the detection of non-ionic species that neither absorb in the UV region nor fluoresce (Arti *et al*., 2011).

2.4.4.4 Electrochemical Detector

Electrochemical detector depends primarily on the oxidizing or reducing potential of compounds (Arti *et al*., 2011). An oxidation or reduction reaction which occurs at the surface of an electrode leads to the production of electrons and subsequent generation of electrical signals (Arti *et al*., 2011). An ideal electrochemical detector depends on voltammetric, conductometric or potentiometric properties of the analyte molecules in aqueous or aqueousorganic mobile phase (Arti *et al*., 2011; Skoog *et al*., 2004). Three electrodes namely the indicator (working) electrode for oxidation or reduction processes, auxiliary and reference electrodes both of which compensate for changes in background conductivity of the eluent are required for effective and efficient functioning of an electrochemical detector (Arti *et al*., 2011; Kenkel, 2003). The two types of electrochemical detector are the dynamic or amperometric detector whose operation principally involves electron transfer and the equilibrium detector which do not promote oxidation or reduction of analyte molecules (Arti *et al*., 2011).

2.4.4.4.1 Dynamic or Amperometric Detector

Detection by this detector principally depends on oxidation or reduction of an analyte at a voltage equal to the energy required to cause loss or gain of an electron in a molecule (Arti *et al*., 2011; Kenkel, 2003). The amperometric detector can detect several analytes at a time due

to its multi electrode array detection system which allows the operation of each electrode in array at a different potential (Arti *et al*., 2011). This detector is suitable for the detection of neuro-active substances (Arti *et al*., 2011).

2.4.4.4.2 Equilibrium Detector

Detection by this detector is dependent on the conductance of a flowing solvent (mobile phase) stream. Changes in conductance resulting from the presence of analyte molecules are detected and recorded by the detector (Arti *et al*., 2011; Kenkel, 2003).

2.4.4.5 Electrical Conductivity Detector

Electrical conductivity detector is classified as one of the bulk property detectors and suitable for the detection of all ionic species or compounds capable of producing ions in solution such as anions, cations, metals, organic acids and surfactants (Arti *et al*., 2011). Sensors of electrical conductivity detector have a flow cell which has few microliters (µL) of volume containing two electrodes (Arti *et al*., 2011). These electrodes are mostly made of platinum, stainless steel or any other noble metal (Arti *et al*., 2011). This detector is considered very universal, reproducible and highly sensitive towards charged species, hence suitable for the detection of alkali and alkaline earth cations such as Na^+, K^+, Li+, Ca^{2+}, Mg^{2+} etc (Arti *et al*., 2011).

2.4.4.6 Liquid Light Scattering Detector

Detection by this detector is based on the measurement of scattered or dispersed light from a laser (Arti *et al*., 2011). It is suitable for the detection of large molecular weight compounds such as polymers since these compounds have the potential to scatter light (Arti *et al*., 2011).

The scattered light is examined by passing it through an appropriate sensor and illuminated by a high intensity beam of light (Arti *et al*., 2011). The two types of this detector available are low angle laser light scattering (LALLS) detector and multiple angle laser light scattering (MALLS) detector.

2.4.4.6.1 Low Angle Laser Light Scattering (LALLS) Detector

It is also known as the Fraunhofer diffraction or Mie scattering detector (Arti *et al*., 2011). It is suitable for particles which are larger than 1 µ wavelength of light (Arti *et al*., 2011). The sizes of the particles determine the angle to which light is scattered and thus for large particles, scattering of light occurs at the edge of the particles. This means that intensity of light at different angles of scattering can be used to the relative amount of different size of particles (Arti *et al*., 2011). ‘’In LALLS detector, scattered light is measured at a very small angle (almost zero) to the incident light (Arti *et al*., 2011). The detection provides sensitive, simple and accurate molecular weight measurement for large molecules (Arti *et al*., 2011).

2.4.4.6.2 Multiple Angle Laser Light Scattering (MALLS) Detector

In MALLS detector, measurement of scattered radiation (light) is carried out at several different angles, none of which is close to the incident radiation (Arti *et al*., 2011). Variations in the intensities of these scattered radiations are dependent upon size, shape, nature of material, orientation and internal structure of the particle (Arti *et al*., 2011).

2.4.4.7 Aerosol Based Detectors

These detectors include evaporative light scattering (ELS) detector, charged aerosol detector and nano quantity aerosol (NQA) detector.

2.4.4.7.1 Evaporative Light Scattering Detector

This detector operates on the principle of light scattering just as in laser light scattering detectors (Arti *et al*., 2011). The mode of operation of the detector involves nebulization, evaporation and detection processes (Arti *et al*., 2011). The nebulizer converts the eluent from the column into fine spray droplets. To reduce the background noise, small droplets are used (Arti *et al*., 2011). The fine spray droplets are evaporated using a hot evaporation tube at a different temperature. Solute molecules remaining as fine particles after evaporation go through an optical head meant for the measurement of scattered light (Arti *et al*., 2011). This detector is suitable for both gradient and isocratic elution methods (Arti *et al*., 2011). In situation where analytes are fairly sensitive to temperature, low temperature can be used for evaporation in order to prevent thermal decomposition of analytes (Arti *et al*., 2011). ELS detector is highly sensitive, efficient and very reproducible and allows detection of semivolatile and thermo-sensitive substances due to detection through low temperature and gas supported focusing within the optical head (Arti *et al*., 2011). It is suitable for the detection of substances such as carbohydrates, lipids, or triglycerides, polymer blends (copolymers), surfactants and caffeine.

2.4.4.7.2 Charged Aerosol Detector

This detection technique provides effective and efficient analytical results than other HPLC detection techniques such as ELS, UV, refractive index etc (Arti *et al*., 2011). Charged aerosol detection technique has higher sensitivity, broad dynamic range, excellent reproducibility, consistent response and wide applicability (Arti *et al*., 2011). In charged aerosol detection technique, nitrogen gas is used to nebulize the eluent. The nebulized eluent is dried to form a stream of analyte particles which are allowed to bombard a stream of positive ions produced by corona discharge (Arti *et al*., 2011). The impact of collision leads to the formation of charged

analyte particles which are detected by an electrometer. Detection is dependent on particle size and rate of mobility of analyte particles (Arti *et al*., 2011). A secondary stream of nitrogen gas becomes positively charged as it passes through a high voltage corona wire (Arti *et al*., 2011). This detection technique is suitable for the detection of non-volatile or semi-volatile substances such as pharmaceuticals, lipids, protein, steroids, surfactants, polymers and carbohydrates particularly oligosaccharides. Drugs such as propranolol, salicylic acid, ibuprofen, amitriptyline, dextrin and estradiol can be analysed by this detection technique (Arti *et al*., 2011).

2.4.4.7.3 Nano Quantity Aerosol (NQA) Detector

This is a new aerosol based HPLC detection technique which operates using condensation nucleation (Arti *et al*., 2011). It is highly sensitive and has a wide range of linearity than the already existing aerosol-based HPLC detectors (Arti *et al*., 2011). NQA detector makes use of water condensation particle counter which selectively grows particles through water vapour condensation (Arti *et al*., 2011). The droplets evaporate leaving particles in the form of non-volatile residue. As the level of non-volatile residue increases the sizes of particles also become large enough to be detected optically (Arti *et al*., 2011). The particle count rate is then converted into a chromatogram output signal by the detector (Arti *et al*., 2011). NQA detector is suitable for the detection of drug impurities, degradation products and excipients hence employ in the analysis of non-volatile and semi-volatile substances such as carbohydrates, amino acids, steroids, amines, cholesterol, caffeine, artemisinin, dihydroartemisinin, sucrose, saccharin, theophylline, sulphonic acid and sulphanilamide (Arti *et al*., 2011).

2.4.4.8 Chiral Detectors

Chiral detectors are also known as optical activity detectors (Skoog *et al*., 2004). They are suitable for the detection of optically active compounds or compounds containing anomeric (asymmetric) carbon such as amino acids, sugars, terpenes etc (Arti *et al*., 2011). Chiral detectors include polarimetric detectors (optical rotary dispersion (ORD) detectors) which operate based on changes or differences in refractive index and circular dichroism detectors which also differentiate enantiomers by measuring differences in the absorption of right and left-handed circularly polarized light (Arti *et al*., 2011).

2.4.4.8.1 Polarimetric Detector

Chiral molecules have the property of rotating plane-polarized light either to the left or right direction (Arti *et al*., 2011). The degree of polarized light is measured using a polarimeter, hence chiral molecules for HPLC analysis are detected using the polarimetric detector (Arti *et al*., 2011). The degree of optical rotation, θ is dependent on the number of optically active species that come into contact with the radiation and this in turn is dependent on the analyte concentration and path length of the sample (Arti *et al*., 2011). The degree of optical rotation, θ can be determined from specific rotation, θs expression;

Θs = θ/l.d where l = sample path length (dm) and d = density if sample is a pure liquid or concentration (g/100 ml) if sample is a solution (Arti *et al*., 2011). Polarimetric detector is suitable for the detection of compounds such as diuretics, analgesics, vitamins, antibiotics, amino acids, naphthalene, orange oil, lemon oil etc (Arti *et al*., 2011).

2.4.4.8.2 Circular Dichroism Detector

This detection technique differentiates enantiomers by measuring differences in the absorption of right and left-handed circularly polarized light due to the presence of chiral chromophores (Arti *et al*., 2011). Circular dichroism detector is strongly selective and sensitive to chiral molecules that exhibit strong absorption bands within 220-420 nm in the UV region of the electromagnetic spectrum (Arti *et al*., 2011). This means that a UV signal is provided in addition to the circular dichroism signal, hence has an advantage of optically determining the purity of compounds without chiral separation (Arti *et al*., 2011). It also has the advantage of not being affected by gradient elution or changes resulting from non-chiral components during elution process (Arti *et al*., 2011). This detection technique is suitable for the detection of compounds such as trans-stilbene oxide, pindolol, ibuprofen, flubiprofen, sugar, naproanilide, indapamide, flavanone and triadimefon (Arti *et al*., 2011).

2.4.4.9 Pulsed Amperometric Detector

This detection technique is a three step waveform system termed pulsed amperometric detection and was developed to remove poisons from the surface of electrode during detection (Arti *et al*., 2011). This waveform system which involves the measurement and recording of current at an electrode followed by oxidation to remove foreign particles and then oxidation stripping, is applied to the electrode at least once every second to remove poisons from the electrode surface (Arti *et al*., 2011). This detection technique is suitable for the detection of polar aliphatic compounds which have poor detection characteristics, hence require derivatization for optical measurement. These compounds include amines, alcohols, sulphur moieties and carbohydrates (Arti *et al*., 2011).

The detection limit and linearity range of some groups of HPLC detectors are indicated in the table below;

Table 2.3: Detection limit and linearity range of some groups of HPLC detectors.

Detector	Limit of Detection	Range of Linearity (decades)
UV-Visible	1-10 pg	3-4
Fluorescence	10 fg	5
Electrochemical	100 pg	4-5
Refractive Index	1 ng	3
Electrical Conductivity	100 pg-1 ng	5
Mass Spectrometer	< 1 pg	5
FTIR	1 μg	3
Light Scattering	1 μg	5
Chiral	1 ng	4

Source: (Skoog *et al*., 2004)

2.4.5 HPLC Method Development

Analytical method development and validation are essential components in many pharmaceutical and chemical development programs (Shrivastava and Gupta, 2011; Singh, 2013). This has led to the development and emergence of many analytical methods for the analysis of drugs, foods and other chemical compounds (Santos *et al*., 2009). The quality of a developed analytical method is dependent on the suitability for its intended purpose, recovery, requirement for standardization, sensitivity, analyte stability, ease of analysis, skill subset required, time and cost (Shrivastava and Gupta, 2011). Analytical methods help in the process of new drug synthesis, screen potential drug candidates, support formulation studies, monitor the stability of bulk pharmaceuticals and test final product for release (Shrivastava and Gupta, 2011).

Most analytical method developments are based on spectrophotometry and chromatography with High Performance Liquid Chromatography (HPLC) constituting the greatest percentage

in most chromatographic analytical method development (Suresh *et al*., 2010). HPLC has become a method of choice for drug analysis especially in the analysis of multi-component drugs, hence several researches are on-going for HPLC method development for drug analysis (Suresh *et al*., 2010). Examples of some HPLC methods that have been developed for the analysis of multi-component formulations include method for the simultaneous determination of paracetamol and caffeine in tablet formulation (Tsvetkova *et al*., 2012), method for the simultaneous estimation of paracetamol, caffeine, cetirizine and phenylephrine in tablet dosage form (Malakar *et al*., 2013), method for the analysis of acetylsalicylic acid in the presence of its degradation products (Suresh *et al*., 2010), method for simultaneous quantitation of aspirin, salicylic acid and caffeine in effervescent tablets etc
(Sawyer and Kumar, 2003).

HPLC method development involves several important procedures or processes such as sample pre-treatment, detection of sample bands, choice of separation conditions, quantitation and validation of the developed method (Snyder *et al*., 2005).

HPLC method helps in the identification, quantification and purification of analytes of interest (Singh, 2013). Effective and accurate method development provides optimization of laboratory resources and ensures that the developed method is reliable, cost effective, precise and less time consuming (Singh, 2013). Again, for accurate method development, it is important that the physical and chemical characteristics such as solubility, molecular mass, structure and function, pKa, UV spectra of the compound(s) of interest are known. These allow the choice of most appropriate conditions such as sample preparation and standardization, types of mobile and stationary phases etc that are required during the method development (Singh, 2013). The goal of analytical method development in relation to drugs is to make known the identity, purity, physical characteristics and potency of drugs (Singh, 2013).

2.4.5.1 Components of HPLC Method Development

There are three important or critical components during HPLC method development (Singh, 2013). These are sample preparation, analysis condition and standardization. Sample preparation includes percentage organic, pH, shaking/sonication, sample size and sample age Analysis condition includes percentage organic, pH, flow rate, temperature, wavelength and column dimensions. Standardization includes wavelength, standard concentration and response factor correction (Singh, 2013).

At the primary stage of method development, every individual component must be properly and critically investigated before the final method is optimized. This provides an advantage of carefully evaluating the performance of the method for each component and streamlining the final method for optimization (Santos *et al*., 2009; Singh, 2013).

2.4.5.2 Factors to Consider for Effective Method Development

Many factors such as resolving power, specificity and speed contribute to effective and accurate HPLC method development (Suresh *et al*., 2010; Singh, 2013). However, selectivity is manipulated by a combination of several different factors such as solvent composition, type of stationary/mobile phases, buffers and pH (Suresh *et al*., 2010; Singh, 2013). Variations in mobile phase composition and stationary phase are the best ways to obtain a better separation (Suresh *et al*., 2010; Singh, 2013). For example proper pH range is suitable for the separation of ionisable substances, low pH favours the retention of acidic substances whereas high pH on the other hand favours the retention of basic compounds, neutral compounds are not affected by pH changes i.e whether the medium is acidic or basic (Singh, 2013). pH range between 4 and 8 is not commonly used because a slight change in pH within this range results in

uncontrollable shift in retention of compounds. However, pH range between 2 and 4 and 8 and 10 are most suitable and desirable in HPLC method development (Singh, 2013).

2.4.5.3 Method Validation

According to International Organization for Standardization (ISO), validation is simply ‘’verification, where the specified requirements are adequate for an intended use ’’(ISO/IEC, 2007). The term verification is defined as ‘’ the provision of objective that a given item fulfils the required specifications ‘’ (ISO/IEC, 2007). ‘’ Validation of analytical procedure, therefore, refers to the process of verifying, confirming and ascertaining the suitability of an analytical procedure for its intended use *(*Chanda *et al*., 2010) or ‘’the process by which it is established, by laboratory studies, that the performance characteristics of a method meet the requirements for the intended application’’ (Ahuja and Rasmussen, 2007). The aim of method validation is to identify the critical parameters and to establish acceptance criteria for method system suitability ‘’ (Singh, 2013).

Validation of analytical method demonstrates the accuracy and precision of the method and its acceptability by the scientific community (Suresh *et al*., 2010; Singh, 2013). This means that an analytical method provides measurement of the exact substance in the correct concentration or amount and within which appropriate range for the analyte of interest can be measured (Singh, 2013; Sivakumar *et al*., 2007). Validation also provides means for the analyst to ascertain or understand the nature of the method and to establish the performance limits of the method (Singh, 2013; Sivakumar *et al*., 2007).

Before a validation process begins, the applicability and scope of the analytical method must be clearly defined. This definition includes the analytes, concentration range, description of equipment and procedures, level of validation and the criteria required for validation (Singh,

2013). The validated range is defined by the International Union of Pure and Applied Chemistry (IUPAC) as '' the interval of analyte concentration within which the method can be regarded as validated '' (Singh, 2013). This range is not actually the lowest and highest levels of the analyte that can be determined by the method but it defines the basis of the intended purpose of the method (Singh, 2013).

Methods are validated for qualitative or quantitative information or both. Methods are also validated for use on single equipment, different equipment in the laboratory or different laboratories (Singh, 2013). Method validation takes into account parameters such as accuracy, precision, linearity, selectivity, specificity etc (Crevar *et al*., 2008; Singh, 2013).

2.4.5.4 Method Validation Parameters

Method validation is considered to be the final step in method development (Ahuja and Rasmussen, 2007). Validation assesses the performance and suitability of the method for its intended purpose (Ahuja and Rasmussen, 2007; Singh, 2013). For a method to be accepted as validated for its intended purpose, it must have been statistically evaluated based on parameters such as specificity, accuracy, precision, linearity, limit of detection (LOD) and limit of quantitation (LOQ) etc (Ahuja and Rasmussen, 2007; Singh, 2013).

2.4.5.4.1 Specificity

Specificity refers to the ability to critically assess the analyte of interest in the presence of other components of the sample (Ahuja and Rasmussen, 2007; Singh, 2013). These other components may be impurities, degradation products, placebo ingredients or even the matrix itself which can interfere with the analytical procedure and affect the validity of the analytical results and the method as a whole (Ahuja and Rasmussen, 2007; Singh, 2013). Specificity of an analytical method is determined through comparison of the results from analysis of sample containing

impurities, degradation products or placebo ingredients to the results of the sample without impurities, degradation products or placebo ingredients (Singh, 2013). The lack of specificity of an analytical method is indication of the lack of accuracy, precision and linearity of that analytical method (Ahuja and Rasmussen, 2007).

