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HPLC METHOD DEVELOPMENT FOR THE QUANTIFICATION AND STABILITY STUDIES OF AMOXCILLIN AND CLAVULANIC ACID IN LIQUID ORAL FORMULATIONS

BY

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DECLARATION

The research work reported in this thesis was carried out at the Department of Pharmaceutical Chemistry, KNUST. Any assistance obtained has been duly acknowledged. This work has not been submitted for any other degree.

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(Head of Department) Signature Date

ABSTRACT

Amoxicillin-Clavulanic acid combinations have become first line medicines on the essential medicines list of the National Health Insurance Scheme for the treatment of infections caused by beta-lactamase producing micro-organisms. These organisms have become resistant to amoxicillin. As a result, clavulanic acid which is a beta-lactamase inhibitor is combined with amoxicillin to extend the antibacterial properties of amoxicillin and thus the synergy produced by the two drugs are exploited to treat infections caused by beta-lactamase resistant bacteria. It is essential that the two compounds maintain their stability throughout the period of use to obtain the expected synergy. However, no studies have been conducted on the liquid oral suspension of these drugs under the local Ghanaian temperature storage conditions to ascertain their stability in liquid oral suspension throughout the period of use. Also, few methods that are stability indicative are available for the assay of these liquid oral formulations. To assist in assessing the stability and the quality of these formulations, a simple and cost effective High Performance Liquid Chromatographic (HPLC) method for assay and assessment of stability of the two compounds in liquid oral formulations fixed-dose was developed and validated.

The HPLC method employed a C18 reverse phase column Phenomenex, Ultracarb ODS 20micron with an isocratic mixture of methanol, water and sodium acetate buffer pH 4.37 in the ratio of 5:90:5 as the mobile phase. The flow rate was 1ml/min and detection was by means of a UV detector at a wavelength of 220nm. The LOD of amoxicillin and clavulanic acid were 0.00202%w/v and 0.000264%w/v respectively. The LOQ of amoxicillin and clavulanic acid were also 0.00674%w/v and 0.000879%w/v. The method was accurate when compared to a standard method in the British Pharmacopoeia. Five brands of the Amoxicillin-Clavulanic acid combinations in liquid suspensions were analysed. Four out of the five brands passed the assay. Both amoxicillin and Clavulanic acid in reconstituted liquid oral suspensions were not stable under temperatures of 30˚C and 40˚C but were however stable at a temperature of 10˚C in a refrigerator over a period of seven days. However, Clavulanic acid was less stable than

amoxicillin at both temperatures of 30˚C and 40˚C. It was also established that the innovator brand was more stable than the most commonest and highly stocked generic brand on the Ghanaian market.

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DEDICATION

I dedicate this work to my parents, John Yaw Danso and Rebecca Achiaa, my wife, Henrietta Owusu-Danso and my children.

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ABBREVIATIONS

AMX: Amoxicillin Trihydrate

ANOVA: Analysis of Variance

BP: British Pharmacopoeia

CLV: Clavulanic Acid

HPLC: High Performance Liquid Chromatography

ICH: International Conference on Harmonization

IR: Infra Red Spectroscopy

LOD: Limit of Detection

LOQ: Limit of Quantification

ODS: Octadecylsilane

RP-HPLC: Reversed-Phase High Performance Chromatography

RSD: Relative Standard Deviation

SD: Standard Deviation

Syx: Residual Standard Deviation

TLC: Thin Layer Chromatography

USP: United States Pharmacopoeia

UV: Ultra Violet

WHO: World Health Organisation

CHAPTER 1.0 – INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Sir Alexander Fleming's accidental discovery of the antibacterial properties of penicillin in 1929 is largely credited with initiating the modern antibiotic era [1].The penicillins belong to the beta-lactam group of antibiotics which are the dominant class of agents currently used for the chemotherapy of bacterial infections [1]. Penicillins are inactivated by beta-lactamase producing micro-organisms which compromise their antibacterial activity [1]. The strategy used to overcome this is to use a β–lactamase inhibitor in combination with the penicillin in treating infections caused by β–lactamase producing micro-organisms [1]. Amoxicillin is active against certain Gram-positive and Gram-negative organisms but is inactivated by penicillinases including those produced by *Staphylococcus aureus* and by common Gram-negative bacilli such as *Escherichia coli* [3].

Clavulanic acid which is a β-lactamase inhibitor is added to amoxicillin to inhibit β-lactamase and increase the antibacterial effect of amoxicillin, and the combination is used as a broad spectrum antibiotic for treatment of a wide range of bacterial infections, including upper and lower respiratory tract infections and infections of the skin and soft tissue structures [4].

In Ghana, fixed dose combinations of amoxicillin and clavulanic acid is one of the first line medicines on the essential drug list of the National Health Insurance Scheme for the treatment of a wide range of bacterial infections and post-operative prohylaxis[8]. Among

these bacterial infections are upper and lower respiratory tract infections, infections of the skin and other soft tissues [9].

Several methods have been reported for the analyses of amoxicillin and clavulanic acid, such as microbiological assay, enzymatic assay, ultraviolet spectrometry or polarography [15]. Since these methods are generally known not to be specific enough, High Pressure Liquid chromatography (HPLC) assays have also been developed [6]. Some involved pre-treatment of amoxicillin and clavulanic acid with imidazole, pre-column and post column derivatization or ion-pair HPLC.

Amoxicillin and Clavulanic acid combinations are available in oral solid dosage form, powder for reconstitution as suspension and injectable [7]. These two drugs act synergistically to produce the desired therapeutic effect and the potency depends on content of the active moiety in these dosage forms. Thus the availability of very simple but highly sensitive and cost effective analytical methods to quantify the content of amoxicillin and clavulanic acid in liquid dosage forms and established the stability of both compounds under the temperature conditions in Ghana which can go as high as 42˚C at certain times of the year is imperative if the synergistic efficacy of the two drugs is to be exploited in the treatment beta-lactamase infections.

1.2 STATEMENT OF THE PROBLEM

Mehta et al carried out a study using High Performance Liquid Chromatography to determine the stability of amoxicillin and clavulanic acid in co-amoxiclav oral suspension at temperatures of 8˚C and 20˚C [11]. Both compounds were found to be stable at 8˚C over a period of seven days while amoxicillin was more stable than clavulanic acid at 20˚C over the same period of days [11]. In-use stability studies of the two compounds have also been conducted under temperature conditions of -7˚C and 5˚C which are prevailing temperature

conditions in the temperate regions [12]. However, studies on the in-use stability of the two compounds in reconstituted liquid orals formulations under the local Ghanaian temperature condition which can go as high as 42˚C at some times in the year have not been studied. The rate at which these compounds are breaking down and which of the two compounds breaks down faster must be investigated.

The instability of these two compounds in reconstituted suspensions from the powdered formulation could result in the breakdown of clavulanic acid and amoxicillin that are supposed to work synergistically and thus reduce the amount of the two compounds in the suspension required to elicit the desired therapeutic response and lead to treatment failures. These treatment failures could also lead to the emergence of resistant strains of bacteria to these two compounds. The development of a High Pressure Liquid Chromatography (HPLC) method that is specific and highly sensitive to separate and quantify these two compounds, determine which compound breaks down faster and establish the appropriate local temperature conditions for liquid oral suspension of amoxicillin and clavulanic acid is thus imperative.

Secondly, High Pressure Liquid Chromatography (HPLC) is one of the most sensitive analytical techniques employed to simultaneously assay the content of amoxicillin and clavulanic acid in dosage forms recently in most pharmacopoeias [7]. HPLC apparatus is very expensive and requires a very effective preventive maintenance scheme to derive the expected full benefit after purchase and installation [13]. However, the use of inorganic buffer systems such as phosphate buffer in the mobile phase system of most pharmacopoeial HPLC method leads to the formation of crystals of these buffers which damage analytical columns and the moving parts of HPLC pumps [13].

The usage of these buffers require long hours of wash out periods after analyses and this becomes disincentive to analytical chemist especially when several batches of medicines are to be analysed [13]. Moreover, these buffers also support fungal growth coupled with the crystals formed, can generate back pressure in pumps and increase the pressure of the pump beyond acceptable limits and thus affect the efficiency of the pump and the overall performance of the HPLC chromatograph [14].

However, Pharmacopoeia methods employ phosphate buffer as part of the mobile phase system. An alternate buffer system that is devoid of the aforementioned problems associated with the use of a phosphate buffer in an HPLC mobile system is considered imperative in the development of an HPLC Method in this study.

1.3 OBJECTIVES

The general objective of this study is to develop, validate and report of a highly sensitive and cost effective HPLC method to separate and simultaneously quantify amoxicillin and clavulanic acid in liquid oral dosage forms and employ the method to assess the intermediate/in-use stability of amoxicillin and clavulanic acid in liquid oral suspension of the two compounds reconstituted from powdered formulations

1.4 SPECIFIC OBJECTIVES

To develop and validate a reversed-phase HPLC method using isocratic elution to separate amoxicillin from clavulanic and quantify the two compounds in dosage forms.

To obtain a very efficient solvent system for amoxicillin and clavulanic acid that can be used for sample preparation and an organic buffer for the mobile phase.

To study the in-use stability of amoxicillin and clavulanic acid in reconstituted oral liquid suspensions from powdered formulations for paediatric use.

1.5 JUSTIFICATION OF THE STUDY

In Ghana, formulated products of amoxicillin and clavulanic acid is one of the first line medicines on the essential drug list of the National Health Insurance Scheme for the treatment of a wide range of bacterial infections and post-operative prohylaxis [8]. The stability of the clavulanic acid especially in liquid oral suspension has to be monitored to determine the exact local storage conditions and intermediate shelf life of the product. This could reduce the incidence of therapeutic failures as a result of inappropriate storage conditions. Moreover, amoxicillin and clavulanic acid must meet standards of quality as stipulated in the pharmacopoeias, if their synergistic efficacy are to be of great benefit [16]. It is therefore necessary for health practitioners to know the quality of co-amoxiclav they prescribe for their patients. It is only through the use of simple, sensitive and cost effective analytical methods that the quality of these compounds can be rapidly, routinely and consistently assessed.

Secondly, HPLC is considered a first line technique in pharmaceutical analyses due to its sensitive and robust nature especially for the analyses of multi-component products [16]. However, the major challenge that has prevented most local pharmaceutical companies from acquiring an HPLC chromatograph is the high cost of acquisition and maintenance. The design of this HPLC analytical method will not in any way contribute to the maintenance cost of the HPLC chromatograph as often happens with methods that employ phosphate buffer and other inorganic buffers as part of the mobile phase system [16].This study would therefore enable local pharmaceutical practitioners to reduce the cost of using and maintaining the HPLC chromatograph. Finally the study would provide information on

the stability of the two compounds in liquid oral suspensions and the appropriate storage conditions. This would in turn improve therapeutic outcomes and reduce the emergence of resistant bacteria strains in particular and reduce the overall economic cost of healthcare to the nation as a whole.

1.6 SCOPE OF THE STUDY

The study involved liquid oral dosage form combinations of amoxicillin and clavulanic acid on the local market. Certified Reference Standards (CRS) of the two compounds were obtained from United States Pharmacopoeia (USP) and the Food and Drugs Boards.

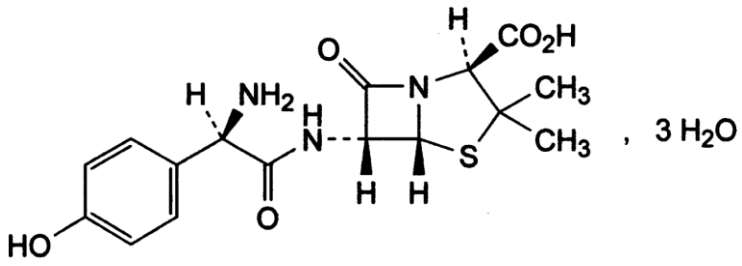
Five (5) different brands of the powder for reconstitution oral liquid suspensions were used for the study. The stability of the samples was assessed at temperatures of 10˚C, 30˚C, and 40˚C.

1.7 LITERATURE REVIEW

1.7.1 AMOXICILLIN

1.7.1.1 Chemistry of Amoxicillin

Amoxicillin is chemically named or designated as (2-S,5R.6R)-6-[[(2R)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid Trihydrate [10]. Amoxicillin belongs to a class of antibiotics known as penicillins. Penicillins have the basic structure, 6-aminopenicillanic acid [1]. This basic structure consists of a thiazolidine ring attached to a beta lactam ring that carries a secondary amino group (RNH-) [1]. Substituents R can be attached to the amino group but the structural intergrity of the 6-aminopenicillanic acid nucleus is essential for the structural integrity of these compounds[1].



Molecular Weight: 419.4[10]

Fig. 1.0 Chemical structure of Amoxicillin Trihydrate.

1.7.1.2 Stereochemistry of Amoxicillin

The penicillin molecule contains three chiral carbon atom(C-3. C-5. and C-6). All naturally occurring and microbiologically active synthetic and semi-synthetic penicillins have the same absolute configuration about these three centers[1]. The carbon atom bearing the acylamino group (C-6) has the L- configuration, whereas the carbon to which the carboxyl group is attached has the D- configuration. Thus, the acylammo and carboxyl groups are trails to each other, with the former in the $\propto$ and the latter in theβ orientation relative to the penam ring system[1]. The atoms composing the 6-aminopenicillanic acid portion of the structure are derived biosynthetically from two amino acids, L-cysteine (S-I. C-5, C-6. C-7. and 6-amino) and L-valine (2.2-dinlethyl. C-2. C-3, N-4. and 3-carboxyl). The absolute stereochemistry of the penicillins is designated 3S:5R:6R [1].

1.7.1.3 Physical appearance of Amoxicillin

Amoxicillin trihydrate is a white or almost white crystalline powder, slightly soluble in water, very soluble in ethanol (96%) but practically insoluble in fatty oils [10]. It also dissolves in dilute acids and dilute solutions of alkali hydroxides. A solution of amoxicillin trihydrate gives an optical rotation of +290 to +315 and a pH of 3.5 to 5.5. [10]

1.7.1.4 Solubility of Amoxicillin

The white crystalline powder of amoxicillin trihydrate has a solubility profile of 1 in 400 of water, 1 in 1000 of ethanol, and 1 in 200 of methanol but is practically insoluble in chloroform and ether [17]

1.7.1.5 Partition Coefficient of Amoxicillin

The Log *P*(octanol/water) of amoxicillin is 0.87. It has three dissociation constants with pKa values of 2.4, 7.4, 9.6, corresponding to the –COOH, -OH, and $–NH_2$ functional groups respectively[17]

1.7.1.6 Ultraviolet Spectrum of Amoxicilin.

Amoxicillin trihydrate: aqueous acid—230nm (A^1_1=225a), 272nm (A^1_1=26a); aqueous alkali—247nm (A^1_1=286b), 291 nm (A^1_1=62a) [17].

1.7.1.7 Infra–red Spectrum.

Principal peaks at wavenumbers 1775, 1583, 1684, 1248, 1613, 1313 cm^{-1} (amoxicillin trihydrate, KBr disk) [17].

1.7.2 CLAVULANIC ACID

1.7.2.1 Chemistry of Clavulanic acid

Clavulanic acid is available as the potassium salt and its named as Potassium (2*R*,3*Z*,5*R*)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo [3.2.0] heptane-2-carboxylate.

Clavulanate potassium is a 1-oxopenam lacking the 6-acylamino side chain of penicillins but possesses a 2-hydroxy ethyldene moiety at C-2[10]

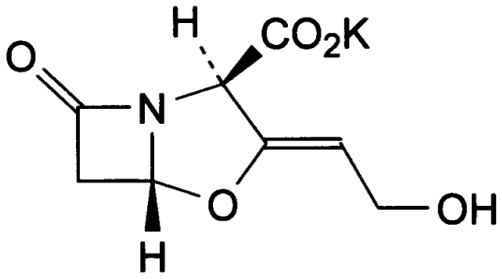


Fig. 2.0 Chemical Structure of Clavulanic acid

1.7.2.2 Physical Appearance

Clavulanate potassium is a white or almost crystalline powder. It is hygroscopic and has a percentage purity of 96.5 to 102.0 percent of the anhydrous powder. It has a pKa value of 2.7 [10].

1.7.2.3 Solubility

It is freely soluble in water, slightly soluble in alcohol and very slightly soluble in acetone.

A solution of 0.400g of potassium clavulanate in 20ml of carbondioxide free water gives a pH of 5.5 to 8.0. A solution of potassium clavulanate as prepared above gives a specific optical rotation of +53 to +63[10]

1.7.2.4 Mechanism of action of Amoxicillin and Clavulanic acid

The Beta-lactam antibiotics are useful and frequently prescribed antimicrobial agents that share a common structure and mechanism of action—inhibition of synthesis of the bacterial peptidoglycan cell wall [18]. The cell walls of bacteria are essential for their normal growth and development. Peptidoglycan is a heteropolymeric component of the bacteria cell wall that provides rigid mechanical stability by virtue of its highly cross-linked latticework structure [18]. In gram-positive microorganisms, the cell wall is 50 to 100 molecules thick, but it is only 1 or 2 molecules thick in gram-negative bacteria [19]. The peptidoglycan is composed of glycan chains, which are linear strands of two

alternating amino sugars (*N*-acetylglucosamine and *N*-acetylmuramic acid) that are cross-linked by peptide chains [18].

The biosynthesis of the peptidoglycan involves about 30 bacterial enzymes and may be considered in three stages. The first stage involves precursor formation and it takes place in the cytoplasm [19]. The product, uridine diphosphate (UDP)–acetylmuramyl-pentapeptide, accumulates in cells when subsequent synthetic stages are inhibited. The last reaction in the synthesis of this compound is the addition of a dipeptide, D-alanyl-D-alanine. Synthesis of the dipeptide involves prior racemization of L-alanine and condensation catalyzed by D-alanyl-D-alanine synthetase. D-Cycloserine is a structural analog of D-alanine and acts as a competitive inhibitor of both the racemase and the synthetase. During reactions of the second stage, UDP-acetylmuramyl-pent peptide and UDP-acetylglucosamine are linked (with the release of the uridine nucleotides) to form a long polymer [18].

The third and final stage involves completion of the cross-link. This is accomplished by a transpeptidation reaction that occurs outside the cell membrane. The transpeptidase itself is membrane-bound [18]. The terminal glycine residue of the pentaglycine bridge is linked to the fourth residue of the pentapeptide (D-alanine), releasing the fifth residue (also D-alanine).

It is this last step in peptidoglycan synthesis that is inhibited by the beta-lactam antibiotics and glycopeptide antibiotics such as *vancomycin* (by a different mechanism than the beta - lactams; Stereomodels reveal that the conformation of penicillin is very similar to that of D-alanyl-D-alanine. The transpeptidase probably is acylated by penicillin; that is, penicilloyl enzyme apparently is formed, with cleavage of the —CO—N— bond of the beta-lactam ring [19].

Although inhibition of the transpeptidase just described is demonstrably important, there are additional, related targets for the actions of penicillins, these are collectively termed *penicillin-binding proteins* (PBPs). All bacteria have several such entities; for example, *S. aureus* has four PBPs, whereas *Escherichia coli* has at least seven [19].

The PBPs vary in their affinities for different beta-lactam antibiotics, although the interactions eventually become covalent. The higher-molecular-weight PBPs of *E. coli* (PBPs 1a and 1b) include the transpeptidases responsible for synthesis of the peptidoglycan[23]. Other PBPs in *E. coli* include those that are necessary for maintenance of the rod-like shape of the bacterium and for septum formation at division. Inhibition of the transpeptidases causes spheroplast formation and rapid lysis [19]. However, inhibition of the activities of other PBPs may cause delayed lysis (PBP 2) or the production of long, filamentous forms of the bacterium (PBP 3). The lethality of penicillin for bacteria appears to involve both lytic and non-lytic mechanisms [18]. Penicillin's disruption of the balance between PBP-mediated peptidoglycan assembly and murein hydrolase activity results in autolysis. Non-lytic killing by penicillin may involve holin-like proteins in the bacterial membrane that collapse the membrane potential [18].

1.7.2.5 Pharmacokinetics of Amoxicillin and Clavulanic acid

Amoxicillin is resistant to inactivation by gastric acid. It is more rapidly and more completely absorbed than ampicillin when given orally [4]. Peak plasma-amoxicillin concentrations of about 5 micrograms/mL have been observed 1 to 2 hours after a dose of 250 mg, with detectable amounts present for up to 8 hours [9]. Doubling the dose can double the concentration. The presence of food in the stomach does not appear to diminish the total amount absorbed. Concentrations of amoxicillin after intramuscular injection are similar to those achieved with oral doses. About 20% is bound to plasma proteins and

plasma half-lives of 1 to 1.5 hours have been reported [4]. The half-life may be prolonged in neonates, the elderly, and patients with renal impairment; in severe renal impairment the half-life may be 7 to 20 hours [4]. Amoxicillin is widely distributed at varying concentrations in body tissues and fluids. It crosses the placenta; small amounts are distributed into breast milk. Little amoxicillin passes into the CSF unless the meninges are inflamed [9].

Amoxicillin is metabolised to a limited extent to penicilloic acid which is excreted in the urine. About 60% of an oral dose of amoxicillin is excreted unchanged in the urine in 6 hours by glomerular filtration and tubular secretion [4]. Urinary concentrations above 300 micrograms/mL have been reported after a dose of 250 mg. Probenecid reduces renal excretion. Amoxicillin is removed by haemodialysis. High concentrations have been reported in bile; some may be excreted in the faeces [4].

1.7.2.6 Stability of Amoxicillin

The main cause of degradation of amoxicillin is the reactivity of the strained lactam ring, particularly to hydrolysis [1]. The course of the hydrolysis and the nature of the degradation products are influenced by the pH of the solution [1]. The 3-lactam carbonyl group of amoxicillin readily undergoes nucleophilic attack by water or especially hydroxide ion to form the inactive penicilloic acid, which is reasonably stable in neutral to alkaline solutions but readily undergoes decarboxylation and further hydrolytic reactions in acidic solutions [4]. Other nucleophiles, such as hydroxylamines, akylamines, and alcohols, open the 3-lactam ring to form the corresponding hydroxamic acids, amides, and esters [4]. It has been speculated that one of the causes of penicillin allergy may be the formation of antigenic penicilloyl proteins in vivo by the reaction of nucleophilic groups.

In strongly acidic solutions pH <3, amoxicillin undergoes a complex series of reactions leading to a variety of inactive degradation products involve rearrangement to the

penicillanic acid. This processes initiated by protonation of the 8-lactam nitrogen, followed by nucleophilic attack of the acyl oxygen atom on the lactam carbonyl carbon [1]. The subsequent opening of the lactam ring destabilizes the thiazolidine ring, which then also suffers acid-catalyzed ring opening to form the penicillanic acid. The latter is very unstable and experiences two major degradation pathways. The most easily understood path involves hydrolysis of the oxazolone ring to form the unstable penamaldic acid [1]. Because it is an enamine, penamaldic acid easily hydrolyzes to penicillamine a major degradation product and penaldic acid [4]. The second pathway involves a rearrangement of penicillanic acid to a penillic acid. Penillic acid (an imidazoline-2-acid) readily decarboxylates and suffers hydrolytic ring opening under acidic conditions to form a second major end product of acid-catalyzed penicillin degradation penilloic acid [1]. Penicilloic acid, the major product formed under weakly acidic to alkaline (as well as enzyme hydrolytic conditions), cannot be detected under strongly acidic conditions. It exists in equilibrium with penamaldic acid, however, and undergoes decarboxylation in acid to form penilloic acid [1]. The third product of the degradation is penicilloaldehyde [4].

Amoxicillin is inactivated by some metallic ions such as copper and zinc. This can be avoided by using phosphate and citrate to combine with these ions to prevent their free existence in solution [1]. Oxidizing agents also inactivate penicillins, but reducing agents have little effect on them. Temperature affects the rate of deterioration: although the dry salts are stable at room temperature and do not require refrigeration, prolonged heating inactivates the penicillins [1].

1.7.2.6.1 Chemical reactions and pathway of the degradation of Amoxicillin

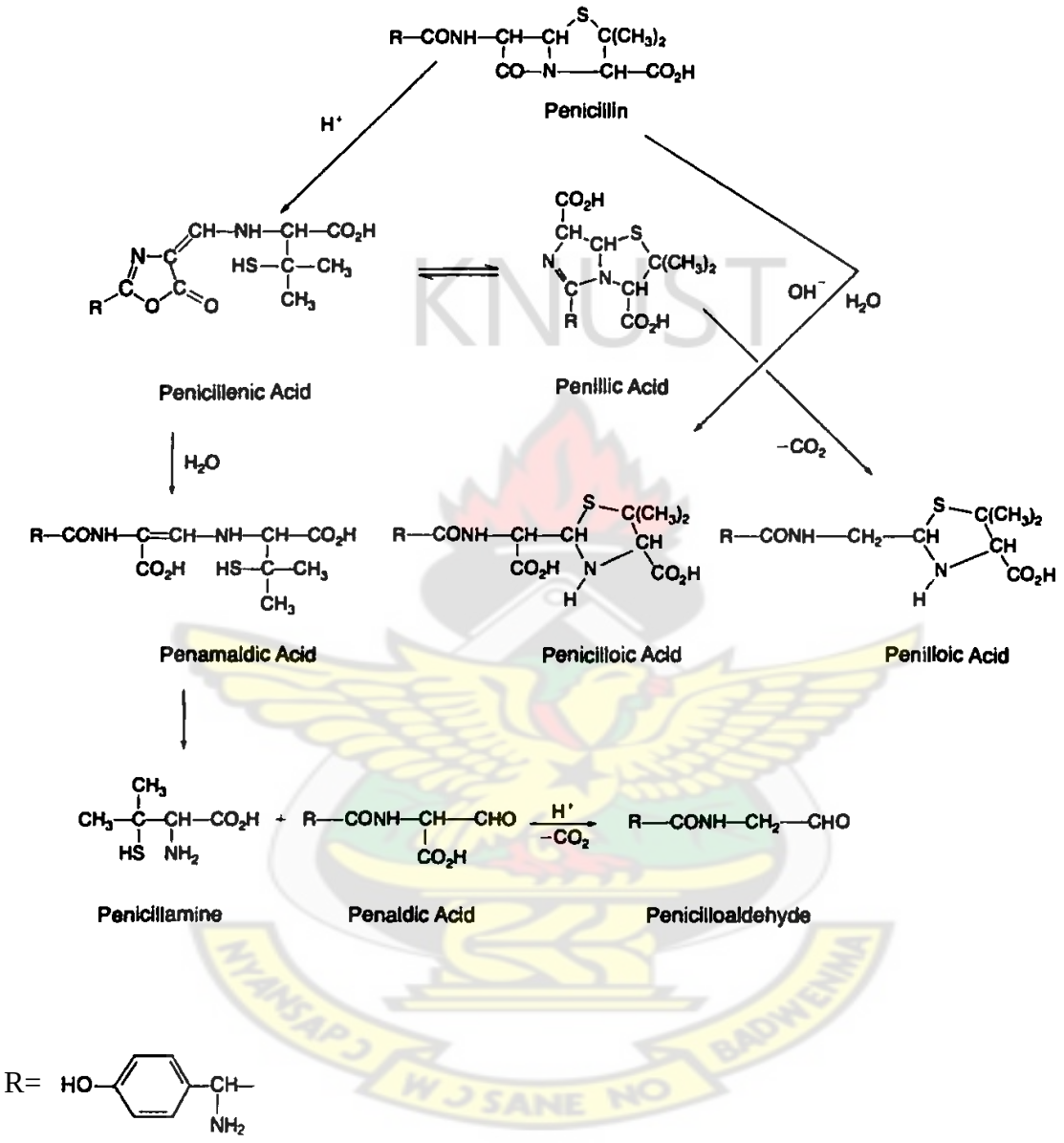


Fig. 3.0 Degradation of Amoxicillin

1.7.2.7 Stability of Clavulanic acid

Clavulanic acid undergoes chemical breakdown to form five major degradation products.The major cause of degradation of clavulanic acid is the reactivity of the strained lactam ring, particularly to hydrolysis [1]. The pathway of the hydrolysis and the nature of the degradation products are affected by the pH of the solution [1]. The 3-lactam carbonyl group of clavulanic acid readily undergoes nucleophilic attack by water or especially hydroxide ion. Clavulanic acid is also affected by temperature to form amine derivatives. Clavulanic acid also readily undergoes decarboxylation and further hydrolytic reactions in acidic solutions [4].

