DETERMINATION OF POTENCY AND QUALITY OF SOME SELECTED PENICILLINS ON THE GHANAIAN MARKET USING MICROBIOLOGICAL AND DEVELOPED AND VALIDATED HPLC METHODS

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Declaration

I hereby declare that this submission is my own work towards the MPhil and that, to the best of my knowledge, it contains no materials previously published by another person nor material which has been accepted for the award of another degree of the University, except where due acknowledgement has been made in the text.

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Abstract

The use of antibiotics in health delivery is inevitable since it is one of the most prescribed medications. The quality and efficacy of these medications are crucial in health systems since they can affect the quality of healthcare delivery. The study was designed to determine the quality and potency of some penicillins on the Ghanaian market. A total of 54 samples (29 capsules and 25 suspensions), of different brands and batches were sampled from different pharmacies in Accra and Kumasi, Ghana, from October, 2011 to May, 2012. The potency, activity and minimum inhibitory concentration (MIC) of the samples were determined by the agar well diffusion and micro-dilution methods against selected Gram-negative and Gram-positive bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 4853, *Staphyloccocus aureus* ATCC 25923 and *Bacillus subtilis* NTCC 10073). The quality of the samples was determined quantitatively by developed and validated HPLC method. The MICs of flucloxacillin and cloxacillin samples were ≥ 1400 µg/mL, whiles that of amoxicillin samples were ≥ 200 µg/mL, with reference to the standards which gave MICs of 200 to 800 µg/mL against all the test bacteria with the suspensions exhibiting higher antimicrobial activity. The biological assay results revealed higher MICs for all the various penicillins evaluated but were much higher in flucloxacillin samples. The United State Pharmacopoeia (2011) methods of assay of the selected samples were slightly modified, making use of the available materials in the laboratory. The methods were well validated using the International Conference on Harmonization (ICH) guidelines, British Pharmacopoeia (BP) and USP. Specificity, linearity, precision and accuracy of the HPLC method were determined. HPLC analysis of the samples revealed that 75% of amoxicillin capsule samples and 92.3% of amoxicillin suspension samples contained the right amount of active pharmaceutical ingredient (API) with percentages ranging from 93.2 to 104.3% and 81.0 to 104.1% respectively. For samples of flucloxacillin

capsules, 62.5% of the samples revealed API's within 96 to 120.5%. All flucloxacillin suspension samples were below the British Pharmacopoeia (BP) and United State Pharmacopoeia (USP) specifications. None of the cloxacillin capsule samples contained the right active pharmaceutical ingredient and all the suspension samples have their API within BP and USP specification of 114.4 to 120.0%. Variation within same brand was observed in some of the samples but were not significant ($p>0.05$). For some of the samples, only one batch could be sampled within the period of the study. Consequently, no data from these have been analyzed. Variations in microbiological evaluation and HPLC analysis were observed. In general, 58.6% of the capsules of all the samples contained the right API whereas 64% of them were recorded for suspensions. Out of the 54 samples evaluated, 61.1% were within BP and USP specifications.

The biological assay revealed higher MIC values for all the penicillin samples evaluated compared with the reference samples. Among the samples evaluated, amoxicillin showed better quality of 82.8% as compared to flucloxacillin (31.3%) and cloxacillin (44.4%) samples. Efforts should therefore be made to improve the quality and storage conditions of these antibiotics and also constant monitoring and surveillance of activity and potency of these antibiotics should be done. These results suggest the need for increased monitoring and surveillance of these antibiotics by their manufacturers and regulatory bodies.

List of Publication

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Dedication

I dedicate this work to my father, Mr. Patrick Osei-Assibey and my mother, Madam Veronica Twum.

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Abbreviations and Prefixes

ATCC	American Typed Culture Collection
API	Active Pharmaceutical Ingredient
AUC	Area under curve
BP	British Pharmacopoeia
°C	Degree Celsius
Cfu	Colony forming unit
HPLC	High performance liquid chromatography
G	Gram
H	Hour
IS	Internal standard
KH_2PO_4	Potassium dihydrogen orthophosphate
MeCN	Acetonitrile
MeOH	Methanol
MIC	Minimum inhibitory concentration
Mg	Milligram
Min	Minutes
Ml	Millilitre
MRVP	Methyl Red-Voges Proskauer
MTT	Methylthiazolyl tetrazolium chloride
NCCLS	National Committee for Laboratory Standards
NTCC	National Typed Culture Collection
RS	Reference standard
SDEV	Standard deviation
SEM	Standard error mean
RSD	Relative standard deviation
µg	Microgram
µL	Microliter
UV	Ultra violet
ZOI	Zones of inhibition
R^2	R spuare

CHAPTER 1

INTRODUCTION

1.0 GENERAL INTRODUCTION

The World Health Organization (WHO) defines counterfeit products as those which are deliberately and fraudulently mislabeled with respect to identity and/or source (Shakoor *et al.*, 1997; Wondemagegnehu, 1999). Substandard medicines, on the other hand, are medicines that do not meet official standards and specification for strength, quality, purity, packaging, and/or labeling and their presence are one of the latest threats facing the pharmaceutical industry and healthcare globally. As a result of weak or no regulatory systems in many low and middle income countries (Caudron *et al.*, 2008; WHO, 2010), most of the medicines in circulation in these countries do not meet internationally accepted quality and specification and may be detrimental to patients.

The total worldwide trade in counterfeit medicines is estimated to be 5 to 7% of the pharmaceutical market (Gibson, 2004). The problem is more severe in developing countries. More than 30% of all medicines sold in Africa are counterfeit medicines (Moore *et al.*, 2012). Counterfeit and/or substandard medicines are not only available in the developing countries but also in the developed world (Vila and Pal, 2010). In 1999, 22% of the 771 reports of counterfeited medicines received by WHO came from the industrialized countries, the remaining 78% were from the developing countries (Caudron *et al.*, 2008).

Prevalence of counterfeit and/or substandard medicines has a major effect on the health delivery system. They can result in treatment failure, toxicity or severe side effects thereby increasing mortality rate (Kelesidis *et al.*, 2007). Counterfeit and/or substandard medicines may be found in all classes of medicines. The two major classes most counterfeited in the developing countries are anti-parasitic and anti-infectives (Wondemagegnehu, 1999).