2.4.5.4.2 Accuracy

Accuracy is simply the closeness of an experimental value to the true value (Ahuja and Rasmussen, 2007; Chandra and Sharma, 2013; Singh, 2013). The experimental value is the value obtained from practical experiments and the true value is the one that is generally accepted as either a conventional true value or a reference value (Ahuja and Rasmussen,

2007). Evaluation of accuracy is done by analysing test samples at different concentrations (Singh, 2013). According to ICH accuracy evaluation is performed using a minimum of nine determinations corresponding to three concentration levels over a specified range (Singh, 2013). The methods of determining accuracy include analysis of an analyte of known purity as a reference material and comparison of the results of the proposed analytical method to those of an already existing, well characterised analytical procedure and standard addition method (Singh, 2013). Accuracy is usually expressed as percentage recovery (Ahuja and Rasmussen, 2007; Chandra and Sharma, 2013; Singh, 2013).

2.4.5.4.3 Precision

Precision refers to the reproducibility or repeatability of experimental measurements that are obtained from various or multiple sampling of a particular homogenous sample under the same experimental conditions (Ahuja and Rasmussen, 2007; Israel *et al*., 2013; Singh, 2013).

Precision is determined at three levels and these include repeatability, intermediate precision and reproducibility (Ahuja and Rasmussen, 2007; Singh, 2013).

Repeatability also termed as intra assay precision is when a particular person carries out analysis in a particular laboratory using a particular set of equipment over a short period of time (Singh, 2013). Repeatability can further be divided into system repeatability which is concerned with carrying out various injections of the same reference solution and expressing the results in terms of RSD on the measured peak areas and analysis repeatability which is also concerned with the precision of the analytical procedure or method under the same working conditions over a short period of time (Ahuja and Rasmussen, 2007).

Intermediate precision also called inter-day precision is related to all the laboratory variations that occur in the analytical method. For example analysis run on different days by different analyst with different equipment i.e it seeks to reflect discrepancies in results when experimental conditions change as a result of different equipment, different analyst and other related experimental conditions (Ahuja and Rasmussen, 2007; Singh, 2013). ‘’The objective of intermediate precision validation is to verify that the analytical method will provide same results in the same laboratory once the development phase is over’’(Singh, 2013).

Reproducibility is concerned with the analysis of the same sample by different analyst in different laboratories under different experimental conditions although experimental conditions may be within the specified parameters of the method (Ahuja and Rasmussen, 2007; Singh, 2013). The objective is to verify that same results can be obtained irrespective of the differences in room temperature, humidity, analyst, equipment etc (Singh, 2013).

2.4.5.4.4 Linearity

An analytical method is said to be linear when test results have a direct relation or proportion to the amount of analyte in the sample (Ahuja and Rasmussen, 2007; Gangishetty and Verma, 2013; Singh, 2013). It is always important to determine the suitable range at which instrumental response directly relates to analyte concentration (Singh, 2013). A minimum of five data points which are obtained from three to six injections of five or more standards are usually required to determine the linearity of an analytical method (Ahuja and Rasmussen, 2007; Singh, 2013). In general a correlation coefficient (R^2) value > 0.998 is considered an acceptable fit of data for the regression line when peak area or height values are plotted against concentrations of standard solutions (Ahuja and Rasmussen, 2007; Singh, 2013).

Although R^2 is used as basis for linearity, linearity can also be determined statistically from the intercept (y-intercept) of the regression line and slope of the regression line (Ahuja and Rasmussen, 2007).

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

LOQ refers to the lowest amount of analyte in a sample that can be determined quantitatively based on suitable level of precision and accuracy. LOD on the other hand refers to the lowest amount of analyte that can be detected (Ahuja and Rasmussen, 2007; Devi *et al*., 2013; Singh, 2013). According to ICH, LOQ and LOD can be determined through visual evaluation, signal-to-noise ratio and standard deviation of the response and slope but the most commonly used is the signal-to-noise ratio (Ahuja and Rasmussen, 2007; Singh, 2013).

Visual evaluation can be applied to both instrumental and non-instrumental methods (Singh, 2013). It involves the use of standard solution of the analyte and establishment of the minimum

concentration at which the analyte can be easily detected or quantified based on suitable level of precision and accuracy (Singh, 2013).

Signal-to-noise (S/N) ratio may be used for analytical procedures that exhibit baseline noise (Singh, 2013). ‘’It is determined by comparing measured signals from samples with known low concentrations of the analyte with those of blank samples and establishing minimum concentration at which the analyte can be reliably detected’’(Singh, 2013). A S/N ratio of 3:1 with RSD ≤ 10 % is considered appropriate in determining LOD while that of LOQ is 10:1 with RSD ≤ 3 % (Singh, 2013).

Standard deviation of the response and slope involves using formula to express LOD and LOQ (Singh, 2013). LOD is expressed as 3.3 x θ/S and LOQ = 10 x θ/S where θ = standard deviation of the response which is taken as standard deviation of analytical background responses of an appropriate number of blank samples and S = slope of the calibration curve (Singh, 2013).

2.4.5.4.6 Range

This refers to the interval between the highest and lowest amount of the analyte in the sample as well as amount at which the analytical method has been shown to have a suitable level of precision, accuracy and linearity (Ahuja and Rasmussen, 2007; Singh, 2013). The range of an analytical method is determined based on the intended purpose of the method (Singh, 2013). ‘’It is generally 80 to 120 % of the test and for assay of a drug substance or a finished drug product, 70 to 130 % of the test concentration for content uniformity, ± 20 % over the specified range for dissociation testing, reporting level of an impurity and it should fall in line with LOD and LOQ for impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects’’ (Singh, 2013).

2.4.5.4.7 Robustness

Robustness determines the ability of an analytical method to be unaffected by small and deliberate variations in method parameters and gives certainty for the normal use of the analytical method (Singh, 2013). Robustness testing involve a variation in a number of chromatographic parameters such as column temperature, injection volume, mobile phase composition, flow rate, detection wavelength etc (Singh, 2013). These parameters are varied within a reasonable, acceptable and realistic range so that quantitative influence on the parameters can be determined (Singh, 2013). A parameter is considered to be within robustness range if the influence on the parameter is within a previously specified tolerance level (Singh, 2013). Data on robustness enable the analyst to determine whether or not the analytical method has to be revalidated if one or more of the parameters is/are changed, for example to assess or compensate for column performance with time (Singh, 2013). Variation in method condition for robustness should be small and reflect typical day-to-day variation (Singh, 2013). Identification of critical parameters during the method development is essential for robustness testing (Singh, 2013). The two types of critical parameters are:

a.) HPLC conditions which include HPLC column (Lot, age and brand), composition of mobile phase (pH ± 0.05 unit, organic content ± 2 %) and HPLC instrument (injection volume, detection wavelength ± 2 nm, column temperature ± 5 ^{o}C, flow rate).

b.) Sample preparation variations which include sample solvent (pH ± 0.05 unit, organic content ± 2 %), sample preparation procedure (shaking time, different membrane filters) and HPLC solution stability (Singh, 2013). Below is a table for variations in robustness parameters during method development.

Table 2.4: Variations in robustness parameters for method development.

Parameter	Change
Detection wavelength	± 5 nm
Flow rate	± 0.05 ml/min
Buffer pH	± 0.1 unit
Mobile phase	± 2 ml
Column	Different brand/batch number

Source: (Singh, 2013).

2.4.5.4.8 System Suitability

This forms or constitutes an essential stage in the method validation process (Ahuja and Rasmussen, 2007). It defines the adequacy in terms of the performance of the chromatographic system (Ahuja and Rasmussen, 2007). System suitability is dependent on factors such as retention time, tailing factor, number of theoretical plates, resolution, symmetry and peak area (Singh, 2013).

2.4.5.5 HPLC Methods Developed for Simultaneous Identification and Quantification of Active Ingredients in Multi-component Drug Formulations

Table 2.5: Some already developed HPLC methods for the estimation of multicomponent drug formulations.

Authors and Year	Stationary Phase	Mobile Phase	Flow Rate	Components Separated	Detector (Wavelength, nm)
Tsvetkova *et al*., (2012)	Phenomenex-Luna C8 (125x 4.6 mm)	Acetonitrile-Phosphate buffer (60:40)	1ml/min	ASA and Paracetamol	UV at 230

Sawyer and Kumar, (2003)	Hypersil C18 (5 um, 150x 4.6 mm)	Water-Methanol-Acetic acid	-	ASA, Salicylic acid and Caffeine	UV at 275
Altun, (2002)	µ-bondapack C8	0.01M KH_2PO_4Methanol-Acetonitrile-Isopropyl alcohol (420:20:30:30)	1 ml/min	Paracetamol, Caffeine and Dipyrone	UV at 215

Bharate and Bharate, (2012)	SMT-C18, ODS-5, 100/25 (250x4.6 mm)	Methanol	-	ASA and Caffeine	UV at 240
Chandra and Sharma, (2013)	C18	Methanol-Water (40:60)	1ml/min	Paracetamol and Caffeine	UV at 243
Xu and Stewart, (2000)	Non-porous ODS 3 um	Acetonitrile-50mM Phosphate buffer (2:98)	1.5ml/min	ASA, Caffeine and Butalbital	-
		Methanol-50 Mm Phosphate buffer (3:97)	2 ml/min	Acetaminophen, Caffeine and Butalbital	

Hadad and Mahmoud, (2011)	Conventional C18 Monolithic C18	Methanol10mM NaH_2PO_4, 3 Mm Heptane Sulfonic acid sodium salt, 0.1 % Triethylamine buffer (50:50) Same composition (40:60)	-	Caffeine, Paracetamol, Pseudoephrine, ASA, Dextromethorphan and Chlorpheniramine	UV at 214 nm
Golubitskii *et*	Adsorbent with immobilised nitrile groups	Acetonitrile-	-	Paracetamol, ASA	-
al., (2007)		KH_2PO_4 buffer		and Caffeine	
Devi *et al*., (2013)	C18 (250 x 4.6 mm, 5 um)	Acetonitrilewater (25:75) + Orthophosphoric acid	-	Paracetamol	UV at 207

2.5 HPLC QUALITATIVE AND QUANTITATIVE DETERMINATIONS

HPLC can be employed for both qualitative and quantitative analyses of almost all compounds in the field of science. As a result HPLC has a very wide applicability in many areas of science such as environmental, pharmaceutical, forensic, industrial, research and even in clinical analysis of blood, plasma, serum, urine and other biological samples (Harvey, 2000). Qualitative determination describes the identity of a particular analyte in a given sample while

quantitative determination expresses how much or the amount of a particular analyte available in a given sample (Harvey, 2000; Kenkel, 2003; Skoog *et al*., 2004).

Besides diode array, IR and mass spectrometer detectors which give additional identification information such as finger-printing in mass spectrometry, qualitative determination with HPLC are solely provided by retention time of analytes or components in a sample mixture (Arti *et al*., 2011; Kenkel, 2003; Skoog *et al*., 2004). Retention time is the time taken for the components of a sample mixture to be eluted from the column or to be detected after injection into the chromatograph (Harvey, 2000; Kenkel, 2003; Skoog *et al*., 2004). Retention time is characteristic of a particular analyte and is dependent on factors such as mobile and stationary phase compositions, flow rate of the mobile phase, column length, column temperature and dead volume of the instrument. Retention time of a particular analyte changes only when any of these factors changes. Therefore retention time provides the required identification information in most HPLC analysis (Kenkel, 2003). Like gas chromatography, retention time in HPLC analysis is determined from standard solutions of the analytes or components of interest (Kenkel, 2003).

HPLC quantitative information on the other hand is obtained from the peak size or peak area or peak height (Kenkel, 2003). This is achieved by injecting standard solutions with varying concentrations of the analyte of interest and generating a standard or calibration curve of peak size or area against concentration of standards (Jain *et al*., 2012; Kenkel, 2003; Vidhya and Sunil, 2012). The standard curve is then used to compute for the amount (concentration) of each analyte by extrapolating from the peak size axis to the concentration axis (Jain *et al*., 2012; Kenkel, 2003; Vidhya and Sunil, 2012). The concentration of the analyte in the sample can be obtained by either external standard method or internal standard method or area normalization method or standard addition method but the most commonly used methods are the external and

internal standard methods (Center for Drug Evaluation and Research (CDER), 1994; Kupiec, 2004).

2.5.1 External Standard Method

This is the simplest and most widely used HPLC quantitation method (Kupiec, 2004). The accuracy of the external standard method depends on the reproducibility of the injection volume (Kupiec, 2004). For the external standard method, the standard or known material is separate from the unknown or sample and both known data from a calibration standard and unknown data from the sample are used to generate a quantitative report through a plot of peak area versus concentration of standard solutions (CDER, 1994; Kupiec, 2004). The amount of analyte in the sample is determined by extrapolating from the peak area axis to the concentration axis or by the formula:

$$Conc._{(analyte)} = [Peak\ area_{(analyte)}/Peak\ area_{(standard)}] \times Conc._{(standard)}$$ (Kupiec, 2004).

External standard method is suitable for: samples with narrow concentration range, simple sample preparation procedure, increased baseline time for detection of potential extraneous peaks eg impurities test (CDER, 1994). Advantage of external standard method is its simplicity and wide application to a variety of procedures. The disadvantage is that it is adversely affected by the stability of the chromatographic system and the presence of chromatographic interferences in a sample or sample extract (Kupiec, 2004).

2.5.2 Internal Standard Method

In the internal standard method, a compound that is not present in the sample is added in equal amount to both the sample and the standard solutions to compensate for losses during sample preparation and variability during analytical determination (Kupiec, 2004). This compound is

termed the internal standard and should have similar physical and chemical properties (especially in relation to retention time and structure) to the analyte of interest (Kupiec, 2004). In addition, the internal standard must be of high purity and should show no interference with the components of the sample mixture (Kupiec, 2004). In cases where analyte extraction is required, the internal standard is added to the sample before extraction is carried out (Kupiec, 2004). Quantitation of the analyte can be achieved through a calibration plot of the ratio of peak area or height of the standards and the internal standard versus the ratio of the concentration of the standards and the internal standard (Kupiec, 2004). The analyte concentration is then determined by extrapolating from the peak area ratio axis to the concentration ratio axis or by the formula:

$Conc._{(sample)}$ = [$Area_{(IS)}$ of standard solution/$Area_{(IS)}$ of sample] x [$Area_{(sample)}$/$Area_{(standard)}$] x $Conc._{(standard)}$ where IS = internal standard (Kupiec, 2004).

The internal standard method has an advantage of yielding the most accurate and precise results as compared to external standard (Kupiec, 2004). This method is more suitable for: complex samples preparation procedures especially the ones involving multiple extractions, low concentration samples especially in situations where sensitivity is a problem eg pharmacokinetic studies and wide range of concentrations expected in the sample for analysis e.g pharmacokinetic studies (CDER, 1994). The properties of a good internal standard include:

1. A retention time close to that of the analyte of interest.
2. Physical and chemical similarity to the analyte of interest.
3. Being absent as a component in the original mixture.
4. Must be available in a high state of purity
5. Must not be reactive towards any of the sample component
6. Is added in a concentration range of 0.3 to 0.5 of the analyte nominal concentration (CDER, 1994).

CHAPTER THREE

3.0 METHODOLOGY

3.1 CHEMICALS/REAGENTS, APPARATUS/GLASSWARES AND EQUIPMENT

3.1.1 Chemicals/Reagents

Chemical/Reagent	Source
1. Concentrated Hydrochloric acid	Analar Normapur, Analyrical grade
2. Potassium dichromate Crystals	Analar Normapur, Analytical grade
3. Sodium Hydroxide pellets	Analar Normapur, Analytical grade
4. Calcium Hydroxide	‘’ ‘’
5. Nitrobenzaldehyde	‘’ ‘’
6. Sulphamic acid crystals	BDH Chemicals, Analytical grade
7. Iodine crystals	BDH Chemicals, Analytical grade
8. Potassium Iodide crystals	BDH Chemicals, Analytical grade

9. Chloroform BDH Chemicals, Analytical grade

10. Acetone '' ''

11. Diethyl ether Analar Normapur, Analytical grade

12. Methanol '' HPLC grade

13. Glacial Acetic Acid '' Analytical grade

14. 0.0167 M Potassium dichromate solution

15. Sodium hydroxide solutions (5, 1, 0.25, 0.1 M) 16. 0.25 M Sulphamic acid solution

17. Hydrochloric acid solution (1, 0.25 M) 18. 0.05 M Iodine solution

19. Iron (iii) chloride solution 20. Phenol red solution

21. Phenolpthalein indicator 22. Distilled water

23. Tablet Samples

24. Reference standards (ASA, Paracetamol and Caffeine)

3.1.2 Apparatus/Glassware

1. Volumetric flasks (25, 50, 100, 200, 250, 500, 1000 and 2000 ml)

2. 50 ml burette 3. 25 ml Pipette

4. 250 ml conical flask 5. Glass beakers (50, 100, 150 and 250 ml)

6. Glass rod/stirrer 7. Spatula

8. Separating funnel 9. Glass Funnels

10. 5 ml Graduated pipette 11. Whatsman filter paper

12. Retort stand and clamp
13. Ice bath

14. Thin Layer Chromatography Plates
15. Capillary Tubes

16. Chromatographic Tank

3.1.3 Equipment

1. Bellstone Analytical Balance/Satorius CPA623S Analytical Balance

2. Labinco Hot Plate Stirrer, Model L-81

3. Fast Thermostat Digital Display Water bath, Model HH-42

4. Shimadzu UV/Visible 1240 Mini Spectrophotometer

5. Perkin Elmer HPLC Chromera Version 4.1.0.6386

6. Perkin Elmer Flexar Photodiode Array (PDA) LC Detector, N3896

7. Refrigerator

8. Haraeus Klasse 2 Oven

Table 3.1: Information on tablet samples

Sample	Manufacturer	Manufactured Date	Expiry Date	Batch Number	Source
A = Effpac	Amponsah Effah Pharmaceuticals Ltd, Kumasi-Ghana	June, 2014	June, 2017	BN244	Elite Pharmacy, Bomso-Kumasi
B = Cafalgin	M&G Pharmaceuticals Ltd, Accra-Ghana	May, 2014	May, 2016	CF 255V	Elite Pharmacy, Bomso-Kumasi

Table 3.2: Information on reference standards of ASA, Paracetamol and Caffeine

Reference Standard	Manufacturer	Manufactured Date	Expiry Date
ASA	Alta Labs Ltd, India	March, 2013	March, 2018

Paracetamol	Hebei Jiheng (Group) Pharmaceutical Co. Ltd, China	May, 2014	May, 2018
Caffeine	AARTI Industries Ltd, India	January, 2014	December, 2018

3.2 PREPARATION OF SOLUTIONS/REAGENTS

3.2.1 Preparation of 500 ml 5 M NaOH Solution

Approximately 101.0000 g of NaOH pellets were weighed accurately into a beaker, dissolved in some quantity of distilled water and transferred into a 500 ml volumetric flask. The solution was well shaken, topped up to the 500 ml mark of the volumetric flask with distilled water and labelled accordingly.