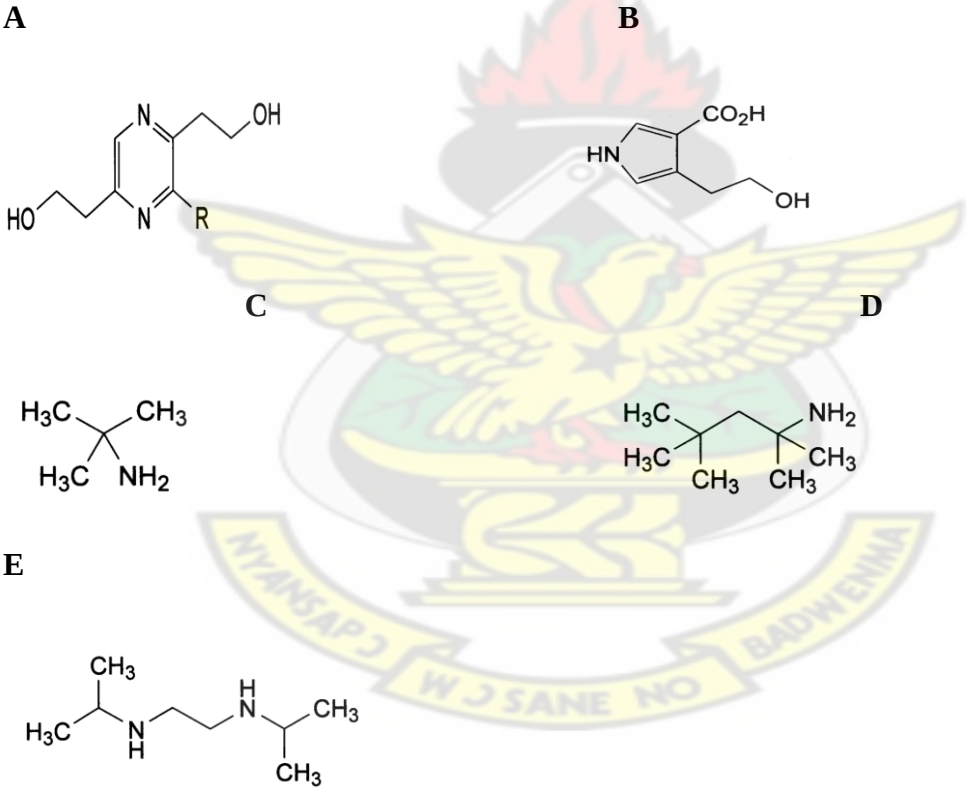


Fig.4.0 Degradation Products of Clavulanic acid

A=3-[3,6-bis(2-hydroxyethyl)pyrazin-2-yl] propanoic acid,

B=4-(2-hydroxyethyl)pyrrole-3-carboxylic acid,

C=2-amino-2-methylpropane (1,1-dimethylethylamine),

D=2-amino-2,4,4-trimethylpentane (1,1,3,3-tetramethylbutylamine),

E=*N*,*N*¢-bis(1-methylethyl)-1,2-ethanediamine (*N*,*N*¢-diisopropylethylenediamine),

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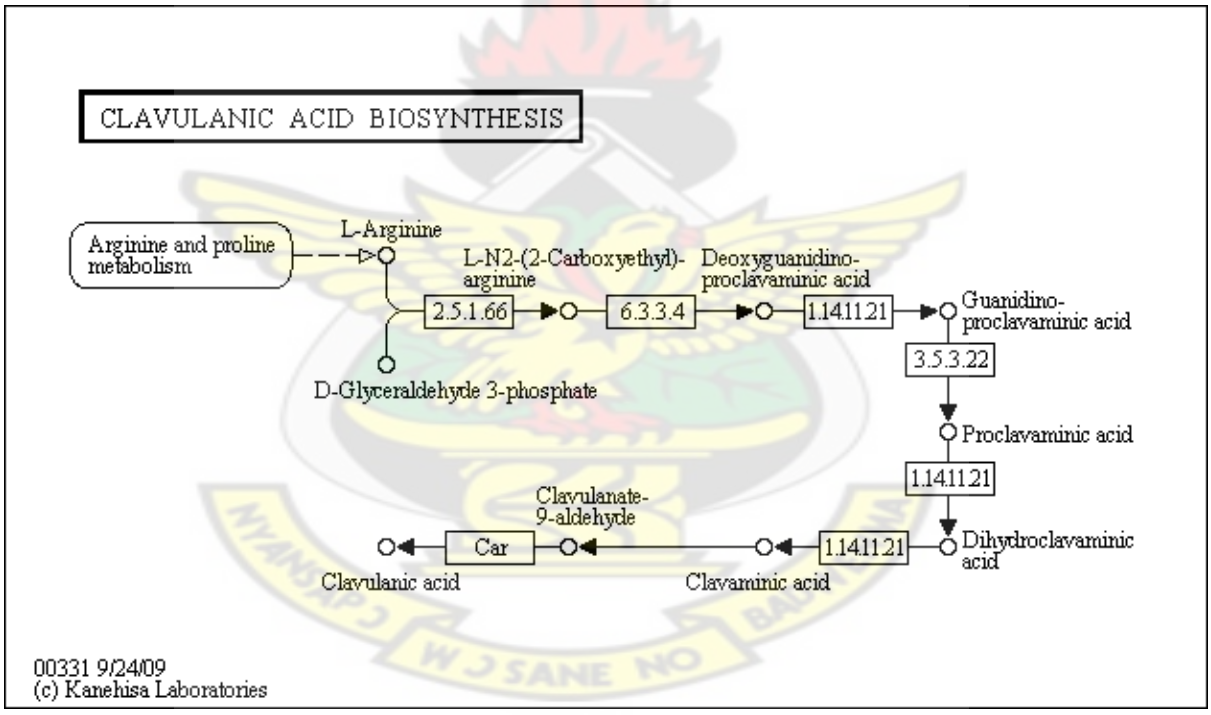

Fig.5.0 Biosynthesis of Clavulanic acid

1.7.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

1.7.3.1 Introduction

In the modern pharmaceutical industry, high-pressure liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, development, analysis and production [20]. Throughout this drug discovery and drug development paradigm, rugged analytical HPLC separation methods are developed and are tailored by each development group (i.e., early drug discovery, drug metabolism, pharmokinetics, process research, preformulation, and formulation) [20]. At each phase of development the analyses of a myriad of samples are performed to adequately control and monitor the quality of the prospective drug candidates, excipients, and final products. Effective and fast method development is of paramount importance throughout this drug development life cycle [13]. This requires a thorough understanding of HPLC principles and theory which lay a solid foundation for appreciating the many variables that are optimized during fast and effective HPLC method development and optimization [21].

1.7.3.2 Chromatographic Process

Chromatographic separations are based on a forced transport of the liquid (mobile phase) carrying the analyte mixture through the porous media and the differences in the interactions at analytes with the surface of this porous media resulting in different migration times for a mixture of components [14]. In the above definition the presence of two different phases is stated and consequently there is an interface between them. One of these phases provides the analyte transport and is usually referred to as the mobile phase, and the other phase is immobile and is typically referred to as the stationary phase [13].

A mixture of components, usually called analytes, are dispersed in the mobile phase at the molecular level allowing for their uniform transport and interactions with the mobile and stationary phases[13].

High surface area of the interface between mobile and stationary phases is essential for space discrimination of different components in the mixture [21]. Analyte molecules undergo multiple phase transitions between mobile phase and adsorbent surface. Average residence time of the molecule on the stationary phase surface is dependent on the interaction energy. For different molecules with very small interaction energy difference the presence of significant surface is critical since the higher the number of phase transitions that analyte molecules undergo while moving through the chromatographic column, the higher the difference in their retention [14]. The nature of the stationary and the mobile phases, together with the mode of the transport through the column, is the basis for the classification of chromatographic methods [35].

1.7.3.3 Classification of Chromatography

The mobile phase could be either a liquid or a gas, and accordingly we can subdivide chromatography into liquid chromatography (LC) or gas chromatography (GC) [14]. Apart from these methods, there are two other modes that use a liquid mobile phase, but the nature of its transport through the porous stationary phase is in the form of either (a) capillary forces, as in planar chromatography (also called thin-layer chromatography, TLC), or (b) electro osmotic flow, as in the case of capillary electrochromatography (CEC) [14]. The next classification step is based on the nature of the stationary phase. In gas chromatography it could be either a liquid or a solid. Gas–liquid chromatography (long capillary coated with a thin film of relatively viscous liquid or liquid-like polymer; in older systems, liquid-coated porous particles were used) and gas–solid chromatography

(capillary with thin porous layer on the walls or packed columns with porous particles) [13].

In liquid chromatography a similar distinction historically existed, since to a significant extent the development of liquid chromatography reflected the path that was taken by gas chromatography development [13]. Liquid–liquid chromatography existed in the early 1970s, but was mainly substituted with liquid chromatography with chemically bonded stationary phases. Recently, liquid–liquid chromatography resurfaced in the form of counter current chromatography with two immiscible liquid phases of different densities [20]. The other form of LC is liquid–solid chromatography. Liquid chromatography was further diversified according to the type of the interactions of the analyte with the stationary phase surface and according to their relative polarity of the stationary and mobile phases [20].

Since the invention of the technique, adsorbents with highly polar surface were used (CaCO3—Tswett, porous silica—most of the modern packing materials) together with relatively non-polar mobile phase. In 1964, Horvath introduced a chemically modified surface where polar groups were shielded and covered with graphitized carbon black and later with chemically bonded alkyl chains [21]. The introduction of chemically modified hydrophobic surfaces replaces the main analyte—surface interactions from polar to the hydrophobic ones, while mobile phase as an analyte carrier became polar [14]. The relative polarity of the mobile and stationary phases appears to be "reversed" compared to the historically original polar stationary phase and non-polar mobile phase used by M. S. Tswet [14]. This new mode of liquid chromatography became coined as reversed-phase liquid chromatography (RP), where "reversed-phase" referred to the reversing of the relative polarity of the mobile and stationary phases. In order to distinguish this mode from the old form of liquid chromatography, the old became known as normal-phase (NP*)* [8].

The third mode of liquid chromatography, which is based on ionic interactions of the analyte with the stationary phase, is called Ion-exchange (IEX) [7]. The separation in this mode is based on the different affinity of the ionic analytes for the counterions on the stationary phase surface [21]. Specific and essentially stand-alone mode of liquid chromatography is associated with the absence or suppression of any analyte interactions with the stationary phase, which is called size-exclusion chromatography (SEC) [21]. In SEC, the eluent is selected in such a manner that it will suppress any possible analyte interactions with the surface, and the separation of the analyte molecules in this mode is primarily based on their physical dimensions (size [21]. The larger the analyte molecules, the lower the possibility for them to penetrate into the porous space of the column packing material, and consequently the faster they will move through the column.

1.7.3.4 HPLC Chromatograph

Typical HPLC Chromatograph consists of the following main components:

Solvent Reservoirs. Storage of sufficient amount of HPLC solvents for continuous operation of the system. Could be equipped with an online degassing system and special filters to isolate the solvent from the influ-ence of the environment [22].

Pump. This provides the constant and continuous flow of the mobile phase through the system; most modern pumps allow controlled mixing of different solvents from different reservoirs [22].

Injector. This allows an introduction (injection) of the analytes mixture into the stream of the mobile phase before it enters the column; most modern injectors are autosamplers, which allow programmed injections of different volumes of samples that are withdrawn from the vials in the autosampler tray [14].

Column. This is the heart of HPLC system; it actually produces a separation of the analytes in the mixture. A column is the place where the mobile phase is in contact with the stationary phase, forming an interface with enormous surface. Most of the chromatography development in recent years went toward the design of many different ways to enhance this interfacial contact [14].

Detector. This is a device for continuous registration of specific physical (sometimes chemical) properties of the column effluent. The most common detector used in pharmaceutical analysis is UV (ultraviolet), which allows monitoring and continuous registration of the UV absorbance at a selected wavelength or over a span of wavelengths (diode array detection). Appearance of the analyte in the detector flowcell causes the change of the absorbance. If the analyte absorbs greater than the background (mobile phase), a positive signal is obtained [14].

Data Acquisition and Control System. Computer-based system that controls all parameters of HPLC instrument (eluent composition (mixing of different solvents); temperature, injection sequence, etc.) and acquires data from the detector and monitors system performance (continuous monitoring of the mobile-phase composition, temperature, backpressure, etc.) [22].

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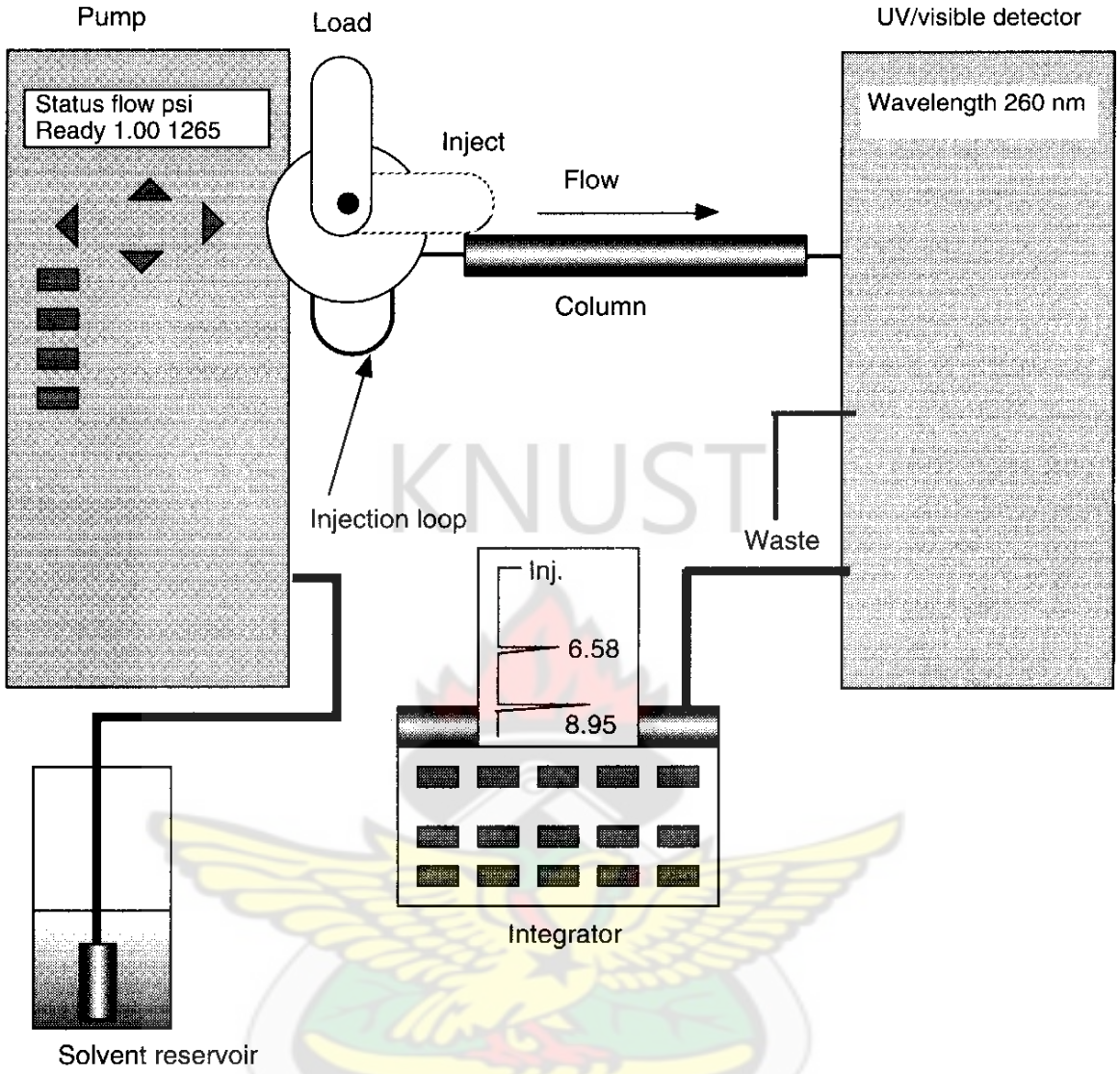

Fig. 6.0 HPLC Chromatograph

1.7.3.5 Types of HPLC

The four main types of HPLC techniques are

Normal Phase, Reversed Phase, Ion Exchange, and Size Exclusion Chromatography

The principal characteristic defining the identity of each technique is the dominant type of molecular interactions employed.

1.7.3.5.1 Normal-Phase Chromatography (NP HPLC)

Normal-phase HPLC explores the differences in the strength of the polar interactions of the analytes in the mixture with the stationary phase [21]. The stronger the analyte–stationary phase interaction, the longer the analyte retention [13]. As with any liquid chromatography technique, NP HPLC separation is a competitive process. Analyte molecules compete with the mobile-phase molecules for the adsorption sites on the surface of the stationary phase [21]. The stronger the mobile-phase interactions with the stationary phase, the lower the difference between the stationary-phase interactions and the analyte interactions, and thus the lower the analyte retention [13]. Mobile phases in NP HPLC are based on nonpolar solvents (such as hexane, heptane, etc.) with the small addition of polar modifier (i.e., methanol, ethanol) [13].

Variation of the polar modifier concentration in the mobile phase allows for the control of the analyte retention in the column [14]. Typical polar additives are alcohols (methanol, ethanol, or isopropanol) added to the mobile phase in relatively small amounts [22]. Since polar forces are the dominant type of interactions employed and these forces are relatively strong, even only 1 v/v% variation of the polar modifier in the mobile phase usually results in a significant shift in the analyte retention [14]. Packing materials traditionally used in normal-phase HPLC are usually porous oxides such as silica (SiO_2) or alumina (Al_2O_3). Surface of these stationary phases is covered with the dense population of OH groups,

which makes these surfaces highly polar [13]. Analyte retention on these surfaces is very sensitive to the variations of the mobile-phase composition [13]. Chemically modified stationary phases can also be used in normal-phase HPLC [22]. Silica modified with trimethoxy glycidoxypropyl silanes (common name: diol-phase) is typical packing material with decreased surface polarity [21]. Surface density of OH groups on diol phase is on the level of 3–4μmol/m2, while on bare silica silanols surface density is on the level of 8μmol/m2 [9]. The use of diol-type stationary phase and low-polarity eluent modifiers [esters (ethyl acetate) instead of alcohols] allow for increase in separation ruggedness and reproducibility, compared to bare silica [14].

Selection of using normal-phase HPLC as the chromatographic method of choice is usually related to the sample solubility in specific mobile phases [13]. Since NP uses mainly non polar solvents, it is the method of choice for highly hydrophobic compounds (which may show very stronger interaction in reversed-phase HPLC), which are insoluble in polar or aqueous solvents [14].

1.7.3.5.2 Reversed-Phase HPLC (RP HPLC or RPLC)

As opposed to normal-phase HPLC, reversed-phase chromatography employs mainly dispersive forces (hydrophobic or Van der Waals interactions) [14]. The polarities of mobile and stationary phases are reversed, such that the surface of the stationary phase in RP HPLC is hydrophobic and mobile phase is polar, where mainly water-based solutions are employed [14]. Reversed-phase HPLC is by far the most popular mode of chromatography [22]. Almost 90% of all analyses of low-molecular-weight samples are carried out using RP HPLC [9]. One of the main drivers for its enormous popularity is the ability to discriminate very closely related compounds and the ease of variation of retention and selectivity [14]. The origin of these advantages could be explained from an

energetic point of view: Dispersive forces employed in this separation mode are the weakest intermolecular forces, thereby making the overall background interaction energy in the chromatographic system very low compared to other separation techniques [22]. This low background energy allows for distinguishing very small differences in molecular interactions of closely related analytes [14]. Adsorbents employed in this mode of chromatography are porous rigid materials with hydrophobic surfaces [22]. In all modes of HPLC with positive analyte surface interactions the higher the adsorbent surface area, the longer the analyte retention and in most cases the better separation [14]. The majority of packing materials used in RP HPLC are chemically modified porous silica [13].

1.7.3.5.3 Ion-Exchange Chromatography (IEX)

Ion-exchange chromatography, as indicated by its name, is based on the different affinities of the analyte ions for the oppositely charged ionic centers in the resin or adsorbed counterions in the hydrophobic stationary phase [21].

Four major types of ion-exchange centers are usually employed:

1. SO_3^- : Strong cation-exchanger
2. CO_2^- : weak cation-exchanger
3. Quaternary amine : strong anion-exchanger
4. Tertiary amine : weak anion-exchanger

Analyte retention and selectivity in ion-exchange chromatography are strongly dependent on the pH and ionic strength of the mobile phase [14].

1.7.3.5.4 Size-Exclusion Chromatography (SEC)

SEC is the method for dynamic separation of molecules according to their size; as indicated by its name, the separation is based on the exclusion of the molecules from the porous space of packing material due to their steric hindrance [21]. Hydrodynamic radius

of the analyte molecule is the main factor determining its retention [21]. This is the only chromatographic separation method where any positive interaction of the analyte with the stationary phase should be avoided [21]. In size-exclusion chromatography, the higher the molecular weight of the molecule, the greater its hydrodynamic radius, which results in faster elution [21].

1.7.3.6 Basic Chromatographic Descriptors

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Four major descriptors are commonly used to report characteristics of the chromatographic column, system, and particular separation:[20]

1. Retention factor (*k*)
2. Efficiency (*N*)
3. Selectivity (□)
4. Resolution (*R*)

1.7.3.6.1 Retention factor (k)

It is the unit less measure of the retention of a particular compound on a particular chromatographic system at given conditions defined as:

$$k = \frac{V_R - V_o}{V_o} = \frac{t_R - t_o}{t_o}$$

where V_R is the analyte retention volume, V_O is the volume of the liquid phase in the chromatographic system, t_R is the analyte retention time, and t_O is sometimes defined as the retention time of non retained analyte [6]. Retention factor is convenient because it is independent on the column dimensions and mobile phase flow rate. Note that all other chromatographic conditions significantly affect retention factor [20].

1.7.3.6.2 Efficiency

It is the measure of the degree of peak dispersion in a particular column; as such, it is essentially the characteristic of the column. Efficiency is expressed in the number of theoretical plates (*N*) calculated as: [14]

$$N = 16\left[\frac{t_R}{w}\right]^2$$

where *tR* is the analyte retention time and *w* is the peak width at the baseline.

Column efficiency is mainly dependent on the kinetic factors of the chromatographic system such as molecular diffusion, mass-flow dynamics, properties of the column packing bed, flow rate, and so on [14]. The smaller the particles and the more uniform their packing in the column, the higher the efficiency [13]. The faster the flow rate, the less time analyte molecules have for diffusive band-broadening. At the same time, the faster the flow rate, the further analyte molecules are from the thermodynamic equilibrium with the stationary phase. This shows that there should be an optimum flow rate that allows achievement of an optimum efficiency for a given column [13].

1.7.3.6.3 Selectivity (□)

It is the ability of chromatographic system to discriminate two different analytes. It is defined as the ratio of corresponding retention factors:[22]

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

Where k_1 and k_2 are the retention factors of the two analytes.

The selectivity is primarily dependent on the nature of the analytes and their interaction with the stationary phase surface [22]. If a dramatic change of the selectivity is needed for

a particular separation, the best solution is the replacement of the type of the stationary phase [21]

1.7.3.6.4 Resolution (*R*)

It is a combined measure of the separation of two compounds which include peak dispersion and selectivity. Resolution is defined as:

$$R = 2\,\frac{(t_2 - t_1)}{(w_2 + w_1)}$$

t_1 and t_2, w_1 and w_2 are the respective retention times and the

The distance between the peak maxima reflects the selectivity of the system. The greater the distance, the higher the selectivity [14].

Improvement of the resolution of poorly resolved analytes then could be pursued in two different ways: either by increasing the efficiency or by improving the selectivity [14]. The resolution value equal to 1.5 is usually regarded as sufficient for the baseline separation of closely eluted peaks; and the typical average efficiency of modern HPLC column is equal to 10,000 theoretical plates, then the selectivity necessary for this separation to get a resolution of 1.5 can be calculated. It will be also useful to compare what would be required in terms of efficiency and selectivity to improve the resolution from1 to 1.5 [22].

1.7.4 HPLC DETECTORS

1.7.4.1 UV-Visible Detectors

The most widely used detectors in modern HPLC are photometers based on ultraviolet (UV) and visible light absorption. These detectors have a high sensitivity for many solutes, but samples must absorb in the UV (or visible) region [20].

UV-visible spectrophotometric detectors can respond throughout a wide wavelength range (e.g., 190–600 nm), which enables the detection of a broad spectrum of compound types [21].

Reversed- phase mobile phases of acetonitrile plus water or phosphate buffer can be used routinely for detection at 200 nm, whereas methanol-containing mobile phases cannot be used below ≈210 nm, depending on methanol concentration [13]. The proper selection of the mobile phase makes it possible to operate UV detectors in a near-universal detection mode in the 200-nm to 215-nm region, where most organic compounds exhibit some UV absorbance [21]. UV detectors come in three common configurations. Fixed-wavelength detectors rely on distinct wavelengths of light generated from the lamp, whereas variable-wavelength and diode-array detectors select one or more wavelengths generated from a broad-spectrum lamp [21]. UV-detector characteristics include but are not limited to the following:

Capable of very high sensitivity (for samples that absorb in the UV), good linear range (>105), can be made with small cell volumes to minimize extra-column band broadening, relatively insensitive to mobile-phase flow and temperature changes, very reliable, easy to operate, non destructive of sample, widely varying response for different solutes, compatible with gradient elution and detection wavelength can be selected [22].