Exposure of microorganisms to counterfeit and/or substandard anti-infectives leads to antimicrobial resistance, thereby putting health of patients at risk (Yankus, 2006). Antimicrobial resistance contributes to high cost of healthcare as patients using these counterfeit and /or substandard medicines do not respond to treatment and have to resort to higher doses and, or/ newer medicines. Additionally, patients remain ill for longer period leading to the loss of productivity (Aldhous, 2005; Shakoor *et al.*, 1997). Infectious diseases are taking lives of people and believed to be the world's leading cause of death. It is estimated that 50,000 people die a day out of infectious diseases (Ahmad and Beg, 2001).

Medicines need to be of acceptable quality, safety and efficacy, especially antibiotics (Diaz *et al.,* 2011). The appropriate active pharmaceutical ingredients (API) quantity and its efficacy to effect treatment must be ascertained. This is achieved through analysis and comparison to the manufacturer's specifications or standard specification in the pharmacopoeias. Consequently, there is the need to sample and evaluate some of the antibiotics on the Ghanaian market to ensure that they meet the required specifications as spelt out in the USP and BP to avoid all the problems associated with counterfeit and/or substandard medicines.

1.2 LITERATURE REVIEW

1.2.1 Overview of Antibiotics

Antibiotics are natural or synthetic chemical agents that can inhibit the growth or kill microorganisms (Gallo *et al.*, 1995). Antibiotics are one class of antimicrobials and are either referred to as bactericidal or bacteriostatic when they kill or inhibit growth or bacteria respectively (Pankey and Sabath, 2004). They are heterogeneous and the only common property is that they are all organic in nature. A required feature of any antibiotic is its effect on bacteria at low concentration since that differentiate antibiotics from other compounds which have antimicrobial effect at higher concentrations e.g. ethanol**.** The

discovery of antibiotics have significantly reduced mortality resulting from infectious diseases and also facilitated the success rates of many medical procedures such as surgery (Lohsiriwat *et al.*, 2009; Spielholz, 2011). They are also employed extensively to prevent and to treat infectious diseases in humans and animals (McEwen and Fedorka-Cray, 2002). These agents are mostly directed against some targets that are peculiar to bacteria, interfering with the growth of sensitive structures or processes that are critical to the survival and/or growth of the bacteria. Antibiotics inhibit sensitive bacteria by blocking important macromolecules like enzymes and nucleic acid activity which are very important in cell multiplication (Keyes *et al.*, 2008). In effect, they are able to bind to specific site on the macromolecule to form a complex, different from the original entity and are unable to perform its function. The main targets are bacterial cell wall synthesis (peptidoglycan), bacterial protein synthesis (bacterial ribosome), bacterial DNA replication (bacterial enzymes involved in DNA supercoiling) and cytoplasmic membrane function (Walsh, 2003).

1.2.2 Types of Antibiotics

Each type of antibiotic affects different bacteria in different ways. For example, an antibiotic might inhibit a bacterium's ability to turn glucose into energy, or its ability to construct its cell wall, e.g. the penicillin (amoxicillin). This action leads to the death of the bacterium (Sefton, 2002). Some antibiotics can be used to treat a wide range of infections and are known as 'broad-spectrum antibiotics, an example is amoxicillin. Others are only effective against a few types of bacteria and are called narrow-spectrum antibiotics e.g. ticarcillin, cefazolin and vancomycin.

1.2.2.1 Aminoglycosides

Aminoglycosides are broad-spectrum antibiotics and among the most commonly used antibiotics. They are highly potent and effective in the treatment of life threatening infections such as Gram-negative infections (Gilbert, 1997; Langslet and Habel, 1981). They are poorly absorbed when given orally and are therefore given by injection. Aminoglycosides may be used along with penicillins or cephalosporins to give synergetic effect on the bacteria (Finch *et al.*, 2010; Greenwood, 2007). An example of amonoglycocide is gentamicin with ototoxicity and nephrotoxicity being the main side effects (Kaloyanides, 1984; Shetty *et al.*, 2009).

1.2.2.2 Fluoroquinolones

These are broad-spectrum antibiotics effective against different types of bacteria. They are mainly used in the treatment of urinary tract infections (UTI's), skin infections and respiratory infections (pneumonia and bronchitis) (Scholar, 2002; Walters, 2010). Some of the common side effects include diarrhoea and mild stomach upset. Examples include ciprofloxacin and ofloxacin.

1.2.2.3 Tetracyclines

These are broad spectrum antibiotic discovered in the 1940s. They have favourable antibiotic properties with less adverse reactions. This has made them the antibiotic of choice and has been extensively used both in human and animal infections (Chopra and Roberts, 2001). They are commonly used for upper respiratory infections, urinary tract infections and sexually transmitted diseases (MacGregor and Graziani, 1997). Some examples of tetracyclines are doxycycline, tetracycline and oxytetracycline. The commonly used tetracyclines are doxycycline and minocycline (Greenwood, 2007). Some of the side effects are epigastric discomfort, nausea and vomiting (Walters, 2010).

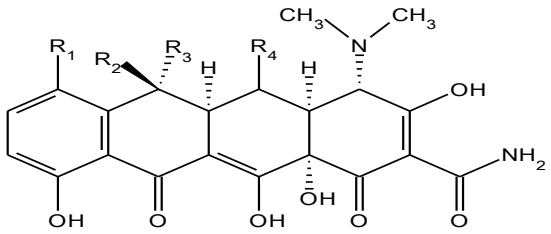

Figure 1.1: Parent structure of tetracyclines

1.2.2.4 Macrolide

The macrolides belong to the polypeptide class of natural products and they bind to ribosomes from susceptible bacteria which lead to inhibition of protein synthesis. They are mainly bacteriostatic, but can also be bactericidal in high concentrations or over time. They are able to penetrate tissue and are stable for the treatment of respiratory and soft tissue infections including genital, gastrointestinal tract, caused by susceptible strains of specific bacteria (Fiol and O'Connor, 2005; Van Bambeke *et a*l., 2010). Some of commonly used macrolides are erythromycin, clarithromycin and azithromycin. The common side effect is gastrointestinal disturbances and it occurs in 15 to 20% of patients on erythromycin (Periti *et al.*, 1993).

1.2.2.5 Beta-lactam antibiotics

This group includes the cephalosporins, penicillins and other compounds that share structural features of the beta-lactam ring (Baldo *et al.*, 2001; Wilke *et al.*, 2005). They are basically bactericidal and interfere with the final cross-linking reaction of bacterial cell wall formation. They are differentiated based on the form of enzyme needed to maintain the complex form of the cell (Hussain, 2012; Jordan *et al.*, 2008).