NB: Lower concentrations of 0.10, 0.25 and 1 M NaOH solutions were prepared from the 5 M NaOH solution using the serial dilution formula: $V1 = \frac{C2V2}{C1}$.

Where C_1 and C_2 are initial and final concentrations and V_1 and V_2 are initial and final volumes respectively.

3.2.2 Preparation of 250 ml 1 M HCl Solution

Approximately 21.20 ml of concentrated HCl solution (11.80 M) was measured accurately and mixed with some quantity of distilled water in a 250 ml volumetric flask. The solution was well shaken for complete mixing, topped up to the 250 ml mark of the volumetric flask with distilled water and labelled accordingly.

NB: Lower concentration of 0.25 M HCl solution was prepared from the 1 M HCl solution using the serial dilution formula: $V1 = \frac{C2V2}{C1}$.

Where C_1 and C_2 are initial and final concentrations and V_1 and V_2 are initial and final volumes respectively.

3.2.3 Preparation of 100 ml 0.0167 M $K_2Cr_2O_7$ Solution

Approximately 1.2311 g of $K_2Cr_2O_7$ was weighed accurately into a beaker, dissolved in some quantity of distilled water and transferred into a 100 ml volumetric flask. The solution was well shaken, topped up to the 100 ml mark of the volumetric flask with distilled water and labelled accordingly.

3.2.4 Preparation of 250 ml 0.25 M H_2NSO_3H Solution

Approximately 6.1237 g of H_2NSO_3H was weighed accurately into a beaker, dissolved in some quantity of distilled water and transferred into a 250 ml volumetric flask. The solution was well shaken, topped up to the 250 ml mark of the volumetric flask with distilled water and labelled accordingly.

3.2.5 Preparation of 50 ml Ferric Chloride Reagent

Approximately 7.0000 g of $FeCl_3.6H_2O$ was weighed accurately into a 50 ml beaker, dissolved in some quantity of distilled water and 1 ml of concentrated HCl added. The solution was transferred into a 50 ml volumetric flask, shaken well, topped up to the 50 ml mark of the volumetric flask with distilled water and labelled accordingly.

3.2.6 Preparation of 50 ml 0.05 M Iodine (I_2) Solution

Approximately 0.6500 g of I_2 and 1 g of KI crystals were accurately weighed and put into a beaker. Minimum amount of water was added to aid the dissolution of the I_2 in KI. The solution was transferred into a 50 ml volumetric flask, topped up to the 50 ml mark with distilled water and labelled appropriately.

3.3 IDENTIFICATION TEST

3.3.1 Identification of Reference Standards of Active Drugs

3.3.1.1 Colour Identification Test

For ASA, few grams of the ASA reference standard was heated to boiling with about 10 ml of 5 M NaOH solution for about 2-3 minutes. The solution was cooled, an excess of iron (iii) chloride solution was added and the observation recorded (BP, 2009).

Again, few grams of ASA reference standard was mixed with 0.5000 g calcium hydroxide. The mixture was heated and the fumes produced were exposed to a piece of filter paper which had been soaked in nitrobenzaldehyde solution. The observation was then recorded (BP, 2009).

For paracetamol, few grams of the reference standard of paracetamol was heated to boiling with 1 ml concentrated HCl for about three minutes. Approximately 10 ml of water was added and the solution cooled in an ice bath. This was followed by the addition of approximately 0.05 ml of 4.9000 g/L (0.0167 M) $K_2Cr_2O_7$ solution and the observation recorded (BP, 2009).

Colour test for caffeine was carried out by adding a few millilitres of saturated pure caffeine solution to 0.05 ml of 0.05 M iodine solution followed by 0.1 ml of dilute HCl. The solution was neutralized using dilute NaOH solution and the observation recorded (BP, 2009).

3.3.1.2 Specific Absorbance Identification Test for Paracetamol and Caffeine

For paracetamol, a 0.1500 g quantity of the reference standard of paracetamol was accurately weighed and dissolved in 50 ml of 0.10 M NaOH and 100 ml of water was added. The solution was shaken for about fifteen minutes and the solution topped up to the 200 ml mark with water. The resulting solution was filtered and 10 ml of the filtrate was diluted to 100 ml with water. Ten millilitres (10 ml) of the resulting solution was then added to 10 ml of 0.10 M NaOH solution and the mixture diluted to 100 ml with water. The absorbance of the resulting solution was then measured at a wavelength of 257 nm and the specific absorbance A (1 %, 1 cm) value determined from the measured absorbance and the concentration of the solution (BP, 2009).

For caffeine, a 0.0300 g of caffeine reference standard was accurately weighed, dissolved in 200 ml of water and shaken for about 30 minutes. Sufficient water was then added to produce 250 ml of the solution, after which the solution was filtered. Approximately 10 ml of 1 M NaOH solution was added to 10 ml of the filtrate and the mixture diluted to 100 ml with water. The absorbance of the resulting solution was measured at a wavelength of 273 nm and the specific absorbance value was determined (BP, 2009).

3.3.2 Identification of Active Pharmaceutical Ingredients (APIs) in Tablet Samples

3.3.2.1 Colour Identification Test

For ASA, few grams of the powdered tablets was heated to boiling with about 10 ml of 5 M NaOH solution for about 2-3 minutes. The solution was cooled, excess iron (iii) chloride solution was added and the observation recorded (BP, 2009).

Another test was also performed by mixing few grams of powdered tablets with 0.5000 g calcium hydroxide. The mixture was heated, the fumes produced were exposed to a piece of

filter paper which had been soaked in nitrobenzaldehyde solution and the observation recorded (BP, 2009).

Paracetamol was identified by boiling few grams of the powdered tablets with 1 ml concentrated HCl for about three minutes. Approximately 10 ml of water was added and the solution cooled in an ice bath. This was followed by the addition of approximately 0.05 ml of 4.9000 g/L (0.0167 M) $K_2Cr_2O_7$ solution and the observation recorded (BP, 2009).

Caffeine was also identified by adding few millilitres of powdered tablets sample solution to 0.05 ml of 0.05 M iodine solution followed by 0.1 ml of dilute HCl. The solution was neutralised using dilute NaOH solution and the observation recorded (BP, 2009).

3.3.3 Identification of APIs in Tablet Samples by Thin Layer Chromatography (TLC)

Solutions of reference standards of ASA, paracetamol and caffeine and the powdered tablet samples were made using methanol. ASA and tablet sample solutions were spotted on the TLC plate placed in a chromatographic tank containing mixture of chloroform and acetone as the mobile phase in a ratio of 4:1 respectively. The spots on the TLC plates were identified using iodine vapour as colour developing agent. The retention factors (R_f) for the reference standard and the API in the tablet samples were then determined.

The above method was repeated for paracetamol and caffeine but in the case of caffeine, the mobile phase was a mixture of chloroform and methanol in a ratio of 9:1 respectively.

3.4 ASSAY OF REFERENCE STANDARDS OF ACTIVE DRUGS

3.4.1 Standardization of Solutions A. Standardization of 0.25 M Sodium Hydroxide Solution Using Sulphamic Acid

Solution as Primary Standard

A 25 ml aliquot of standard sulphamic acid solution was pipetted into a 250 ml conical flask and two drops of phenolphthalein indicator added. The solution was titrated against approximately 0.25 M sodium hydroxide solution until a faint pink colour which indicated the end point was observed. The procedure was repeated two more times to obtain consistent titre values and the average titre determined (Olaniyi and Ogungbamila, 2012).

B. Standardization of 0.25 M Hydrochloric Acid Solution Using 0.25 M Sodium Hydroxide Solution as Secondary Standard

A 25 ml aliquot of standard sodium hydroxide solution was pipetted into a 250 ml conical flask and two drops of phenolphthalein indicator added. The solution was titrated against approximately 0.25 M hydrochloric acid solution until a colourless solution which indicated the end point was observed. The procedure was repeated two more times to obtain consistent titre values and the average titre determined (Olaniyi and Ogungbamila, 2012).

3.4.2 Back-Titration to Determine the Percentage Purity of Reference Standard of ASA

A quantity of 0.2500 g of reference standard of ASA was accurately weighed, transferred into a 250 ml conical flask and 25 ml of 0.256 M sodium hydroxide solution was added. The solution was heated on a water bath for about 10 minutes at 60 ^{o}C, cooled, 2 drops of phenol red indicator added and the solution titrated against 0.213 M hydrochloric acid solution from a burette until a yellow colour which indicated the end point was observed. The titre value was then recorded. A blank determination was carried out by pipetting 25 ml of 0.256 M sodium hydroxide solution and heating for about 10 minutes at 60 ^{o}C. The solution was cooled, 2 drops of phenol red indicator were added and the solution titrated against 0.213 M hydrochloric acid

solution from a burette until a yellow colour which indicated the end point was observed. The titre value for the blank was also recorded and the difference between the blank titre and the test titre represented the amount of 0.256 M sodium hydroxide solution required by ASA. The procedure was repeated two more times and the percentage purity of the ASA reference standard was determined (BP, 2009).

3.4.3 Assay of Reference Standard of Paracetamol Using UV Spectrophotometry

A quantity of 0.1520 g of reference standard of paracetamol was weighed accurately, dissolved in 50 ml of approximately 0.10 M sodium hydroxide solution and 100 ml of water was added. The solution was transferred into a 200 ml volumetric flask, shaken for about 15 minutes and topped up with water to the 200 ml mark of the volumetric flask. The solution was filtered and 10 ml of the filtrate was diluted to 100 ml with distilled water in a 100 ml volumetric flask. Ten millilitres (10 ml) of the resulting solution was added to 10 ml of approximately 0.10 M NaOH solution in a 100 ml volumetric flask and the solution diluted to 100 ml with distilled water. The absorbance of the resulting solution was then measured at a wavelength of 257 nm. The percentage purity of the reference standard was calculated from the measured absorbance value and specific absorbance, A (1 %, 1 cm) value of 715 (BP, 2009).

3.4.4 Assay of Reference Standard of Caffeine Using UV Spectrophotometry

A 0.0310 g quantity of reference standard of caffeine was weighed accurately, dissolved in 200 ml of distilled water, transferred into a 250 ml volumetric flask and the solution shaken for about 30 minutes. The solution was topped up to the 250 ml mark of the volumetric flask and filtered. Ten millilitres (10 ml) of the filtrate was added to 10 ml of approximately 1 M NaOH solution and the mixture diluted to 100 ml with distilled water. The absorbance of the resulting solution was measured at maximum wavelength of 273 nm. The percentage purity of the

reference standard was calculated from the measured absorbance value and specific absorbance, A (1 %, 1 cm) value of 504 (BP, 2009).

3.5 HPLC METHOD DEVELOPMENT

3.5.1 Considerable Parameters

HPLC method development takes into consideration certain physico-chemical properties of the APIs under consideration. Therefore, before the method development, certain physicochemical properties such polarity, purity, solubility, chemical structures, wavelength for maximum absorption, ionization/dissociation constants etc of the APIs under consideration were carefully studied. These aided in the choice of the mobile phase, stationary phase and the wavelength for the detection of all APIs. In the case of solubility, several solvent systems such as water, isopropyl alcohol, chloroform, ethanol, acetic acid, methanol and a combination of two or more of these solvents were tried. The solvent system that was very inert and aided complete dissolution of the APIs in the shortest possible time was chosen.

3.5.2 Establishment of Chromatographic Conditions

3.5.2.1 Mobile Phase Determination

To obtain a suitable mobile phase to aid complete resolution of the three APIs, different ratios such as 90:10, 80:20, 75:25, 70:30, 50:50 of acidic water-methanol solvent system were tried. Finally, a ratio of 60:40 acidic water-methanol solvent system aided the complete and better resolution of all the three components within the shortest period, hence was used as the mobile phase. The acidic water was a combination of distilled water-acetic acid in 75:0.2 ratio.

3.5.2.2 Stationary Phase Selection

In RP-HPLC, the best columns especially for drug analysis are Octylsilane (OS) columns (C8) or Octadecyl Silane (ODS) columns (C18). Therefore, based on the polarities of the three active components and the mobile phase, an OS column, Brownlee Analytical C8, 5 µm, 150x4.6 mm was chosen for the method development.

3.5.2.3 Detection Wavelength Determination

The 270 nm wavelength was selected after a UV-Visible spectrophotometer scan within a wavelength range of 200 nm to 400 nm. The 270 nm was the common wavelength at which all three components were suitably detected.

3.5.2.4 Flow Rate Determination

The liquid chromatograph pump was set to deliver the mobile phase at flow rates of 0.5 ml/min, 0.75 ml/min, 1 ml/min, 1.5 ml/min and 2 ml/min. However, the 1 ml/min produced the best separations within reasonable retention times and best peak resolutions, hence was selected as the flow rate for the method development.

3.5.3 Validation of the Developed Method

The method was validated based on ICH guidelines, using validation parameters such as accuracy, linearity, precision, specificity, robustness, LOQ and LOD.

3.5.3.1 Accuracy

The accuracy of the developed method was determined by calculating the percentage recovery by using the five (5) different concentrations of the standard solutions for the three active components. Percent recovery (% R) was calculated based on the formula:

$$\% \text{ R} = \frac{Amount\ recovered\ (ppm)}{Injected\ amount\ (ppm)}\ x\ 100\ \%$$

The accuracy of the developed method was evaluated statistically. The assay values of the tablet samples were also used as a measure of accuracy of the developed method.

3.5.3.2 Linearity

The linearity of the developed method for each API was determined from the calibration plot of peak area against concentrations of standard solutions for each API. The R^2 values obtained for each curve depicted the linear relationship existing between the peak areas and the concentration of the standard solutions. The linearity of the developed method was also analysed statistically.

3.5.3.3 Precision

Precision of the developed method was determined based on intra-day and inter-day parameters. The intra and inter-day parameters were carried out for reference standards using six determinations each. Precision was determined by carrying out six (6) determinations each of 20 and 100 ppm of ASA, 15 and 20 ppm for paracetamol and 2.5 and 5 ppm for caffeine and calculating the actual concentrations of these standard solutions. The precision was also evaluated statistically.

3.5.3.4 Specificity

Specificity of the developed was evaluated by preparing a solution of the reference standards of the three APIs in the presence of excipients or impurities. Five (5) injections of this solution were carried out to observe any interfering peaks.

3.5.3.5 Robustness

Robustness was established by varying some parameters of the HPLC conditions or the established chromatographic conditions. The parameters that were varied included the flow rate, mobile phase composition, mobile phase pH, wavelength, column and column temperature. Robustness was carried out by preparing six sample solutions. These solutions were ran by varying the above mentioned parameters to determine their influence on the developed method.

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

The LOQ and LOD of the developed method was established based on the calibration curve and the following formulae:

LOD = $\frac{3.3\sigma}{s}$ and LOQ = $\frac{10\sigma}{s}$ where σ = standard deviation of the calibration curve and s = slope of the calibration curve.

3.6 ASSAY OF TABLET SAMPLES USING BP, (2009) METHOD

3.6.1 Determination of the Content (mg and %) of ASA in Tablet Samples by Back-Titration

Forty (40) tablets each of both tablet samples were ground to powder and an average weight equivalent to the content (mg) of ASA in both tablet samples was accurately weighed and dissolved in 25 ml of

0.256 M NaOH solution. The mixture was heated on a water bath for about ten minutes at 60 ºC and cooled. Two drops of phenol red indicator were added and the mixture titrated against 0.213 M HCl solution from the burette until a yellow colour which indicated the end-point was observed. A blank determination was carried out by pipetting 25 ml of 0.256 M sodium hydroxide solution and heating for about 10 minutes at 60 ºC. The solution was cooled, 2 drops of phenol red indicator were added and the solution titrated against 0.213 M hydrochloric acid solution from a burette until a yellow colour which indicated the end point was observed. The procedure was repeated two more times and the content (mg and %) of ASA in both tablet samples was calculated using the expression 0.0225 g ASA ≡ 1 ml 0.25 M NaOH solution (BP, 2009).

3.6.2 Determination of the Content (mg and %) of Paracetamol in Tablet Samples by UV Spectrophotometry

Forty (40) tablets each of both tablet samples were ground to powder and an average weight equivalent to the content (mg) of paracetamol in both tablet samples was accurately weighed. Five millilitres of water was added to the powdered tablets followed by 10 ml of diethyl ether. The mixture was shaken vigorously and continuously for about 30 minutes and allowed to stand for 2 hours. The organic layer was separated from the aqueous layer and the organic solvent was evaporated to obtain the residue. The residue was washed severally with water and dried in an oven at 60 ºC for 1 hour. The dried residue was dissolved in 50 ml of approximately 0.1 M NaOH solution and 100 ml of water was added. The solution was shaken for about 15 minutes and then topped up to 200 ml with water. The resulting solution was filtered and 10 ml of the filtrate was diluted to 100 ml with water. Ten millilitres of the resulting solution was then added to 10 ml of approximately 0.1 M NaOH solution and the mixture diluted to 100 ml with water. The absorbance of the resulting solution was then measured at 257 nm taking 715 as the specific absorbance A (1 %, 1 cm) value of paracetamol at 257 nm. The content (mg and %) of paracetamol in each tablet sample was calculated (BP, 2009).

3.6.3 Determination of the Content (mg and %) of Caffeine in Tablet Samples by UV Spectrophotometry

Forty (40) tablets each of both tablet samples were ground to powder and an average weight equivalent to the content (mg) of Caffeine in both tablet samples was accurately weighed. Five millilitres of water was added to the powdered tablets followed by 10 ml of chloroform. The mixture was shaken vigorously and continuously for about 30 minutes and allowed to stand for 2 hours. The organic layer was separated from the aqueous layer and the organic solvent was evaporated to obtain the residue. The residue was washed severally with water and dried in an oven at 60 ^{o}C for 1 hour. The dried residue was dissolved in 200 ml of water and shaken for about 30 minutes. Sufficient water was added to produce 250 ml and the solution filtered. Ten millilitres of approximately 1 M NaOH solution was added to 10 ml of the filtrate and the mixture diluted to 100 ml with water. The absorbance of the resulting solution was then measured at 273 nm taking 504 as the specific absorbance A (1 %, 1 cm) value of caffeine at 273 nm. The content (mg and %) of caffeine in each tablet sample was calculated (BP, 2009).