1.7.4.2. Florescence detectors

Fluorescence detectors are very sensitive and selective for solutes that fluoresce when excited by UV radiation [21]. Sample components that do not fluoresce do not produce a detector signal, so sample cleanup may be simplified [14]. Because of its high sensitivity the fluorescence detector is particularly useful for trace analysis, or when either the sample size is small or the solute concentration is extremely low [13]. The linear dynamic range of

the fluorescence detector usually is smaller than that of UV detectors, but it is more than adequate for most trace analysis applications [13]. While the dynamic range (the range over which a change in sample concentration produces a change in the detector output) of fluorescence detectors can be fairly large (e.g., 104), the *linear* dynamic range may be restricted for certain solutes to relatively narrow concentration ranges (as low as 10-fold) [22]. In comparison to other detection techniques, fluorescence generally offers greater sensitivity and fewer problems with instrument instability (e.g., from temperature and flow changes) [22]. The major disadvantage of the fluorescence detector is that not all compounds fluoresce [14].

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1.7.4.3 Electrochemical detectors

Many compounds that can be oxidized or reduced in the presence of an electric potential can be detected at very low concentrations by selective electrochemical (EC) measurements [20]. By this approach the current between polarizable and reference electrodes is measured as a function of applied voltage [20]. By fine-tuning the detector potential, one can achieve great selectivity for electro-active compounds. The EC detector's sensitivity makes it one of the most sensitive of all HPLC detectors [13]. However, to operate under high sensitivity, extra care must be taken to use highly purified mobile phases to reduce background noise [13]. In order to reduce the background noise, in some applications the mobile phase is routed through a high-potential pre-treatment cell so as to oxidize or reduce background interferences before the mobile phase reaches the autosampler [21].

1.7.4.4 Refractive Index detectors

The differential refractive index (RI) detector responds to a difference in the refractive index of the column effluent as it passes through the detector flow cell [7]. The RI detector is a bulk-property detector that responds to all solutes, if the refractive index of the solute is sufficiently different from that of the mobile phase [14].Refractive index detector characteristics include but are not limited to the following:

Excellent versatility; all solutes can be detected, moderate sensitivity but generally not useful for trace analyses, not useful for gradient elution, efficient heat-exchanger required, sensitive to temperature changes [14]. It is also reliable, fairly easy to operate, non destructive [22].

1.7.5 pH CONTROL AND SELECTIVITY IN RP-CHROMATOGRAPHY

1.7.5.1 Ionization and pH

The retention of an ionisable analyte depends on its degree of ionization [21]. For simple substances, one can state that the non-ionic form of the analyte always has a much higher retention than the ionic form [21]. If the analyte has multiple stages of ionization, the form with the higher degree of ionization usually exhibits lower retention [13]. The degree of ionization of the analyte depends on the pH of the solution and on the p*Ka* values of the ionization stages of the analyte [14]. The dependence of the retention on the degree of ionization is shown in Fig. 3.0 for both an acidic and a basic analyte [13]. For both compounds, the retention changes by more than an order of magnitude. This is typical: for most compounds, the change in retention between the ionized and the non-ionized form is of the order of 10 to 30-fold. The ionized form always has lower retention in RP-chromatography [21]. Therefore, the retention is lowest under acidic conditions for a basic

analyte and under basic conditions for an acidic analyte [7]. On the other hand, high retention is observed when the analyte is in its neutral form [21]

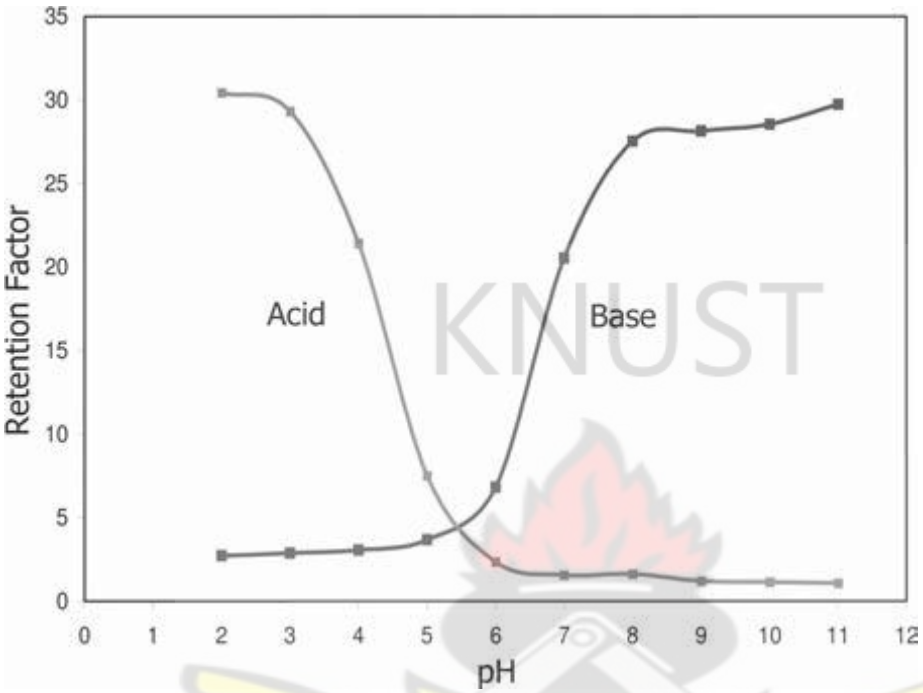

Fig.7.0 Effect of pH on retention factor

The p*Ka* values of the analyte determine the pH range in which the charge, and therefore the retention, changes [14]. If the pH value is outside ± 2 pH units around the p*K*a of the compound, the analyte is either 99% dissociated or 99% undissociated [13]. This means that outside this pH range the retention does not change with a change in pH [14]. However, within this range, especially within ± 1.5 pH units around the p*K*a, the retention changes markedly [13]. For the practice of chromatography, this means that good pH control is absolutely essential in order to achieve reproducible retention times [13].

1.7.5.2 Changes of pKa and pH Value in the Presence of an Organic Solvent

The pH and p*K*a values of buffers as well as the p*K*a values of analytes found in the literature have commonly been measured using water as the solvent. It is known that both the pH values and the p*Ka* values change when an organic solvent is added [13].

Generally, the p*K*a values of acids increase, while the p*K*a values of bases decrease with the addition of the organic modifier [13]. However, the buffer capacity does not change in the presence of the organic solvent [21]. A buffer that is a good buffer in water is also a good buffer in the presence of an organic solvent [14]. The most important value for the practitioner of HPLC is the buffer capacity, and this does not change with the addition of the organic solvent, while the p*K*a value of the buffer and the optimal pH do change [22].

1.7.5.3 HPLC Buffers

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Which buffers are preferred for an application depends primarily on the choice of detector. Phosphate buffers are buffers used for UV detection [13]. Their p*K*a values are 2.15 and 7.20. They can be used without difficulty at low UV wavelength, for example 210 nm [21]. At weakly alkaline pH, buffers based on the ammonium ion (p*K*a = 9.24) with a suitable counter ion with low UV absorption can be used [22]. Borate buffers can be used in the same pH range and also have a low UV absorption [21]. In the more alkaline pH range, some simple amines can be used: pyrrolidine has a p*K*a of 11.3, and triethylamine a p*K*a of 10.7, and both can be used at low UV wavelengths [13]. With all amines, their purity is an important factor for their use at such UV wavelengths. Other buffers often exhibit a substantial UV absorption below 215 nm [14]. However, for the normal UV detection range around 254 nm a large number of buffers is available [13]. Acetate with its p*K*a of 4.75 is probably the most popular buffer, since its p*Ka* is exactly intermediate between the first and second dissociation constants of phosphate [13]. Ammonium hydrogen carbonate is a preferred buffer for the pH range around 9 to 10, since the buffering ranges of the ammonium ion (p*K*a =9.24) and the hydrogencarbonate ion (p*K*a = 10.25) overlap [21].

1.7.6 REVIEW OF OTHER ANALYTICAL TECHNIQUES

1.7.6.1 Infra red Spectroscopy

Qualitative Analysis

The value of infrared spectrometry as a means of identification of unknown compounds and to investigate structural features is immense [23]. Spectra are used in an empirical manner by comparison of samples with known materials and by reference to charts of group frequencies [24].

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Principles

Absorption of electromagnetic radiation in the infrared region of the spectrum resulting in changes in the vibration energy of molecules is the underlying principle behind the technique [25].

Instrumentation

The main instrumentation types are the Fourier transform spectrometer or double-beam spectrophotometer that incorporates prism or grating monochromatic, thermal or photon detector and alkali halide cells [23].

Applications

Infra red spectroscopy is widely used, for the identification and structural analysis of organic materials; useful for quantitative analysis but less widely used than UV and visible spectrometry in analytical chemistry. Near infrared region used increasingly for industrial quality control [24].

Disadvantages

Difficult to analyse mixtures and special cells required are for aqueous samples [25].

A molecule can absorb energy only if there is a net change in the *dipole moment* during a particular vibration, a condition fulfilled by virtually all polyatomic molecules [23]. The absorption spectra of such molecules are often very complex and the underlying reason for

this complexity is best understood by first considering the spectrum of a heteronuclear diatomic molecule in the gaseous state [25].

1.7.6.2 UV-Visible Spectroscopy

Principle

The underlying principle behind the technique is the absorption of electromagnetic radiation in the visible and ultraviolet regions of the spectrum resulting in changes in the electronic structure of ions and molecules [24].

Instrumentation

It consist of a light source, slit, monochromator, a cell and a detector [25].

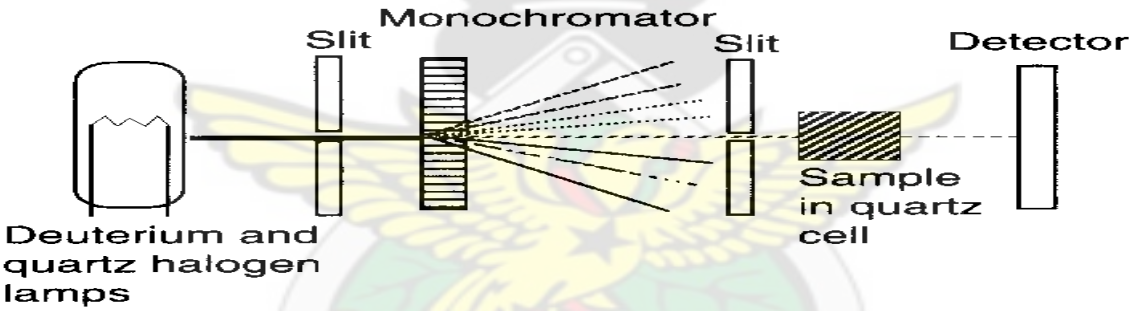

Fig. 8.0 Single beam UV/Vis spectrophotometer

Applications

A most widely used technique for quantitative analysis. Used as an adjunct to other spectrometric techniques in the identification and structural analysis of organic materials [24].

Quantitative Analysis

Quantitative methods based on the absorption of electromagnetic radiation involve measurement of the reduction in intensity of the radiation on passage through an absorbing medium, i.e. the sample [24]. The degree of absorption is determined by comparing the intensity of the transmitted beam when no absorbing species is present, i.e. a blank, with

that transmitted by the sample [24]. For monochromatic, collimated radiation passing through a homogeneous liquid sample, the reduction in intensity of the incident radiation can be related to the concentration of absorbing species and to the thickness of the absorbing medium, both relations being embodied in the *Beer-Lambert law* [23].

The *Beer-Lambert* law may be expressed in the form $\log_{10}(I_0/I) = A = \varepsilon Cl$

where log10 (*I*0/*I*) is defined as the *absorbance A* and $\mathcal{E}$ is a constant known as the *molar absorptivity*. The value of $\mathcal{E}$ (the absorbance of a 1 M solution in a 1 cm cell) depends upon the nature of the absorbing species and on the wavelength of the incident radiation. Absorbance is thus seen to be directly proportional both to the concentration of the absorbing species and to the thickness of the absorbing medium [23].

The usual quantitative procedure is to prepare a calibration graph, or Beer's law plot, by plotting absorbance against concentration for a series of standards [23]. This should give a straight line passing through the origin [23]. Measurements are generally made at a maximum in the absorbance curve to maximize sensitivity and to mini-mize errors in setting the instrument at the chosen wavelength [24]. This also minimizes apparent deviations from Beer's law for incident radiation of wide bandwidth (*vide infra*) [24]. The concentrations of unknowns can then be read directly from the graph or calculated using a factor, i.e. the absorbance reading is divided by the slope[23]. The composition of mixtures of two or more absorbing materials can be established by measuring standards and samples at two or more wavelengths, preferably corresponding to the absorbance maximum of each component [23]. The concentrations of the components can be calculated from a set of simultaneous equations once the respective molar absorptivities at each wavelength are known [22]

Disadvantages

Samples should be in solution. Mixtures can be difficult to analyse without prior separation of the constituents [25].

1.7.6.3 Nuclear Magnetic Resonance

Principles

Absorption of electromagnetic radiation in the radio-frequency region of the spectrum resulting in changes in the orientation of spinning nuclei in a magnetic field is underlying principle behind the technique [24].

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Instrumentation

It consists of a powerful and highly homogeneous electromagnet, radio-frequency signal generator and detector circuit, electronic integrator and glass sample tubes [24].

Applications

It is used for the identification and structural analysis of organic materials and study of kinetic effects, mainly from proton and carbon-13 spectra [24]. Useful for quantitative analysis but not widely applied [25].

Disadvantages

Expensive and complex instrumentation [24]. Moderate to poor sensitivity with continuous wave (scanning) instruments, but greatly enhanced by Fourier transform instruments [23]. Limited range of solvents for studying proton spectra unless they are deuterated [24]. Absorption of radiation in the radio-frequency, RF, region of the electromagnetic spectrum can be observed for those nuclei which are considered to spin about their own axes [25].

1.7.7 STABILITY STUDIES

1.7.7.1 Introduction

Chemical degradation and physical degradation of drug substances may change their pharmacological effects, resulting in altered therapeutic efficacy as well as toxicological consequences [27]. Because pharmaceuticals are used therapeutically based on their efficacy and safety, they should be stable and maintain their quality until the time of usage or until their expiration date [28]. The quality should be maintained under the various conditions that pharmaceuticals encounter, during production, storage in warehouses, transportation, and storage in hospital and community pharmacies, as well as in the home [26]. Therefore, understanding the factors that alter the stability of pharmaceuticals and identifying ways to guarantee their stability are critical [28].

1.7.7.2 Factors affecting stability of pharmaceutical products

pH

pH is perhaps the most important parameter which affects the hydrolysis rate of drugs in liquid formulations; it is certainly the one which has been most widely examined [27]. Studying the influence of pH on degradation rate is not as simple as might at first be imagined [47]. If the hydrolysis rate of the drug in a series of solutions buffered to the required pH is measured and the hydrolytic rate constant is then plotted as a function of pH, a pH–rate profile will be produced, but this will almost certainly be influenced by the buffers used to prepare the solutions [29].

Temperature

Increase in temperature usually causes a very pronounced increase in the hydrolysis rate of drugs in solution, a fact which is used to good effect in the experimental studies of drug stability described above [29]. Such studies are usually carried out at high temperatures, say 60 or 80°C, because the hydrolysis rate is greater at these temperatures and can

therefore be measured more easily [26]. Of course, if a formulation has to be heat sterilized then its stability will, in any case, have to be measured at elevated temperatures [26].

Oxygen

Since molecular oxygen is involved in many oxidation schemes, oxygen could be used as a challenge to find out whether a particular drug is likely to be affected by oxidative breakdown [28]. This can be done by storing solutions of the drug in ampoules purged with oxygen and then comparing their rate of breakdown with similar solutions stored under nitrogen [26]. Formulations that are shown to be susceptible to oxidation can be stabilized by replacing the oxygen in the storage containers with nitrogen or carbon dioxide, by avoiding contact with heavy metal ions, and by adding antioxidants [29].

Light

Photo labile drugs are usually stored in containers which exclude ultraviolet light, since exposure to light in this wavelength range is the most usual cause of photo degradation [29]. Amber glass is particularly effective in this respect because it excludes light of wavelength of less than about 470 nm [26]. As an added precaution, it is always advisable to store photolabile drugs in the dark [28].

Moisture

Water-soluble drugs present in a solid dosage form will dissolve in any moisture which has adsorbed on the solid surface [30]. The drug will now be in an aqueous environment and its decomposition could be influenced by many of the factors which we have already discussed when dealing with liquid dosage forms [45]. For example, decomposition could now occur by hydrolytic cleavage of ester or amide linkages in the drug molecule and hence will be affected by the pH of the adsorbed moisture film [46]. It is not surprising, therefore, that moisture is considered to be one of the most important factors that must be controlled in order to minimise decomposition [29].

1.7.7.3 General principles

The purpose of stability testing is to provide evidence of how the quality of an Active Pharmaceutical Ingredient (API) or Finished Pharmaceutical Product (FPP) varies with time under the influence of a variety of environmental factors such as temperature, humidity and light [28]. The stability programme also includes the study of product-related factors that influence its quality, for example, interaction of API with excipients, container closure systems and packaging materials [30]. In fixed-dose combination (FDCs) the interaction between two or more APIs also has to be considered [26]. As a result of stability testing a re-test period for the API (in exceptional cases, e.g. for unstable APIs, a shelf-life is given) or a shelf-life for the FPP can be established and storage conditions can be recommended [30]. Various analyses have been done to identify suitable testing conditions for WHO Member States based on climatic data and are published in the literature on the basis of which each Member State can make its decision on long-term (real-time) stability testing conditions [28]. The choice of test conditions is based on an analysis of the effects of climatic conditions as classified by the WHO into zones [30]. The mean kinetic temperature in any part of the world can be derived from climatic data, and the world can be divided into four climatic zones, I-IV [28].

1.7.7.4 In-use stability

The purpose of in-use stability testing is to provide information for the labelling on the preparation, storage conditions and utilization period of multi-dose products after opening, reconstitution or dilution of a solution, e.g. an antibiotic suspension or injection supplied as a powder for reconstitution [28]. As far as possible the test should be designed to simulate the use of the FPP in practice, taking into consideration the filling volume of the container and any dilution or reconstitution before use [26]. At intervals comparable to those which

occur in practice appropriate quantities should be removed by the withdrawal methods normally used and described in the product literature [30].

1.7.8 VALIDATON OF ANALYTICAL METHOD

1.7.8.1 Introduction

Method validation is the process of demonstrating that analytical procedures are routinely suitable for their intended use [32]. The method validation process for analytical procedures begins with the planned and systematic collection by the applicant of the validation data to support analytical procedures [33]. Validation of an analytical method includes testing of its important characteristics. The final aim is to be certain that the analysis process is reliable and precise, remains under total control of the operator, and leads to reliable results [34].

The following are typical analytical performance characteristics which may be tested during methods validation: Specificity, Precision, Accuracy, Linearity, Range, Limit of detection, Limit of quantitation and Robustness

1.7.8.2 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present such as impurities, degradation products, and excipients [35]. Specificity measures only the desired component without interference from other species that might be present.

To determine specificity during the validation, sample matrix (placebo), and known related impurities are analyzed to determine whether interferences occur.

1.7.8.3 Precision

Precision consists of two components: repeatability and intermediate precision. Repeatability is the variation experienced by a single analyst on a single instrument [36]. Repeatability does not distinguish between variation from the instrument or system alone and from the sample preparation process [32]. During the validation, repeatability is performed by analyzing multiple replicates of an assay composite sample by using the analytical method [33]. The recovery value is calculated and reported for each value. Intermediate precision refers to variations within a laboratory such as different days, with different instruments, and by different analysts [34]. During the validation, a second analyst repeats the repeatability analysis on a different day using different conditions and different instruments [32]. Recovery values are calculated and reported. A statistical comparison is made to the first analyst's results [30].

1.7.8.4 Linearity

Linearity evaluates the analytical procedure's ability (within a give range) to obtain a response that is directly proportional to the concentration (amount) of analyte in the sample [33]. If the method is linear, the test results are directly or by well-defined mathematical transformation proportional to the concentration of analyte in samples within a given range [36]

1.7.8.5 Range

Range is defined as the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity [33]. Range is normally expressed in the same units as test results (e.g., percent, parts per million) obtained by the analytical method [32]. During validation, range (sometimes

referred to as *linearity of method*) is evaluated using samples (usually spiked placebos) and must encompass the specification range of the component assayed in the drug product [34].

1.7.8.6 Limit of Detection (LOD)

The limit of detection (LOD) of an individual procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value [34]. The LOD is a parameter of limit tests (i.e., tests that only determine if the analyte concentration is above or below a specification limit). In analytical procedures such as HPLC that exhibit baseline noise, the LOD can be based on a signal-to-noise (S/N) ratio (3:1), which is usually expressed as the concentration (e.g., percentage, parts per billion) of analyte in the sample [33]. There are several ways in which it can be determined, but it usually involves injecting samples, which generate an S/N of 3:1, and estimating the LOD [11]. The LOD can also be obtained by: Calculation from the standard deviation of the blank, calculation from the calibration line at low concentration, standard deviation of the blank, standard deviation of the ordinate intercept, or residual standard deviation of the linear regression b: slope of the regression line [33]. The estimated limits should be verified by analysing a suitable number of samples containing the analyte at the corresponding concentrations [32].

1.7.8.7 Limit of Quantitation (LOQ)

The limit of quantitation (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy [35]. The LOQ is a parameter of quantitative assays for low concentrations of compounds in sample matrices and is used particularly for the determination of impurities and/or degradation products [34]. It is usually expressed as the concentration (e.g., percentage, parts per million) of analyte in the sample [36]. For analytical procedures such as HPLC that exhibit baseline noise, the LOQ is generally estimated from a determination

of S/N ratio (10:1) and is usually confirmed by injecting standards which give this S/N ratio and have an acceptable percent relative standard deviations (%RSDs) as well [34].

1.7.8.8 Robustness

Robustness is defined as the measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters (e.g., pH, mobile-phase composition, temperature, and other instrumental parameters) and provides an indication of its reliability during normal usage [35]. This is an important parameter with respect to the transferability of the method following validation [38].

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CHAPTER 2.0 – EXPERIMENTAL WORK

2.1 MATERIALS AND EQUIPMENT

- Adam Analytical Balance PW124 (Max 120g, d=0.0001g)
- pH Meter- Eutech Instruments pH 510
- Double Beam UV-VIS Spectrophotometer T90+ PG Instruments Limited
- Clifton Sonicator (Nickel Electro Limited)
- HPLC Chromatograph:
    - LC 20AB Binary Pump- Schimadzu
    - SPD-20AB UV Detector
    - DGU-20A_3 Degasser
    - LC Solutions Software
    - ODS Column C18 Phenomenex 250x4.6mm(Ultracarb 5.0)
- Stuart Melting Point Apparatus SMP(10)
- Stability Chamber
- Referigerator
- Vacuum pump
- Dessicator
- Pre-coated TLC Plates (Merck)
- Volumetric Flask (10ml,25ml,50ml,100ml,250ml,500ml)
- Transfer pipette (1.0ml, 2.0ml, 5.0ml, 10.0ml)
- Beakers
- Plastic Funnel
- Conical Flasks
- Polarimeter (Atago Polax-2L)

2.2 REAGENTS AND SAMPLES

- Sodium Acetate (99.6%) (BDH)
- Glacial Acetic Acid Analar (99.8%)
- Methanol (HPLC Grade)
- 1.0N Sodium Hydroxide
- 1.2N Hydrochloric Acid
- 0.01N Iodine VS
- 0.01N Sodium thiosulphate VS
- Starch Iodide Paste Ts
- Sodium Hydrogen Carbonate solution
- Acetone R
- Ammonium Acetate
- Ferric Chloride
- 0.5M Hydrochloric Acid
- Sodium Carbonate Solution
- Ferric Chloride
- Dilute Ammonia R
- Sodium Carbonate Solution R
- Sodium Sulphide Solution
- Tartaric acid

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Table 1 Certified Reference Standard

Name of standard	Source	Mfg Date	Exp Date
Amoxicillin Trihydrate	FDB Labs	April 2010	August 2013
Clavulanic acid	FDB Labs	July 2010	May2012

Table 2 Brands of reconstituted oral suspensions of Co-amoxiclav 457mg/5ml

Brand	**Code**	**Batch No**	**Exp Date**	**Country of Origin**
Augmentin	A	BN 431896	Jul 2012	United Kingdom
Amoksiklav	B	BN 190461	Jun 2012	England
Fleming	C	Lot BB1810	Aug 2012	Slovenia
Clavudor	D	BN2346	May2012	China
Co-amox	E	BN56432	Sep 2012	Switzerland

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2.3 METHODS

2.3.1PREPARATION OF REAGENTS

2.3.1.1 Preparation and Standardization of 0.1M Sodium thiosulphate

Preparation

6.25g of Analar sodium thiosulphate crystals was accurately weighed and dissolved together with 0.05g of sodium carbonate in freshly boiled and cooled distilled water. The solution was transferred into a 250ml volumetric flasks and made up to volume with the same solvent.

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Standardization

0.4g of pure dried potassium iodate was accurately weighed into a 250ml volumetric flask, dissolved in water and make up to volume. 25.0ml of this solution was pipetted into a conical flask. 2g of KI and 5ml of 2MHCl were added. The liberated iodine was titrated with 0.1M sodium thiosulphate solution with constant stirring. The liquid mixture was diluted to about 200ml with water when the colour had become pale yellow. 2ml of starch mucilage was subsequently added and titration continued until the colour changed from blue to colourless. The titration was repeated to obtain three replicate results.

Preparation and standardization of 0.25M Sodium Hydroxide

Preparation

10.0g of Analar sodium hydroxide was weighed into a beaker and dissolved with distilled water and transferred into a 250ml volumetric flask. The solution was made up to volume with distilled water.

Standardization

6.075.0g of Sulphamic acid was accurately weighed and dissolved in about 50ml of recently boiled and cooled water. The solution was transferred into a 250ml volumetric flask and diluted to the mark with distilled water. The solution was titrated with the 0.25M

solution of sodium hydroxide using methyl orange as indicator. Two replicate determinations were made [37].

2.3.2 IDENTIFICATION TESTS

2.3.2.1 Identification tests for Amoxicillin Trihydrate (BP)

a) About 5mg of Amoxicillin trihydrate was placed in test tube and 0.5ml of water was used to moisten it. 2ml of sulphuric acid-formaldehyde was added and placed on a water bath for 1 minute.

b) About 5mg of amoxicillin was dissolved in water and 1-2drops of neutral ferric chloride solution. The colour was observed.

c) Melting point determination

10.0mg of Amoxicillin reference was packed into a capillary tube sealed at one end. It was placed in the sample holder of the melting point apparatus and the temperature range over which the sample melted was recorded. This was repeated and the average temperature determined

d) With the aid of a sonicator, 0.1g of amoxicillin was dissolved in carbondioxide free water and the solution was diluted with 50ml of the same solvent. The specific optical rotation was then determined.