In Gram-negative bacteria, beta lactam antimicrobials enter the cell through pore channels in the outer membrane and bind to penicillin binding proteins (PBPs), which are the enzymes required for cell wall synthesis in susceptible microorganisms. The attachment of the beta-lactam molecules to the PBPs, located on the surface of the cytoplasmic membrane, blocks their function as they form complex different from the normal cell component. This complex cannot be recognized by the bacterium. This causes weakened or defective cell walls and leads to cell lysis and death. The activity is similar in Gram-positive bacteria where the transpeptidases located in the periplasmic space are directly accessible. This is protected by the outer membrane in Gram-negative bacteria (Gallo *et al.*, 1995; Torrence and Isaacson, 2008; Van Bambeke *et al.*, 2010).

Cephalosporins

The cephalosporins inhibit the synthesis of bacteria cell wall and their bactericidal effect is closely related to the penicillins. They bind to bacterial cell and disrupt the peptidoglycan synthesis (Torrence and Isaacson, 2008). There are four different generations of cephalosporins as a result of their antimicrobial properties. They are the first (e.g. cephazolin and cephalexin), second (e.g. cefuroxime and cefprozil), third (e.g. cefotaxime and cefixime, cefpodoxime), and fourth generations (cefepime) (Van Bambeke *et al.*, 2010). They are used to treat diseases like pneumonia, staphylococcal infections and bronchitis. Some of the common side effects are vomiting, headache and nausea (Walters, 2010).

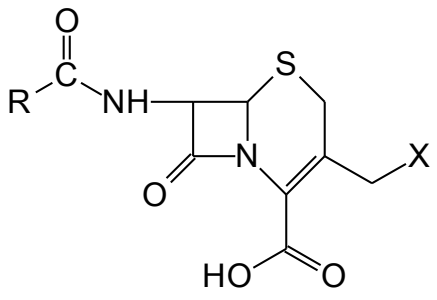

Figure 1.2: Parent structure of cephalosporin

Penicillins

Discovered in 1929 by Alexander Fleming, they are the first and well-known type of antibiotics (Bentley, 2009). These are mainly used to treat ear infections, dental infections, respiratory tract infections, gonorrhoea, urinary tract infections and skin infections. They are sometimes combined with beta-lactamase inhibitors, which protect the penicillin from bacterial enzymes such as beta-lactamases or penicillinases that may destroy it. Examples of penicillins include amoxicillin, flucloxacilin, cloxacillin, ampicillin, and those combined with beta-lactamase enzyme inhibitor, e.g. amoxicillin + clavulinic acid.

Amoxicillin

This is a broad-spectrum semi-synthetic, ß-lactam antibiotic (Figure 1.1) and it is bactericidal. It is the most preferred among the beta-lactam antibiotics because it is better absorbed when taken orally and resistant to gastric acid. This permits higher serum levels with oral administration.

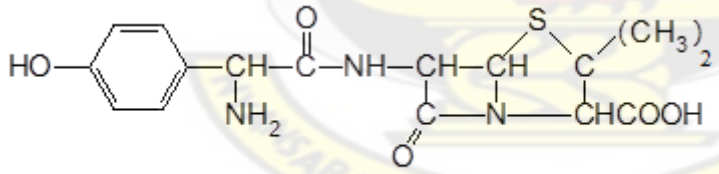

Figure 1.3 Chemical structure of amoxicillin

Amoxicillin is a bactericidal antibiotic. It binds to the penicillin binding proteins in the bacteria cell wall and prevents the cross-linking which keeps the cell rigid (Hett and Rubin, 2008).

Resistance to amoxicillin began rapidly after its introduction. Bacteria produce enzyme called a beta-lactamase which inactivates the beta-lactams rings of the penicillins by hydrolyzing the peptide bond in the beta-lactam ring (Hazir *et al.*, 2002). The main side effect is gastrointestinal disturbances (Casiano, 1991).

Cloxacillin

Cloxacillin is a semi-synthetic antibiotic used for the treatment of infections caused by beta-lactam producing strains of *staphylococcus aureus* (Pawar *et al.*, 2010). They act by binding to specific penicillin-binding proteins (PBPs) located inside the bacterial cell wall and it inhibits the third and last stage of bacterial cell wall synthesis. Intrahepatic cholestasis is the side effect (Enat *et al.*, 1980; Westphal *et al.*, 1994).

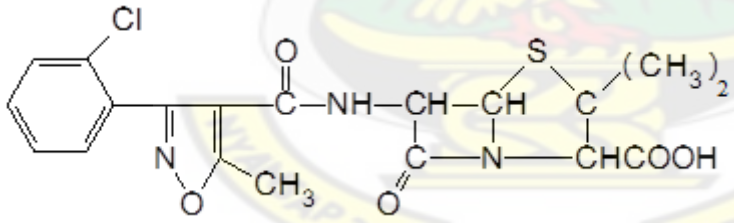

Figure 1.4 Chemical structure of cloxacillin

Flucloxacillin

Flucloxacillin is a derivative of 6-amino-penicillanic acid (Figure 1.5). It is semi-synthetic penicillin with a narrow spectrum of bactericidal activity. Flucloxacillin, by its action on the synthesis of the bacterial wall, exerts a bactericidal effect on streptococci, staphylococci, (including the beta-lactamase-producing strains) clostridia and neisseria (Greenwood,

2007). Cholestasis liver disease has been reported with its use (Eckstein *et al.*, 1993; Russmann *et al.*, 2005).

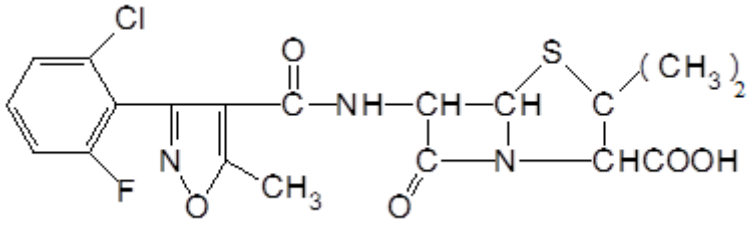

Figure 1.5 Chemical structure of flucloxacillin

1.2.3 Antibiotic resistance

Antimicrobial resistance can be defined as the ability of bacteria to survive even at the exposure to a minimum inhibitory concentration of an antimicrobial agent (Acar and Rostel, 2001). The use of antibiotics over the years has also triggered the appearance of resistance in bacteria (Davies and Davies, 2010). For instance in the case of methicillin-resistant *Staphylococcus aureus* (MRSA) studies have repeatedly shown the mortality in severe infections to be twice as high as in infections with non-resistant strains (Nordberg *et al.*, 2005).