3.7 ANALYSIS OF TABLET SAMPLES USING THE DEVELOPED HPLC METHOD

3.7.1 Preparation of Mobile Phase/Dissolution Medium

A 1000 ml mobile phase solution was prepared by mixing and shaking carefully 598.40 ml of distilled water, 1.60 ml of glacial acetic acid and 400 ml of methanol in a 1000 ml volumetric flask.

3.7.2 Preparation of Standard Stock Solutions

3.7.2.1 For ASA

Approximately 0.2000 g of reference standard of ASA was accurately weighed, dissolved in some quantity of the mobile phase, transferred into a 100 ml volumetric and topped up to the 100 ml mark of the volumetric flask using the mobile phase to make a 2000 ppm ASA stock solution. Serial dilutions were carried out to obtain standard solutions of concentrations 20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm. The standard solutions were filtered and 10 µL of each was injected into the HPLC to obtain the chromatograms. Six (6) injections were carried out for each standard solution.

3.7.2.2 For Paracetamol

Approximately 0.2000 g of reference standard of paracetamol was accurately weighed, dissolved in some quantity of the mobile phase and transferred into a 100 ml volumetric flask. The solution was topped up to the mark of the volumetric flask to obtain a 2000 ppm standard stock solution of paracetamol. Approximately 2.5 ml of the 2000 ppm solution was further diluted to 100 ml to obtain a 50 ppm stock solution. Serial dilutions of the 50 ppm solution were carried out to obtain standard solutions of concentrations 2.5 ppm, 5 ppm, 10 ppm, 15 ppm and 20 ppm. The standard solutions were filtered and 10 µL of each was injected into the HPLC to obtain the chromatograms. Six (6) injections were carried out for each standard solution.

3.7.2.3 For Caffeine

A 0.1000 g quantity of reference standard of caffeine was weighed accurately, dissolved in some quantity of the mobile phase, transferred into a 100 ml volumetric flask and topped up to

the 100 ml mark of the volumetric flask using the mobile phase to make a 1000 ppm caffeine stock solutions. Exactly 5 ml of the 1000 ppm stock solution was further diluted to 100 ml to obtain a 50 ppm stock solution. Serial dilutions of the 50 ppm stock solution were carried out to obtain standard solutions of concentrations 1.25 ppm, 2.5 ppm, 5 ppm, 7.5 ppm and 10 ppm. The standard solutions were filtered and 10 µL of each was injected into the HPLC to obtain the chromatograms. Six (6) injections were carried out for each standard solution.

3.7.3 Calibration Curves

The measured peak area (mean peak area) values were plotted against their respective standard concentration values to obtain the calibration curve for each API. The calibration curve was then used to estimate or determine the concentration of each API in each tablet sample.

3.7.4 Preparation of Tablet Samples Solutions/Assay of Tablet Samples

Approximately 0.1000 g quantity of each powdered tablet sample was accurately weighed, dissolved in some quantity of the mobile phase, transferred into a 100 ml volumetric flask and topped up to the 100 ml mark of the volumetric flask using the mobile phase. Approximately 1.5 ml of each tablet sample solution was further diluted to 50 ml, filtered and 10 µL of each was injected into the HPLC to obtain the chromatograms. Six (6) injections were carried out for each tablet sample solution.

The content (mg) and percentages of each API in each tablet sample was computed for using the equations of straight line from the calibration curve for each respective component.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSIONS

4.1 IDENTIFICATION TESTS

4.1.1 For Reference Standards

4.1.1.1 Results of Colour Identification Test

The inferences and observations (results) obtained for the colour identification test carried out on the reference standards of the APIs under consideration are indicated in Table 4.1 below;

Table 4.1: Results of colour identification test for reference standards of APIs

Reference standard	Test	Observation	Inference
ASA	BP,(2009)	**A.)** Formation of a deep violet colour upon addition of excess iron (iii) chloride solution. **B.)** Formation of a greenish-yellow colour on the filter paper which became blue upon moistening the filter paper with dilute HCl solution	ASA present

Paracetamol	BP,(2009)	Violet colour was formed slowly upon addition of 0.05 ml of 0.0167 M potassium dichromate solution. The violet colour did not turn red.	Paracetamol Present
Caffeine	BP,(2009)	Formation of a brown precipitate which dissolved upon addition of dilute NaOH solution.	Caffeine present

4.1.1.2 Results for Specific Absorbance Identification Test

The results obtained from the specific absorbance identification test for paracetamol and caffeine reference standards are shown in Table 4.2 below;

Table 4.2: Results of identification test of reference standards of APIs by Specific Absorbance.

Reference standard	Specific Absorbance	Inference
Paracetamol	718.42	Paracetamol present
Caffeine	506.45	Caffeine present

Colour identification and specific absorbance tests were used to identify the reference standards of active drugs in the tablet samples. In the case of colour identification, the colours obtained correspond to that stated in the BP, (2009). Specific absorbance of paracetamol and caffeine on the other hand were in close conformity to that stated in the BP, (2009). The BP, (2009) indicates specific absorbance of 715 and 504 for paracetamol and caffeine respectively. The specific absorbance values obtained from experiment are 718.42 and 506.45 respectively. From Tables 4.1 and 4.2, both colour and specific absorbance tests confirmed the reference standards as ASA, paracetamol and caffeine.

4.1.2 Identification Test Results for APIs in Tablet Samples

The observations and inferences (results) obtained when colour identification test was used to detect or identify the presence of the APIs in the tablet samples are indicated in Table 4.3;

Table 4.3: Identification test results of APIs in Tablet Samples.

API	Test	Observation	Inference
ASA	BP,(2009)	**A.)** Formation of a deep violet colour upon addition of excess (iii) chloride solution. **B.)** Formation of a greenish-yellow colour on the filter paper which became blue upon moistening the filter paper with dilute HCl solution.	ASA present
Paracetamol	BP, (2009)	Violet colour was formed slowly upon addition of 0.05 ml of 0.0167 M potassium dichromate solution. The violet colour did not turn red.	Paracetamol present

Caffeine	BP, (2009)	Formation of a brown precipitate which dissolved upon addition of dilute NaOH solution.	Caffeine present

Qualitative test on tablet samples based on colour identification was carried out to confirm the presence of the active drugs in the tablet samples. The results obtained agreed with that stated in the BP, (2009), hence confirmed the presence of ASA, paracetamol and caffeine in the tablet samples.

4.1.3 Thin Layer Chromatography (TLC) Identification Test Results for APIs

Thin layer chromatography (TLC) was also used to identify the APIs in the tablet samples and the results obtained are indicated in Table 4.4. The retention factor (R_f) was calculated as the ratio of the analyte (API) distance moved to that moved by the solvent. Figures 4.1 and 4.2 on the other hand, show thin layer results (chromatograms) for paracetamol and acetylsalicylic acid (ASA) respectively.

Table 4.4: Identification test results for APIs by TLC

Reference standard	Distance Moved (mm)	API	Distance Moved (mm)	Solvent Distance (mm)	Retention Factor (R_f) for reference standard	Retention Factor (R_f) for API
ASA	1.70	ASA	1.80	5.70	0.30	0.32
Paracetamol	2.10	Paracetamol	2.20	5.85	0.36	0.38
Caffeine	3.10	Caffeine	2.90	5.80	0.53	0.50

Figure 4.1: Thin layer chromatogram of paracetamol

Figure 4.2: Thin layer chromatogram of ASA

Key:

Ps = Chromatogram of reference standard of paracetamol

As = Chromatogram of reference standard of ASA

Sp = Chromatogram of API in tablet sample

The APIs in the tablet samples were also identified by thin layer chromatography. The R_f values of the APIs were compared with that of the reference standards to identify the APIs in the tablet

samples. From Table 4.4, there are not much significant differences between the R_f values of the APIs and that of the reference standards. The closeness of the R_f values of the APIs to that of the reference standards indicate the presence of the APIs in the tablet samples.

4.2 ASSAY OF REFERENCE STANDARDS OF ACTIVE DRUGS

4.2.1 Assay of Reference Standard of ASA by Back-Titration

4.2.1.1 Standardization of Solutions

Results obtained upon standardization of approximately 0.25 M sodium hydroxide and 0.25 M hydrochloric acid solutions are shown in Table 4.5. Sulphamic (H_2NSO_3H) acid was used as the primary standard for the standardization of sodium hydroxide solution. The standard sodium hydroxide solution was in turn used as secondary standard to standardize approximately 0.25 M hydrochloric acid solution.

Table 4.5: Standardization parameters for NaOH and HCl solutions

Solution	Primary/Secondary Standard used	Nominal Conc. (M)	Average Titre (ml)	Factor	Actual Conc. (M)
NaOH	H_2NSO_3H	0.25	24.50	1.024	0.256
HCl	NaOH	0.25	29.70	0.862	0.216

4.2.1.2 Determination of the Purity of ASA Reference Standard by Back-Titration

Equation of reaction;

$$C_9H_8O_4 + 2NaOH \rightarrow CH_3COONa + C_6H_3COONa + 2H_2O$$

From the equation of reaction; 1 mole $C_9H_8O_4 \equiv 2$ moles NaOH

∴ 180 g $C_9H_8O_4$ ≡ 2000 ml 1 M NaOH Solution

90 g $C_9H_8O_4$ ≡ 1000 ml 1 M NaOH Solution

0.090 g $C_9H_8O_4$ ≡ 1 ml 1 M NaOH Solution

0.0225 g $C_9H_8O_4$ ≡ 1 ml 0.25 M NaOH Solution

0.2483 g $C_9H_8O_4$ ≡ 12.80 ml (0.862) 0.25 M NaOH Solution

⇒Percentage purity of ASA reference standard $= \frac{Actual\ weight}{Nominal\ weight} x\ 100\ \%$

$= \frac{0.2483g}{0.2500g}\ x\ 100\ \% = \mathbf{99.32}\ \%$

NB: This same approach was used in calculating percentage purities of W_2 and W_3. The average percentage purity representing the percentage purity of reference standard of ASA was then calculated. Results are summarised in Table 4.6; Table 4.6: Assay parameters of the reference standard of ASA

Nominal Weight (g)	Titre (ml)	Blank Titre (ml)	Actual Weight (g)	Purity (%)	Mean Purity (%)
W_1 = 0.2500	16.60		0.2483	99.32	
W_2 = 0.2500	16.50	29.40	0.2502	100.08	100.07
W_3 = 0.2500	16.40		0.2520	100.80	

4.2.2 Assay of Reference Standards of Paracetamol and Caffeine by UV Spectrophotometry

4.2.2.1 Assay of Reference Standard of Paracetamol

Weight of reference standard of paracetamol used = 0.1520 g

Specific Absorbance of paracetamol at 257 nm = 715

Mean Absorbance value of paracetamol at 257 nm = 0.546

Using the relation; $A = Asbc$; where A = absorbance, As = specific absorbance (1 %, 1cm), b = cell pathlength = 1cm and c = concentration (g/100 ml).

Making c the subject of the formula gives;

$$c = \frac{A}{Asb}$$

$\Rightarrow c = \frac{0.546}{1\ cm\ x\ 715} = 7.636\text{x}10^{-4}$ g/100 ml

Actual weight of paracetamol reference standard used = $concentration\ x\ dilution\ factors$

$$= 7.636\text{x}10^{-4}\ x\ (\tfrac{200}{10})\ x\ (\tfrac{100}{10}) = 0.1527\ g$$

∴ Percentage purity of paracetamol reference standard $= \frac{Actual\ weight}{Nominal\ weight}\ \mathbf{x\ 100\ \%}$

$$= \frac{0.1527\ g}{0.1520\ g}\ x\ 100\ \% = 100.46\ \%$$

∴ Percentage purity of paracetamol reference standard = **100. 46 %**

This same approach was used in calculating for the percentage purity of reference standard of caffeine and the results are summarized in Table 4.7. Table 4.8 is a summary of the average percentage purity values of the reference standards of APIs. Table 4.7: Assay parameters of reference standards of paracetamol and caffeine

Reference Standard	Nominal weight (g)	Mean Absorbance	Specific Absorbance (1 %, 1 cm)	Actual weight (g)	Purity (%)
Paracetamol	0.1520	0.546	715	0.1527	100.46
Caffeine	0.0310	0.628	504	0.0312	100.65

Table 4.8: Summary of assay values of reference standards of APIs

Reference Standard	Assay Value (% Purity)	Assay Value, BP Range (%)
ASA	100.07	99.50 – 101.00 99.00
Paracetamol Caffeine	100.46	– 101.00
	100.65	98.50 – 101.50

From Table 4.8, the assay values of the reference standards indicate that all the three reference standards fall within the assay value range specified by BP, (2009). This shows that all the three reference standards contained acceptable limit of impurities and certified as pure, hence were suitable to be used as reference standards for HPLC method development.

4.3 HPLC METHOD DEVELOPMENT

4.3.1 Solubility of ASA, Paracetamol and Caffeine

For a suitable dissolution medium, various solvents were tried. Table 4.9 indicates the various solvents used and their respective length of time for the dissolution of the reference standards. The dissolution medium was also used as the mobile phase for the method development.

Table 4.9: Degree of dissolution of the reference standards in some selected solvents.

		Time (min) for Dissolution

Reference Standard	Solvent	Without Heat	Heated at 60 °C /min
ASA	Water	More than 60	25
Paracetamol	Water	5	-
Caffeine	Water	5	-
ASA	Methanol	40 to 45	10
Paracetamol	Methanol	2	-
Caffeine	Methanol	2	-
ASA	Water + Methanol	37 to 40	7 to 10
Paracetamol	Water + Methanol	Less than 2	-
Caffeine	Water + Methanol	Less than 2	-

From Table 4.9, water + methanol was chosen as the dissolution medium and mobile phase because that solvent system could cause the dissolution of all the three components within the least period. Isopropyl alcohol, ethanol and chloroform could cause dissolution of paracetamol and caffeine but not ASA. The addition of acetic acid to the methanol-water solvent system was to provide a suitable pH of the mobile phase in order to obtain complete resolution of symmetric peaks (Ahuja and Rasmussen, 2007; Kenkel, 2003). However, the water-methanol solvent was found to be very much cost effective than the other solvent systems (Ahuja and Rasmussen, 2007; Kenkel, 2003).

4.3.2 Wavelength Determination

Results of wavelength scan carried out within the UV region (200-400 nm) of the electromagnetic spectrum are shown in Table 4.10.

Table 4.10: Selection of suitable wavelength for maximum absorption of the three components

Peak	Wavelength of Absorption (nm)														
	200	210	220	230	240	250	257	260	265	266	270	273	276	300	330
ASA	X	X	X	√	√	X	X	√	√	√	√	X	√	X	X
Paracetamol	X	X	X	X	√	X	√	√	X	X	√	X	X	X	X
Caffeine	X	X	X	X	X	X	X	X	X	X	√	√	√	X	X

Key:

X = No peak detection at that wavelength

√ = Peak detection at that wavelength

The UV-Visible spectrophotometer was used to determine the common absorption wavelength of the three components when a scan was carried out within a wavelength range of 200 to 400 nm. Although there was peak detection at certain wavelengths for some of the components, 270 nm was the wavelength at which all the three components showed peak detection, hence chosen as the suitable wavelength for optimum resolution of all the three components. The differences in the absorption of radiation by the three components at certain wavelength is due to the differences in the number of chromophores in the three compounds absorbing radiation at different wavelengths. However, absorption of radiation at 270 nm by all three components is due to the presence of same chromophores common to all the three components or similar chromophores that exhibit radiation absorption at that wavelength (Ahuja, 2003; Kenkel, 2003).

4.3.3 Established Chromatographic Conditions for the Developed Method

Mobile Phase/Dissolution Medium: Acidic Water + Methanol (HPLC grade)

Mobile Phase Ratio: 60 : 40

Acidic Water: Mixture of Distilled Water and Acetic Acid (75:0.2)

Column: Brownlee Analytical C8, 5 um, 150 x 4.6 mm

Pump: Flexar LC Pump

Injection volume: 10 uL

Flow rate: 1 ml/min

Analysis time: 5.5 minutes

Detector: Perkin Elmer Flexar Photodiode Array (PDA) LC Detector, N3896

Wavelength: 270 nm

Elution Mode: Isocratic

4.3.3.1 Retention Time of Components

Table 4.11 shows the mean retention time which indicate the order of elution of the three components.

Table 4.11: Mean retention times of the three components.

Component	Mean Retention Time (Min)

ASA	5.03 ± 0.0140
Paracetamol	2.05 ± 0.0062
Caffeine	2.45 ± 0.0030

The elution of the three components is in the order: paracetamol, caffeine and ASA. This is due to their levels of affinities for both stationary and the mobile phases and their solubility in the mobile phase (Ahuja, 2003; Kenkel, 2003; Skoog *et al*., 2004). Paracetamol was eluted first because it has greatest affinity for the mobile phase and least affinity for the stationary phase than ASA and Caffeine. This greatest affinity of paracetamol for the mobile phase caused it to have the least of interaction with the stationary phase, hence resulting in its fastest elution. ASA on the other hand was eluted last because of its high affinity for the stationary phase and least affinity for the mobile phase than paracetmol and caffeine. This means that the level of interaction between ASA and the stationary phase was greater than that of paracetamol and caffeine. Caffeine exhibited a level of interaction between that of paracetamol and ASA, hence became the second component to be eluted (Ahuja, 2003; Kenkel, 2003; Skoog *et al*., 2004).

On the basis of solubility, it can be explained that paracetamol is more readily soluble in the mobile phase followed by caffeine and lastly ASA. The degree of solubility of a compound in a solvent, determines the level of affinity of the compound for the solvent, hence the reason for the elution order of the three components (Ahuja, 2003; Kenkel, 2003; Skoog *et al*., 2004). However, of the three components, paracetamol is the most polar since it had the greatest of interaction with the mobile phase whereas ASA is the least polar (Ahuja, 2003; Kenkel, 2003; Skoog *et al*., 2004).