2.3.2.2 Identification test for Clavulanic Acid (BP)

a) About 10mg of potassium clavulanate was dissolved in 2ml of freshly boiled and cooled distilled water, 1ml of sodium carbonate solution was added and heated.0.05ml of sodium sulphide solution was added to the hot solution and cooled. 2ml of tartaric acid solution was added and observed

b) 0.1g of potassium clavulanate was dissolved in freshly boiled and cooled distilled water and diluted to 20ml. 5ml of the resulting solution was diluted to 10ml. The specific optical rotation of this solution was determined.

c) Melting point determination

10.0mg of Clavulanic acid reference was packed into a capillary tube sealed at one end. It was placed in the sample holder of the melting point apparatus and the temperature range over which the sample melted was recorded. This was repeated and the average temperature determined

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2.3.2.2 THIN LAYER CHROMATOGRAPHY (TLC)

Amoxicillin

The mobile phase was prepared by mixing 15ml of ethyl acetate, 5ml of glacial acetic acid and 5ml of water. The mixture was stored in a chamber lined with filter paper and adequate time allowed for the chamber to be saturated with solvent vapour. The stationary phase was made of pre-coated silica plate. A line parallel to and 1.5cm from the bottom edge of chromatoplate.

15mg of the amoxicillin reference standard was weighed and dissolved in 10ml of acidified aqueous acetone solution and shaked for five minutes.1ml of this solution was pipetted and 7ml of the acidified aqueous acetone solution added. This solution was spotted on the chromatoplate four times using a microcapillary pipette. The chromatoplate was then allowed to dry off all extraction solvent and carefully placed in the mobile phase. After 30 minutes the chromatoplate was removed and solvent front. All residual solvent was dried and the chromatoplate observed under UV light.

Clavulanic Acid

Similarly, a mobile phase consisting of 15ml of ethylacetate and 5ml of methanol and 20ml of distilled water. The mobile phase was stored in a chamber lined with filter and allowed to be saturated with the solvent. The stationary phase was also made of pre-coated silica plate. 10mg of potassium clavulanate was dissolved in 15ml of distlled water. The solution was spotted on the chromatoplate four times, allowing all solvent to dry off. The chromatoplate was developed in the mobile phase and observed under UV light after 30 minutes.

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2.3.3 ASSAY OF REFERENCE STANDARDS

2.3.3.1 Assay of Amoxicillin reference standard by Iodometry.

0.1g of the reference amoxicillin powder was weighed into a beaker, 25mls of water was added and sonicated in a water bath at 25˚C for 45minutes.2.0ml of this solution was pipetted into a conical flask and 2.0ml of a 1M sodium hydroxide solution was added and mixed by swirling. The solution was allowed to stand for 15minutes in the dark. This procedure was repeated for two more conical flasks. To each of the three conical flasks, 2.0ml of a 1.2N hydrochloric acid was added. 10.0ml of a 0.01M solution of iodine was added and allowed to stand for 15minutes. The resulting solution was titrated with 0.1M sodium thiosulphate. As the endpoint was approached one (1) drop of starch iodide paste was added and the titration is continued to discharge the blue colour. The titration was repeated for the two remaining flasks.

2.3.3.2 Assay of Clavulanic acid reference standard.

0.45g of clavulanic acid reference standard was accurately weighed and dissolved in a beaker with distilled water. The solution was then transferred quantitatively into a 100ml volumetric flask and made up to volume with distilled water. 20.0ml of this solution was pipetted into a conical flasks and titrated with a 0.25M solution of Sodium Hydroxide already standardised with 0.1M solution of sulphamic acid. Phenolpthalein was used as indicator. Two replicate determintations were made and the average titre was determined.

2.4 HPLC METHOD DEVELOPMENT

2.4.1 Preliminary Studies In Establishing Conditions For Final Method Development.

2.4.1.1 Solubility studies of amoxicillin and clavulanic acid

To establish a suitable solvent system for both amoxicillin and clavulanic acid five different solvents were employed. The solvents used in assessing solubility included water, methanol, isopropyl alcohol, ethanol and combinations of water: methanol in varying ratios of 80:20, 50:50 and 20:80. To 10ml each of these solvents and their combinations 5mg of amoxicillin was first added and sonicated at 26˚C. The time it took for the sample to dissolve completely was recorded.

2.4.1.2 UV investigation of wavelength of detection of Amoxicillin and Clavulanic acid

In establishing the wavelength of detection of the two compounds, 0.00004%w/v of solutions of amoxicillin and clavulanic acid were prepared using different solvent such as methanol and a combination of methanol and water in the ratios of (80:20) and (10:90) . The solutions were scanned with a UV spectrophotometer between a wavelength of 200-

400nm and the wavelength of maximum absorption were identified from the spectra obtained.

2.4.2 Establishment of chromatographic conditions

2.4.2.1 Determination of mobile phase conditions

In establishing optimal mobile phase conditions to separate the two compounds, different buffers were employed and the one that gave optimal resolution of the two compounds was selected. Also, the buffer was used in combination with methanol at varying ratios of methanol/buffer such as 60:40, 50:50, 30:70, 15:85 and 5:95. The ratio of methanol/buffer combination that gave an optimal resolution of the two compounds was selected.

2.4.2.2 Selection of Stationary Phase

The chromatographic mode used in the method development is reversed-phase. As a result, an Octadecyl silane (ODS) 20 ultracarb 5micron column 250 x 4.6mm was used.

2.4.2.3 Determination of flow rate of mobile phase

Different flow rates of the mobile phase were investigated ranging from 0.5m/min, 0.8ml/min, 1.0ml/min,1.2ml/min and 1.5ml. Flow rate that yield the best separation and retention times of the two compounds were selected.

2.4.2.4 Determination of wavelength of detection of amoxicillin and clavulanic acid

From the preliminary investigations on wavelength of detection made with the UV spectrophotometer, a wavelength range of 218 to 270nm were selected and used on the UV detector to determine the wavelength at which the two compounds could be detected.

2.4.2.5 Studies on sensitivity of the method

The sensitivity of the method was investigated by varying the recorder range of the detector (AUFS) to determine which value of AUFS would give the best response. The range was varied from 0.01 to 0.08 AUFS.

2.4.2.6 Selection of Internal Standard

In selecting an internal standard for the method, five compounds were investigated and the compound that gave the best response was selected. The selection was based on parameters such as stability of the compound, chemical structure, resolution from the analyte, and detector response being close to that produced by analyte for a given weight.

2.4.3 Preparation of Mobile Phase and solution of reference standards

2.4.3.1 Preparation of Mobile Phase (Methanol:Water:Acetate Buffer/ 5:90:5)

An acetate buffer of pH 4.34 is prepared. This was done by accurately weighing 0.299g of sodium acetate and dissolving it in 25ml of distilled water in a beaker. The solution was transferred into a 100ml volumetric flasks. 0.9ml glacial acetic acid was added to the solution gradually and mixed by swirling. The solution was then made up to the 100ml mark with distilled water. 500.0ml of the mobile phase was prepared by measuring 450.0ml of distilled water into a 500ml volumetric flask. 25.0ml the acetate buffer was added and mixed. 25.0ml of HPLC grade methanol was then added and mixed thoroughly.

2.4.3.2 Preparation of solution of reference standards of amoxicillin and clavulanic acid

Amoxicillin Trihydrate

10mg of amoxicillin trihydrate was accurately weighed and transferred into a 25.0ml beaker. 6.0ml of a solution of methanol:water in a ratio of 80:20 was added and sonicated at 26˚C for 5minutes to completely dissolve the amoxicillin. The solution was transferred

into a 10.0ml volumetric flask and made up to the mark with the mobile phase. This solution was diluted with the mobile phase to obtain a solution of concentration 50µg/ml.

Potassium Clavulanate

10mg of potassium clavulanate was accurately weighed and transferred into 25ml beaker. 5.0ml of distilled water was added and sonicated at 26˚C for 5minutes to completely dissolve the potassium clavulanate. The solution was transferred into a 10.0ml volumetric flask and made up to the mark with the mobile phase. The solution was diluted with the mobile phase to obtain a solution of concentration 20µg/ml.

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2.4.3.3 Stability of solutions of reference standards of Amoxicillin and Clavulanic acid.

To demonstrate validity, precision and accuracy of result, the stability of the solutions of amoxicillin, clavulanic acid and metronidazole were investigated over a period of twenty-four (24Hrs) hours. Solutions of concentrations 0.04%, 0.004% and 0.0005% for amoxicillin, clavulanic acid and metronidazole reference standards respectively were prepared. 20µL of each solution was injected after 5.0min, 15.0min, 30.0min.45min, 1.0hr, 2.0hr, 5.0hr, 8.0hr, 10.0hr and recovery determined.

2.4.4 Calibration curve for Amoxicillin and Clavulanic acid

2.4.4.1 Amoxicillin

Preparation of stock solution

A stock solution of amoxicillin was prepared by weighing 100mg of amoxicillin reference powder and dissolving it in 20.0ml of a solution of methanol:water in a ratio of 80:20. The solution was then made up to the 100ml mark in a 100ml volumetric flask to obtain a stock solution of 0.1%w/v

Preparation of Calibration Curve

To obtain a calibration curve, the stock solution prepared was used to prepare dilute solutions of concentrations 0.018%w/v, 0.027%w/v, 0.036%w/v, 0.045%,w/v, 0.0540%w/v and 0.063%w/v. 20µL of these solutions were sequentially injected three times to obtain their corresponding peak areas using metronidazole as an internal standard at a final concentration of 0.0004%w/v. The mean peak area ratios were calculated and plotted against their corresponding concentration to obtain the calibration curve

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2.4.4.2 Clavulanic acid

Preparation of stock solution

A stock solution of clavulanate potassium was prepared by weighing 100mg of reference powder and dissolving in 25.0ml of distilled water. The solution was then made up to the 100ml mark in a 100ml volumetric flask with the mobile phase to obtain a final concentration of 0.1%w/v

Preparation of Calibration curve

To obtain a calibration curve, the stock solution prepared was used to prepare dilute solutions of concentrations 0.001%w/v, 0.002%w/v, 0.003%w/v, 0.004%w/v, 0.005%w/v and 0.006%w/v. 20µL of these solutions were sequentially injected three times to obtain their corresponding peak areas using metronidazole as an internal standard at a final concentration of 0.0004%w/v. The mean peak area ratios were calculated and plotted against their corresponding concentration to obtain the calibration curve.

2.4.5 Assay of Amoxicillin and Clavulanic acid combinations in liquid oral suspension

62.0ml of distilled water was used to reconstitute five different brands of Co-amoxiclav paediatric powder for reconstitution as suspension. 0.57ml of each of the reconstituted suspension was taken with a graduated pipette and accurately transferred quantitatively

into a 100ml volumetric flask. 25ml of distilled water added and well shaken for 5minutes to completely dissolve all the suspended particles. More distilled water was added and made up to the 100ml mark. The solutions were filtered using a membrane filter. The concentration of final solution expected was 0.0456w/v% and 0.0057%w/v for amoxicillin and clavulanic acid respectively. The solutions were injected using the same internal standard. Three replicate injections were performed and the average percentage content calculated for each brand.

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2.5 HPLC METHOD VALIDATION

2.5.1 AMOXICILLIN

2.5.1.1 Precision-Repeatability (Intra-day precision)

To establish intra-day precision of the method, six different amoxicillin samples were analysed at 100% concentration of the test (0.06%w/v). Three replicate injections were performed for each sample and the mean peak area ratio determined. The relative standard deviations of the concentrations were calculated.

2.5.1.2 Reproducibility (Inter-day precision)

The inter-day precision was established by analysing six different amoxicillin samples on three consecutive days. Three replicate injections were performed for each sample and the mean peak area ratio determined. The relative standard deviations of their corresponding concentrations were calculated.

2.5.1.3 Linearity

Linearity was demonstrated by establishing a linear relationship between concentration and Mean peak area ratio. A series of six different concentrations of amoxicillin reference powder were prepared and injected. The concentration of the solutions prepared ranges from 0.018%w/v to 0.063%w/v. A calibration curve of peak area against concentration was obtained and analyzed statistically using Excel and Graph Pad Prism

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2.5.1.4 Accuracy

In establishing accuracy of the method, a known amount of the amoxicillin reference powder was added to three different solution prepared from the reconstituted suspension at 80%, 100% and 120% concentrations corresponding to 0.045%, 0.054%and 0.063%. The solutions were injected sequentially three times and the mean percentage recovery and relative standard deviations calculated at these concentration levels.

To further establish accuracy of the method, this method was compared to a standard Pharmacopoeial method. The reconstituted suspension was assayed using the standard pharmacopoeial method in the BP and the result obtained compared statistically to that of an assay of the same sample using the method developed. The means of replicate determinations of the percentage content were calculated. The two means were compared using the student *t-test.*

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

The LOD and LOQ were calculated from the calibration curve using the residual standard deviation of the calibration curve.

2.5.1.5 Specificity

To establish specificity, a solution of the pure powder was prepared in the presence of the excipients of the powder for reconstitution. The solutions were injected and observed for any interfering peaks.

2.5.1.6 Robustness

To demonstrate robustness, some conditions of the HPLC method for the quantification of amoxicillin were varied while all other conditions were kept constant. The conditions varied include: pH of mobile phase and the column used (the same ODS but from a different manufacturer).

2.5.2 CLAVULANIC ACID

2.5.2.1 Precision-Repeatability (Intra-day precision)

To establish intra-day precision of the method, six different amoxicillin samples were analysed at 100% concentration of the test (0.005%w/v). Three replicate injections were performed for each sample and the mean peak area ratio determined The relative standard deviations of their corresponding concentrations were calculated.

2.5.2.2 Reproducibility (Inter-day precision)

The inter-day precision was established by analysing six different clavulanic acid samples on three consecutive days. Three replicate injections were performed for each sample and the mean peak area ratio determined The relative standard deviations of their corresponding concentrations were calculated.

2.5.2.3 Linearity

Linearity was demonstrated by establishing a linear relationship between concentration and peak area ratio. A series of six different concentrations of clavulanic reference powder were prepared and injected. The concentration of the solutions prepared ranges from 0.001%w/v to 0.006%w/v. A calibration curve of peak area ratio against concentration was obtained and analyzed statistically.

2.5.2.4 Accuracy

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In establishing accuracy of the method, a known amount of the reference powder was added to three different solution prepared from the reconstituted suspension at 80%, 100% and 120% concentrations corresponding to 0.004%, 0.005%and 0.006%. The solutions were injected sequentially for three times. The average percentage recovery and relative standard deviations were calculated at these concentration levels.

To further establish accuracy of the method, this method was compared to a standard Pharmacopoeial method. The reconstituted suspension was assayed using the standard pharmacopoeial method and the result obtained compared statistically to that of an assay of the same sample using the method developed. The means of replicate determinations of the percentage content were calculated. The two means were compared using the student *t-test.*

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

The LOD and LOQ were calculated from the calibration curve.

2.5.2.6 Specificity

To establish specificity, a solution of the pure powder was prepared in the presence of the excipients of the powder for reconstitution. The solutions were injected and observed for any interfering peaks.

2.5.2.7 Robustness

To demonstrate robustness, some conditions of the HPLC method for the quantification of clavulanic acid were varied while all other conditions were kept constant. The conditions varied include: pH of mobile phase and the column used (the same ODS but from a different manufacturer)

2.6 STABILITY STUDIES OF THE RECONSTITUTED SUSPENSION

2.6.1 Conditions of stability studies

2.6.1.1 Temperature

The stability studies was conducted at three different temperatures. The temperatures used are 10˚C, 30˚C and 40˚C. To obtain a temperature of 10˚C the samples were kept in a refrigerator and the temperature in refrigeration compartment was monitored with a calibrated thermometer and temperature recordings were made at 8:00am, 12:00noon and 6:00pm. The highest temperature reading of 12˚C and lowest temperature of 8˚C were recorded with an average temperature of 10˚C. For the temperature of 30˚C, the samples were kept on a bench in a room and the temperature monitored at regular intervals recording the highest temperature of 32˚C and lowest of 28˚C with an average of 30˚C. The temperature of 40˚C was obtained in a chamber where the temperature was set to read 40˚C. A calibrated thermometer was used to measure the temperature of the chamber at regular intervals in a day as stated above throughout the study period.

2.6.1.2 Duration of study

For an in-use stability studies the study was conducted for a period of seven days which is maximum period for which the reconstituted suspension must be used as per the World Health Organization (WHO) guideline for in-use stability studies for reconstituted products The samples were analysed at 0, 24hours, 48hours, 72hours, 4th-day, 5th-day, 6th-day and 7th-day

2.6.1.3 Preparation of samples and reference standard

Two brands of powder for reconstitution of amoxicillin and clavulanic acid combination were used for the stability studies. These two brands include the innovator and one other generic brand that from a survey report was the most highly stocked brand on the Ghanaian local market. The powders for reconstitution as oral suspension of these two brands were reconstituted by adding 62.0ml of distilled mixing thoroughly.0.75ml of the reconstituted suspension was taken with a pipette and accurately transferred together with the washing ensuring complete transfer into a 100ml volumetric flask. The solution was well shaken and made up to the 100ml mark with distilled water, giving a solution with a final concentration of 0.06% and 0.005% for amoxicillin and clavulanic acid respectively. This concentration lies within the linear range of the method. The pH of the final solution was measured with a pH meter and recorded. The reference standards of both amoxicillin and clavulanic acid were also prepared at the same concentrations as the sample and compared.

2.6.1.4 Analysis of samples at the various temperatures

Samples of the two brands stored at the various temperatures of 10°C, 30°C and 40°C were drawn and analysed throughout the study period. The samples were analysed at 0hr, 24hours, 48hours, 72hours, 4th-day, 5th-day, 6th-day and 7th-day. At each time the reference standard was injected and the samples of the two brands were also drawn, filtered and

injected and the percentage content calculated by comparing the peak area ratios of the reference and the samples.

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CHAPTER 3 – RESULTS AND CALCULATIONS

3.1 IDENTIFICATION TESTS

3.1.1 Identification test for Amoxicillin (AX)

Table 3.0 Identification of Amoxicillin by colour reaction tests

Test	Observation	Specification(BP)
About 2mg of AX+1.5ml distilled water +2ml Sulphuric acid- formaldehyde	A dark yellow colour was observed	A dark yellow colour develops
About 5mg of AX + distilled water + 1-2drops of neutral Ferric Chloride solution	A blue colour was observed	A blue colour develops

Table 4.0 Melting Point determination of Amoxicillin

Sample	Melting Point(˚C)			Reference Range
	1st determination	2nd determination	Average	
Amoxicillin	192-196	194-196	193-196	192-196

Table 5.0 Specific optical rotation determination of Amoxicillin (0.25%)

Sample	Specific optical rotation			Reference range
	1stdetermination	2nddetermination	Average	
Amoxicillin	+298	+300	+299	+290 to +315

3.1.2 Identification tests for Clavulanic acid (CLV)

Table 6.0 Identification of Clavulanic acid by colour reaction test

Test	Observation	Reference
About 10mg of CLV + 1ml distilled water+2m Na_2CO_3 solution+heat. Add 0.05ml of sodium sulphide + heat and cool. Add 2m of tartaric acid and allow to stand for 5minutes	A white crystalline precipitate was formed	A white crystalline precipitate develops

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Table 7.0 Melting point determination of Clavulanic acid

Sample	Melting Point (˚C)			Reference range(˚C)
	1stdetermination	2nd determination	Average	
Clavulanic acid	121-125	123-125	12-125	121-125

Table 8.0 Determination of specific optical rotation of Clavulanic acid (0.2%)

Sample	Specific optical rotation			Reference range
	1st value	2nd value	Average	
Clavulanic acid	+56	+58	+57	+53 to +63

Table 9.0 Determination of pH of Amoxicillin and Clavulanic acid solutions

Sample	1st and 2nd pH reading		Average pH	Reference range
Amoxicillin	5.1	5.3	5.2	3.5-5.5
Clavulanic acid	6.4	6.2	6.3	5.5-8

3.1.3 Thin Layer Chromatography of Amoxicillin and Clavulanic Acid under UV light

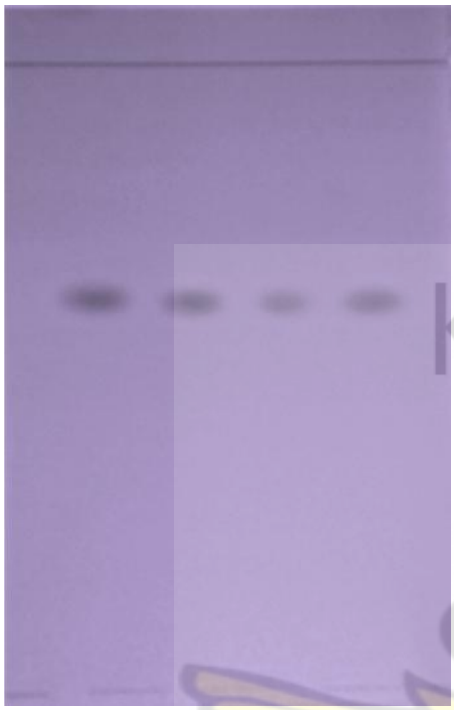

Fig. 9 TLC of Pure Amoxicillin

Fig. 10 TLC of Pure Clavulanic acid

3.1.4 Calculation of Retention Factor (RF) of Amoxicillin and Clavulanic Acid

Retention factor (Rf) is given by

$$Rf = \frac{\text{Distance travelled by sample from baseline}}{\text{Distance travelled by solvent front from baseline}}$$

For Pure Amoxicillin Distance travelled by sample from baseline =4.1cm

Distance travelled by solvent front from baseline= 5.4cm

$$Rf = \frac{4.1cm}{5.4cm} = 0.76$$

Average Rf for the Pure Amoxicillin spots $= \frac{0.76+0.75+0.76+0.75}{4} = 0.755$

Similarly, the average Rf values of the spots of Pure clavulanic acid was calculated as 0.54

3.2 ASSAY RESULTS OF AMOXICILLIN AND POTASSIUM CLAVULANATE REFERENCE STANDARD

3.2.1 Assay Amoxicillin Reference Standard

Table 10.0 Standardization of 0.1M Sodium thiosulphate with potassium iodate

Burette reading	**1st determination**	**2nd determination**	**3rd determination**
Final reading(ml)	21.00	41.00	19.90
Initial reading(ml)	1.00	21.00	0.00
Titre(ml)	**20.00**	**20.00**	**19.90**

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$$\text{Average Titre} = \frac{20.00+20.00+19.90}{3} = 19.97\text{ml}$$

$2Na_2S_2O_3 + I_2 \rightarrow Na_2S_4O_6 + 2NaI$

$KIO_3 + 5I^- + 6H^+ \rightarrow 3I_2 + 3H_2O$

1mole of KIO_3 = 6moles of $Na_2S_2O_3$

214g of KIO_3 in 1000ml = 6M $Na_2S_2O_3$

35.666g of KIO_3 in 1000ml = 1M $Na_2S_2O_3$

3.5666g of KIO_3 in 1000ml = 0.1M $Na_2S_2O_3$

0.8917g of KIO_3 in 250ml = 0.1M $Na_2S_2O_3$

Factor of KIO_3 = Actual weight/Nominal weight Factor of KIO_3 = 0.8920/0.8917

Factor of KIO_3 (F_1) = 1.0003 Factor of $Na_2S_2O_3$(F_2) =

Volume of $Na_2S_2O_3$ (V_2) = 19.97ml

Volume of KIO_3 (V_2) = 20.0ml

$$\text{Factor of } Na_2S_2O_3 = \frac{F_1 V_1}{V_2} = \frac{1.0003 \text{ X } 20}{19.97} = 1.0018$$

Table 11 Results of titration of Amoxicillin with 0.1M sodium thiosulphate

Burette reading	**1st determination**	**2nd determination**	**3rd determination**
Final reading(ml)	14.20	28.50	14.20
Initial reading(ml)	0.00	14.20	0.00
Titre(ml)	**14.20**	**14.30**	**14.20**

Average Titre = $\frac{14.20=14.20}{2}$ = 14.20ml

419.4g of $C_{16}H_{19}N_3O_5S,3H_2O$ in 1000ml = 6M $Na_2S_2O_3$

69.9g of $C_{16}H_{19}N_3O_5S,3H_2O$ in 1000ml = 1M $Na_2S_2O_3$

6.99g of $C_{16}H_{19}N_3O_5S,3H_2O$ in 1000ml = 0.1M $Na_2S_2O_3$

0.00699g of $C_{16}H_{19}N_3O_5S,3H_2O$ = 1ml of 0.1M$Na_2S_2O_3$

Actual volume of $Na_2S_2O_3$ = Factor of $Na_2S_2O_3$ X Titre

= 1.0018 X 14.20

= 14.23ml

1ml of 0.1M$Na_2S_2O_3$ = 0.00699g of $C_{16}H_{19}N_3O_5S,3H_2O$

14.23ml of 0.1M$Na_2S_2O_3$ = 14.23 X 0.00699

=0.0995g

Weight of $C_{16}H_{19}N_3O_5S,3H_2O$ taken = 0.1000g

% purity of $C_{16}H_{19}N_3O_5S,3H_2O$ = $\frac{0.0995}{0.100}$X 100%

= 99.5%

Percentage purity of amoxicillin trihydrate reference sample = 99.5%

3.2.2 Assay of Clavulanic acid Reference Standard

Table 12 Standardisation of 0.25M Sodium Hydroxide using Sulphamic acid

Burette reading	**1st determination**	**2nd determination**	**3rd determination**
Final reading(ml)	21.40	42.70	21.5
Initial reading(ml)	0.00	21.40	0.00
Titre(ml)	**21.40**	**21.30**	**21.50**

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Average Titre = $\frac{21.40+21.30+21.50}{3}$ = 21.40ml

1 mole of H_2NSO_3 = 1mole of NaOH

97.09g H_2NSO_3 in 1000ml = 1M NaOH

24.2725g H_2NSO_3 in 1000ml = 0.25M NaOH

6.0681g H_2NSO_3 in 250ml = 0.25M NaOH

Factor of H_2NSO_3 (F_1) = Actual weight/Nominal weight = 6.075/6.0681 = 1.0011

Factor of NaOH (F_2) , Volume of H_2NSO_3 (V_1) = 20.00ml Volume of NaOH (V_2) =

Factor of NaOH(F_2) = $\frac{F1V1}{V2}$ $F_2 = \frac{1.0011\ X\ 20.0}{21.40}$ = 0.9356

Factor of NaOH = 0.9356

Table 13 Results of Titration of Clavulanic acid with 0.1M Sodium Hydroxide

Burette reading	**1st determination**	**2nd determination**	**3rd determination**
Final reading(ml)	9.60	19.40	29.00
Initial reading(ml)	0.00	9.60	19.40
Titre(ml)	**9.60**	**9.80**	**9.60**

Average titre = $\frac{9.6+9.6}{2}$ = 9.60ml

199.3g of $C_8H_9NO_5$ in 1000ml ≡1M NaOH

49.75g of $C_8H_9NO_5$ in 1000ml ≡0.25M NaOH

0.04975g of $C_8H_9NO_5$ ≡ 1ml of 0.25M NaOH

Actual volume of NaOH = Factor of NaOH X Titre

= 0.9356 X 9.6 = 8.98ml

1ml of 0.25M NaOH ≡0.04975g of $C_8H_9NO_5$

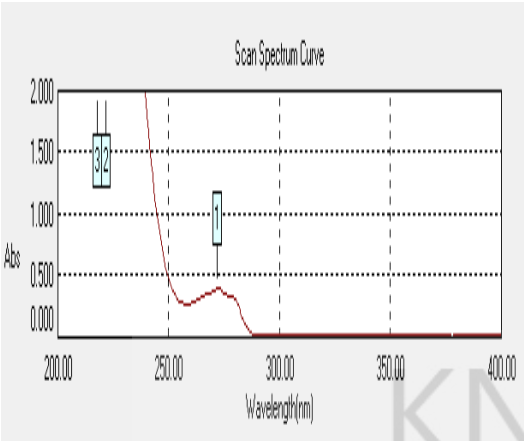
8.98ml of 0.25M NaOH = 8.98 X 0.04975 = 0.44675g