Antibiotics are very important in healthcare but the emergence of bacteria resistant, especially the multi-drug resistance (MDR) in Gram-negative bacteria limits therapeutic options and it is a major cause of mortality in hospital-acquired infections. These pose serious threat to public health because some infectious diseases are becoming more difficult to treat (Mamelli *et al.*, 2009). Effective treatment of infectious diseases is dramatically minimized by resistant bacteria which increase the risk of complications and patients with severe infections with fatal outcomes.

The usefulness of some commonly used antibiotics like amoxicillin and ampicillin is limited by beta-lactamase hydrolysis (Islam *et al.*, 2008). They also possess an internal

mechanism of changing their structure so the antibiotic no longer works. They develop ways to inactivate or neutralize the activity of antibiotic. Gram-negative bacteria are said to be responsible for a large portion of antibiotic-resistant bacterial infections as a result of their complex cell envelope comprising an outer membrane and inner membrane delimiting the periplasm (Mamelli *et al.*, 2009; Nordberg *et al.*, 2005). Bacteria can also transfer the genes coding for antibiotic resistance among them, making it possible for bacteria never exposed to an antibiotic to acquire resistance from those that they have developed resistance.

Resistant bacteria do not respond to the antibiotics and continue to cause infections that are even worse than the previous treated infections (Levy, 1997). Bacteria demonstrate two kinds of resistance to antibiotics, namely intrinsic resistance and acquired resistance (Towner, 1995).

Intrinsic resistance is where bacterial species develop resistance to an antibiotic in their natural state even before their exposure to the agent without acquiring resistant factors. This may be due to the absence of target cell or the inability of the antibacterial to enter the bacterial cell (Bronzwaer, 2003; Schulz-Aellen, 1997).

Acquired resistance is where bacterial species which was originally susceptible to an antibiotic is no longer sensitive to some agents. This could be due to mutation or exchange of genetic material among same or closely related species (Emori and Gaynes, 1993; Towner, 1995). The sudden acquisition of resistance to antibiotics poses difficulties in treating infections. When bacteria are exposed to the same antibiotics over and over, the bacteria can change and will no longer be affected by the antibiotic (Levy and Marshall, 2004; Tapsall *et al.*, 2009). This can also be as a result of the administration of sub-standard and/or non-efficacious antibiotics to patients. In the case of non-efficacious antibiotics,

higher doses are prescribed to the patient making them resistant to lower doses (Kelesidis *et al.*, 2007; Santoso *et al.*, 2008).

Mechanism of Antibiotic Resistance

The major ways by which bacteria develop resistance are by:

- Limiting the intercellular concentration of antibiotics by increased efflux (they are pumped out of the cell) of the agent (Lewis, 2001; Shetty *et al.*, 2009).
- Modification or neutralization of the antibiotic by enzymes that reversibly or irreversibly inactivate the agent (Russell, 2001).
- Alteration of the target of the agent so that it no longer interferes with its activity and (Sebolt-Leopold and Herrera, 2004);
- Eliminating the target altogether by the utilization of different metabolic pathways. The bacteria may use one or more of these mechanisms against a particular class or different classes that leads to resistance (Kaloyanides, 1984).

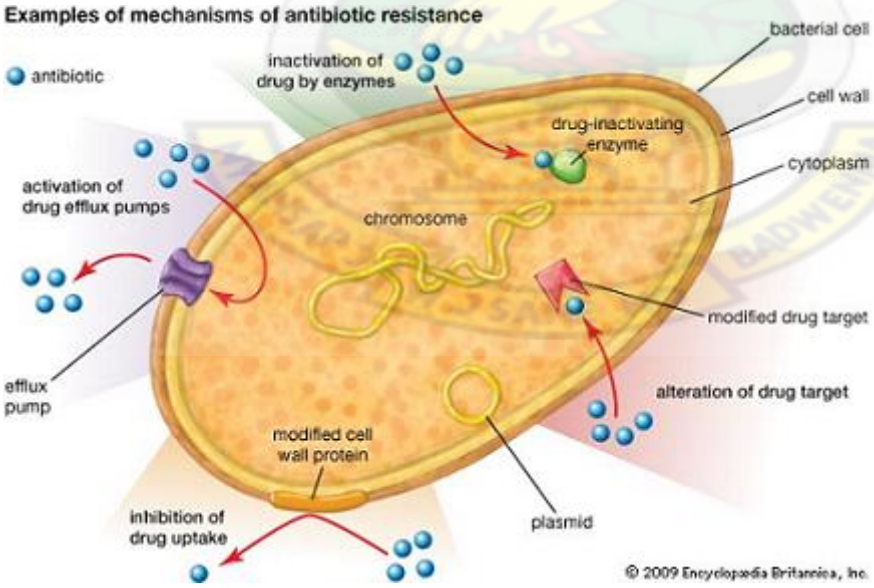

Figure 1.6 Structural mechanism of antibiotic resistance

(source: http://www.britannica.com/media/full/129670)

Beta-lactamases are enzymes produced by bacteria that inactivate beta-lactam antibiotics by hydrolyzing the beta-lactam ring of the beta-lactam molecules. Most beta-lactamases inactivate either penicillins or cephalosporins but some can inactivate both classes of antibiotics (Bush, 1988).

Bacteria

Bacteria are microscopic organisms (Figure 1.7) with their body made of pili (fimbriae), flagellum, capsule, cell wall, cytoplasmic membrane, polysomes and plasmids. Most of them reproduce by simple cell division. Their growth rate is much dependent on certain conditions such as changes in temperature and nutrition. They possess the ability to adapt their shape or functions to environmental changes and some potentially lethal substances, like antibiotics by bacteria (Clément, 2011).