4.3.3.2 Mobile Phase

In the selection of mobile phase for RP-HPLC method development, certain factors such as cost of solvent(s), polarities of solvent(s) and that of the analyte(s) of interest, pKa and the solubility of the analyte(s) must be considered (Ahuja and Rasmussen, 2007; Kenkel, 2003; Skoog *et al*., 2004). These factors actually aided in the choice of acidic water-methanol solvent system as the mobile phase. In terms of the above mentioned factors, this solvent system was found to be satisfactory as against other solvents such as ethanol, isopropyl alcohol and chloroform that were tried. The ratio of 60:40 acidic water-methanol solvent system was chosen because that combinational ratio aided the best resolution of peak symmetry and separation of all components within the least retention times. However, glacial acetic acid was added to provide an optimum pH of the mobile phase since pH has a lot of influence on the retention times especially of ionisable compounds (Ahuja and Rasmussen, 2007; Kenkel, 2003; Skoog *et al*., 2004; Singh, 2013; Suresh *et al*., 2010).

4.3.3.3 Stationary Phase

The stationary phase was also chosen based on the polarities of the analytes of interest (Ahuja and Rasmussen, 2007; Kenkel, 2003; Skoog *et al*., 2004). Since the analytes of interest are drug molecules, hence very polar, a non-polar OS C8 column was chosen in order to reduce the time of interaction between the stationary phase and the analytes (Ahuja and Rasmussen, 2007; Kenkel, 2003; Skoog *et al*., 2004). This reduces the affinity of the analytes for the stationary phase and increases interaction of the analytes with the mobile phase so that the analytes can be eluted within the least period, hence reducing the total time for the analysis (Ahuja and Rasmussen, 2007; Kenkel, 2003; Skoog *et al*., 2004). However, in RP-HPLC, non-polar stationary phases are the best stationary phases to use because the mobile phases are always polar (Ahuja and Rasmussen, 2007; Kenkel, 2003; Skoog *et al*., 2004).

4.3.4 Validation of the Developed HPLC Method

Validation parameters used were accuracy, precision, linearity, robustness, specificity, LOD and LOQ.

4.3.4.1 Accuracy

The standard deviation (SD) and relative standard deviation (%RSD) values in terms of accuracy of the developed method for the three components are indicated in Table 4.12 below;

Table 4.12: Accuracy of the developed method for the three APIs

API	Injected amount (ppm)	Mean peak area	S	X	Mean amount (ppm) recovered	Mean amount (%) recovered	SD	% RSD
ASA	20 40 60 80 100	38.50 68.52 109.20 157.05 185.51	1.9128	-3.009	21.70 37.39 58.66 83.67 98.55	101.50 98.52 97.77 100.59 98.55 ā = **99.39**	1.58	1.59
Paracetamol	2.5 5.0 10.0 15.0	34.85 69.09 147.25 222.94	14.428	-0.1026	2.42 4.79 10.21 15.45	98.85 99.20 100.75 101.60	1.45	1.45
	20.0	282.84			19.61	98.05 **ā = 99.69**		
Caffeine	1.25 2.50 5.00 7.50 10.00	33.96 62.58 140.56 211.68 279.44	28.498	-3.9728	1.33 2.34 5.07 7.57 9.95	102.51 98.45 101.40 100.93 99.50 **ā = 100.56**	1.60	1.59

Where S = Slope of the calibration curve

X = Intercept of the calibration curve

SD = Standard deviation given by SD = $\sqrt{\sum(a-\bar{a})^2/n-1}$ a = Individual experimental values for percentage amount recovered

$\bar{a}$ = Mean of the individual experimental values for the percentage amount recovered

n= Number of runs or determinations, n =5.

RSD = Relative standard deviation given by RSD = $\frac{SD}{\bar{a}} x\ 100\ \%$

$\sum$ = Summation sign

Accuracy expresses the closeness of an experimental value to the already established value termed as the "conventional true value" or accepted reference value (Ahuja and Rasmussen, 2007; Chandra and Sharma, 2013; Singh, 2013). For a developed method to be accepted as accurate, the percent recovery must fall within a range of 90 to 110 % (Ahuja and Rasmussen, 2007; Chandra and Sharma, 2013; Singh, 2013). From Tables 4.12, the mean accuracy values of ASA, paracetamol and caffeine are 99.39 ± 1.58, 99.69 ± 1.45 and 100.56 ± 1.60 respectively. These values fall within the range indicated, hence the developed HPLC method is accurate. The mean recovery values of ASA, paracetamol and caffeine conform to those of already developed HPLC methods by Tsvetkova *et al*., (2012), Malakar *et al*., (2013), Suresh *et al*., (2010) etc in Literature.

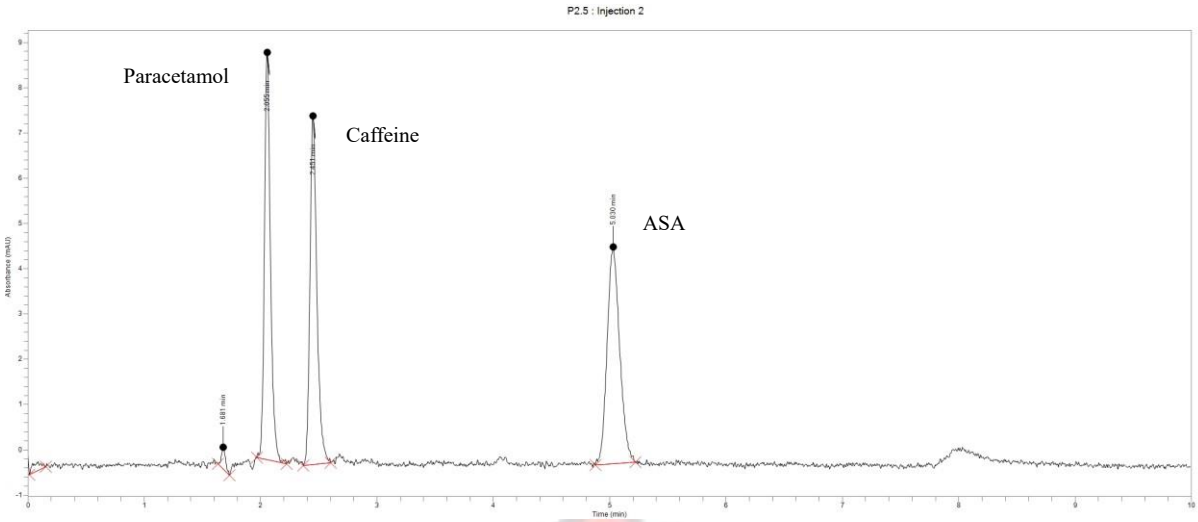

Figure 4.3: A chromatogram of a single injection for the determination of accuracy

4.3.4.2 Linearity

The relationship between the concentration and the peak areas of analytes were used to establish the linearity (R^2 value) of the developed method. The R^2 values were obtained from the graphical plot in Figures 4.4 to 4.6. The results obtained are summarized in Table 4.13.

Table 4.13: Linearity of the developed method for the three APIs.

API	Concentration (ppm)	Mean peak area	R^2 value
ASA	20 40 60 80 100	38.50 68.50 109.20 157.05 185.51	0.9933
Paracetamol	2.5 5.0 10.0 15.0 20.0	34,85 69.09 147.25 222.94 282.84	0.9978
	1.25	33.96	
Caffeine	2.50 5.00 7.50 10.00	62.58 140.56 211.68 279.44	0.9991

Figures 4.4-4.6 show a graphical plot of the linear relationship existing between the concentrations and the peak area values of the analytes.

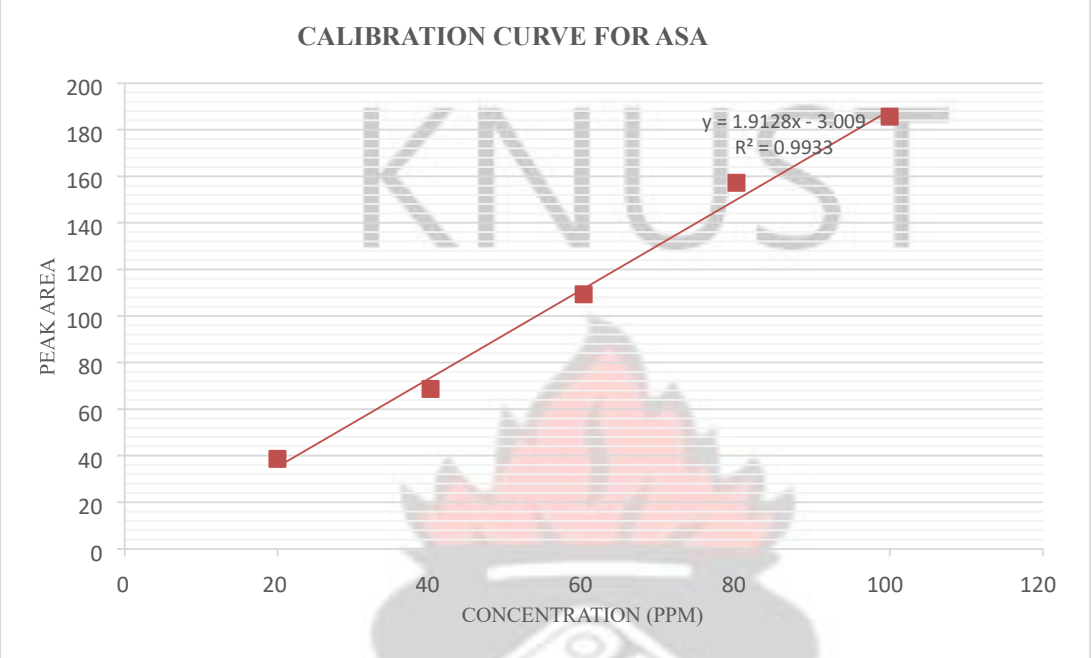

Figure 4.4: Calibration curve for ASA

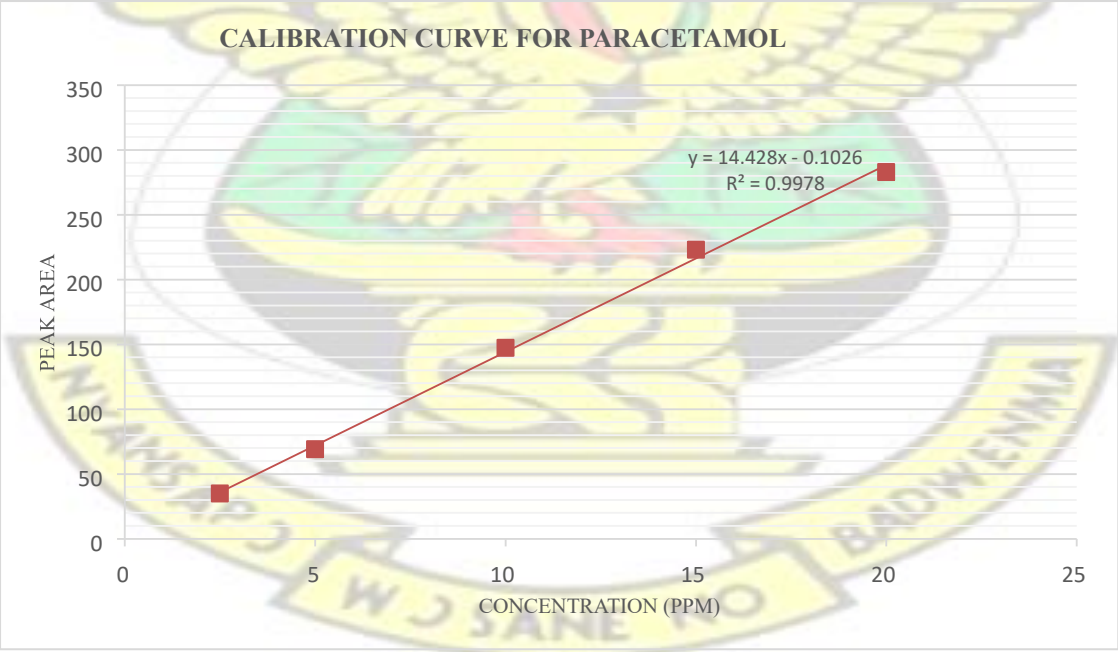

Figure 4.5: Calibration curve for paracetamol

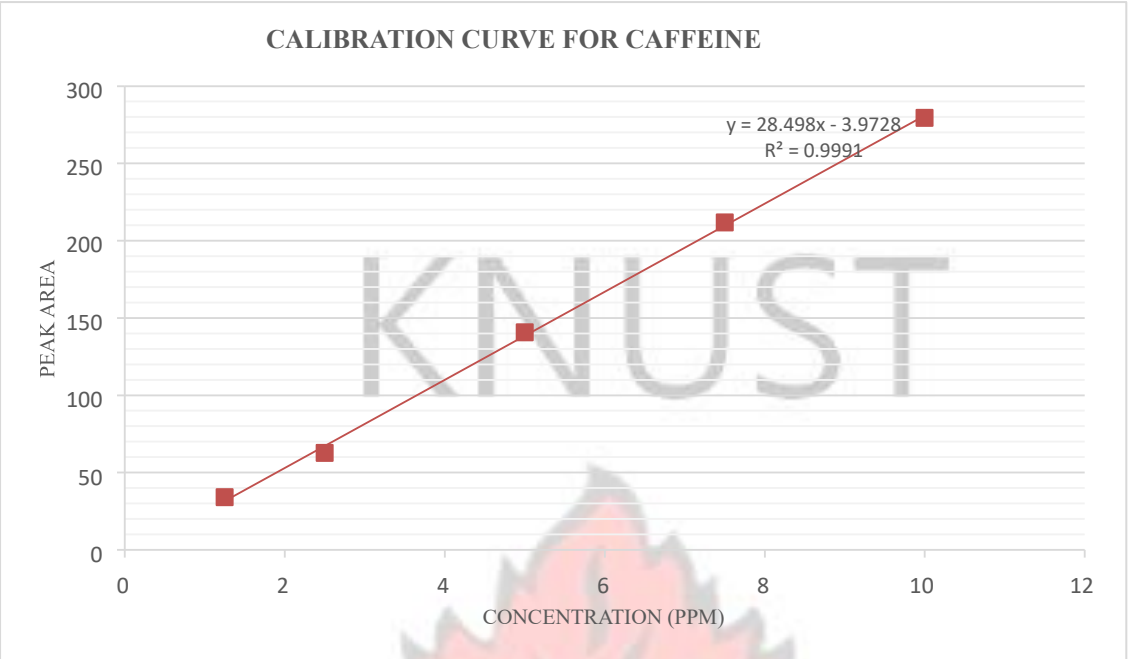

Figure 4.6: Calibration curve for caffeine Analytical method is said to be linear when test results have a direct relation or proportion to the amount of analyte in the sample (Ahuja and Rasmussen, 2007; Gangishetty and Verma, 2013; Singh, 2013). Linearity is expressed as a correlation co-efficient (R^2) value between

0.99 and 1 (Ahuja and Rasmussen, 2007; Singh, 2013).

From Table 4.13, the developed method shows linearity over the concentration range of 20 to 100 ppm for ASA, 2.5 to 20 ppm for paracetamol and 1.25 to 10 ppm for caffeine with R^2 values of 0.9933, 0.9978 and 0.9991 for ASA, paracetamol and caffeine respectively. The developed method is linear since the R^2 values are almost equal to 1. The R^2 values of ASA, paracetamol and caffeine conform to those of already developed HPLC methods by Tsvetkova *et al*., (2012), Malakar *et al*., (2013), Suresh *et al*., (2010) etc in Literature.

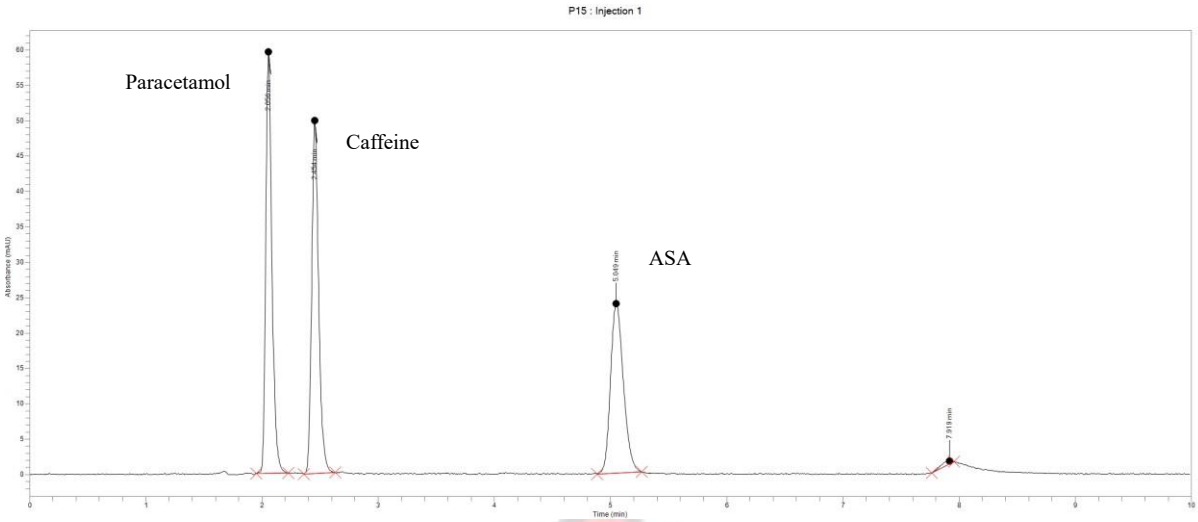

Figure 4.7: Chromatogram of a single injection of standard solution containing 80, 15 and 7.5 ppm of ASA, paracetamol and caffeine respectively.

4.3.4.3 Precision

4.3.4.3.1 Intra-day Precisions for ASA, Paracetamol and Caffeine

The standard deviation (SD) and relative standard deviation (% RSD) values (statistical results) for intra-day precision of the developed method are summarised in Table 4.14;

Table 4.14: Intra-day precision for ASA, Paracetamol and Caffeine.