% Purity = $\frac{\text{Weight Obtained}}{\text{Weight taken}}$ X 100%

% Purity = $\frac{0.44675}{0.45}$ X 100%

% purity of clavulanic acid reference standard = 99.3%

3.2.3 UV Spectra of Amoxicillin and Clavulanic Acid in different solvent systems



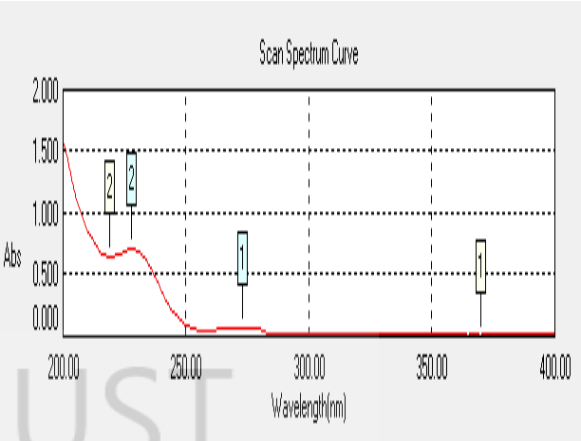
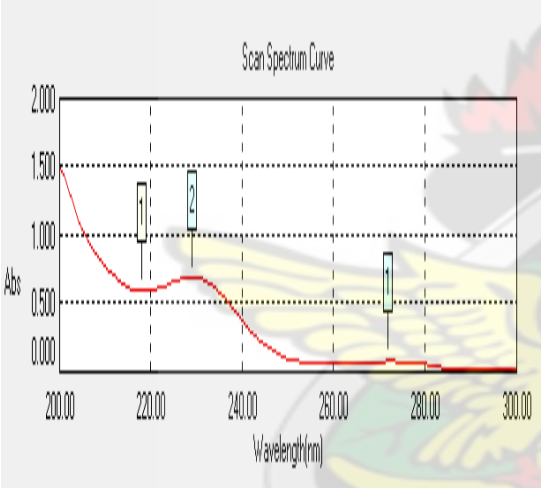


Fig. 11 Amoxicillin in methanol:water (10:90) Fig. 12 Amoxicillin in methanol:buffer (20:80)



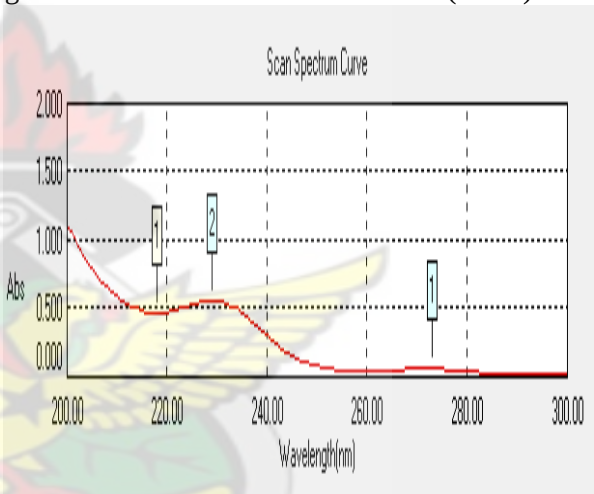


Fig.13 Clavulanic acid in methanol:buffer (20:80) Fig.14 Clavulanic acid in methanol:water (10:90)

Table 14 UV absorption pattern of Amoxicillin and clavulanic acid

Drug	Solvent	Wavelength	Absorbance
Amoxicillin	Methanol:water	272nm	0.388
Amoxicillin	Methanol:Buffer	228nm	0.703
Clavulanic acid	Methanol:water	229nm	0.552
Clavulanic acid	Methanol:Buffer	229nm	0.693

3.3 HPLC METHOD DEVELOPMENT

3.3.1 Solubility studies of Amoxicillin trihydrate and Potassium Clavulanate

Table 15 Results of solubility studies of Amoxicillin and Potassium Clavulanate

Sample	**Solvent**	**Time dissolved (Sonicatedat 26˚C)**	**Solvent**	**Time dissolved (Sonicated at 26˚C)**
Amoxicillin Trihydrate 2mg/ml	Water	45.0 Minutes	Methanol:Water (80:20)	5.0 Minutes
Potassium Clavulanate 2mg/ml	Water	5.0 Minutes	Methanol:Water (80:20)	15.0 Minutes

3.3.2 Wavelength Selection

Table 16 Selection of Optimum wavelength of detection

Peaks	**Wavelength of Absorption(nm)**						
	275.0	265.0	255.0	245.0	230.0	225.0	220.0
Amoxicillin	√	√	√	√	√	√	√
Clavulanic acid	X	X	X	X	x	X	√

KEY:

√→Peak is Detected at that wavelength,

X→Peak is not detected at that wavelength

3.3.3 Mobile Phase Selection

Chromatograms of different ratios of Methanol:Water:Acetate Buffer combinations

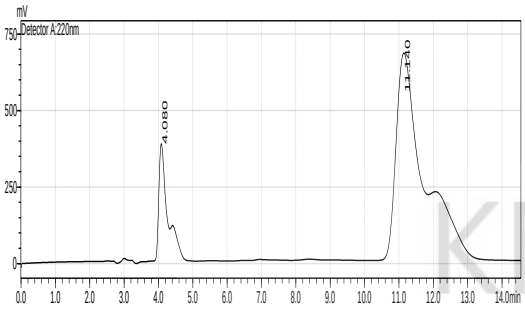


Fig. 15 Methanol:Acetate Buffer(40:60)

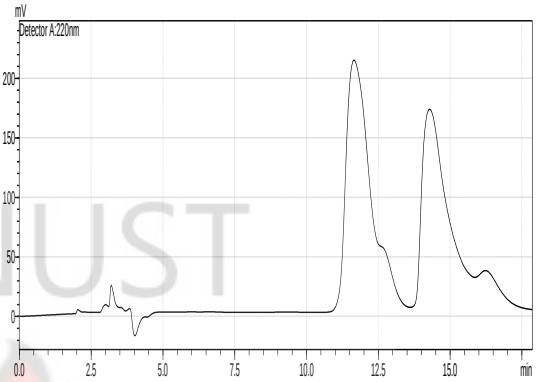


Fig. 16 Methanol:Acetate Buffer(30:70)

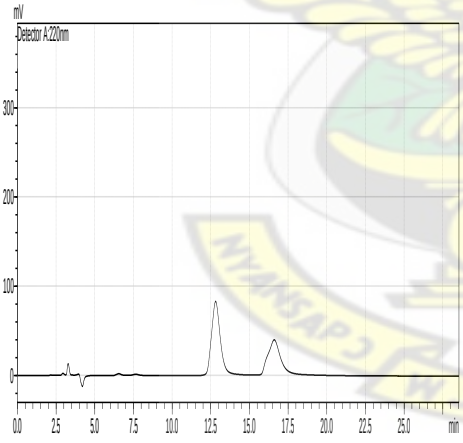


Fig.17 MeOH:H_2O:Acetate Buffer(10:80:10)

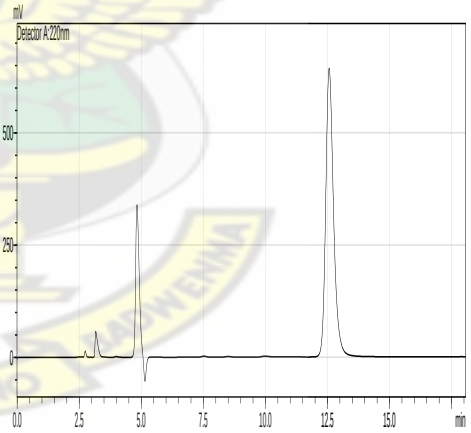


Fig. 18 MeOH:H_2O:A.Buffer(5:90:)

3.3.4 Optimal HPLC conditions established for Amoxicillin and Clavulanic acid

- Stationary phase:Phenomenex, Ultracarb 5µODS(20), 250 X4.6mm column.
- Mobile Phase: Methanol:Water:Acetate Buffer pH4.34 (5:90:5)
- Flow rate: 1.0ml/min
- Wavelength of detection: 220nm
- Sensitivity(AUFS) : 0.05 and Pump pressure:4.3-5.6MPa

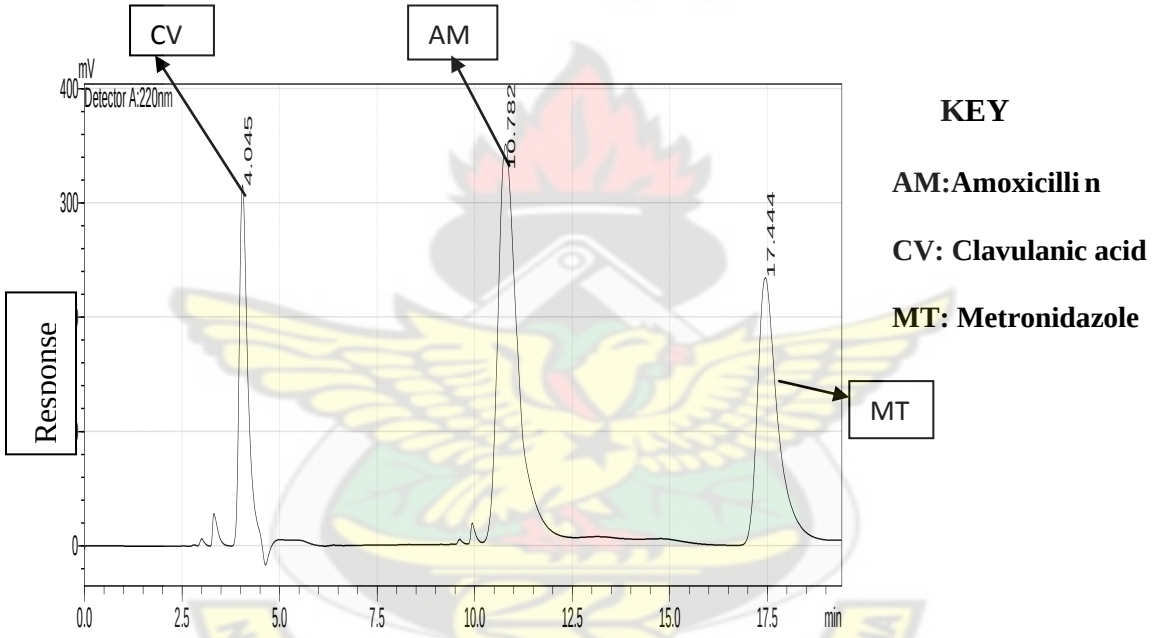


Fig. 19 Chromatogram of Amoxicillin and Clavulanic acid with metronidazole as internal standard

Table 17 Parameters of chromatogram of Amoxicillin and Clavulanic acid

Name of Drug	Mean Retention time/min	Mean Peak Area
Amoxicillin	10.68±0.01	14234567±0.4
Clavulanic acid	4.12±0.03	9879544±0.2
Metronidazole	17.10±0.02	8130642±0.5

3.3.5 Stability of solutions of reference standards

Table 18 Stability of solutions of Amoxicillin, Clavulanic acid and Metronidazole

Time	Reference standards		
	Amoxicillin %content	**Clavulanic acid %content**	**Metronidazole% content**
0	102.7	99.8	100.7
5min	102.5	99.1	100.5
15min	102.4	98.8	100.3
30min	102.2	98.6	100.2
45min	102.1	98.4	100.1
1hr	101.9	98.2	99.8
2hr	101.1	81.3	99.7
5hr	90.1	73.6	99.4
8hr	68.9	51.4	71.9
10hr	58.5	40.2	62.4

Table 19 Internal standards investigated and their retention times

Sample	Internal Standard	Retention Time/min
1	Paracetamol	3.5
2	Ampicillin	10.7
3	Metronidazole	17.2
4	Metformin	5.5
5	Caffeine	23.6

3.4 HPLC METHOD VALIDATION

3.4.1 Calibration curve for Amoxicillin

Table 20 Calibration curve of Amoxicillin

Mean Peak Area Amoxicillin	Mean Peak Area Internal Standard	Concentration %w/v	Mean Peak Area Ratio
5112426	8216693	0.018	0.6222
7979692	8327794	0.027	0.9582
9889643	8145658	0.036	1.2141
12643730	8220356	0.045	1.5381
14733341	8164325	0.054	1.8046
17796497	8300218	0.063	2.1441

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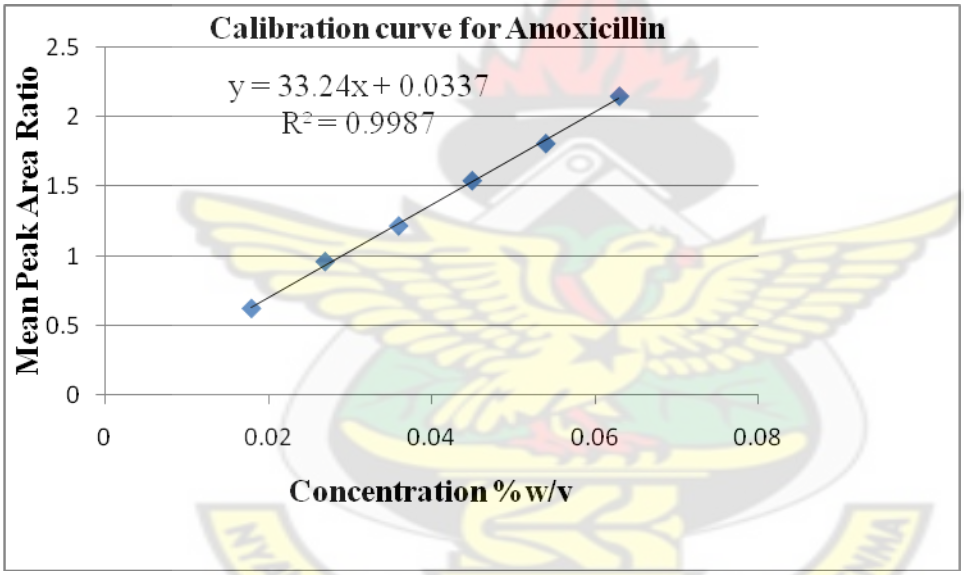

Fig. 20 Calibration curve for Amoxicillin

Table 21 Linear regression of calibration curve of Amoxicillin

Best-fit values	
Slope	33.24 ± 0.5950
Y-intercept when X=0.0	0.03373 ± 0.02578
X-intercept when Y=0.0	-0.001014
1/slope	0.03008
95% Confidence Intervals	
Slope	31.59 to 34.90
Y-intercept when X=0.0	-0.03783 to 0.1053
X-intercept when Y=0.0	-0.003322 to 0.001087
Goodness of Fit	
r^2	0.9987
Sy.x	0.02240
Is slope significantly non-zero?	
F	3122
DFn, DFd	1.000, 4.000
P value	< 0.0001
Deviation from zero?	Significant

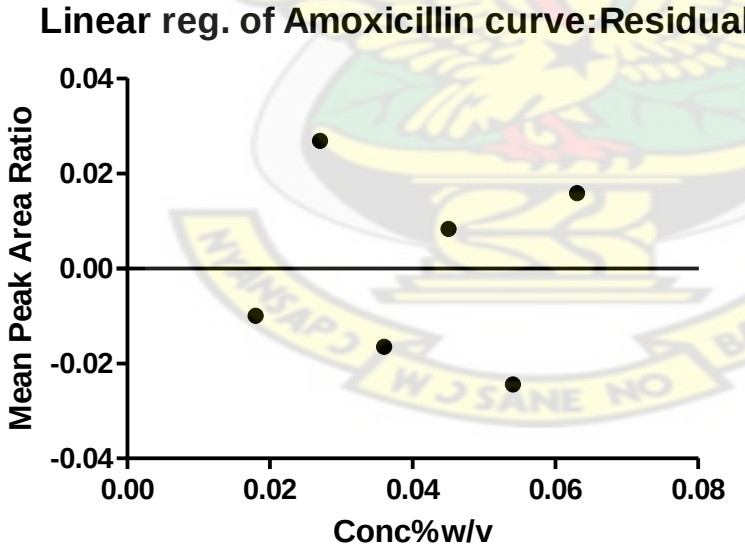

Fig. 21 Residual plot of the calibration curve for Amoxicillin

3.4.2 Calibration curve for Clavulanic acid

Table 22 Calibration curve of Clavulanic acid

Mean Peak Area Clavulanic	Mean Peak Area Internal standard	Concentration (%w/v)	Mean Peak Area Ratio
5916753	8304215	0.001	0.7125
9869162	8174573	0.002	1.2073
13247323	8287346	0.003	1.5985
16417704	8169231	0.004	2.0097
20898763	8312622	0.005	2.5141
24608180	8352624	0.006	2.9815

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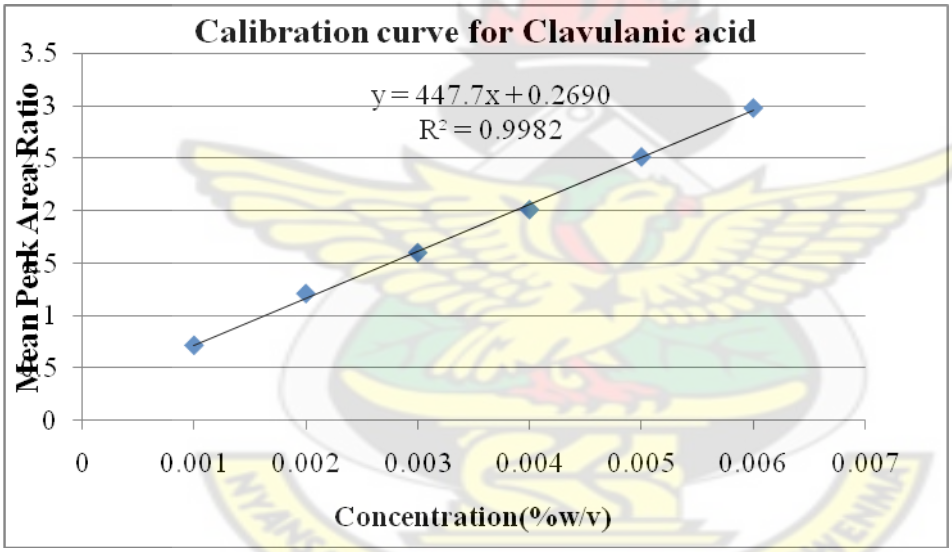

Fig. 22 Calibration curve for Clavulanic acid

Table 23 Linear Regression for calibration of Clavulanic acid

Best-fit values	
Slope	447.7 ± 9.412
Y-intercept when X=0.0	0.2690 ± 0.03665
X-intercept when Y=0.0	-0.0006010
1/slope	0.002234
95% Confidence Intervals	
Slope	421.5 to 473.8
Y-intercept when X=0.0	0.1673 to 0.3708
X-intercept when Y=0.0	-0.0008751 to -0.0003549
Goodness of Fit	
r^2	0.9982
Sy.x	0.03937
Is slope significantly non-zero?	
F	2262
DFn, DFd	1.000, 4.000
P value	< 0.0001
Deviation from zero?	Significant

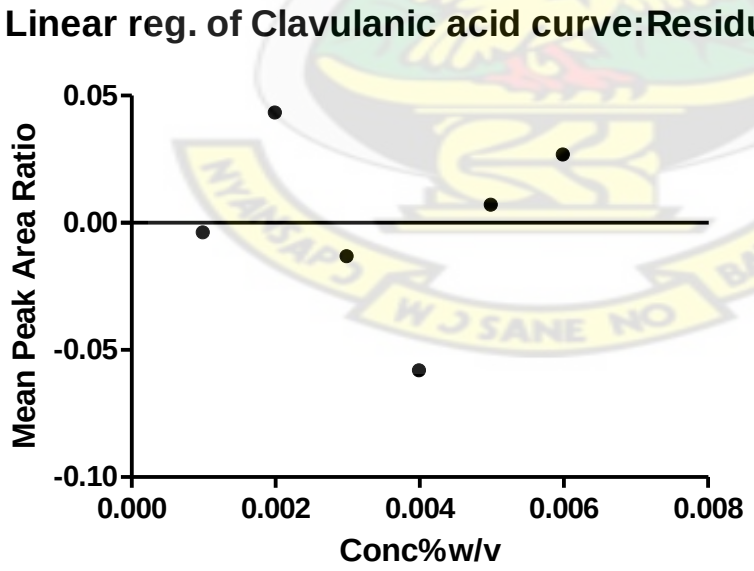

Fig. 23 Residual plot of the calibration curve for Clavulanic acid

3.4.3 Precision-Repeatability (Intra-day Precision) Amoxicillin

Table 24 Intra-day precision for Amoxicillin

Sample	Nominal conc %w/v	Peak Area Ratio	Actual conc. %w/v	
1	0.05400	1.8386	0.0543	
2	0.05400	1.8220	0.0538	
3	0.05400	1.8320	0.0541	
4	0.05400	1.8187	0.0537	
5	0.05400	1.8353	0.0542	**Mean=0.054**
6	0.05400	1.8253	0.0539	**RSD(%)=0.44**

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The mean $\overline{X}$ is given by $\overline{X} = \sum \frac{X}{n}$, where x is concentration and n is number of samples

$$\overline{X} = \frac{0.0543+0.0538+0.0541+0.0537+0.0542+0.0539}{6} = 0.054$$

The mean is thus 0.054%

Standard Deviation *S*, is also given by $S = \sqrt{\frac{\sum(X-\overline{X})2}{n-1}}$

X	$X-\overline{X}$	$(X-X)2$
0.0543	0.0003	0.00000009
0.0538	-0.0002	0.00000004
0.0541	0.0001	0.00000001
0.0537	-0.0003	0.00000009
0.0542	0.0002	0.00000004
0.0539	0.0001	0.00000001

$\sum(X-\overline{X})2$ = 0.00000028, n-1=5, S=0.00000028/5 = $\sqrt{}$0.000000056 S=0.0002366

Relative Standard Deviation (RSD) is given by RSD=100$S/\overline{X}$

RSD = 100x0.0002366/0.054 =0.02366/0.054 RSD =0.44%

3.4.4 Repeatability (Intra-day Precision) Clavulanic acid

Table 25 Analytical data for Intra-day precision for Clavulanic acid

Sample	Nominal conc. %w/v	Peak Area ratio	Actual Conc.%w/v	
1	0.00500	2.5622	0.00510	
2	0.00500	2.5478	0.00509	
3	0.00500	2.5612	0.00512	
4	0.00500	2.5344	0.00506	
5	0.00500	2.5657	0.00513	**Mean=0.005095**
6	0.00500	2.5388	0.00507	**RSD (%) =0.54**

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3.4.5 Inter- day Precision for Amoxicillin-Day 1

Table 26 Analytical data for Inter-day Precision of Amoxicillin

Sample	Nominal Conc %w/v	Peak Area Amoxicillin	Peak Area Internal std.	Peak Area Ratio	Actual Conc %w/v
1	0.0540	15152405	8241273	1.8386	0.0543
2	0.0540	14659205	8045667	1.8220	0.0538
3	0.0540	14626175	8042104	1.8187	0.0537
4	0.0540	15238286	8257891	1.8453	0.0545
5	0.0540	15120921	8238937	1.8353	0.0542
6	0.0540	14661027	8046667	1.8220	0.0538

Mean =0.05405	RSD(%) = 0.61

3.4.6 Inter-day Precision for Amoxicillin-Day 2

Table 27 Analytical Data for Inter day Precision of Amoxicillin

Sample	Nominal Conc%w/v	Peak Area Amoxicillin	Peak Area Internal std	Peak Area Ratio	Actual Conc %w/v
1	0.0540	15089581	8236671	1.8320	0.0541
2	0.0540	14639949	8049678	1.8187	0.0537
3	0.0540	15154177	8242237	1.8386	0.0543
4	0.0540	14669361	8051241	1.8220	0.0538
5	0.0540	14872201	8147812	1.8253	0.0539
6	0.0540	15233178	8255123	1.8453	0.0545

Mean =0.05405	RSD(%) = 0.57

Table 28 Analytical data for Inter-day Precision of Amoxicillin for Day 3

Sample	Nominal Conc %w/v	MPAR AMX	MPAR Internal Std.	Peak Area Ratio	Actual Conc %w/v
1	0.0540	14583109	8047896	1.8120	0.0535
2	0.0540	15148071	8238916	1.8386	0.0543
3	0.0540	14698068	8052412	1.8253	0.0539
4	0.0540	14957084	8149667	1.8353	0.0542
5	0.0540	14667324	(8149667)8050123	1.8220	0.0538
6	0.0540	14955819	(8050123)8148978	1.8353	0.0541

Mean =0.05397	RSD (%) =0.55

Table 29 Inter-day precision of Amoxicillin for all the three (3) days

Sample No	Day 1 Conc(%w/v)	Day 2 Conc (%w/v)	Day3 Conc (%w/v)
1	0.0543	0.0541	0.0535
2	0.0538	0.0537	0.0543
3	0.0537	0.0543	0.0539
4	0.0545	0.0538	0.0542
5	0.0542	0.0539	0.0538
6	0.0538	0.0545	0.0542

Mean of 18 samples = 0.05403	RSD for 18 samples =0.55%

3.4.7 Analysis of Variance (ANOVA) Amoxicillin (Inter-day Precision)

Table 30 Analysis of Variance for Amoxicillin

One-way analysis of variance			
P value	0.9654		
P value summary	Ns		
Are means signif. different? (P < 0.05)	**No**		
Number of groups	3		
F	0.03527		
R squared	0.004681		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)	0.006087		
P value	0.9970		
P value summary	Ns		
Do the variances differ signif. (P < 0.05)	**No**		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.000000004444	2	0.000000002222
Residual (within columns)	0.0000009450	15	0.000000063

3.4.8 Inter-day Precision for Clavulanic acid

Table 31 Inter-day Precision Clavulanic acid Day 1

Sample	Nominal Conc %w/v	Peak Area Clavulanic acid	Peak Area Internal std	Peak Area Ratio	Actual Conc %w/v
1	0.0050	21005306	8215467	2.5568	0.00511
2	0.0050	20755834	8146571	2.5478	0.00509
3	0.0050	20385768	8043627	2.5344	0.00506
4	0.0050	21170219	8251245	2.5657	0.00513
5	0.0050	20808762	8167345	2.5478	0.00509
6	0.0050	20496283	8073217	2.5388	0.00507

Mean =0.005092	RSD(%) = 0.50

Table 32 Inter-day Precision Clavulanic acid Day 2

Sample	Nominal Conc%w/v	Peak Area Clavulanic acid	Peak Area Internal std	Peak Area Ratio	Actual Conc %w/v
1	0.0050	21112533	8243219	2.5612	0.00512
2	0.0050	21244723	8265786	2.5702	0.00514
3	0.0050	20382180	8042211	2.5344	0.00506
4	0.0050	20860515	8187658	2.5478	0.00509
5	0.0050	21011447	8217869	2.5568	0.00511
6	0.0050	20519676	8082431	2.5388	0.00507

Mean = 0.005098	RSD(%) = 0.60

Table 33 Inter-day Precision Clavulanic acid Day 3

Sample	Nominal Conc%w/v	Peak Area Clavulanic acid	Peak Area Internal std	Peak Area Ratio	Actual Conc %w/v
1	0.0050	20390860	8045636	2.5344	0.00506
2	0.0050	21358131	8281234	2.5791	0.00516
3	0.0050	21178743	8254567	2.5657	0.00513
4	0.0050	21077098	8243546	2.5568	0.00511
5	0.0050	20597589	8098765	2.5433	0.00508
6	0.0050	20849812	8183457	2.5478	0.00509

Mean = 0.005105	RSD(%) =0.71

Table 34 Inter-day precision of Clavulanic acid for all the 3 days

Sample	Day 1 Mean conc. %w/v	Day 2 Mean Conc %w/v	Day3Mean Conc %w/v
1	0.00511	0.00512	0.00506
2	0.00509	0.00514	0.00516
3	0.00506	0.00506	0.00513
4	0.00513	0.00509	0.00511
5	0.00509	0.00511	0.00508
6	0.00507	0.00507	0.00509

Mean of 18 samples = 0.005098	RSD(%) for 18 samples = 0.58

Table 35 Analysis of Variance (ANOVA) Clavulanic acid (Inter-day Precision)

Table Analyzed	Results		
One-way analysis of variance			
P value	0.7629		
P value summary	Ns		
Are means signif. different? (P < 0.05)	**No**		
Number of groups	3		
F	0.2755		
R squared	0.03544		
Bartlett's test for equal variances			
Bartlett's statistic (corrected)	0.5406		
P value	0.7632		
P value summary	Ns		
Do the variances differ signif.? (P < 0.05)	**No**		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.000000000533	2	0.000000000267
Residual (within columns)	0.00000001452	15	0.000000000968
Total	0.00000001505	17	

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3.4.9 Accuracy

Table 36 Recovery studies for determination of accuracy of method –Amoxicillin

Concentration	Amount added	Amount recovered	%Recovery	Mean/RSD
0.0450%	0.012	0.0123	102.5	**Mean = 101.9**
	0.018	0.0182	101.1	**RSD = 0.7%**
	0.020	0.0204	102.0	
0.0540%	0.014	0.0144	102.9	**Mean = 101.9**
	0.018	0.0181	100.6	**RSD = 1.17%**
	0.022	0.0225	102.3	
0.0630%	0.012	0.0122	101.7	**Mean =101.9**
	0.016	0.0164	102.5	**RSD =0.52%**
	0.020	0.0203	101.5	

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Table 37 Recovery studies for determination of accuracy of method –Clavulanic acid

Concentration	Amount added	Amount recovered	%Recovery	Mean/RSD
0.0040%	0.001	0.00102	102.0	**Mean = 101.2**
	0.002	0.00201	100.5	
	0.003	0.00303	101.0	**RSD = 0.75%**
0.0050%	0.001	0.00101	101.0	**Mean =101.6**
	0.002	0.00205	102.5	
	0.003	0.00304	101.3	**RSD = 0.78%**
0.0060%	0.001	0.00103	103.0	**Mean = 101.8**
	0.002	0.00204	102.0	
	0.003	0.00301	100.3	**RSD = 1.34%**

Table 38 Comparison of method developed with a standard method

Sample	Amoxicillin		Clavulanic Acid	
	New Method	Standard Method	New Method	Standard Method
	% Content	%Content	% Content	%Content
1	101.3	99.5	98.5	98.9
2	101.0	99.8	99.1	99.0
3	99.8	100.7	98.8	98.7
4	101.4	99.3	99.2	99.4
5	99.6	101.2	98.9	98.6
6	100.6	99.7	99.3	98.5

From the table above, using the student *t* test to compare the two methods. The following results were obtained for Amoxicillin

Table 39 Statistical comparison of New method with a standard method.