There are three principal forms of classification of bacteria, namely; (a) Spherical or ovoid bacteria which occur as single cells (*micrococci*), or in pairs (*diplococci*), clusters (*staphylococci*), chains (*streptococci*) or cubical groups (*sarcinae*), (*b*) *Rod-shaped* bacteria are termed as *bacilli*, more oval ones are known as *coccobacilli*, and those forming a chain are called as *streptobacilli* and (*c*) *Spiral* bacteria are rigid (*spirilla*), flexible (*spirochaetes*) or curved (*vibrios*).

Bacteria are either Gram-negative or Gram-positive. These two groups are distinguished based on their Gram stain characteristics. Gram-negative bacteria do not retain the primary stains like crystal-violet when washed with alcohol. They are believed to possess cell wall membrane that prevents the penetration of the stain. Pink colonies are observed when counterstained with a secondary dyes such as safranin and they are more resistant to antibiotics (Maczulak, 2010; Schaechter *et al.*, 2012). Examples of this group of bacteria are *Escherichia coli* and *Pseudomonas aeruginosa*. Gram-positive bacteria on the other

hand are able to retain crystal-violet stain in even after washing with alcohol. Examples are *Bacillus subtilis* and *Staphylococcus aureus.*

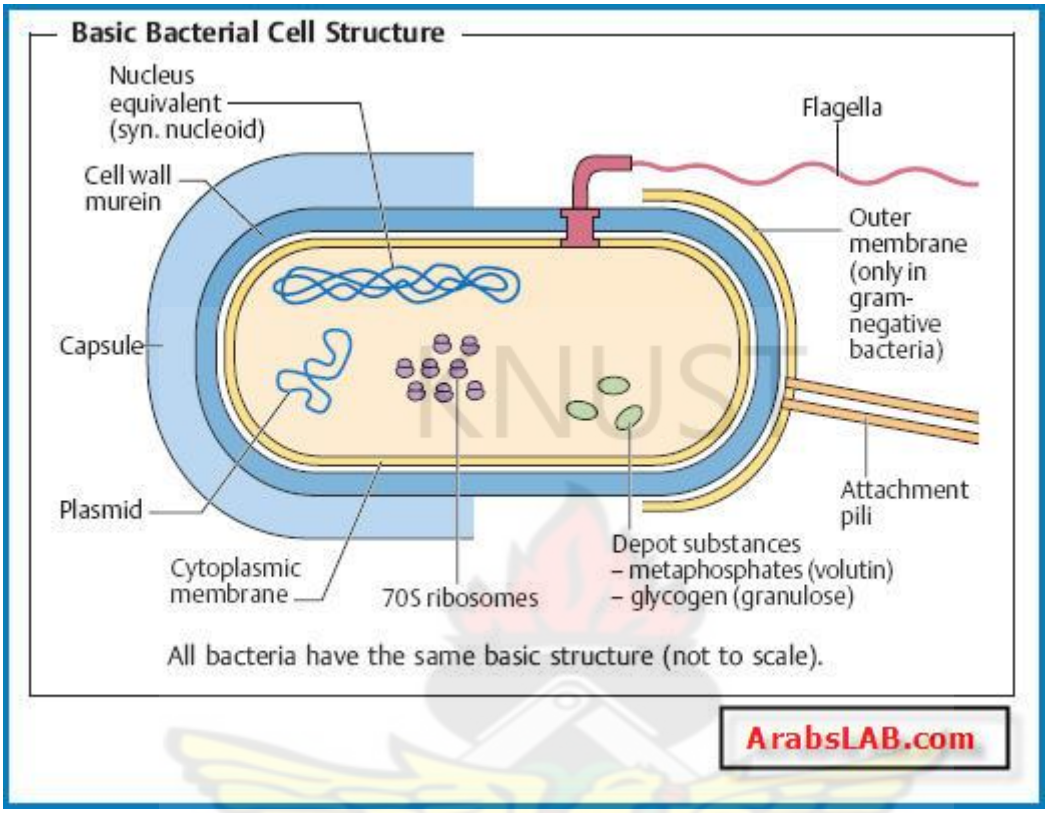

Figure 1.7 Cell structure of bacteria
(Source: http://www.arabslab.com/vb/showthread.php?t=577)

Pseudomonas aeruginosa

This is a Gram-negative bacterium. It is considered resistant opportunistic human pathogen causing serious nosocomial infections (Palleroni, 1984; Rusin *et al.*, 1997). Its pathogenicity is as a result of secretion of numerous toxic compounds and hydrolytic enzymes as well as its ability to resist phagocytosis, however, only few species of this genus cause diseases, the rest are believed to be saprophytic (Adedeji *et al.*, 2007).

Staphylococcus aureus

This is a Gram-positive bacterium which selectively grows on mannitol salt agar and produces colonies surrounded by bright yellow zones. It is a catalase and coagulase

producing and frequently occurs in human nasal passages, mucous membrane or skin of carriers. It is one of the most virulent human pathogens and a leading cause of bone, joint and soft-tissue infections acquired in hospital and in the community. It also causes blood stream infections and endocarditis, and it is a frequent cause of food poisoning. It is the causative agent of boils and variety of infections in both healthy and immune-compromised individuals (Bratu *et al.*, 2005; Nordberg *et al.*, 2005). The level of resistance of *Staphylococcus* to beta-lactams is as high as 60 to 70% (Bratu *et al.*, 2005) which is alarming. Studies have also shown a steadily increasing trend within European countries with MRSA levels around 40% (Nordberg *et al.*, 2005).

Bacillus subtilis

These are common soil micro-organisms, usually recovered from water, air and decomposing plant residues. They are Gram-positive bacterium, rod shaped and chemotrophic, 0.5 to 2.5 µm wide and 1.2 to 10 µm long. Some species are strictly aerobes and others, facultative anaerobes. Their cell walls are typically made up of 20 to 25 layers of peptidoglycan, some lipids, proteins and teichoic acid (which are a distinctive anionic polymer of glycerol phosphate, ribitol phosphate and other sugar phosphate which are not found in Gram-negative). They are distinct from *E. coli* by their cell wall and the ability to produce spores (Waites *et al.*, 2009).

Escherichia coli

Escherichia coli is a Gram-negative bacterium, the only specie contained in the genus Escherichia, in the genera Escherichieae (Qrskov and Orskov, 1984). It is rod-shaped, about 2.5 µm long, 0.8 µm wide and inhabits the gut. Some of them are associated with community and hospital-acquired urinary tract infections, whilst others cause diarrhoeal diseases (Berg, 2003). *E. coli* is said to be one of the most important food-borne pathogens and resistant to broad-spectrum penicillins such as amoxicillin (Nordberg *et al.*, 2005).