			Conc. (ppm)		Statistical parameters	
API	**Run**	**Mean peak area**	**Nominal**	**Actual**	**SD = $\sqrt{\sum}$(a-ā)^2/n-1**	**%RSD = $\frac{SD}{\bar{a}}$ x 100 %**

ASA	1	36.91	20	20.87	0.0845	0.408
	2	36.22	20	20.51		
	3	36.00	20	20.39		
	4	36.62	20	20.72		
	5	36.84	20	20.83		
	6	36.95	20	20.89		
				ā=20.70		
Paracetamol	1	216.42	15	15.00	0.0084	0.056
	2	216.97	15	15.05		
	3	216.37	15	15.00		
	4	216.54	15	15.02		
	5	216.67	15	15.02		
	6	216.48	15	15.01		
				ā=15.02		
Caffeine	1	62.93	2.5	2.35	0.0109	0.47
	2	62.46	2.5	2.33		
	3	62.11	2.5	2.32		
	4	62.87	2.5	2.34		
	5	62.63	2.5	2.33		
	6	62.55	2.5	2.33		
				ā=2.33		

4.3.4.3.2 Inter-day Precisions for ASA, Paracetamol and Caffeine

The standard deviation (SD) and relative standard deviation (% RSD) values (statistical results) for inter-day precision of the developed method are summarised in Table 4.15;

Table 4.15: Inter-day precision for ASA, Paracetamol and Caffeine.

			Conc. (ppm)		Statistical parameters	
API	**Run**	**Mean peak area**	**Nominal**	**Actual**	**SD = $\sqrt{\sum(a- \bar{a})^2/n-1}$**	**%RSD = $\frac{SD}{\bar{a}} x\ 100\ \%$**
ASA	1	188.53	100	100.14	0.143	0.143
	2	188.01	100	99.91		
	3	188.79	100	100.27		
	4	188.09	100	99.90		
	5	188.38	100	100.06		
	6	188.27	100	99.99 **ā=100.05**		
Paracetamol	1	282.84	20	19.61	0.0179	0.091
	2	282.51	20	19.59		
	3	282.17	20	19.56		
	4	282.34	20	19.58		
	5	282.19	20	19.57		
	6	282.60	20	19.59 **ā=19.58**		

Caffeine	1	140.60	5	5.07	0.0105	0.207
	2	140.78	5	5.08		
	3	140.01	5	5.05		
	4	140.44	5	5.06		
	5	140.35	5	5.06		
	6	140.58	5	5.07		
				ā=5.065		

Where RSD = Relative Standard Deviation.

SD = Standard Deviation.

ā= Mean of actual concentration values.

a= Individual experimental values for nominal concentration.

n= Number of runs or determinations, n = 6.

∑ = Summation sign.

Precision refers to the reproducibility or repeatability of experimental measurements that are obtained from multiple sampling of a particular homogenous sample under the same experimental conditions. It is expressed as intra and inter-days precisions (Ahuja and Rasmussen, 2007; Singh, 2013).

The developed method is very precise as particular nominal concentration of ASA, paracetamol and caffeine show almost equal repeated experimental measurements for both intra and inter-days precision as indicated in Tables 4.14 and 4.15. The Relative Standard Deviations (% RSD) for both intra and inter-days precisions for ASA, paracetamol and caffeine are 0.408; 0.143, 0.056; 0.091 and 0.470; 0.207 respectively. The intra and interdays RSD values of ASA,

paracetamol and caffeine are far less than 2.0 and thus an indication of the precision of the developed method. This is also in conformity to those of already developed HPLC methods by Tsvetkova *et al*., (2012), Malakar *et al*., (2013), Suresh *et al*., (2010) etc in Literature.

4.3.4.4 Robustness

The developed method shows a high level of robustness as changes in some parameters of the chromatographic conditions such as stationary phase (column, C18 from a different manufacturer), mobile phase pH (± 0.1 pH units), mobile phase composition (methanol content of ± 2 %), wavelength (± 2 nm), temperature (± 3 ^{o}C), detector (UV detector), HPLC machine (Varian prostar), different laboratory and environmental conditions did not adversely affect the developed method. Statistical results obtained with these changes in chromatographic conditions were almost the same as statistical results from the original chromatographic conditions.

4.3.4.5 Specificity

The method is very specific to the three APIs under consideration. It shows no interfering peaks on the retention times of the APIs in the presence of excipients or impurities. This is very evident in the chromatograms of the two tablet samples in Figures 4.8 and 4.9.

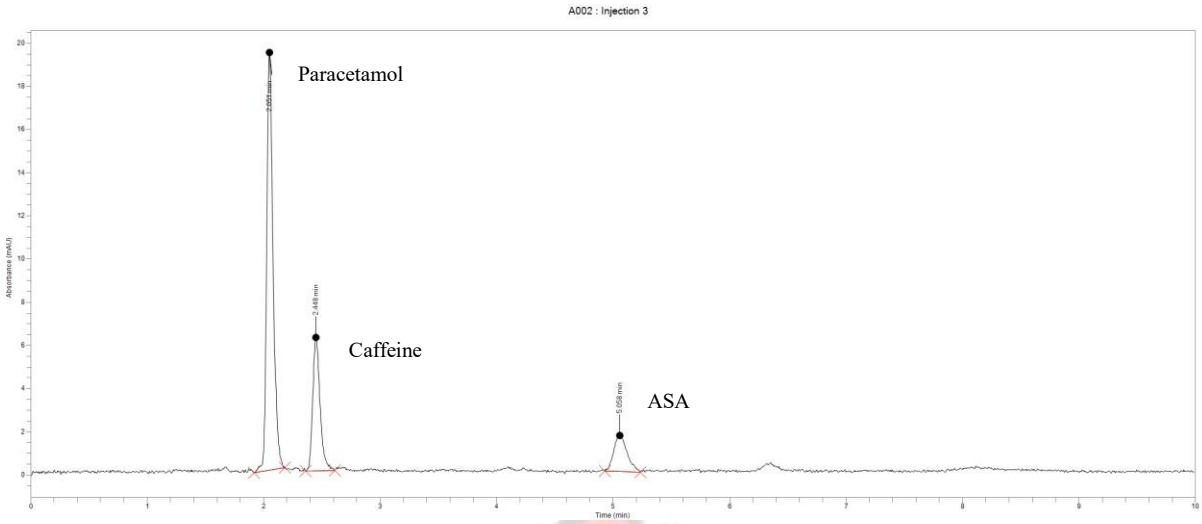

Figure 4.8: A chromatogram of a single injection of tablet sample B to demonstrate the specificity of the developed method.

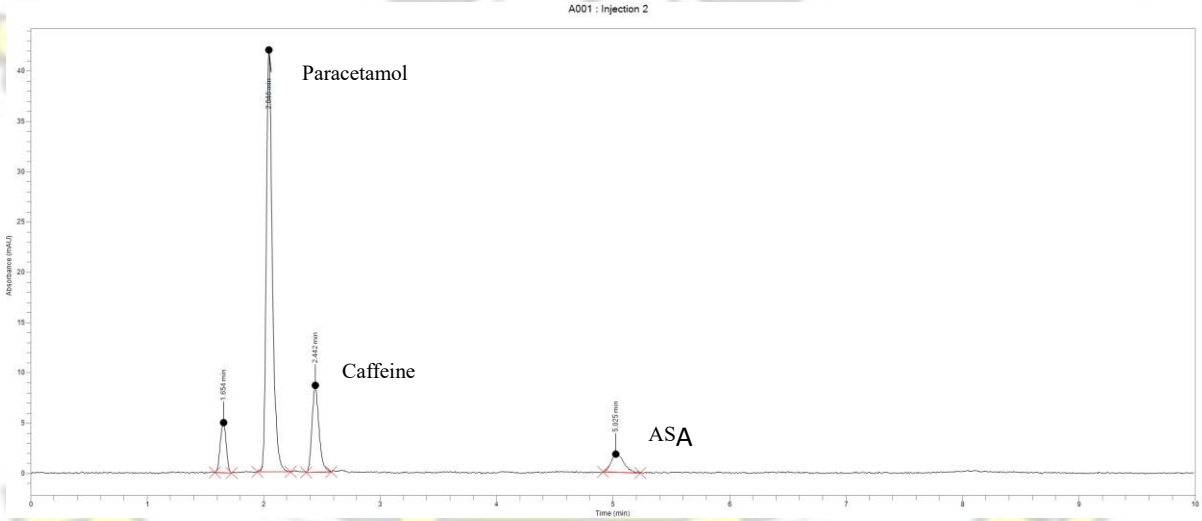

Figure 4.9: A chromatogram of a single injection of tablet sample A to demonstrate the specificity of the developed method.

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

The LOD and LOQ values of the developed method for the components are shown in Table 4.16;

Table 4.16: LOD and LOQ of ASA, Paracetamol and Caffeine

API	Conc./ppm	Peak area (P_E)	Peak area (P_T)	P_E-P_T	σ_{res}	LOD (ppm) / LOQ (ppm)
ASA	20	38.50	35.25	3.25	6.25×10^{-6}	1.078×10^{-5}
	40	68.52	73.50	-4.98		
	60	109.20	111.76	-2.56		
	80	157.05	150.01	7.04		3.267×10^{-5}
	100	185.51	188.27	-2.76		
				∑= **-0.01**		
Paracetamol	2.5	34.85	35.97	-1.12	0.00	0.00
	5.0	69.09	72.04	-2.95		
	10.0	147.25	144.18	3.07		
	15.0	222.94	216.32	6.62		0.00
	20.0	282.84	288.46	-5.62		
				∑= **0.00**		
Caffeine	1.25	33.96	31.65	2.31	6.25×10^{-6}	7.237×10^{-7}
	2.50	62.58	67.27	-4.69		
	5.00	140.56	138.52	2.04		
	7.50	211.68	209.76	1.92		2.193×10^{-6}
	10.00	279.44	281.01	-1.57		
				∑= **-0.01**		

Where;

Equation of straight line for the calibration curve of ASA is y = 1.9128x - 3.009.

Equation of straight line for the calibration curve of paracetamol is y = 14.428x – 0.1026.

Equation of straight line for the calibration curve of caffeine is y = 28.498x - 3.9728.

P_E = Peak area values obtained from the experiment.

P_T = Theoretically calculated peak area values obtained by using the calibration curve σ_{res} = Residual standard deviation of the calibration curve which is obtained using the relation; $\boldsymbol{\sigma_{res}} = [\frac{\sum(PE-PT)}{n-1}]^2$.

S = Slope of the calibration curve n=

Number of determinations, n = 5.

LOD was obtained using the relation; LOD = (3.3 x σ_{res})/S

LOQ was obtained using the relation; LOQ = (10 x σ_{res})/S ∑

= Summation sign.

Limit of detection and limit of quantification expresses the lowest amount of analyte(s) that can be detected and quantified by a particular method. This is a measure of the sensitivity of the developed method and the HPLC equipment (Chromera version 4.1.0.6386) used for the experiment (Ahuja and Rasmussen, 2007; Singh, 2013). From Tables 4.16, the lowest amount of ASA, paracetamol and caffeine that can be detected and quantified are 1.078 x 10^{-5}; 3.267 x 10^{-5} ppm, 0.00; 0.00 and 7.237 x 10^{-7}; 2.193 x 10^{-6} ppm respectively. These values are very small and indicate that even the smallest amount of the analyte(s) can be detected using the developed method and the HPLC equipment under consideration, hence the method and the equipment are sensitive. The zero LOD and LOQ of paracetamol indicates that paracetamol has no detection and quantification limit, hence the smallest amount can be detected and quantified by the developed method.

4.3.4.7 Summary of Validation Parameters

Results obtained for the validation parameters upon validation of the developed method are summarized in Table 4.17;

Table 4.17: Summary of validation parameters of ASA, Paracetamol and Caffeine.

Parameter	ASA	Paracetamol	Caffeine
Linearity (R^2)	0.9933	0.9978	0.9991
Slope of Calibration curve	1.9128	14.428	28.498
Y-intercept of calibration curve	-3.009	-0.1026	-3.9728.
Intra-day precision (% RSD)	0.408	0.056	0.47
Inter-day precision (% RSD)	0.143	0.091	0.207
LOD (ppm)	1.078×10^{-5}	0.00	7.237×10^{-7}
LOQ (ppm)	3.267×10^{-5}	0.00	2.193×10^{-6}
Accuracy (%)	99.39 ± 1.58	99.69 ± 1.45	100.56 ± 1.60
Robustness	Robust	Robust	Robust
Specificity	Specific	Specific	Specific

4.4 ASSAY OF TABLET SAMPLES USING BP, (2009) METHOD

4.4.1 Determination of the content in milligrams (mg) and percentages (%) of APIs in Tablet Samples

4.4.1.1 Content in (mg) and (%) of ASA by Back-Titration

Data:

Weight of 40 tablets of sample A = 19.9574 g

Weight of 40 tablets of sample B = 31.8580 g

Weight of 40 powdered tablets of sample A used (average weight) = 0.4989 g

Weight of 40 powdered tablets of sample B used (average weight) = 0.7965

Content (mg) of ASA in sample A tablets (average weight) = 150.00 mg

Content (mg) of ASA in sample B tablets (average weight) = 230.00 mg

Calculating for the content (mg) of ASA in sample A tablets;

Using the milli-equivalent relation;

0.0225 g $C_9H_8O_4$ ≡ 1 ml 0.25 M NaOH Solution (Refer to 4.2.1.2)

0.1552 g $C_9H_8O_4$ ≡ 8.00 ml (0.862) 0.25 M NaOH Solution

∴ Content (mg) of ASA in sample A tablets will be;

$$= Actual\ weight\ (g)\ x \frac{1000\ mg}{1\ g} = 0.1552\ g\ x \frac{1000\ mg}{1\ g} = \mathbf{155.20\ mg}$$

⇒ Content (mg) of ASA in sample A tablets = **155.20 mg**

Percentage (%) content of ASA in sample A tablets;

$$= \frac{Actual\ weight\ (mg)}{Nominal\ weight\ (mg)}\ x\ 100\ \% = \frac{155.20\ mg}{150.00\ mg}\ x\ 100\ \% = \mathbf{103.47}\ \%$$

NB: This same approach was used in calculating the actual content (mg) and (%) of

ASA in sample B and the results are summarized in Table 4.18;

Table 4.18: Content in milligram (mg) and percentage (%) of ASA in tablet samples.

Sample	API	Nominal weight (g)	Titre (ml)	Blank titre (ml)	Actual weight (g)	Content (mg)	% content	Mean % content
A	ASA	0.4989	8.20	16.20	0.1552	155.20	103.47	103.44
		0.4989	8.10		0.1571	157.10	104.73	
		0.4988	8.30		0.1532	153.20	102.13	
B	ASA	0.7965	14.00	26.20	0.2366	236.60	102.87	101.74
		0.7966	14.20		0.2327	232.70	101.17	
		0.7965	14.20		0.2327	232.70	101.17	

4.4.1.2 Content in (mg) and (%) of Paracetamol and Caffeine by UV Spectrophotometry

Data:

Weight of 40 tablets of sample A = 19.9574 g

Weight of 40 tablets of sample B = 31.8580 g

Weight of 40 powdered tablets of sample A used (average weight) = 0.4989

Weight of 40 powdered tablets of sample B used (average weight) = 0.7965

Content (mg) of paracetamol in sample A (average weight) = 250.00 mg

Content (mg) of paracetamol in of sample B (average weight) = 150.00 mg

Content (mg) of caffeine in both tablet samples (label claim) = 30.00 mg

Mean absorbance value of paracetamol (sample A tablets) at 257 nm = 0.867

Specific absorbance of paracetamol at 257 nm = 715

Calculating for the content (mg) of paracetamol in sample A;

Using the relation; $A = Asbc;$ where A = absorbance, As = specific absorbance (1 %, 1cm), b = cell pathlength = 1cm and c = concentration (g/100 ml).

Making c the subject of the formula gives;

$$c = \frac{A}{Asb}$$

$\Rightarrow c = \frac{0.867}{1\ cm\ x\ 715} = 1.2126 \text{ x } 10^{-3}$ g/100 ml

∴ Content (mg) of paracetamol in sample A tablets will be;

$= \frac{\boldsymbol{Concentration}}{\boldsymbol{1\ g}}\ x\ dilution\ factor(s)\ x\ 1000\ mg$

$= 1.2126 \text{ x } 10^{-3}\ x \left(\frac{200}{10}\right) x(\frac{100}{10})\ x\ 1000\ mg = \mathbf{242.50\ mg}$

⇒ Content (mg) of Paracetamol in sample A tablets = **242.50 mg Percentage**

(%) content of paracetamol in sample A tablets;

$$= \frac{Actual\ weight\ (mg)}{Nominal\ weight\ (mg)}\ x\ 100\ \% = \frac{242.50\ mg}{250.00\ mg}\ x\ 100\ \% = \mathbf{97.00}\ \%$$

NB: This same approach was used in calculating the actual content (mg and %) of other APIs in both tablet samples and the results are summarised in Table 4.19;

Table 4.19: Content in milligram (mg) and percentage (%) of Paracetamol and Caffeine

Sample	API	Nominal weight (g)	Mean Absorbance	Specific Absorbance	Actual weight (g)	Content (mg)	% Content
A	Paracetamol	0.4989	0.867	715	0.2425	242.50	97.00
	Caffeine	0.4989	0.585	504	0.0290	29.00	96.67
B	Paracetamol	0.7965	0.530	715	0.1483	148.30	98.87
	Caffeine	0.7965	0.584	504	0.0289	28.90	96.33

NB: Results obtained upon the assay of both tablet samples using the standard BP, (2009) method are summarised in Table 4.20;

Table 4.20: Summary of Content (mg) and (%) of APIs in tablet samples by BP, (2009) method.

Sample	API	Label claim [Content (mg) of API]	Content (mg) of API	% Content of API	BP, (2009) % Content Range

A	ASA Paracetamol Caffeine	150 250 30	155.17 242.50 29.00	103.44 97.00 96.67	95.00 – 105.00
B	ASA Paracetamol Caffeine	230 150 30	234.00 148.30 28.90	101.74 98.87 96.33	

From Table 4.20, all the three APIs in both tablet samples A and B fall within the percentage content range of 95 to 105 % specified by BP, (2009). This means that both tablet samples contain the right quantities of APIs as indicated on their labels, hence satisfy the BP, (2009) specification in terms of assay and are of the required purity.