Table Analyzed	Data 1
New Method	
Vs	Vs
Standard Method	
Unpaired t test	
P value	0.2112
P value summary	Ns
Are means signif. different? (P < 0.05)	**No**
One- or two-tailed P value?	Two-tailed
t, df	t=1.336 df=10
*t*critical at 95% confidence interval	*t*crit = 2.23
How big is the difference?	
Mean ± SEM of New Method	100.6 ± 0.3124 N=6
Mean ± SEM of Standard Method	100.0 ± 0.3051 N=6
Difference between means	0.5833 ± 0.4367
95% confidence interval	-0.3897 to 1.556
R squared	0.1514
F test to compare variances	
F,DFn, Dfd	1.048, 5, 5
P value	0.9599
F critical	7.146
Are variances significantly different?	**No**

The Null Hypothesis is denoted by H_o

H_o: There is no significant difference between the new method and the standard method.

H_o is accepted when the experimental value of t denoted by t_{exp} is less than the critical value of t which is also denoted by $t_{crit.}$

From the table 39 above t_{exp} is 1.336 and that of t_{crit} is 2.23 at 95% confidence interval.

The F values for experimental and critical are also 1.048 and 7.146

Using the students *t*-test to compare the New method to a standard method, the following results were obtained for Clavulanic acid

Table 40 Statistical comparison of New method with a standard method

Table Analyzed	Data 1
New Method	
Vs	Vs
Standard Method	
Unpaired t test	
P value	0.5307
P value summary	Ns
Are means signif. different? (P < 0.05)	**No**
One- or two-tailed P value?	Two-tailed
t, df	t=0.6494 df=10
t critical at 95% confidence interval	*t*crit= 2.23
How big is the difference?	
Mean ± SEM of New method	98.97 ± 0.1202 N=6
Mean ± SEM of Standard method	98.85 ± 0.1335 N=6
Difference between means	0.1167 ± 0.1797
95% confidence interval	-0.2836 to 0.5170
R squared	0.04046
F test to compare variances	
F,DFn, Dfd	1.235, 5, 5
P value	0.8227
F critical	7.146
Are variances significantly different?	**No**

The Null Hypothesis is denoted by H_o

H_o: There is no significant difference between the new method and the standard method.

H_o is accepted when the experimental value of t denoted by t_{exp} is less than the critical value of t which is also denoted by $t_{crit.}$

From the table 39 above t_{exp} is 0.6494 and that of t_{crit} is 2.23 at 95% confidence interval

The F values for experimental and critical are also 1.235 and 7.146

3.5.0 Robustness

Table 41 Effect of Variation of pH of Mobile Phase on percentage content.

pH of Mobile Phase	Mean Peak Area Ratio		%Content	
	Amoxicillin	Clavulanic acid	Amoxicillin	Clavulanic acid
4.36	1.8245	2.5234	100.2	99.7
4.38	1.8412	2.5143	101.4	98.9
4.40	1.8183	2.5376	99.8	100.1
4.42	1.8327	2.5089	100.4	99.4
4.43	1.8212	2.5279	100.1	100.2

Table 42 Using different column as compared to the original column

Sample	Original Column		New column	
	Mean Peak Area Ratio	%content	Mean Peak Area Ratio	% Content
1	1.8276	99.8	1.8256	99.6
2	1.8342	100.1	1.8301	99.9
3	1.8415	100.4	1.8422	100.5
4	1.8235	99.7	1.8198	99.6
5	1.8394	100.6	1.8364	100.3
6	1.8275	99.8	1.8278	100.0

Using the student t- test to compare the data on original column to that of New column the following results were obtained:

Table 43 Statistical Comparison of data on Original column with that of New column

Table Analyzed	Data 1
Original Column	
Vs	Vs
New Column	
Unpaired t test	
P value	0.7018
P value summary	Ns
Are means signif. different? (P < 0.05)	**No**
One- or two-tailed P value?	Two-tailed
t, df	t=0.3940 df=10
How big is the difference?	
Mean ± SEM of Original Column	100.1 ± 0.1498 N=6
Mean ± SEM of New Column	99.98 ± 0.1493 N=6
Difference between means	0.08333 ± 0.2115
95% confidence interval	-0.3878 to 0.5545
R squared	0.01529
F test to compare variances	
F,DFn, Dfd	1.007, 5, 5
P value	0.9937
P value summary	Ns
Are variances significantly different?	**No**

3.5.1 Limit of Detection (LOD)

3.5.1.1 LOD of Amoxicillin

The LOD is given by

LOD = 3 Sy.x / Slope , where Sy.x is the residual standard deviation of the calibration curve and slope is the slope of the calibration curve.

From the calibration curve the residual standard deviation of the calibration curve is 0.02240

LOD = 3X 0.02240/ 33.24

LOD = 0.00202%w/v

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3.5.1.2 LOD of Clavulanic acid

Similarly, LOD = 3 Sy.x / Slope, LOD = 3 X 0.03937/ 447.7

LOD = 0.000264%w/v

3.5.1 Limit of Quantification (LOQ)

3.5.2.1 LOQ of Amoxicillin

LOQ is given by LOQ = 10 Sy.x / Slope

LOQ = 10 X 0.02578/ 33.24

LOQ = 0.00674%w/v

3.5.2.2 LOQ of Clavulanic Acid

LOQ = 10 X 0.03937/ 447.7

LOQ = 0.000879%w/v

3.5.3 Specificity Amoxicillin and Clavulanic acid

The method demonstrated specificity in the presence excipients of the oral suspension as there were no interfering peaks detected as shown in the chromatogram below:

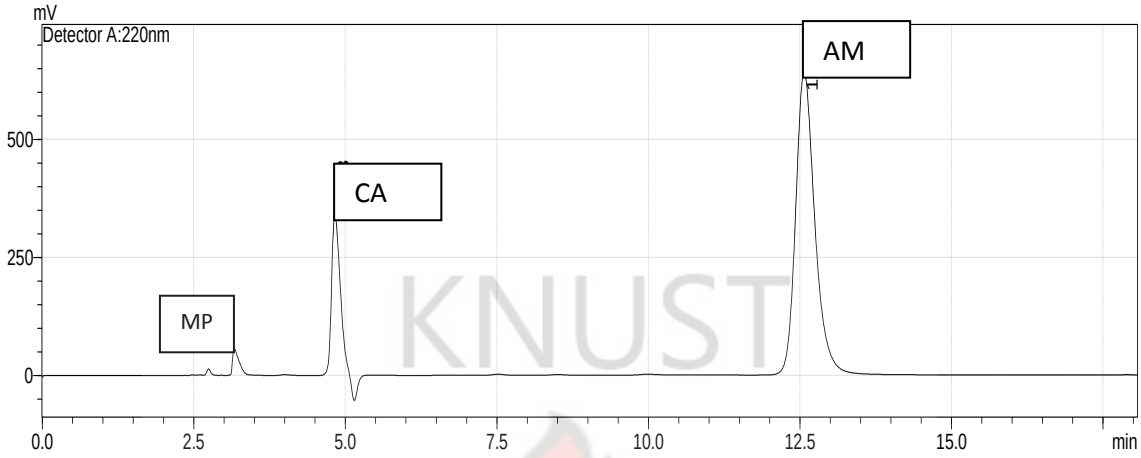

Fig. 24 Chromatogram of single injection of Amoxicillin, Clavulanic acid and Excipients

KEY: AM - Amoxicillin, CA- Clavulanic acid, MP- Mobile phase

3.5.4 Range

From the calibration curve, the range of the HPLC method for amoxicillin is 0.018-0.063%w/v and that of Clavulanic acid is 0.001-0.006%w/v

Table 44 Optimization of peak integration parameters

Peak integration Parameter	**Optimum value**
Width	3sec
Slope	5000uV
Drift	0uV
Min Area/Height	1000counts

Table 45 Summary of Validation Parameters

Validation Parameter	**Amoxicillin**	**Clavulanic acid**
Linearity R^2	0.9987	0.9982
Precision – Repeatability	RSD = 0.44%	RSD =0.54%
Precision – Inter day	RSD = 0.55%	RSD = 0.58%
Slope of calibration curve	33.24 ± 0.5950	447.7 ± 9.412
Y- intercept of calibration curve	0.03373 ± 0.02578	0.2690 ± 0.03665
Limit of Detection	0.00202%w/v	0.000264%w/v
Limit of Quantification	0.00674%w/v	0.000879%w/v
Range	0.018-0.063%w/v	0.001-0.006%w/v
Accuracy	RSD(%)= 0.7,1.17, 0,52 ***t*** crit=2.23, ***t*** exp =1.336 ***f*** crit= 7.146, ***f*** exp=1.048	RSD(%)=0.75,0.78, 1.34 ***t*** crit=2.23, ***t*** exp=0.6494 ***f*** crit=7.146, ***f*** exp=1.235
Robustness	Robust	Robust

3.5.5 Assay of five brands of liquid oral suspensions of Co-amoxiclav

Table 46 Assay of Amoxicillin in liquid oral suspensions of Co-amoxiclav

Brand	Mean Peak Area Amoxicillin	Mean Peak Area Internal standard	Mean Peak Area Ratio	% Content
1	11262333	8221281	1.3699	100.5
2	11125731	8221186	1.3533	99.3
3	11454145	8221465	1.3932	102.3
4	9951169	8221389	1.2104	88.5
5	11344680	8221378	1.3799	101.3

Calculation of % content of assayed brands

From the calibration curve for amoxicillin the equation of the curve is given by:

Y = 33.24x + 0.0337 where y is the Mean peak area ratio, x is the concentration of sample and 33.24 is the slope of the curve.

Sample 1)

When y = 1.5528 , solving for x gives:

$$X = \frac{1.3699 - 0.0337}{33.24} \qquad x = 0.0402$$

Concentration of amoxicillin in undiluted sample is 40.2mg/100ml

Therefore amount of amoxicillin found in assayed syrup is 40.2mg

Expected amount of amoxicillin in syrup is calculated as follows

From the label claim of syrup 5ml of syrup contains 400mg of amoxicillin

0.50ml of the syrup was taken mixed with distilled water and made up to 100ml in a 100ml volumetric flasks.

Concentration of undiluted sample is 0.50 X 400/5 = 40.0mg/100ml

Therefore amount of amoxicillin expected in undiluted sample = 40.0mg

% content of amoxicillin in syrup is given by: $\frac{\text{Amount found}}{\text{Amount Expected}}$ X 100%

$$\frac{40.2}{40.0} \text{ X } 100\% \qquad = 100.5\%$$

Following the same approach the % content of amoxicillin in the other four brands were calculated and the following were obtained: 99.3%, 102.3%, 88.5% and 101.3%

Table 47 Assay of Clavulanic Acid in liquid oral suspensions of Co-amoxiclav

Brand	M. Peak Area CLV Acid	M. Peak Area Internal standard	M. Peak Area Ratio	% Content
1	23218214	8336582	2.7851	98.6
2	23066696	8335753	2.7672	97.9
3	23483521	8338134	2.8164	99.8
4	20382936	8334978	2.4448	85.3
5	23406860	8337261	2.8075	99.5

Calculation of % content of assayed brands

From the calibration curve for clavulanic acid the equation of the curve is given by:

Y = 447.7x + 0.2690 where y is the Mean peak area ratio, x is the concentration of sample and 447.7 is the slope of the curve.

Sample 1)

When the peak area ratio, y = 2.7851 , solving for x gives:

$$X = \frac{2.7851 - 0.2690}{447.7} \qquad x = 0.00562\%$$

Concentration of clavulanic acid in undiluted sample is 5.62mg/100ml

Therefore amount of clavulanic acid found in assayed syrup is 5.62mg

Expected amount of clavulanic acid in syrup is calculated as follows:

From the label claim of syrup 5ml of syrup contains 57mg of clavulanic acid.

0.50ml of the syrup was taken, dissolved with distilled water and made up to 100ml in a 100ml volumetric flasks.

Concentration of undiluted sample is 0.50 X 57/5 = 5.7mg/100ml

Therefore amount of clavulanic acid expected in undiluted sample = 5.7mg

% content of clavulanic acid in syrup is given by: Amount found X 100%
Amount Expected

$$\frac{5.62}{5.70} \text{ X } 100\% \qquad 98.6 = \%$$

Following the same approach the % content of clavulanic acid in the other four brands were calculated and the following were obtained: 97.9%, 99.8%, 85.3% and 99.5%.

Table 48 Summary of assay of five brands of Co-amoxiclav oral suspension

Brands	% Content	
	Amoxicillin	Clavulanic acid
1	100.5±0.12	98.6±0.17
2	99.3±0.10	97.9±0.15
3	102.3±0.11	99.8±0.18
4	88.5±0.13	85.3±0.11
5	101.3±0.21	99.5±0.14