1.2.4 Methods for evaluating antimicrobials

The activity or efficacy of an antibiotic is the ability to inhibit bacterial growth or kill them. This is usually determined by different biological method (Agyare *et al.*, 2013), chemical methods (Abreu *et al.*, 2003) or both (Hsu and Hsu, 1992) This helps in the selection of a specific antimicrobial agent in the treatment of microbial infections. It can also provide information on the chemical properties of compounds and their antibacterial activity. This will serve as a guide on how to use them e.g. those used in the food industry as flavor as well as antimicrobial agent (Cosentino *et al.*, 1999).

1.2.4.1 Biological evaluation of antibiotics

Biological methods for the determination the activity and potency of antibiotics have been in existence after Fleming and Heatley used them in the determination of the activity and assay of lysozyme, respectively (Zuluaga *et al.*, 2009). Some studies also combine both the biological and the chemical assays (Hsu and Hsu, 1992) . The efficacy of some antibiotics like penicillins and vancomycin are usually assessed or evaluated using biological activity and the activity compared with reference standards (Diaz *et al.*, 2011). These biological methods include diffusion and dilution methods.

Diffusion methods

Agar well diffusion is one of the widely used methods in ascertaining the activity and the minimum inhibitory concentration (MIC) of antimicrobials. Sir Alexander Fleming is known to be the first to use this method in 1924 (Piddock, 1990) and has being in use since then by many scientists. It has also been used in the determination of antimicrobial activities of agents and reference antibiotics (Agyare *et al.*, 2012). The antibacterial activity of important medicinal plants on human pathogenic bacteria have also been determined using the agar well diffusion method (Girish and Satish, 2008).

It is described that the diffusion of antimicrobial compound into agar that results in concentration gradient inversely proportional to the distance from the well and it measures the degree of inhibition (Zewge, 2006). These antimicrobials are serially diluted and aliquots put into wells of known diameter in the seeded agar. MIC of the antimicrobial agents can be obtained using this method (Bonev *et al.*, 2008; Griffin *et al.*, 2000). However, inability of agents to diffuse well, agar type, salt concentration, incubation temperature and molecular size of the antimicrobial component are the disadvantages of using agar diffusion in determining activity and efficacy of antimicrobials (Agyare *et al.*, 2013). The disc method involves the carefully placement of antibiotic impregnated disc on seeded agar and the zones of inhibition determined.

Dilution methods

The dilution method is a liquid culture method whereby standardized inocula is dispensed into wells that contain different concentrations of antimicrobial agents (Piddock, 1990). It can be performed using macro test tubes or a 96 well micro-titer plate. Assessing the activity and efficacy antibiotics by this method is much preferred to agar diffusion as antimicrobial agent gets directly in contact with test organism and will not have to diffuse through a solid medium as in agar diffusion (Eloff, 1998).

In this test, the minimum amount of antibiotic that inhibits the visible growth of an isolate or minimum inhibitory concentration (MIC) is determined. Bacterial isolate is subjected to various dilutions of antibiotics. The highest dilution or the minimum concentration of antibiotic that inhibits the growth of bacteria is considered as MIC. These tests categorize bacteria as susceptible with, intermediate resistant with 4 mg/L $\leq$ MIC $\leq$ 32 mg/L, intermediate MIC $\geq$ 4 and resistance with MIC $\geq$ 32 (mg/L) as per National Committee for Clinical Laboratory Standards (NCCLS) guidelines (Rybak and Akins, 2001).

1.2.4.2 Chemical methods

Chemical analysis is basically concerned with the separation, identification and quantification of the chemical components of the analyte or substance of interest. These methods are either instrumental or non-instrumental. Non-instrumental methods make use of separations such as precipitation, extraction, and distillation and qualitative analysis by colour, odour or melting point (Kavittha *et al*., 2012). Quantitative analysis is achieved by measurement of weight or volume. Instrumental methods are based on the measurement of some physical properties of substance using instrument to determine its active compound. The instrumental methods are simple, precise, and reproducible as compared to non-instrumental methods. Therefore, analytical methods developed using sophisticated instruments such as diode array spectrophotometer, high pressure liquid chromatography, gas chromatography and high pressure thin layer chromatography have wide application by ensuring the quality and quantity of raw materials and finished products.

High pressure liquid chromatography method (HPLC)

This was developed from a number of separation methods, e.g. adsorption and partition chromatography. In this method, the resolving agent is packed into a column and the rate of separation depends on the number of theoretical plates. It is the separation method of choice because of its high sensitivity, ability to separate highly volatile compounds and accuracy in quantitation. The method is preferred to thin layer chromatography (TLC) due to its complete control over the mobile phase (Ajibola, 2000).

Method Validation

For a method to be accepted to produce valid results, it must go through a process of validation. Chemical analysis like any other analytical method must be validated before

application in the analysis of sample (Épshtein, 2004) because without it, the outcome will not be reliable (Diaz *et al.*, 2011).

There are various accepted documents that provide the necessary step by step procedure for analytical method validation. The United State Pharmacopoeia and International Conference on Harmonization (ICH-Q2B, 1996) Guideline Validation of Analytical Procedures for industry are some of the documents. They present discussions of the characteristics for consideration during the validation of the analytical procedures included as part of registration applications submitted within the European Union, Japan and the United States.

Analytical Procedure

Analytical procedure basically refers to all detailed activities performed in the validation process which include, but is not limited to, test sample, reference standard and reagent preparation. The use of the apparatus, selection of suitable internal standard, determination of average ratio for the generation of the calibration curve as well as use of the formulae for the calculation are all considered part of the analytical procedures (ICH, 2005a).

Types of analytical procedures that needs to be validated

The following are the four most common types of analytical procedures:

- Identification tests
- Quantitative tests for impurities' content
- Limit tests for the control of impurities
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product (ICH, 2005b).

Identification tests are intended to ensure the identity of an analyte in a sample. This is normally achieved by comparison of a property of the sample (e.g. spectrum,

chromatographic behavior, chemical reactivity etc.) to that of a reference standard. Testing for impurities can be either a quantitative test or a limit test for the impurity in a sample. Either test is intended to accurately reflect the purity characteristics of the sample. Different validation characteristics are required for a quantitative test than for a qualitative test.