4.5 ASSAY OF TABLET SAMPLES BY THE DEVELOPED HPLC METHOD

4.5.1 Determination of the content in (mg and %) of ASA in both Tablet Samples

Data;

Content (mg) of ASA in sample A tablets = 150.00 mg

Content (mg) of ASA in sample B tablets = 230.00 mg

Weight of 40 tablets of sample A = 19.9574 g

Weight of 40 tablets of sample B = 31.8580 g

Weight of 40 powdered tablets of sample A used (average weight) = 0.4989 g

Weight of 40 powdered tablets of sample B used (average weight) = 0.7965 g

Weight of powdered tablets of sample A in 100 ml solution = 0.1004 g

Weight of powdered tablets sample B in 103.60 ml of solution = 0.1003 g

0.5 ml of each tablet sample solution diluted to 50 ml for injection

Mean peak area value of ASA in tablets of sample A = 15.63

Mean peak area value of ASA in tablets of sample B = 12.89 From the calibration curve of ASA, equation of the line is; **y = 1.9128x – 3.009** where y = peak area, x = concentration (ppm) (Refer to Figure 4.4) **Content (mg) of ASA in sample A tablets will be;**

$\Rightarrow x = \frac{14.03+3.009}{1.9128} = 8.908$ ppm

⇒ Actual amount (mg) of ASA in 100 ml solution will be;

$$= weight(mg) x \left(\frac{50\ ml}{1000\ ml}\right) x\ dilution\ factor\ x\ \left(\frac{average\ weight\ of\ 40\ tablets}{weight\ in\ 100\ ml\ solution}\right)$$

8.908 mg x $\left(\frac{50\ ml}{1000\ ml}\right) x\ \left(\frac{100\ ml}{1.5\ ml}\right) x \frac{0.4989\ g}{0.1004\ g} = \mathbf{147.55\ mg}$

Content in percentage (%) of ASA in sample A tablets will be;

$$= \frac{Actual\ weight\ (mg)}{Nominal\ weight\ (mg)}\ x\ 100\ \% = \left(\frac{147.55\ mg}{150.00\ mg}\right) x\ 100\ \% = 98.37\ \%$$

For sample B tablets;

Content (mg) of ASA in sample B tablets will be;

$\Rightarrow x = \frac{12.89+3.009}{1.9128} = 8.312$ ppm

⇒ Actual amount (mg) of ASA in 103.60 ml solution will be;

$$= weight(mg) x \left(\frac{50\ ml}{1000\ ml}\right) x\ dilution\ factor\ x\ (\frac{average\ weight\ of\ 40\ tablets}{weight\ in\ 100\ ml\ solution})$$

8.312 mg x $\left(\frac{50\ ml}{1000\ ml}\right) x\ \left(\frac{103.60\ ml}{1.5\ ml}\right) x \frac{0.7965\ g}{0.1003\ g} = \mathbf{227.94\ mg}$

Content in percentage (%) of ASA in sample B tablets will be;

$$= \frac{Actual\ weight\ (mg)}{Nominal\ weight\ (mg)}\ \mathbf{x\ 100\ \%} = \left(\frac{227.94\ mg}{230.00\ mg}\right) x\ 100\ \% = \mathbf{99.10\ \%}$$

NB: This same approach was used in calculating the actual content (mg and %) of the other APIs in the tablet samples. The results are summarised in Table 4.21;

Table 4.21: Content (mg) and (%) of APIs in tablet samples by the developed HPLC method.

Sample	API	Label claim [Content (mg) of API]	Content (mg) of API	% Content of API	BP % Content Range
A	ASA	150	147.55	98.37	95.00 – 105.00
	Paracetamol	250	242.18	96.87	
	Caffeine	30	28.88	96.27	
B	ASA	230	227.94	99.10	
	Paracetamol	150	143.95	95.97	
	Caffeine	30	29.85	99.50	

From Table 4.21, all the three APIs in both tablet samples A and B fall within the percentage content range of 95 to 105 % specified by BP, (2009). This means that both tablet samples contain the right quantities of APIs as indicated on their labels, hence satisfy the BP, (2009)

specification in terms of assay and are of the required purity. The tablet samples passing the assay test confirms the accuracy of the developed HPLC method.

Figures 4.10 and 4.11 show chromatograms of tablet samples A and B respectively. The order of elution of the components for both tablet samples is the same as that for the standard mixture;

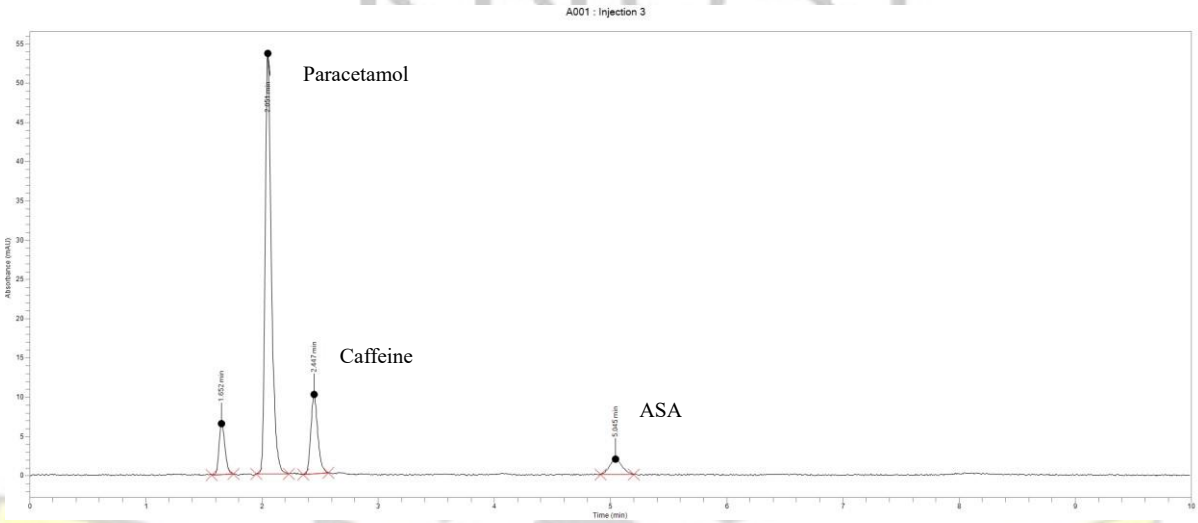

Figure 4.10: Chromatogram of a single injection of sample A tablets

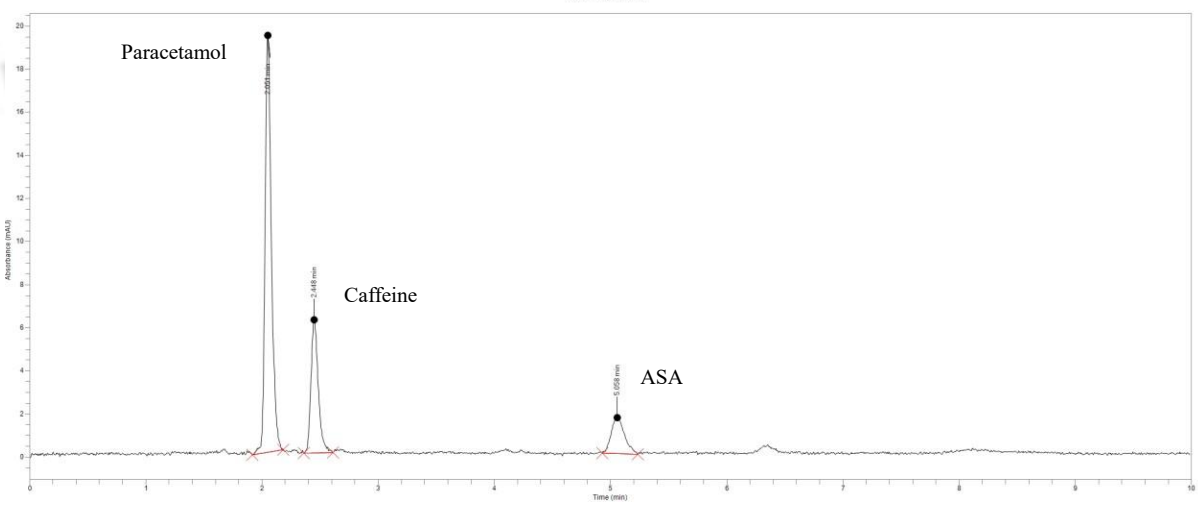

Figure 4.11: Chromatogram of a single injection of sample B tablets

4.5.2 Comparison of BP, (2009) and the developed HPLC methods

Results obtained upon the application of the standard BP, (2009) and the developed and validated HPLC methods in assessing the quality of both tablet samples were compared. The results are summarized in Table 4.22 below;

Table 4.22: BP, (2009) and developed HPLC methods compared.

Sample	API	BP, (2009) Method [Content (%) of API]	Developed HPLC Method [Content (%) of API]
A	ASA	103.44	98.37
	Paracetamol	97.00	96.87
	Caffeine	96.67	96.27
B	ASA	101.74	99.10
	Paracetamol	98.87	95.97
	Caffeine	96.33	99.50

4.6 T-TEST COMPARISON BETWEEN THE STANDARD BP, (2009) AND THE DEVELOPED HPLC METHODS FOR THE APIs IN TABLET SAMPLES

The comparison of the two methods was analysed statistically using two sample t-test statistical analysis at 95 % confidence limit of the mean percentage purities in order to determine the suitability of both methods in assessing the quality of multi-component drugs containing the active ingredients under consideration. Results obtained from the statistical analysis are shown in Table 4.23;

Table 4.23: Two sample t-test statistical comparison at 95 % confidence limit between the BP, (2009) and the developed HPLC methods

	Statistical Parameters			
	BP, (2009) Method	**HPLC Method**		

Sample	API	Mean % purity	SD	Mean % purity	SD	DF	p-value
A	ASA	103.44	1.30	98.59	2.33	1	0.228
	Paracetamol	97.07	0.133	96.87	0.0115	2	0.122
	Caffeine	96.67	0.01	96.27	2.04	2	0.767
B	ASA	101.74	0.981	99.10	1.91	2	0.167
	Paracetamol	98.87	0.01	95.97	1.15	2	0.078
	Caffeine	96.30	0.0577	99.50	2.25	2	0.132

Where SD = Standard deviation and DF = Degree of Freedom

SAMPLE A ASA

```
Two-sample T for UV vs HPLC-ASA

           N    Mean  StDev  SE Mean
UV         3  103.44   1.30     0.75
HPLC-ASA   2   98.59   2.33      1.6


Difference = mu (UV) - mu (HPLC-ASA)
Estimate for difference:  4.85
95% CI for difference:  (-18.18, 27.89)
T-Test of difference = 0 (vs not =): T-Value = 2.68  P-Value = 0.228  DF = 1
```

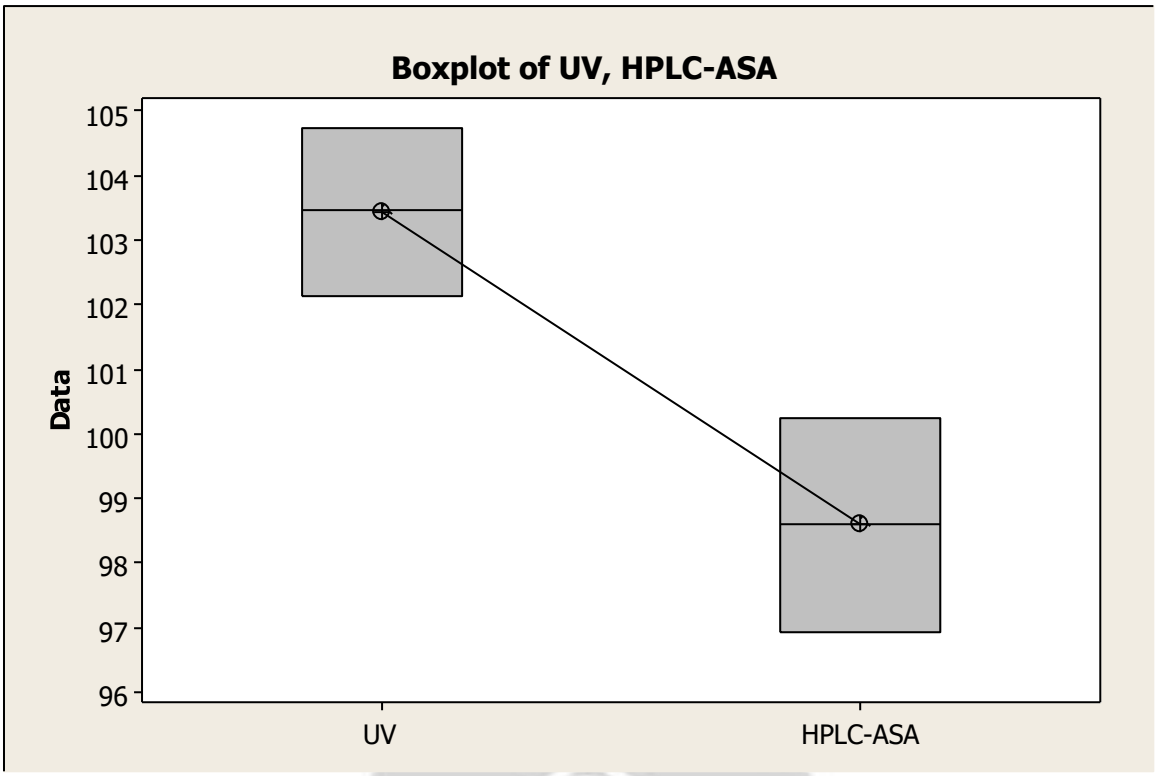

Figure 4.12: Box plot comparison between the mean purity values of ASA for both BP, (2009) and developed HPLC methods for tablet sample A.

The test's p-value (0.228) is greater than the chosen significance level of 0.05 (95% CI). This implies that the developed HPLC method could be used or is appropriate for the analysis of ASA in the sample A tablets just as the standard BP, (2009) method. The box plot indicates that the variation among individual values from the mean is minimal for both methods.

PARACETAMOL

```
Two-sample T for UV-PARA vs HPLC-PARA

            N     Mean   StDev  SE Mean
UV-PARA     3   97.077   0.133    0.077
HPLC-PARA   3  96.8767  0.0115   0.0067


Difference = mu (UV-PARA) - mu (HPLC-PARA)
Estimate for difference:  0.2000
95% CI for difference:  (-0.1311, 0.5311)
T-Test of difference = 0 (vs not =): T-Value = 2.60  P-Value = 0.122  DF = 2
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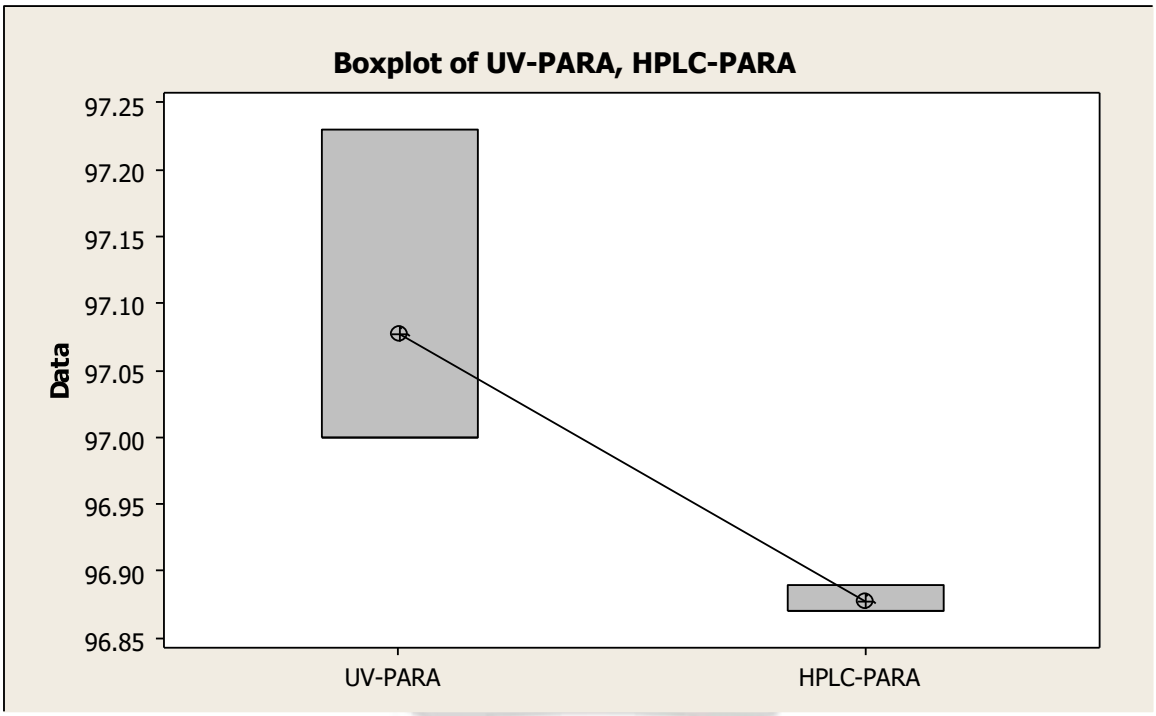

Figure 4.13: Box plot comparison between the mean purity values of paracetamol for both BP, (2009) and developed HPLC methods for tablet sample A.

The test's p-value (0.122) is greater than the chosen significance level of 0.05 (95% CI). This implies that the developed HPLC method could be used or is appropriate for the analysis of paracetamol in the sample A tablets just as the standard BP, (2009) method. From the box plot there is wide variation among individual values from the centre for the BP, (2009) method but the variation for the HPLC is very minimal.

CAFFEINE

```
Two-sample T for UV-CAF vs HPLC - CAF

             N     Mean   StDev  SE Mean
UV-CAF       3  96.6700  0.0100   0.0058
HPLC - CAF   3    96.27    2.04      1.2