Table 49 pH of samples used for the stability studies

Sample	pH
Brand X	6.8-7.1
Brand Y	6.5-7.2

3.5.6 Stability Studies of reconstituted liquid oral suspensions of Co-Amoxiclav

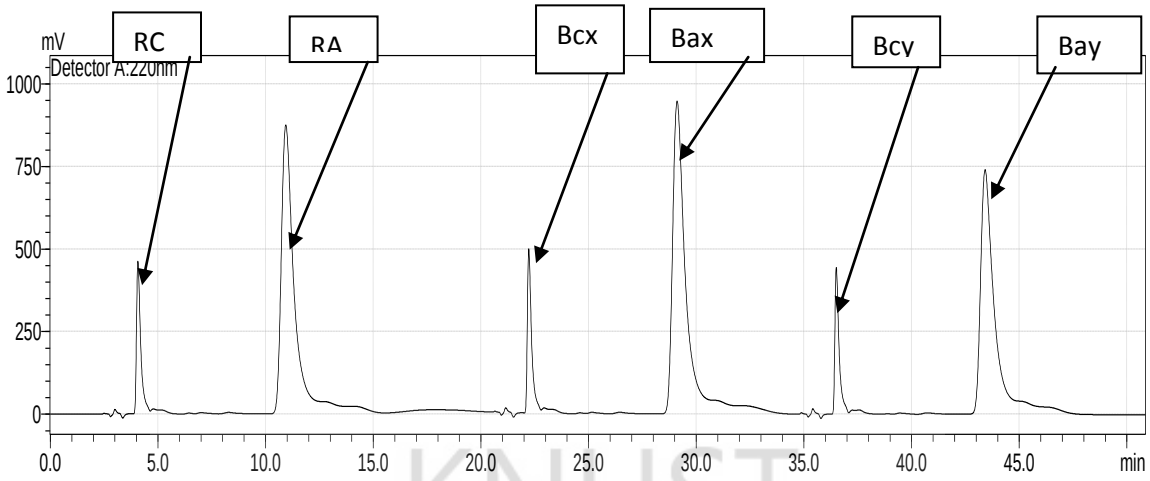

Fig. 25 Chromatogram of samples on Day 1 at 10℃

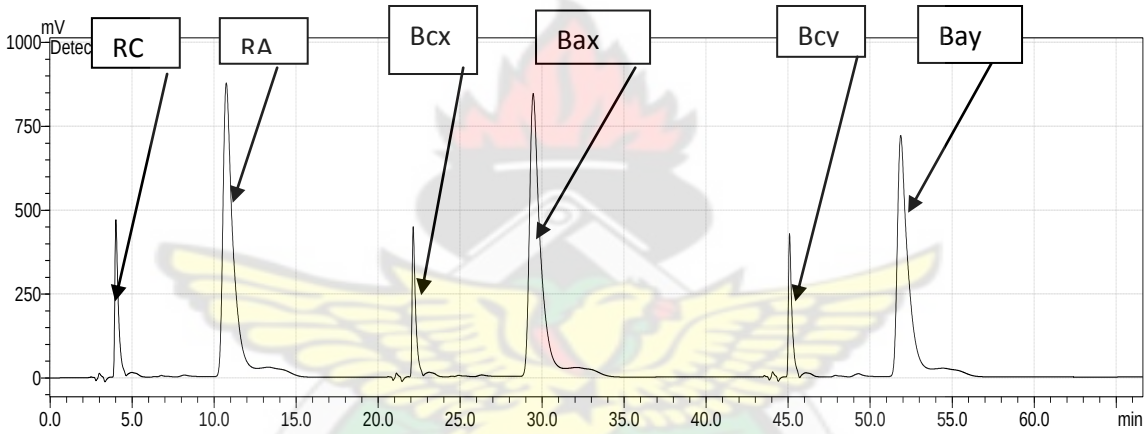

Fig. 26 Chromatogram of samples on Day 3 at 10℃

Key

RA= Reference standard of Amoxicillin Bay= Amoxicillin in brand y

RC= Reference standard of Clavulanic acid Bcy= Clavulanic acid in brand y

Bax= Amoxicillin in Brand X Bcx=Clavulanic acid in Brand X

DP = Degradation Products

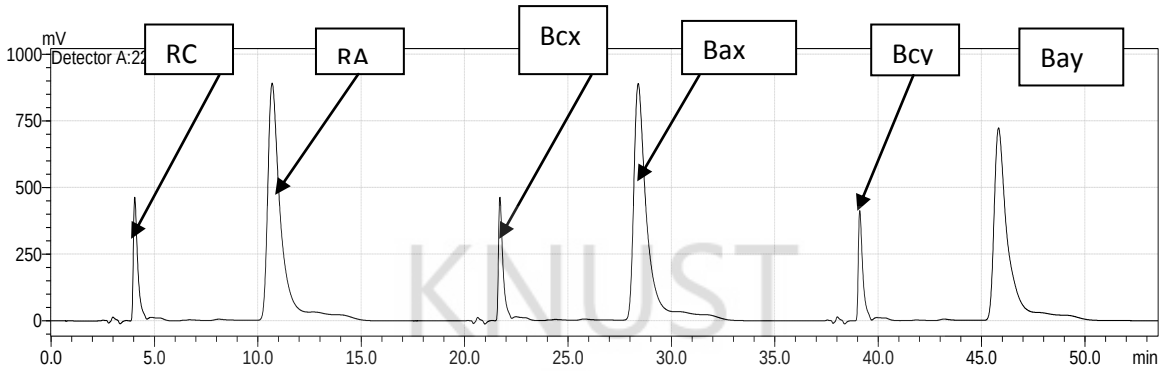

Fig. 27 Chromatogram of samples on Day 5 at 10°C

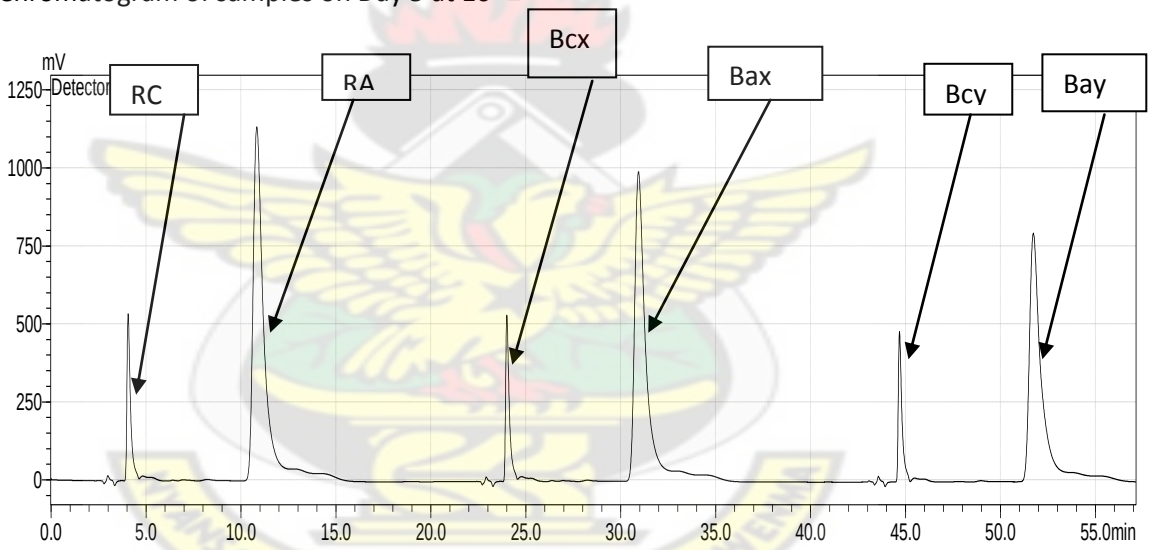

Fig. 28 Chromatogram of samples on Day 7 at 10°C

Key

RA= Reference standard of Amoxicillin Bay= Amoxicillin in brand y

RC= Reference standard of Clavulanic acid Bcy= Clavulanic acid in brand y

Bax= Amoxicillin in Brand X Bcx=Clavulanic acid in Brand X

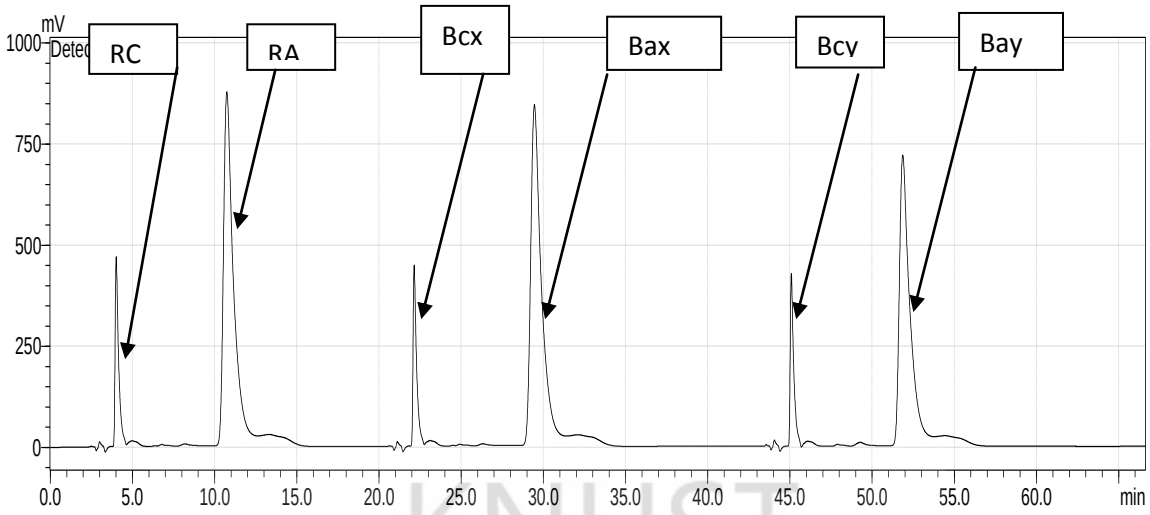

Fig. 29 Chromatogram of samples on Day 1 at 30℃

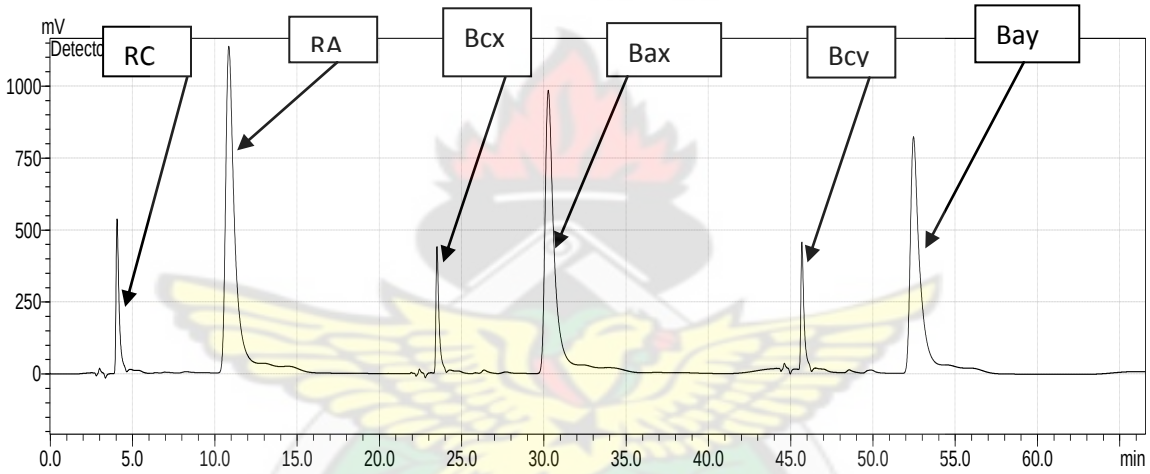

Fig. 30 Chromatogram of samples on Day3 at 30℃

Key

RA= Reference standard of Amoxicillin Bay= Amoxicillin in brand y

RC= Reference standard of Clavulanic acid Bcy= Clavulanic acid in brand y

Bax= Amoxicillin in Brand X Bcx=Clavulanic acid in Brand X

DP = Degradation Products

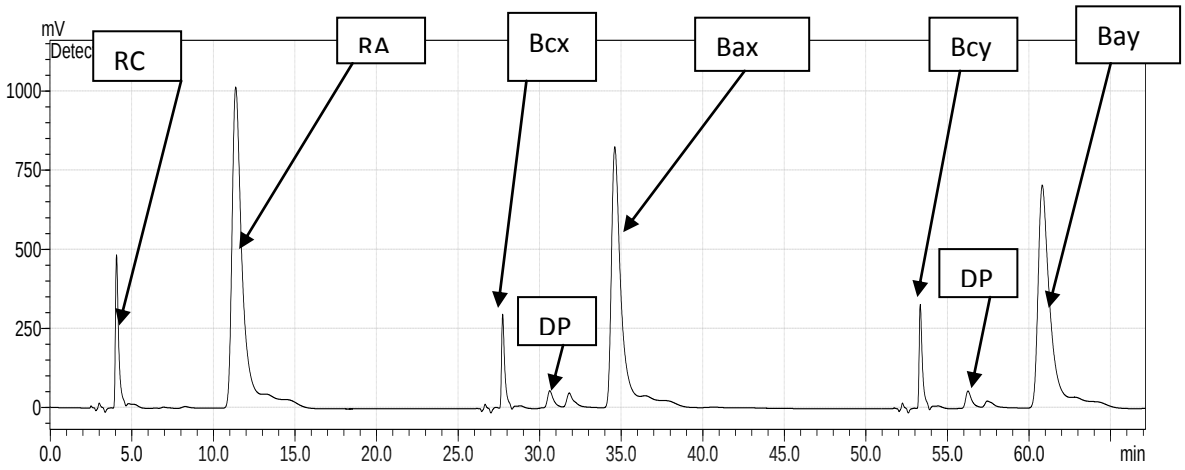

Fig. 31 Chromatogram of samples on Day 5 at 30℃

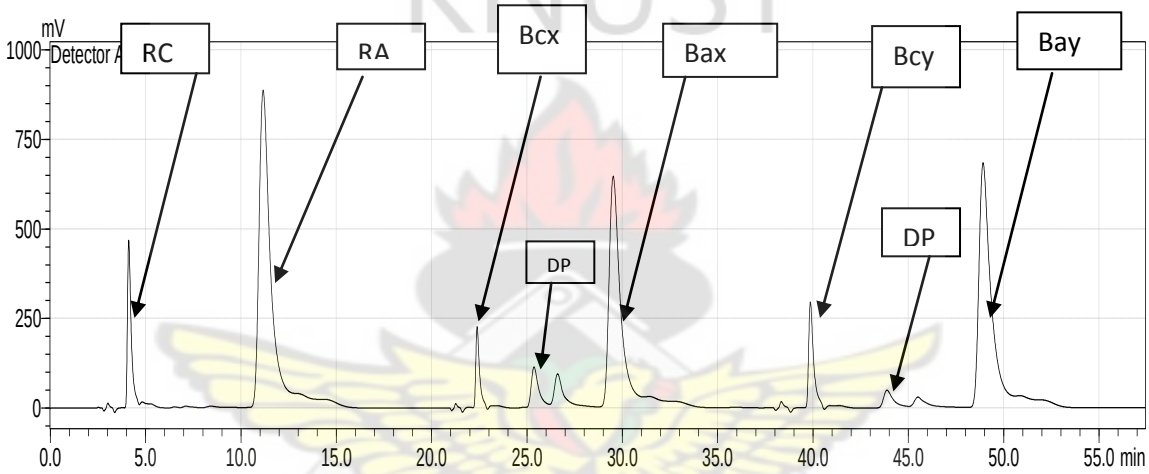

Fig. 32 Chromatogram of samples on Day 7 at 30℃

Key

RA= Reference standard of Amoxicillin Bay= Amoxicillin in brand y

RC= Reference standard of Clavulanic acid Bcy= Clavulanic acid in brand y

Bax= Amoxicillin in Brand X Bcx=Clavulanic acid in Brand X

DP = Degradation Products

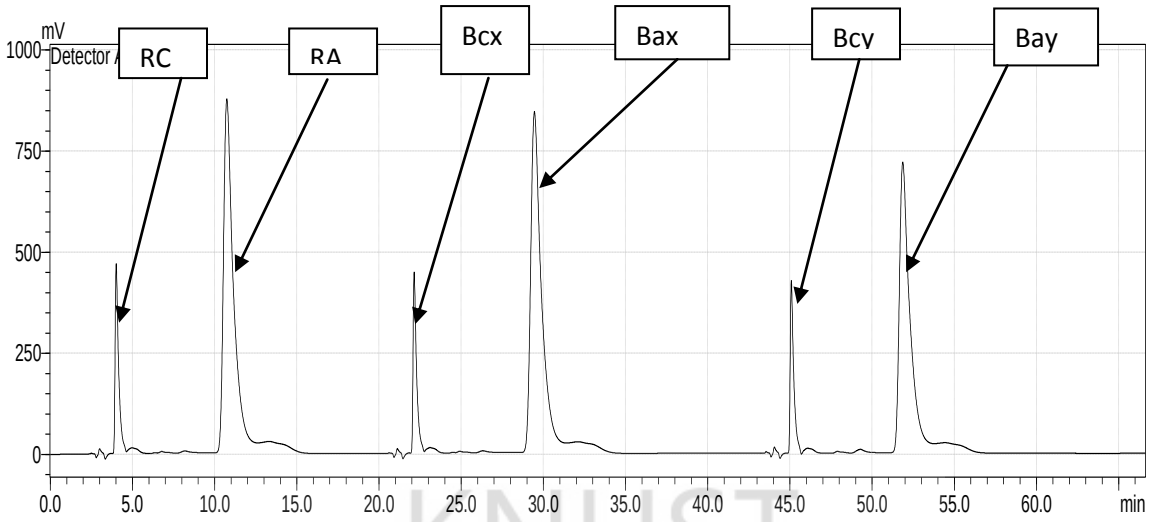

Fig. 33 Chromatogram of samples on Day 1 at 40°C

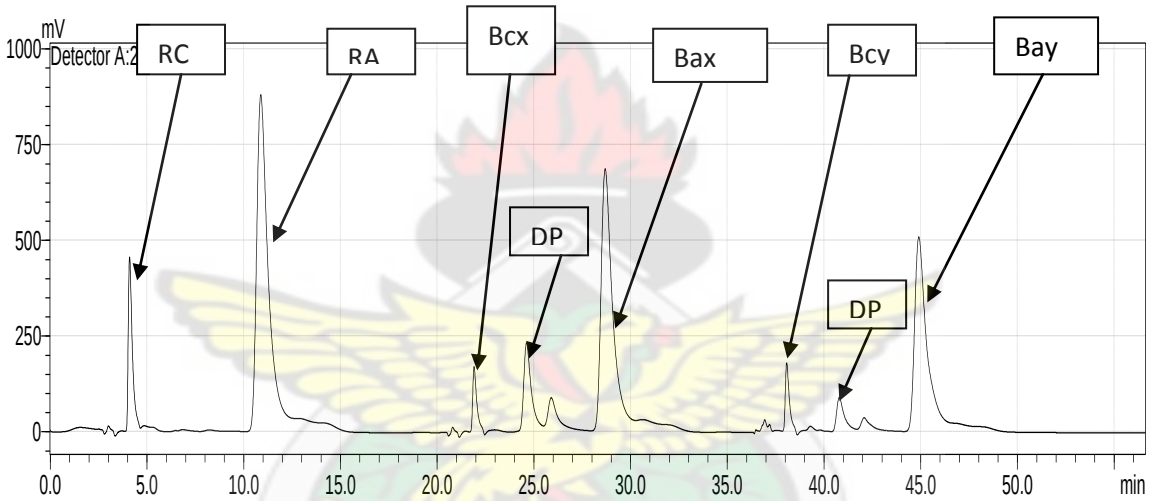

Fig. 34 Chromatogram of samples on Day 3 at 40˚C

Key

RA= Reference standard of Amoxicillin Bay= Amoxicillin in brand y

RC= Reference standard of Clavulanic acid Bcy= Clavulanic acid in brand y

Bax= Amoxicillin in Brand X Bcx=Clavulanic acid in Brand X

DP = Degradation Products

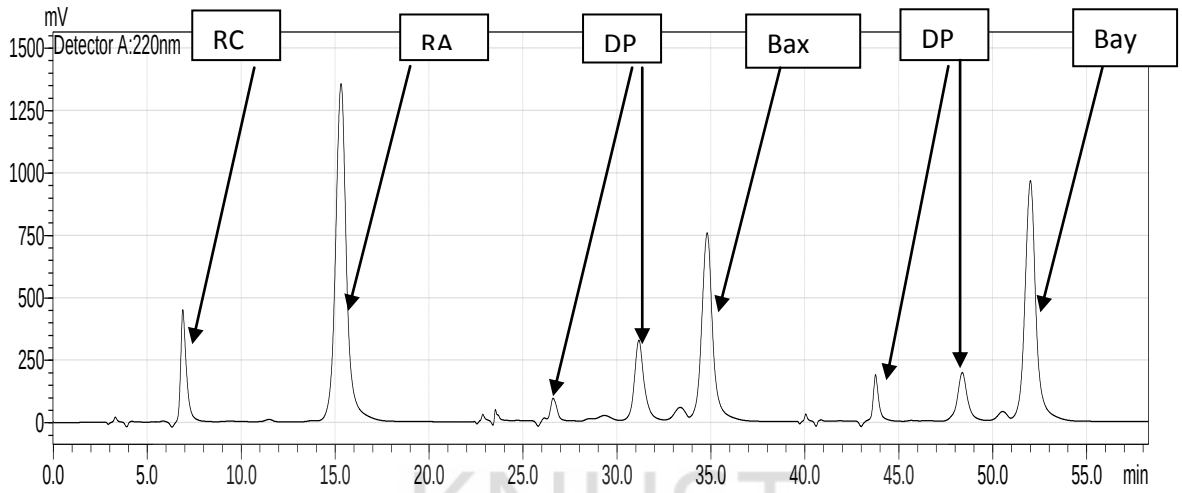

Fig. 35 Chromatogram of samples on Day 5 at 40˚C

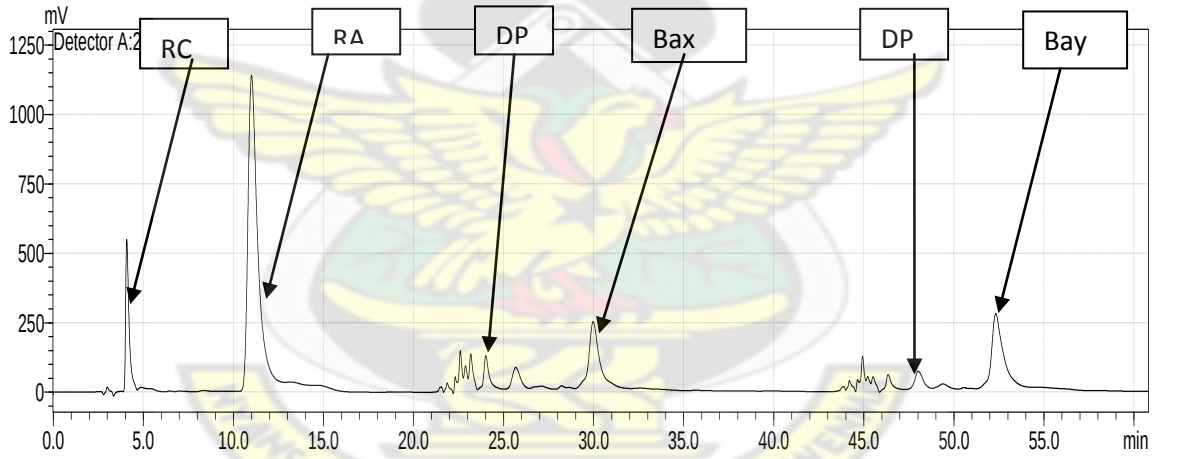

Fig. 36 Stability of samples on Day 7 at 40˚C

Key

RA= Reference standard of Amoxicillin Bay= Amoxicillin in brand y

RC= Reference standard of Clavulanic acid Bcy= Clavulanic acid in brand y

Bax= Amoxicillin in Brand X Bcx=Clavulanic acid in Brand X

DP = Degradation Products

Table 50 Stability studies for Brand X

Time/Hr	% Content of Amoxicillin			% Content of Clavulanic Acid		
	10˚C	30˚C	40˚C	10˚C	30˚C	40˚C
0	102.8	102.8	102.8	101.7	101.7	101.7
24	102.6	94.2	80.2	101.5	66.7	50.3
48	102.4	87.5	72.5	101.4	50.2	30.6
72	102.3	81.3	68.5	101.2	48.3	25.9
96	102.2	76.4	67.8	101.1	45.8	19.4
124	102.1	74.3	59.7	101.0	39.6	16.5
148	102.0	72.6	51.8	100.9	39.4	13.5
172	101.9	71.3	46.8	100.8	38.9	9.4

Table 51 Stability Studies for Brand Y

Time/Hr	% Content of Amoxicillin			% Content of Clavulanic Acid		
	10˚C	30˚C	40˚C	10˚C	30˚C	40˚C
0	99.8	99.8	99.8	96.4	96.4	96.4
24	99.6	91.2	79.9	96.2	62.9	50.1
48	99.3	82.6	71.6	96.0	49.8	30.2
72	99.1	78.3	67.3	95.9	46.5	24.8
96	98.9	72.9	63.4	95.8	43.1	18.6
124	98.8	70.8	54.7	95.7	38.7	14.8
148	98.8	69.7	53.9	95.7	36.5	12.1
172	98.7	69.4	44.9	95.6	34.2	8.4

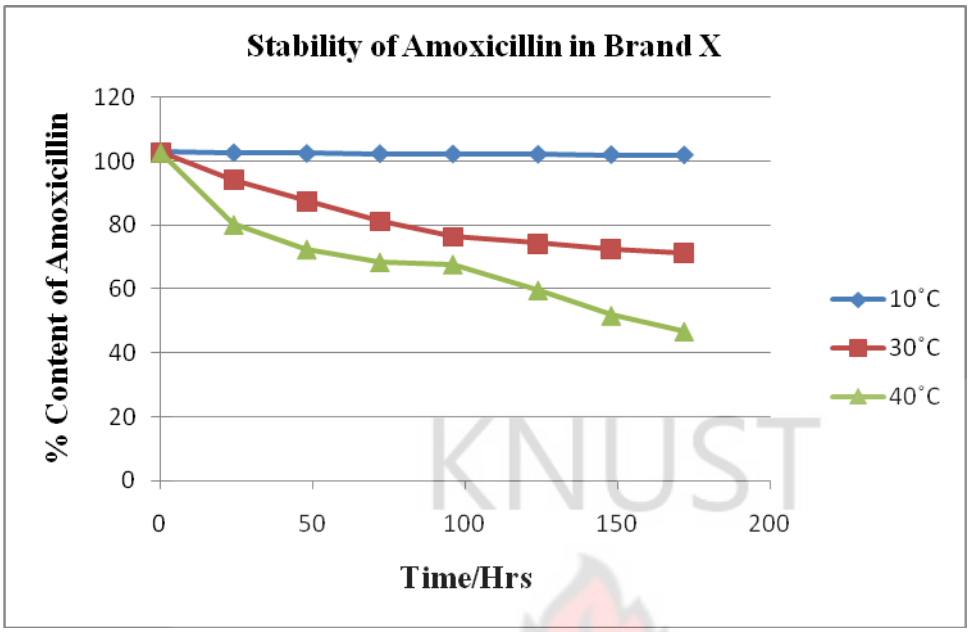

Fig. 37 Stability of Amoxicillin in brand X at 10, 30 and 40˚C

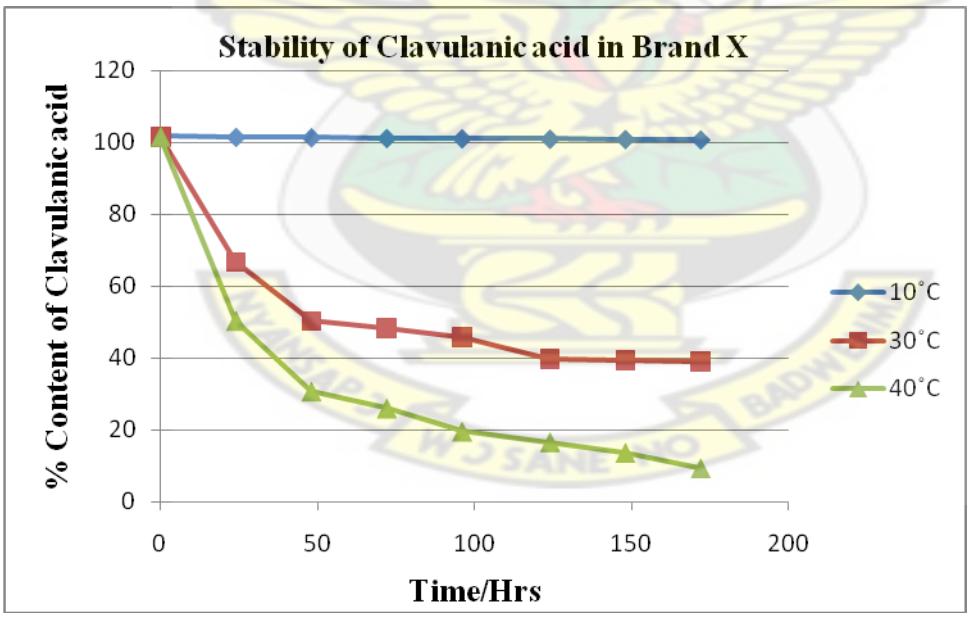

Fig. 38 Stability of Clavulanic acid in brand X at 10, 30, and 40˚C

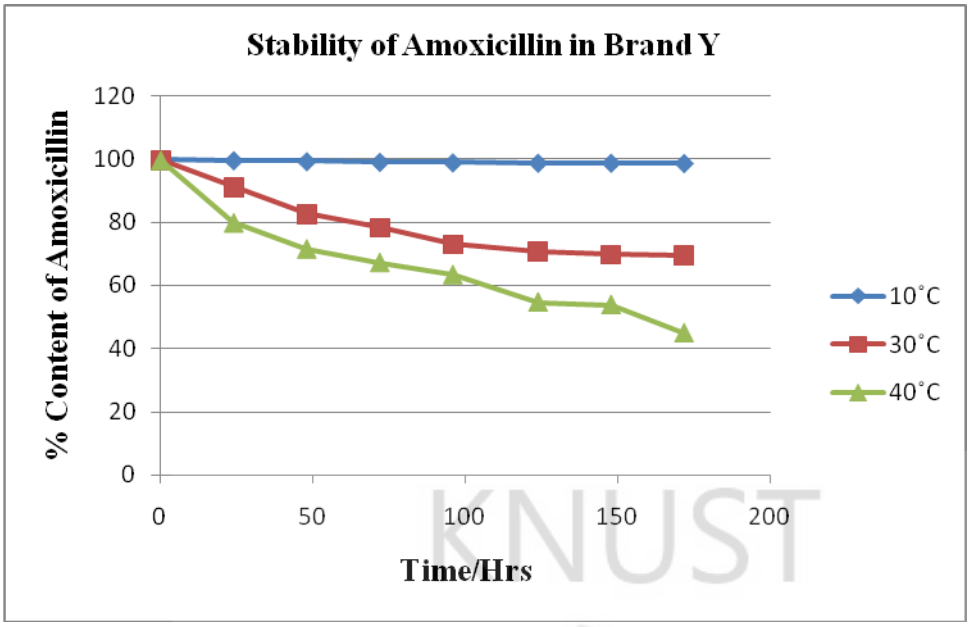

Fig. 39 Stability of Amoxicillin in brand Y at 10, 30 and 40˚C

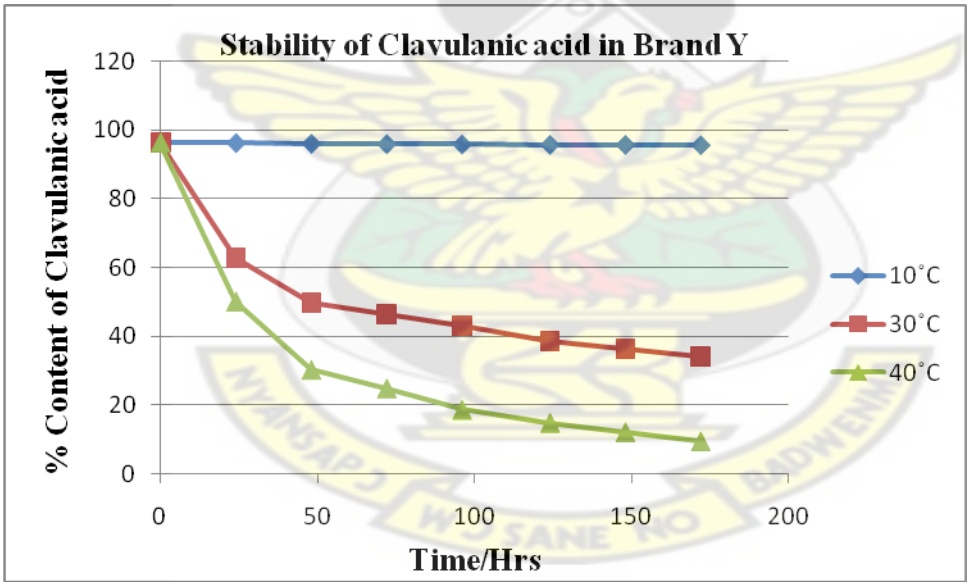

Fig. 40 Stability of Clavulanic acid in brand Y at 10, 30, and 40˚C

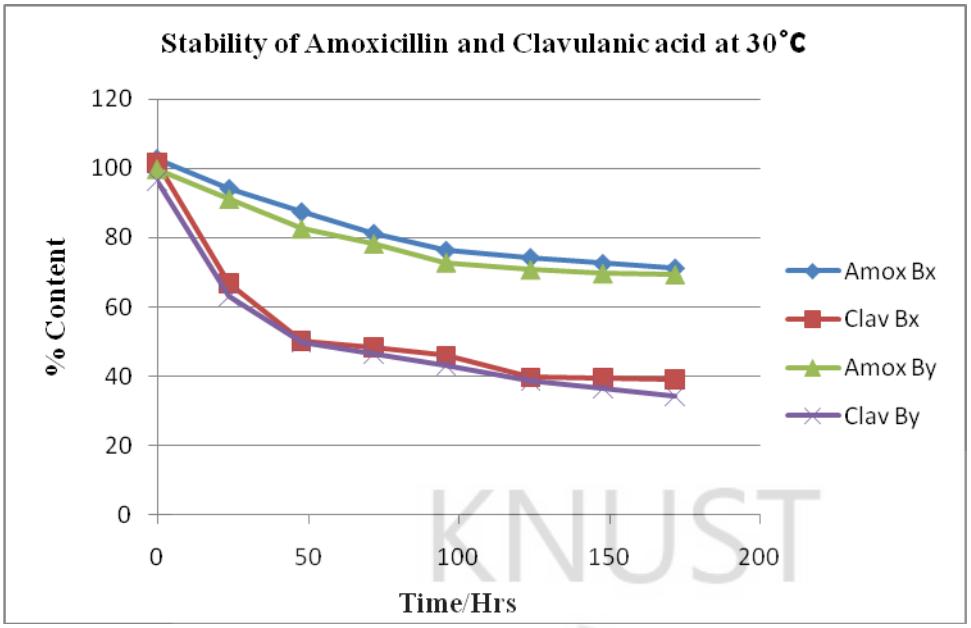

Fig. 41 Comparison of Amoxicillin to Clavulanic acid at 30˚C in brands X and Y

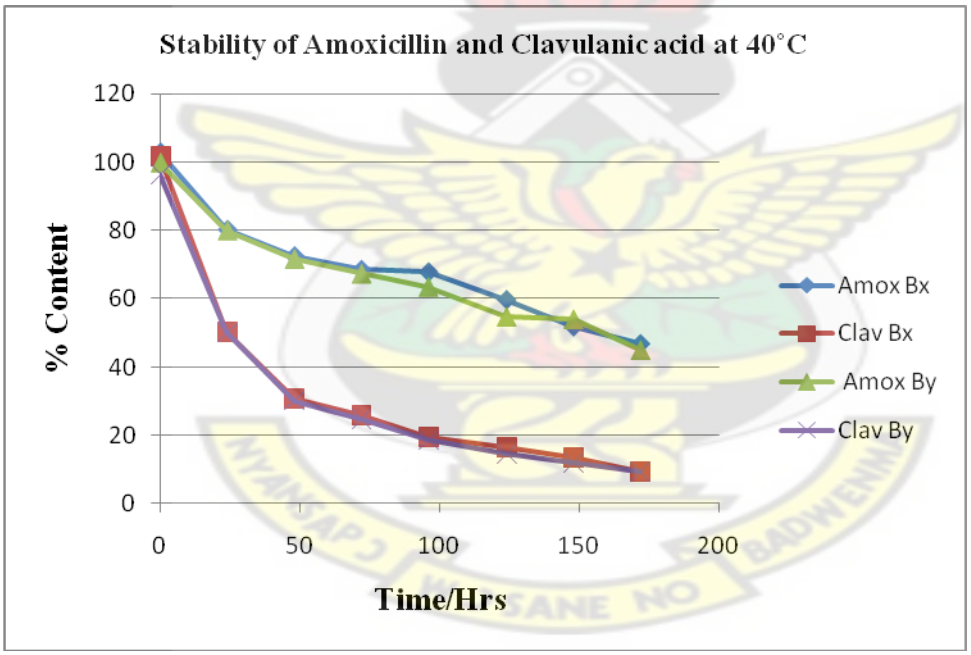

Fig. 42 Comparison of Amoxicillin to Clavulanic acid at 40˚C in brands X and Y

KEY: Amox Bx –Amoxicillin in brand X , Amox By –Amoxicillin in brand Y

Clav Bx –Clavulanic acid in brand X, Clav By –Clavulanic acid in brand

CHAPTER 4 – DISCUSSION, CONCLUSION AND RECOMMENDATION

4.1 DISCUSSION

4.1.1 IDENTIFICATION AND ASSAY OF REFERENCE STANDARD

The importance of the use of reference standards in pharmaceutical analysis as a means of ensuring validity of analytical data cannot be overemphasised. They serve as the basis of comparison with active pharmaceutical ingredients in formulated drug products in establishing the quality of these products both qualitatively and quantitatively. The purity and authenticity of these reference standards are of prime importance and it is imperative that they are investigated and information about their purity confirmed before they are employed in any pharmaceutical analytical work or method development such as conducted in this study. It was to this effect that all reference standards used were qualitatively and quantitatively assessed.