Assay procedures are intended to measure the analyte present in a given sample. Assay represents a quantitative measurement of the major component(s) in the drug substance. For the drug product, similar validation characteristics are also applied when assaying for the active or other selected component(s). The same validation characteristics may also apply to assays associated with other analytical procedures e.g. dissolution (ICH-Q2A, 1995).

Validation characteristics

The following are the characteristic parameters to be considered in method validation ICH.

1. *Specificity*

This is the ability to assess unmistakably the analyte in the presence of other components such as impurities, which may be present (ICH, 2005a). In effect, it is the measure of relative retention of two components in a mixture and shows how selective the method is thus, representing the separation power of particular adsorbent to the mixture of these particular components. This may, however, be compensated by other supporting analytical procedure(s). This definition implies that the identity of the analyte is ensured. There is also the provision of results that give an accurate statement of the content of analyte in a sample. Some of the analytical methods used to determine specificity include percent recovery, minimum difference from baseline and analysis of variance (USP, 2011; Walfish, 2006).

2. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference

value and the value found (ICH, 2005a). The accuracy of a method can be assessed by comparing the results of the method developed with results from an established reference method, by analyzing a sample with known concentrations (e.g. certified reference material) and comparing the measured value with the true value as supplied with the material (Kavittha *et al*., 2012). It may also be inferred when precision, linearity and specificity have been established (ICH, 2005b).

3. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels namely repeatability, intermediate precision and reproducibility. Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of series of measurements.

Repeatability expresses the precision under the same operating conditions over a short interval of time and can also be termed intra-assay precision (ICH, 1996).

Intermediate precision expresses within laboratories variations including different days of analysis, different analysts, different equipment etc.

Reproducibility expresses the precision of method between different laboratories.

4. Limit of detection

The detection limit of an assay is the lowest concentration that can be detected but not necessarily quantified (ICH, 2005a). ICH guidelines suggest three different methods for determining the detection and quantification limits. These methods are visual

determination, signal-to-noise ratio 3 or 2:1 determination, and standard deviation and slope method (Kavittha *et al*., 2012). The signal-to noise ratio method is suggested to be the most logical, since it is based on comparing low levels of the analyte to a blank or background sample (Walfish, 2006).

5. Limit of quantification

The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy (ICH, 2005a) or parameter of quantitative assays for low levels of compounds in sample (Kavittha *et al*., 2012). Methods of determination of the limit if detection can also be applied but at a ratio of 10:1 (ICH-Q2B, 1996).

6. Linearity

An analytical procedure is said to be linear when it has the ability to obtain test results directly proportional to the concentration of analyte in a sample within a given range (ICH, 1996). This can be deduced from the straight line of the calibration curve with an R square close to one. It can be reported by y-intercept, correlation coefficient, residual sum of squares and slope of regression (ICH-Q2B, 1996). This shows how straight the line is with analyte directly proportional to the average area peak ratio (Kavittha *et al*., 2012).

7. System suitability test

This test is commonly used to verify resolution, column efficiency, and repeatability of a chromatographic system to ensure it is suitable for a particular analysis and considered an integral part of many analytical procedures according to the United States Pharmacopeias (USP) and the International Conference on Harmonization (ICH) (Kavittha *et al.*, 2012).

8. Range

The range of an analytical procedure is the interval between the upper and lower concentration (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 (ICH, 1996).

9. Robustness

The robustness of an analytical procedure is its ability to be reliable and remain unaffected by small, except for deliberate variations in method parameters (ICH, 1996).

JUSTIFICATION OF RESEARCH

Although substandard/counterfeit is a problem in almost all categories of antimicrobials, report on counterfeit antibiotics alone is about 8 to 10 times whereas anti-parasitic medicines are about 2 to 3 times as compared to other classes of medicines (Kelesidis *et al.*, 2007). Evaluation of antibiotics to determine their activity and potency to effect treatment is a necessity since that is the ultimate goal for their production, most importantly in the developing countries which have higher percentages of infectious diseases (Okeke *et al.*, 1999). The problem is worsened due to the highest prevalence rates of 1 to 10% of counterfeit medicines in both developed and developing countries (WHO, 2006). Apart from the medical consequences like the high cost involved with the treatment of antibiotic resistant infections as well as finance resource spent on poor quality medicines and ultimately death is alarming (Mali, 2003).

Substandard medicines pose a threat to the lives of patients as evident in various studies (Acar and Rostel, 2001; Lohsiriwat *et al.*, 2009; Mamelli *et al.*, 2009) and needs much attention. When the quality of medicines used against a particular disease is lost due to resistance, steps in prevention and treatment must be revisited. Research and development

of new medicines are expensive and time consuming. Consequently, the quality of the already developed medicines must be protected (Kettler, 2002).

Various chemical analysis including HPLC and microbiological methods confirm the existence of substandard and counterfeit antibacterial agents (Newton *et al.*, 2006). Due to the inevitable variations in using living organisms for biological evaluations, and the resistance of microorganisms to even chemically potent medicines, their evaluation require both chemical and biological approaches. This will provide information on their quality and efficacy (Gilbert *et al.*, 1987).

The choice of the type of antibiotics for the current study was based on the prescription and dispensing pattern observed in series of studies on dispensing patterns of medicines. Penicillins form part of the commonly used antibiotics in Ghana (Helegbe *et al.*, 2009). Amoxicillin was found to be the most frequently dispensed antibiotic in a survey conducted in Australia (McManus *et al.*, 1997). In a study on the prescription pattern in a pediatric outpatient department in Mumbai, India, penicillins constituted 87.6% of the total antibiotics prescribed (Karande *et al.*, 2005). A similar observation has also been made in Ikeja General Hospital in Lagos, Nigeria where the penicillins were again found to be the one of the most prescribed antibiotics (Odusanya, 2005).

AIMS

The aim of this study was to determine the antibacterial activity and develop HPLC methods to analyze various samples of amoxicillin, flucloxacillin and cloxacillin on the Ghanaian market.

The following were the specific objectives:

- To determine activity and minimum inhibitory concentrations (MIC) of samples of amoxicillin, flucloxacillin and cloxacillin against selected bacteria using the agar diffusion and the micro-dilution methods.
- To develop and validate HPLC method for the assay of samples of amoxicillin, flucloxacillin and cloxacillin samples.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Reference drug samples

All reference drug samples used were obtained from Ghana Standard Authority (GSA). The samples and their manufacturers are as follows: Amoxicillin trihydrate and cloxacillin (Unichem Industries Ltd, India), caffeine anhydrous, flucloxacillin BP compacted and acetaminophen (Paracetamol BP) were purchased from Vardhman Chemist Ltd., India.