Difference = mu (UV-CAF) - mu (HPLC - CAF)
Estimate for difference:  0.40
95% CI for difference:  (-4.67, 5.47)
T-Test of difference = 0 (vs not =): T-Value = 0.34  P-Value = 0.767  DF = 2
```

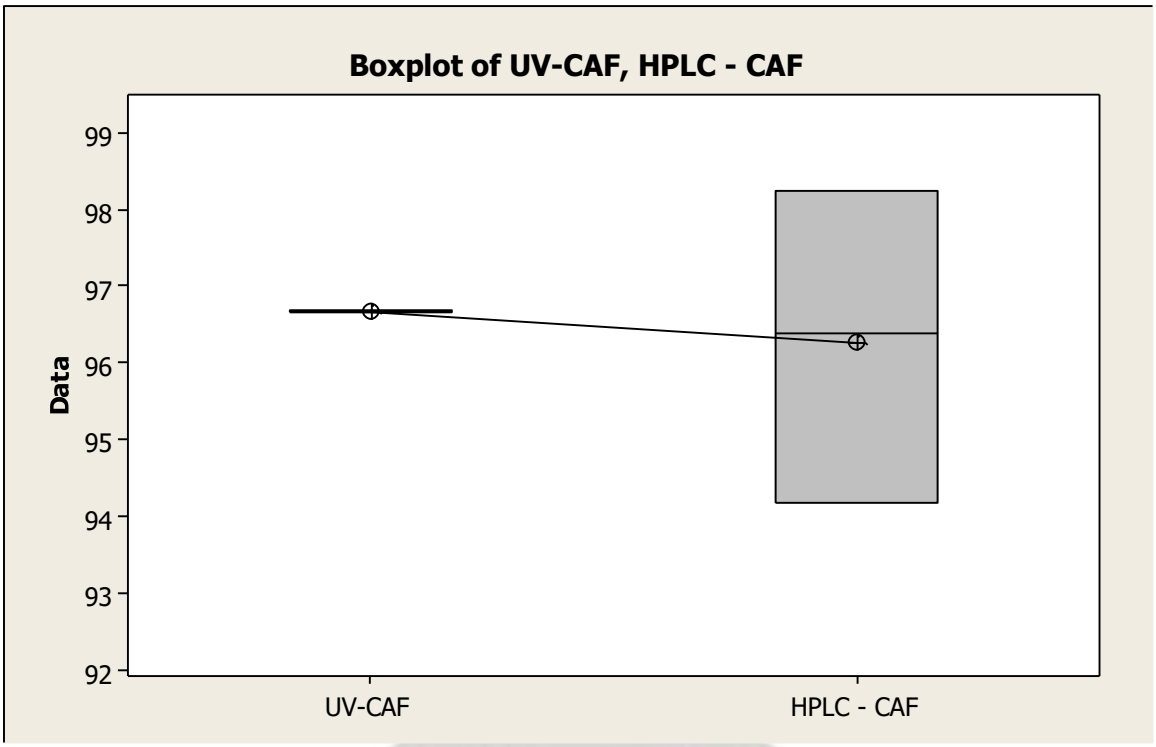

Figure 4.14: Box plot comparison between the mean purity values of caffeine for both BP, (2009) and developed HPLC methods for tablet sample A.

The test's p-value (0.767) is greater than the chosen significance level of 0.05 (95% CI). This implies that the developed HPLC method could be used or is appropriate for the analysis of the caffeine in the sample A tablets just as the standard BP, (2009) method since there is no difference in their means statistically. From the box plot there is wide variation among individual values from the centre for the HPLC method compared to the BP, (2009) method which is very minimal.

SAMPLE B ASA

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Two-sample T for UV-ASA vs HPLC - ASA

             N     Mean  StDev  SE Mean
UV-ASA       3  101.737  0.981     0.57
HPLC - ASA   3    99.10   1.91      1.1


Difference = mu (UV-ASA) - mu (HPLC - ASA)
Estimate for difference:  2.64
95% CI for difference:  (-2.69, 7.96)
T-Test of difference = 0 (vs not =): T-Value = 2.13  P-Value = 0.167  DF = 2
```

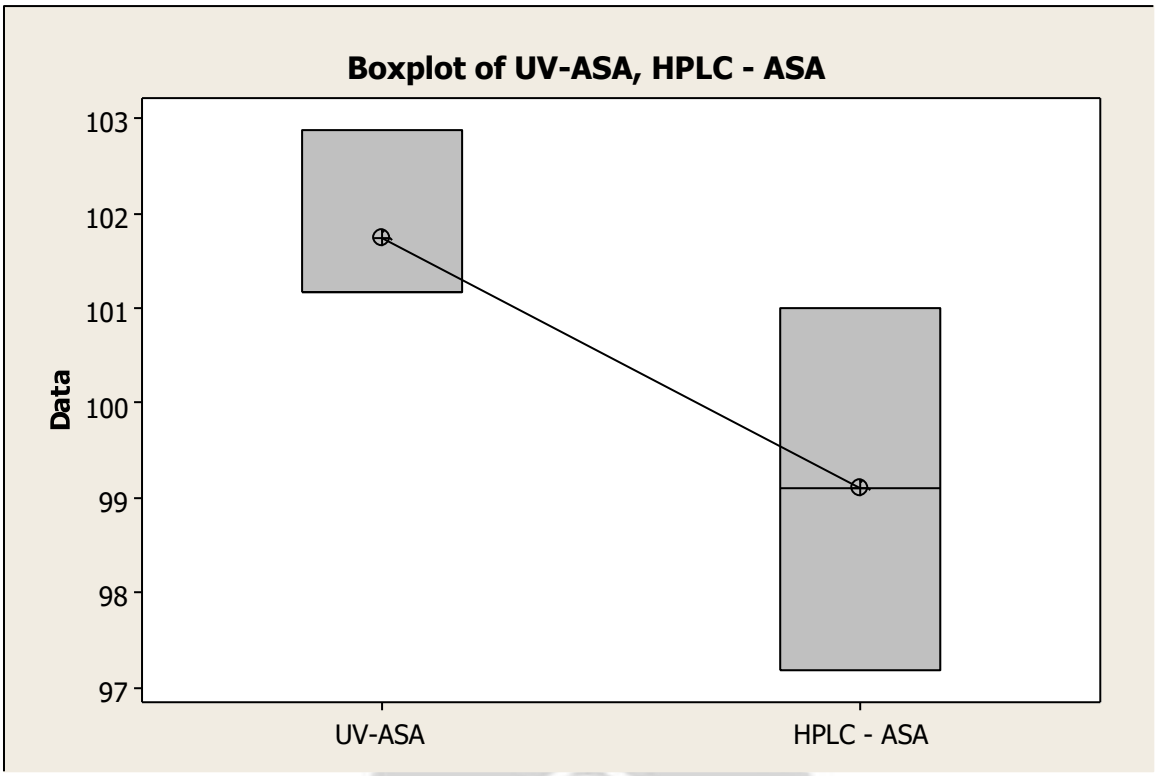

Figure 4.15: Box plot comparison between the mean purity values of ASA for both BP, (2009) and developed HPLC methods for tablet sample B.

The statistical test's p-value (0.126) is greater than the chosen significance level of 0.05 (95% CI). This implies that there is no difference in the means of the results obtained from the two methods statistically. The developed HPLC method is appropriate for the analysis of ASA in sample B tablets just as the accepted standard BP, (2009) method as the test of mean conform to the statistical standard. However, variation among individual values from the mean is relatively minimal for BP, (2009) than HPLC method.

PARACETAMOL

```
Two-Sample T-Test and CI: UV-PARA, HPLC-PARA

Two-sample T for UV-PARA vs HPLC-PARA

            N     Mean   StDev  SE Mean
UV-PARA     3  98.8700  0.0100   0.0058
HPLC-PARA   3    96.63    1.15     0.66


Difference = mu (UV-PARA) - mu (HPLC-PARA)
Estimate for difference:  2.237
95% CI for difference:  (-0.618, 5.091)
T-Test of difference = 0 (vs not =): T-Value = 3.37  P-Value = 0.078  DF = 2
```

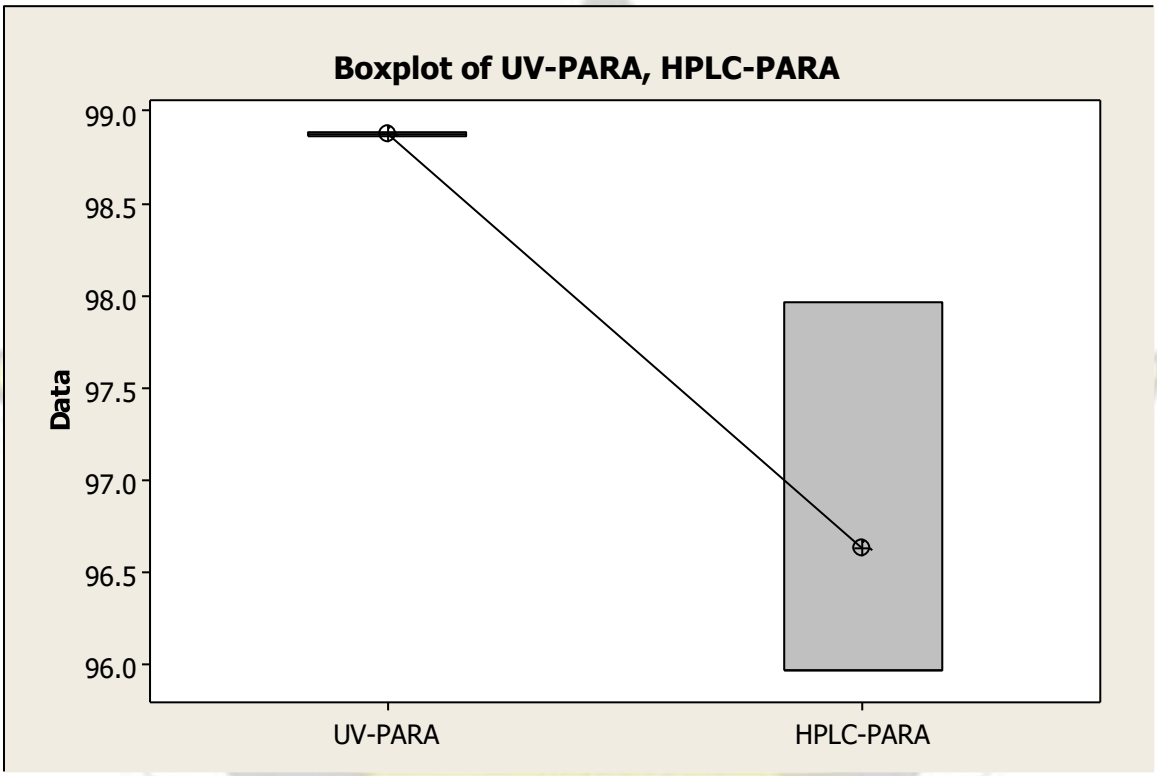

Figure 4.16: Box plot comparison between the mean purity values of paracetamol for both BP, (2009) and developed HPLC methods for tablet sample B.

The test's p-value (0.078) is greater than the chosen significance level of 0.05 (95% CI). This implies that the developed HPLC method could be used or is appropriate for the analysis of the paracetamol in sample B tablets just as the standard BP, (2009) method since there is no difference in the means statistically. From the box plot there is wide variation among individual

values from the centre for the HPLC method but the variation for the BP, (2009) method is very minimal.

CAFFEINE

Two-Sample T-Test and CI: UV-CAF, HPLC - CAF

Two-sample T for UV-CAF vs HPLC - CAF

	N	Mean	StDev	SE Mean
UV-CAF	3	96.2967	0.0577	0.033
HPLC - CAF	3	99.50	2.25	1.3

Difference = mu (UV-CAF) - mu (HPLC - CAF)
Estimate for difference: -3.20
95% CI for difference: (-8.78, 2.38)
T-Test of difference = 0 (vs not =): T-Value = -2.47 P-Value = 0.132 DF = 2

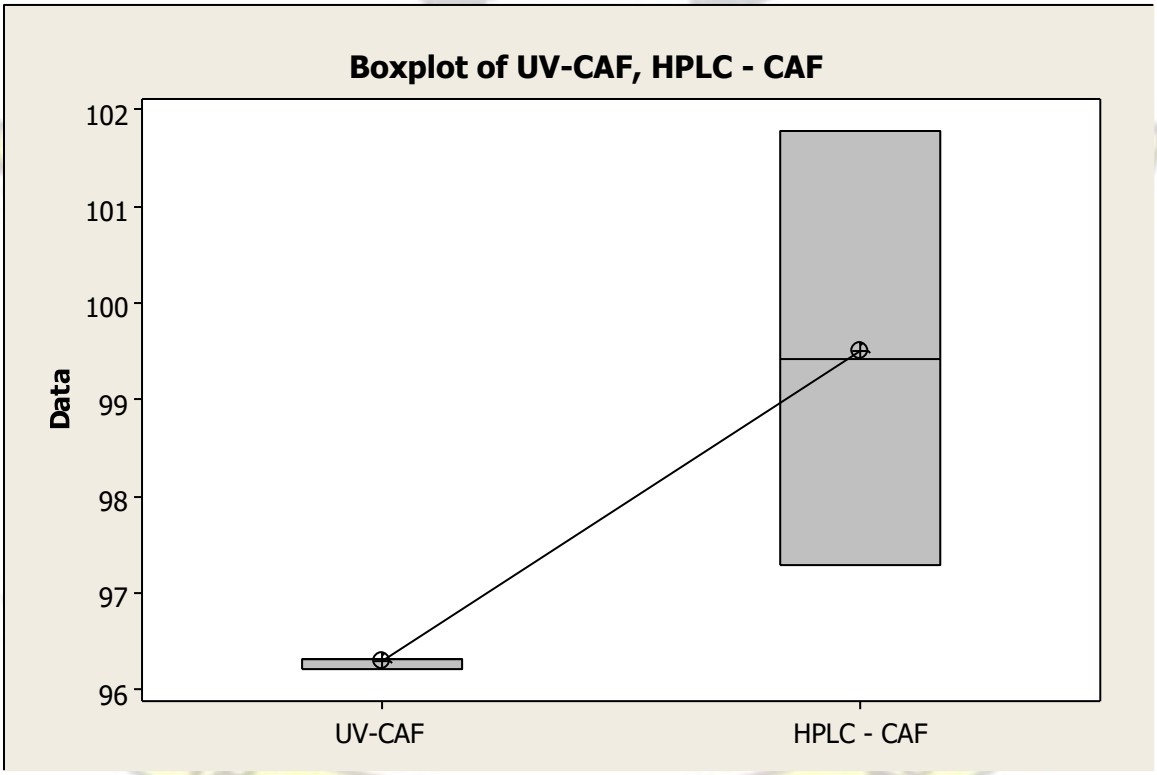

Figure 4.17: Box plot comparison between the mean purity values of caffeine for both BP, (2009) and developed HPLC methods for tablet sample B.

The test's p-value (0.132) is greater than the chosen significance level of 0.05 (95% CI). This implies that the developed HPLC method could be used or is appropriate for the analysis of the caffeine in sample B tablets just as the standard BP, (2009) method since there is no difference

in the means statistically. From the box plot there is wide variation among individual values from the centre for the HPLC method but the variation for the BP, (2009) method is comparatively very minimal.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSION

5.1.1 Identification Tests

Colour identification test and specific absorbance determinations were carried out as indicated in the BP, (2009) official compendium. Both colour test (for reference standards and APIs) and specific absorbance test (for reference standards) conformed to BP, (2009) specifications, hence confirmed the reference standards as ASA, paracetamol and caffeine and the presence of APIs in both tablet samples. Identification test by thin layer chromatography in Table 4.4 also confirmed the presence of the APIs in both tablet samples upon comparison of R_f values of APIs with those of the reference standards.

5.1.2 Assay of Reference Standards

The percentage purity of the reference standards fell within the BP, (2009) specification range as indicated in Table 4.8. This meant that the reference standards were of high purity and contained acceptable limit of impurities and thus were suitable as reference standards for the method development.

5.1.3 HPLC Method Development and Validation

The established chromatographic conditions as well as validation of the developed method signify the suitability of the developed and validated method for its intended use. Upon

validation, the mean recoveries were 99.39 ± 1.58 %, 99.69 ± 1.45 % and 100.56 ± 1.60 % for ASA, paracetamol and caffeine respectively. Linearity of the developed method was in the concentration range of 20-100 ppm with R^2 value of 0.9933 for ASA, 2.5-20 ppm with R^2 value of 0.9978 for paracetamol and 1.25-10 ppm with R^2 value of 0.9991 for caffeine. The % RSD values for inter and intra- days precisions were far below 2.0 % for all the three components. The LOD and LOQ for ASA, paracetamol and caffeine were $1.078x10^{-5}$; $3.267x10^{-5}$, 0.00; 0.00 and $7.237x10^{-7}$; $2.193x10^{-6}$ respectively. Besides these parameters, the developed method demonstrated specificity of components of interest in the presence of excipients and was very robust since variations in certain chromatographic conditions such as mobile phase composition, mobile phase pH, column, HPLC equipment, laboratory and/or environmental conditions did not significantly affect the developed method. Accurate results obtained for the assay of both tablet samples also confirmed the validity of the developed method.

5.1.4 Assay of Tablet Samples by the Developed Method

Results obtained for the assay of both tablet samples using the developed HPLC method fell within the percentage content range of 95-105 % of BP, (2009) specification and thus confirmed the validity of the developed HPLC method (Refer to Table 4.21).

5.1.5 Comparison of the Developed Method with BP, (2009) Method for Analysis

Although statistically both the developed HPLC method and the standard BP, (2009) method can be used in the analysis of multi-component drug formulations containing ASA, paracetamol and caffeine, the standard BP, (2009) method is not suitable for the analysis of multi-component drug formulations since the method involves multiple and repeated extractions of each active

component before actual analysis can be done. This makes the BP, (2009) method very laborious, cumbersome and time consuming. However, there is a possibility of interference or side reactions being produced especially when titrimetry is employed in the analysis. HPLC on the other hand eliminates these interferences since no extractions are involved. The BP, (2009) is therefore suitable for the analysis of single component drug formulations.

The RP-HPLC method developed has no extractions involved, produces no side reactions and no interference. These advantages of RP-HPLC method over the BP, (2009) method of analysis such as UV spectrophotometry, titrimetry etc make RP-HPLC a suitable analytical method for the analysis of multi-component drug formulations. The percentage content assay values obtained by the developed method and the method stated in the BP, (2009) are not statistically significant. The two methods are thus comparable.

5.1.6 Summary of Conclusions

In summary, the developed and validated RP-HPLC method is precise, accurate, linear, cost effective, specific, sensitive and robust and can be used in routine quality control analysis for the simultaneous quantification and estimation of multi-component drug formulations containing ASA, paracetamol and caffeine in tablet dosage forms. Compared to BP, (2009) methods such as UV spectrophotometry, titrimetry etc, RP-HPLC is a preferred method for the analysis of multi-component drug formulations.

5.2 RECOMMENDATIONS

It is recommended that the developed and validated method be used in routine quality control analysis to quantify or assay ASA, paracetamol or caffeine alone or combination of two or all the three components in tablet formulation. Further research can be carried out using the developed and validated HPLC method to detect and quantify the three APIs in urine samples.

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