4.1.1.1 Amoxicillin

The identity of the Amoxicillin reference standard was assessed using colour reaction test, melting point determination and measurement of the specific optical rotation. This was conducted to determine whether it conforms to the specifications in the BP before they could be used for the analytical study. From the results obtained, Amoxicillin solution gave a dark yellow colour with sulphuric-acid formaldehyde. The addition of two drops of a neutral solution of Ferric Chloride also gave a blue colouration [Table 3]. These two tests and their results give an indication that the sample was Amoxicillin as it agrees with the specification of BP identification test for amoxicillin. The blue coloration with ferric chloride is as result of a reaction of the phenolic –OH of amoxicillin, which typical reaction of the phenolic functional group [16]. The identity of Amoxicillin was further verified with a melting point determination which is a measure of the purity of the

compound. The melting point range of 193-196˚C obtained for amoxicillin is in agreement with the literature value of 192-196˚C [Table 4].

The identity was additionally ascertained by determining the specific optical rotation of amoxicillin and the value of +299 obtained [Table 5] is within the acceptable standard literature range of +290 to +315(British Pharmacopoeia 2007)

4.1.1.2 Clavulanic Acid

The identity of the clavulanic acid reference standard was also investigated by carrying out colour reaction tests, melting point and specific optical rotation determinations.

Results of the colour reaction test show that a solution of clavulanic acid produced a white crystalline precipitate upon the addition and heating with a solution of sodium carbonate and sodium sulphide [Table 6]. The melting point determination of clavulanic acid gave a value of 122-125˚C which agrees with the literature range of 121-125˚C [Table 7]. Moreover, the specific rotation of +57 obtained from the results for clavulanic acid was well within the acceptable range of +53 to +63 [Table 8].The results of the colour reaction test, melting point and specific rotation determination were in agreement with the literature values and thus confirms the identity and purity of the clavulanic acid reference standard.

4.1.1.3 Thin Layer Chromatography

The purity of the Amoxicillin and Clavulanic acid reference standards were further assessed by performing a thin layer chromatography. From the chromatoplates, it was found out that there were no impurities or breakdown products for both amoxicillin and clavulanic acid [Fig 9 and Fig 10]. The two brands of the oral suspension used for the stability studies were also assessed for the presence of breakdown products using TLC. From the chromatoplate obtained (Fig 4.3 and Fig 4.4), no breakdown product was found.

4.1.2 Assay of Amoxicillin and Clavulanic acid Reference Standard

The sample of amoxicillin reference standard was assayed by Iodometric titration in which iodine generated from Potassium Iodide was made to react in excess with the amoxicillin and after all the amoxicillin had reacted with the excess iodine was back titrated with Sodium thiosulphate that has already been standardised with Potassium Iodate. From the results of the assay obtained the percentage purity of the amoxicillin reference standard was found to be 99.5%. This value is well within the range of acceptable BP specification of 95-102%.(BP 2007)

Clavulanic acid was also assayed by titrimetry to ascertain its percentage purity before it was used for the method development. The titration involved a simple acid-base reaction of clavulanic acid with sodium hydroxide which had been standardised with sulphamic acid. From the results of the assay, the percentage purity of the clavulanic acid reference standard was found to be 99.3%, which falls within the acceptable range of the BP specification of 96.5 - 102 for clavulanic acid reference standard. Both the amoxicillin and clavulanic acid reference standard passed the assay and therefore their purity are suitable as reference standards in this study.

4.1.3 HPLC METHOD DEVELOPMENT

4.1.3.1 Preliminary Investigations

Solubility Studies

Most HPLC methods designed for the simultaneous determination amoxicillin and clavulanic employ water as the solvent for dissolving amoxicillin trihydrate pure powder. However, amoxicillin trihydrate has a solubility of 1 in 400 of water and 1 in 200 of

methanol [10]. This indicates that amoxicillin trihydrate has a poor solubility in water and this makes sample preparation in such methods difficult and takes longer period. As a result solubility studies was conducted in this study to find out a suitable solvent that can dissolve amoxicillin easily. From the results of solubility studies, it was found out that it takes 45minutes for 2mg of amoxicillin trihydrate to dissolve in 10ml of water after sonicating at 28˚C while it took 5minutes for the same quantity to dissolve in a 10ml mixture of methanol:water in the ratio of 80:20 when it was sonicated at 28˚C. Thus it takes lesser time to prepare the sample with this solvent which makes it more convenient for this method. Clavulanic acid however dissolved within 5 minutes in water as it was in the salt form as Potassium Clavulanate.

4.1.3.2 UV/Vis Spectrophotometric Analysis

An initial UV analysis was performed to determine the wavelength of maximum absorption for both amoxicillin and clavulanic acid. This was done by using different solvents such as water, methanol and a combination of the two (methanol:water 10:90) to determine which wavelength range would be suitable for further study in the HPLC method development. From the result of UV spectra obtained [Table 14], amoxicillin showed maximum absorption at wavelengths of 272nm in methanol:water (10:90) combination with absorbance of 0.388. Clavulanic acid on the other hand showed maximum absorption at wavelengths of 229nm methanol:water (10:90) combination with an absorbance of 0.552 The higher wavelength of maximum absorption of amoxicillin could be due to the structure of amoxicillin which has a good chromophore system with an auxochrome extending the conjugation of carbon-carbon double bond that is responsible for absorbing UV light. Clavulanic acid had shorter wavelength of maximum absorption due to the poor chromophore system in the structure. From this initial wavelength studies a

range of 215 to 270nm was chosen as the range for investigation in the HPLC method development.A final wavelength of detection chosen was 220nm since that gave the optimal resolution peaks of amoxicillin from clavulanic acid.

4.1.3.2 Chromatographic Conditions

Most pharmacopoeial and other HPLC methods for the simultaneous determination of amoxicillin and clavulanic acid employ phosphate buffer as part of the mobile phase system. As mentioned earlier on, the usage of inorganic buffers such as phosphate buffer has caused the breakdown of most HPLC chromatograph [6]. This is because phosphate buffer forms crystals that block the plumbing system of the chromatograph generating undesirable back pressure and causes the wear and tear of the moving parts of HPLC pumps and eventually breaks down the expensive HPLC chromatograph [7]. Therefore long wash out periods are usually required after using mobile phase systems which contains phosphate buffer and inorganic buffers in general [8].

As a result, in designing the conditions of this method organic buffers were considered and after thorough investigation it was Sodium Acetate buffer which gave optimal results. The buffer was used in combination with methanol which is also cheaper as compared with other HPLC organic modifiers such as acetonitrile. From the results a mobile phase system that consist of 5:90:5 of Methanol:Water: Acetate Buffer (pH 4.38-4.41) gave optimal separation of amoxicillin from clavulanic acid with10±0.01 and 4±0.03 minutes with respectively. For any HPLC method the cost of reagents is major challenge especially in developing countries like Ghana. This method was designed using only 5% of methanol (a cheaper solvent in small amount), 90% of distilled water in combination with 5% acetate buffer which is also cheaper and easier to wash out as compared to phosphate buffer. In choosing a stationary phase the polarity of the two compounds were considered. Both

compounds have polar groups and a reversed phase stationary phase was thus suitable. An ODS(20), C-18 phenomenex, Ultracarb 5micron,250x 4.6mm column was used.

From the results obtained, the retention times of amoxicillin and clavulanic acid were 10 and 4 minutes with standard deviations of ±0.01 and ±0.03 respectively. This shows that clavulanic acid was eluted faster than amoxicillin. This could be explained from the mechanism of separation. The main factor underlying the separation of amoxicillin from clavulanic acid is the control of pH of the mobile phase. The compound that is fully ionised easily has a greater affinity for the mobile phase but lower interaction with stationary phase and is thus eluted faster. Also, clavulanic acid was more readily soluble in water than amoxicillin and with the mobile phase composition having more of water; it would be able to elute the more water soluble compound faster. Furthermore, both compounds have ionisable groups in their structures. Amoxicillin has three ionisable groups of –OH, -COOH, and $–NH_2$ which have pKa values of 7.4, 2.4 and 9.6 respectively [10]. Most compounds get ionised at a pH within 1-2 units of their pKa values from literature. Amoxicillin can be fully ionised if at least two of the ionisable groups are ionised. However, at a mobile phase pH of 4.38-4.41 only the –COOH becomes ionised and thus the compound is partially ionised and decreases its affinity for the mobile phase. Clavulanic acid on the other hand has only one ionisable group. With a pKa of 2.7 clavulanic acid becomes fully ionised at a mobile phase pH of 4.38-4.41 and thus has a greater affinity for the mobile phase than amoxicillin. Therefore Clavulanic acid eluted faster than amoxicillin accounting for the retention times of 4 and 10minutes respectively.

From the results obtained both clavulanic acid and amoxicillin were detected at a wavelength of 220nm. Clavulanic acid could not be detected when wavelength was increased to 225, 230, 250 and 270nm [Table 14]. Amoxicillin could however, be detected

at all these wavelengths. This low wavelength of absorption could be due poor chromophore system in clavulanic acid.

Moreover, the minimum flow rate that gave optimal results was selected after different flow rates of 0.5, 0.8. 1.0 and 1.5ml per minute were used. From the results a flow rate of 1.0ml/min gave the optimal conditions [Fig19]. This implies that the quantity of reagents used in this method economically is lower as compared to other methods that used flow rates of 2.0ml/minute [BP 2007].

Other conditions of the method that were established include the sensitivity of the recorder also designated as the Absorbance Units Fraction (AUFS) and pump pressure which is very critical in the design of an HPLC method. A pump pressure of 4.3-5.6Mpa was established and the recorder sensitivity was 0.05AUFS.

Five compounds were investigated with the established HPLC conditions for the selection of an internal standard. From the investigations it was metronidazole that proved to be stable and properly resolved from amoxicillin and clavulanic acid.

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4.1.4 HPLC METHOD VALIDATION

4.1.4.1 Amoxicillin

The reproducibility, validity, precision and accuracy of data obtained after using an analytical method form the basis of analytically method validation. As a result this method was validated as per the ICH guidelines on analytical method validation.

The method demonstrated linearity over a concentration range of 0.018-0.063%w/v of amoxicillin. From the calibration curve the R^2 value over this range was found to be 0.9987. This indicates a linear relationship between the concentrations of amoxicillin and the mean peak areas over the established range.

From the results, the intra-day precision gave an RSD of 0.44% while the inter-day precision gave an RSD value of 0.55%. Thus both the intra-day and inter-day precision RSD were below the maximum RSD limit of 2% as per the ICH standard for analytical method validation. This indicates that the method was precise. The precision of the method is further confirmed statistically by the large F value of 3122, the larger the F value the greater the precision of the method. The inter-day precision was further confirmed by performing a one-way analysis of variance and from the statistical analysis there was no significant difference between the means and the variance.

From the recovery studies performed, the RSD of the % recovery at the three concentration levels were 0.7%, 1.17%, 0.52% which were below 2% and thus indicates good accuracy of the method. The accuracy of the method was also confirmed by comparing this method with a standard pharmacopoeial method (BP 2007). The means obtained for an assay of the drug product for the new method and the standard method were 100.6 ± 0.3124 and 100.0 ± 0.3051 respectively. From the statistical analysis of the assay results from the two methods using the students t-test to compare the means of the new method to the standard

method, a P-value of 0.9599 was obtained. This indicates that there was no significant difference between the means.

The null hypothesis [$\mathbf{H_o}$] is that there is no significant difference between the new method and the standard. The null hypothesis [$\mathbf{H_o}$] is accepted if the experimental value is less than the critical value of *t* at 95% confidence interval. From the results obtained [Table 39] the experimental value of *t* was found to be 1.336 and the critical value of t at 95% confidence interval was also 2.23. The null hypothesis is accepted since the experimental value of *t* is less than the critical value of *t*. Therefore there was no significant difference between the new method and the standard method. From the results obtained, it was also found out that the experimental F-value was 1.048 while the critical F-value was 7.146. This indicates that there was no significant difference between the variances of the two methods.

When some of the conditions of the mobile phase such as pH and column used for the method were varied, there was no statistically significant difference between results of the varied and old conditions of mobile and stationary phases using the student *t*-test. The pH of the mobile phase was varied from 4.34 to 4.48 while a column from a different manufacturer was also used. This indicates that the method is robust under varying conditions of both mobile and stationary phases.

The method proved to be specific since the excipients in the formulated product when injected together did not interfere with the analysis of amoxicillin and clavulanic acid.

From the results and calculations the Limit of Detection (LOD) and the Limit of Quantification (LOQ) of the method for amoxicillin were found to be 0.00202%w/v and 0.00674%w/v respectively.

4.1.4.2 Clavulanic Acid

The method demonstrated linearity with concentrations of clavulanic acid over a range of 0.001%w/v to 0.006%w/v. The R^2 value over this range of concentration from the calibration curve of clavulanic acid was found to be 0.9984 which is very close to 1.0. This demonstrates a very good linear relationship between the concentration of clavulanic acid and peak area.

The intra-day and inter-day precision of the method for clavulanic acid gave RSD values of 0.54% and 0.58% respectively. Both RSD values are well below the maximum limit of 2% and thus the method has good precision. The inter-day precision was further confirmed with one-way analysis of variance which gave no significant difference between the variances. The overall precision of the method was further substantiated by the large F value of 2262 which indicates that the method is very precise.

From the recovery studies performed, the RSD of the % recovery at the three concentration levels were 0.75%, 0.78%, 1.34%.All the three RSD were below 2% and indicates good accuracy of the method. The accuracy of the method was also confirmed by comparing this method with a standard pharmacopoeial method. The means obtained for an assay of the drug product using the new method and the standard method were 98.97 ± 0.1202 and 98.85 ± 0.1335.

From the statistical analysis of the assay results for the two methods using the student *t*-test to compare the means of the new method to the standard method, a P-value of 0.8227 was obtained. This indicates that there was no significant difference between the means.

The null hypothesis [$\mathbf{H_o}$] is that there is no significant difference between the new method and the standard. The null hypothesis [$\mathbf{H_o}$] is accepted if the experimental value is less than the critical value of *t* at 95% confidence interval. From the results obtained [Table 40] the experimental value of *t* was found to be 0.6494 and the critical value of t at 95% confidence interval was also 2.23. The null hypothesis is accepted since the experimental value of *t* is less than the critical value of *t*. Therefore there was no significant difference between the new method and the standard method. From the results obtained, it was also found out that the experimental F-value was 1.235 while the critical F-value was 7.146. This indicates that there was no significant difference between the variances of the two methods.

When some of the conditions of the mobile phase such as pH and column used for the methods were varied there was no statistically significant difference between results of the varied and old conditions of mobile and stationary phases using the student t-test. The pH of the mobile phase was varied from 4.34 to 4.48 while a column from a different manufacturer was also used. This indicates the robustness of the method.

The method proved to be specific since the excipients in the formulated product when injected together did not interfere with the analysis of amoxicillin and clavulanic acid.

From the results and calculations the Limit of Detection (LOD) and the Limit of Quantification (LOQ) of the method for clavulanic were found to be 0.000264%w/v and 0.000819%w/v respectively.

4.1.5 ASSAY OF BRANDS OF ORAL SUSPENSION OF CO-AMOXICLAV.

The USP-30 states that amoxicillin and potassium clavulanate for oral suspension contains the equivalent of not less than 90.0 percent and not more than 120.0 percent of the labelled amount of amoxicillin and the equivalent of not less than 90.0 percent and not more than 125.0 percent of the labelled amount of clavulanic acid.

From the results of the HPLC analysis of the five brands of the oral suspension of Co-amoxiclav, the mean percentage content of amoxicillin in the various brands was found to range from 88.5% to 102.3% of the labelled amount with standard deviations of 0.10 to 0.21. The mean percentage contents of clavulanic acid in the oral suspension range from 85.3% to 99.8% of the labelled amount with standard deviations of 0.11 to 0.18.

Four of the brands analysed had their percentage contents of amoxicillin and clavulanic acid of the labelled amount that conformed to the required amount stated in the USP. One brand, D, however had its percentage contents of both amoxicillin and clavulanic acid being less than 90.0% of the labelled amount. Thus the percentage content of amoxicillin and clavulanic acid in brand D were 88.5%±0.13 and 85.3%±0.11 respectively of the labelled amount. This indicates that four out of the five brands passed the assay as per the USP specifications while one brand failed the assay. The low percentage content of this brand that failed implies that the product is substandard and may not be able to produce the expected therapeutic potency as intended. The failure of brand for the assay could be due to poor storage conditions that might have caused breakdown of the active ingredient. It could also be as a result of the formulation not properly designed and therefore several factors such as purity of active ingredient, stabilizers and other excipients that were used etc may not have been appropriately considered.

5.1.6 STABILITY STUDIES

The antibacterial potency and efficacy of amoxicillin and clavulanic acid combinations in oral suspensions for the treatment of bacterial infections is largely dependent on the synergies derived from the two compounds. It is therefore important that the two compounds remain stable throughout the period of use in order to achieve this synergistic effect. The conditions of storage of reconstituted suspensions of amoxicillin and clavulanic acid have a tremendous effect on the stability of the two compounds.

The stability studies of amoxicillin and clavulanic conducted in this study was based on the World Health Organization's (WHO) protocol for in-use stability studies for reconstituted suspensions using two brands of the product, one being the innovator and other being the highest stocked generic on the local market.

From the results obtained, the percentage contents of both amoxicillin and clavulanic did not show any appreciable change when stored at 10˚C over a period of seven days. The percentage content of amoxicillin stored at 10˚C decreased slightly from 102.8% on day one to 101.9 on day seven for brand X and 99.8% on day one to 98.7% on day seven for brand Y. Also the percentage content of clavulanic acid decreased slightly from 101.7% on day one to 100.8% on day seven for brand X and 96.4% on day one to 95.6% on day seven for brand Y at 10˚C. This shows that both amoxicillin and clavulanic acid demonstrated considerable stability at a temperature of 10˚C over a period of seven days. However, the percentage content of both compounds in brand Y which is the generic brand, was lower than brand X which is the innovator brand. This may be due to differences in formulation of the two brands with respect to stabilizers used in the formulations.

At a temperature of 30˚C, the percentage content of amoxicillin decreased fairly from 102.8% on day one to 71.3% on day seven for brand X and 99.8% on day one to 69.4% on day seven for brand Y. For clavulanic acid, at a temperature of 30˚C, there was an appreciable decrease in percentage content from 101.7% on day one to 38.4% on day seven for brand X and 96.4% on day one to 34.2% on day seven for brand Y. This indicates that both amoxicillin and clavulanic acid are unstable at a temperature of 30˚C. However, the percentage content of both amoxicillin and clavulanic acid in brand Y (the generic brand) were lower than that in brand X which is the innovator brand.

The percentage content of amoxicillin at a temperature of 40˚C however, showed a drastic decrease from 102.8% on day one to 46.8% on day seven for brand X and 99.8% on day one to 44.9% on day seven for brand Y. At a temperature of 40˚C, the percentage content of clavulanic acid decreased drastically from 101.7% on day one to 9.4% on day seven for brand X and 96.4% on day one to 8.4% on day seven for brand Y. This indicates that both amoxicillin and clavulanic acid are very unstable at a temperature of 40˚C.

From Fig.41 and Fig.42, comparing the stability of amoxicillin to clavulanic acid at the temperatures of 30˚C and 40˚C in brand X, it was realised that clavulanic acid breaks down faster than amoxicillin at both temperatures. Analysis of graph in Fig.35, also reveals that at a temperature of 30˚C on day three, the percentage content of amoxicillin was 81.3% while that of clavulanic acid was 48.3%, on day five at the same temperature the percentage content of amoxicillin was 74.3% while that of clavulanic acid was 39.6% and on day seven at 30˚C the percentage content of amoxicillin was 71.3% while that of clavulanic acid was 38.9%

At a temperature of 40˚C on day three, the percentage content of amoxicillin was 68.5% while that of clavulanic acid was 25.9%, on day five the percentage content of amoxicillin

was 59.7% while that of clavulanic acid was 16.5% and on day seven the percentage content of amoxicillin was 46.8% while that of clavulanic acid was 9.4%.

By similar comparison of stability of amoxicillin to clavulanic acid in brand Y, analysis of the graph in Fig.41 revealed that at a temperature of 30˚C on day three ,the percentage content of amoxicillin was 78.3% while that of clavulanic acid was 46.5%, on day five at the same temperature the percentage content of amoxicillin was 70.8% while that of clavulanic acid was 38.7% and on day seven at 30˚C the percentage content of amoxicillin was 69.4 % while that of clavulanic acid was 34.2%. At a temperature of 40˚C on day three, the percentage content of amoxicillin was 67.3% while that of clavulanic acid was24.8 %, on day five the percentage content of amoxicillin was 54.7% while that of clavulanic acid was 14.8% and on day seven the percentage content of amoxicillin was 44.9 % while that of clavulanic acid was 8.4%.From the above analysis, clavulanic acid more unstable than amoxicillin and breaks down twice as faster as amoxicillin at both 30˚C and 40˚C.

From the stability studies it is evident that reconstituted oral suspension of Co-amoxiclav is more stable when stored in refrigerator at 10˚C. This also indicates that at a temperature of 10˚C clavulanic acid which is *B*-lactamase inhibitor can extend the antibacterial properties of amoxicillin and thus give rise to positive therapeutic outcomes.

However, under ambient temperature conditions of 30˚C and 40˚C which are the prevailing temperature conditions in the southern and northern sectors respectively in Ghana, both amoxicillin and clavulanic acid in reconstituted oral suspension stored under these conditions could cause breakdown. This implies that the antibacterial synergies that could be derived from the two compounds would be compromised and this can lead to treatment failures. Also because sub therapeutic doses would be available in oral

suspensions of Co-amoxiclav stored at these temperatures, it could lead to the emergence of resistant strains of B-lactamase producing micro-organisms. Such a situation has cost implications on the health delivery system in Ghana since alternate and potent antibacterial agent have to be procured to treat such infections caused by the resistant strains.

Not all, the presence of degradation products of amoxicillin and clavulanic acid in these oral suspension for paediatric use can have serious health hazards. The major degradation product of amoxicillin, penicilloic acid is known to cause severe allergic reactions and that of clavulanic acid which can also cause cholestatic jaundice. Therefore the presence of these degradation products could compromise the quality of these products and the overall public health.

4.2 CONCLUSION

4.2.1 IDENTIFICATION TEST AND ASSAY OF REFERENCE STANDARDS

The identity of the reference standards of both Amoxicillin and Clavulanic acid were confirmed by the identification test as per the BP specifications. All the samples passed the identification test for colour reaction and specific optical rotation. Percentage purities of 99.5% and 99.3% for Amoxicillin and Clavulanic acid reference samples as well as melting points of 193-196˚C and 121-125 ˚C for Amoxicillin and Clavulanic acid respectively, were within the range specified in the BP .The reference standards were of good purity and this justifies their use in this study.

4.2.2 METHOD DEVELOPMENT AND VALIDATION

A simple Reversed-Phase isocratic HPLC method was developed in this study with a mobile phase system of Methanol:Water:Acetate buffer pH 4.34 (5:90:5) and a stationary phase made of C-18 Phenomenex, Ultracarb 5µODS(20), 250 X4.6mm column. The

method could separate amoxicillin from clavulanic in reconstituted oral suspensions of amoxicillin and clavulanic acid. The method was also used for the simultaneous quantification of amoxicillin and clavulanic acid in reconstituted liquid oral suspension of Co-amoxiclav for paediatric use. The HPLC method was validated against all the parameters such as precision, accuracy, specificity, linearity, and robustness as per the ICH guidelines on analytical method validation.

The method proved to be accurate, precise and linear within the range of 0.018-0.063%w/v for amoxicillin and 0.001-0.006%w/v for clavulanic acid. The method also had an LOD and LOQ of 0.00233%w/v and 0.00775%w/v for amoxicillin and an LOD and LOQ of 0.000246%w/v and 0.000819%w/v for clavulanic acid respectively. Also the method demonstrated specificity in the presence of excipients of the oral suspension and the method proved to be robust under varying mobile phase pH of 4.36 to 4.45 and varying columns from different manufacturers.

4.2.3 ASSAY OF LIQUID ORAL SUSPENSION

According to the USP, amoxicillin and potassium clavulanate for oral suspension contains the equivalent of not less than 90.0 percent and not more than 120.0 percent of the labelled amount of amoxicillin and the equivalent of not less than 90.0 percent and not more than 125.0 percent of the labelled amount of clavulanic acid.

Four out of the five brands of the oral suspensions of amoxicillin and clavulanic analysed passed the assay with a mean percentage content of 99.3-102% ±0.11-21 of the labelled amount of amoxicillin and 97.9-99.8%w/v ±0.14-0.18 of the labelled amount of clavulanic acid. One brand, however, failed the assay with a mean percentage content of 88.5%± 0.10 of the labelled amount of amoxicillin and 85.3% ±0.11 of the labelled amount of clavulanic acid.

4.2.4 STABILITY STUDIES

The HPLC method developed could separate the degradation products of amoxicillin and clavulanic acid from the active compounds and thus no interference with the analysis of active amoxicillin and clavulanic acid occurred. This makes the method suitable for stability studies of the two compounds in liquid oral suspension of amoxicillin and clavulanic acid.

Amoxicillin and clavulanic acid in oral suspension for paediatric use are very stable under temperature conditions of 10˚C over a period of seven days. The two compounds are not stable under temperature conditions of 30˚C and 40˚C. Clavulanic acid however is more unstable than Amoxicillin under both temperature conditions of 30 ˚C and 40˚C with clavulanic acid breaking down almost twice as fast as amoxicillin.

4.3 RECOMMENDATIONS

Further studies could be performed to quantify the amount of degradation products of amoxicillin and clavulanic acid in reconstituted liquid oral suspensions of the two compounds under temperature conditions of 30˚C and 40˚C.

Also, to prevent treatment failures with the use of reconstituted liquid oral suspension of amoxicillin and clavulanic acid, health professional should endeavour to advise patients to store reconstituted oral suspension of amoxicillin and clavulanic acid under refrigeration at a temperature not exceeding 10˚C

To prevent the emergence of resistant strains the general public, especially, patients must be educated on the need to observe a very strict storage precaution of temperature for reconstituted products of amoxicillin and clavulanic acid.

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