Chemicals and equipment

All chemicals used for the HPLC analysis including reference compounds such as amoxicillin trihydtrate, flucloxacillin, cloxacillin, caffeine anhydrous and acetaminophen, solvents etc. were of analytical and chromatographic grade purchased from Sigma-Aldrich, Germany unless otherwise stated and they were available in the Forensic Laboratory, Ghana Standard Authority, Accra, Ghana. All materials and equipment used in the microbiological evaluation are available in the Microbiology Section, Department of Pharmaceutics, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

Chemical/item	Company/Manufacturer	Place/Country
Methanol chromosolv (HPLC grade)	Sigma-Aldrich	Germany
Hydrochloric acid (HPLC grade)	Merck Ltd.	India
Potassium dihydrogen phosphate	VWR Int. BDH Prolaborator	England
Methylthiazolyl tetrazolium chloride	Sigma-Aldrich	Michigan, USA
Finigan SpectraSYSTEM SCM 1000 HPLC	Spectra system	USA
spectra systems FL 3000 detector	Spectra system	USA
UV1000 Detector	Spectra system UV 1000	USA
Quaternary gradient pump	Spectra system P 4000	USA
Degasser	Spectra system SCM1 100	USA
auto sampler	Spectra system AS 3000	Germany
Shim pack CLC-NH_2 C18 column (150 x 4.6) mm, 5 µm	Aloma Shim	USA
Shim-pack CLS ODS (M) (250 x 4.6) mm, 5 µm.	Aloma Shim	USA
Whatman cellulose membrane filter paper of pore size, 0.45 Millipore	Whatman plc	UK
Electronic weighing balance	Ohaus corporation	Pine Brook New Jersey, USA
Volumetric flasks (10, 20, 50, 100 mL 1 L)	Sarstedt, Damstadt	Germany
Injection vials	Danyang Xianghe Pharmaceutical Packaging Ltd	Jiangsu, China
Microbiological materials		
Eosine Methylene Blue agar	Scharlau	UK
Defribrinated Horse blood,	Oxoid	UK
Koser's citrate media	Oxoid	UK
Nutrient broth	Oxoid	UK
Plate Count Agar	Oxoid	UK
A-96 Microtitre plates	Sarstedt	USA
Portable autoclave	Basildon, Ltd.	UK
Hot air oven	OMT Oven,Gallenkamp	UK
Incubator	Gallenkamp	UK
UV spectrophotometer	PG Instruments	UK
UV lamp	UVP	USA
Lamina air flow cabinet	Model T2 2472 Skan, AG,	Switzerland
Thermostatically controlled water bath	R76 New Brunswick, Edison	N.J., USA
Colony counter	Gaber Instruments	AG, Holland
No. 5 Cork borer	Gaber Instruments	AG, Holland
Beakers (10, 20, 50, 500, 1000 mL)	Sarstedt, Damstadt	Germany
Petri dishes	Sarstedt, Damstadt	Germany

Test bacteria

Four bacteria strains consisting of two Gram-negative and two Gram-positive bacteria were used for the microbiological evaluation. All organisms were typed cultures stored at the Microbiology Research Laboratory, Department of Pharmaceutics, KNUST, with the following identities: *Echerichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 4853, *Staphyloccocus aureus* ATCC 25923 and *Bacillus subtilis* NTCC 10073.

Test samples

Imported and locally manufactured antibiotics of interest (Table 2.1) were purchased randomly from different Pharmacies in Accra and Kumasi. The reasons for the choice of samples were to compare different brands and different batches within a brand. Sampling was done between the periods of October 2011 and May 2012.

Table 2.1: Test samples of antibiotics (penicillins) evaluated

Amoxicillin		*Flucloxacillin*		*Cloxacillin*	
Sample code	Expiry date	Sample code	Expiry date	Sample code	Expiry date
Capsules (250 mg)		Capsules (250 mg)		Capsules (250 mg)	
01A	11/2013	FLMG01	11/2014	CLLP	09/2013
01B	03/2012	FLMG02	05/2014	CLLP	11/2013
02A	03/2014	FLMG03	05/2014	CLAR	07/2014
02B	09/2014	FLLP04	10/2013	CLAR	12/2014
03A	05/2014	FLLP05	06/2013	CLMG	01/2014
03B	07/2014	FLLP06	06/2013		
03C	01/2015	FLAR07	11/2015		
04A	01/2013	FLAR08	04/2015		
05A	08/2013				
06A	11/2013				
06B	12/2013				
06A	11/2013				
06C	04/2014				
08A	01/2013				
Capsules (500 mg)					
07A	05/2014				
07B	02/2014				
09A	07/2014				
Suspension (125 mg/ 5 mL)		Suspension (125 mg/ 5 mL)		Suspension (125 mg/ 5 mL)	
SO1	01/2012	FLSMG01	03/2014	CLSLP	12/2013
SO2A	12/2013	FLSMG02	09/2013	CLSLP	10/2014
SO2B	11/2013	FLSMG03	09/2014	CLSLP	11/2013
SO2C	12/2013	FLSLP04	07/2013	CLSMG	11/2012
SO3A	07/2014	FLSLP05	03/2013		
SO4A	08/2013	FLSLP06	02/2014		
SO5A	06/2013	FLSAR07	05/2015		
SO6A	12/2013	FLSAR08	02/2015		
SO6B	12/2013				
SO6C	02/2014				
SO7A	06/2013				
SO8A	03/2015				
SO8B	01/2015				

2.2 METHODS

2.2.1 Antimicrobial evaluation

2.2.1.1 Determination of antimicrobial activity

The antimicrobial activity was determined using modified method described by Agyare et al. (2012) and Girish and Satish (2008). Twenty (20) milliliters stabilized agar at 45°C was seeded with 100 µL of 10^5 colony forming units (cfu)/mL of 18 to 24 h broth culture of *Staphylococcus aureus* and rolled in the palm for uniform distribution and was aseptically poured into sterilized petri dish and allowed to set. Four wells were bored with a sterile cork borer with diameter of 10 mm. The wells were filled with 200 µL each of respective concentrations and allowed to stand for 1 hour on the bench to allow diffusion of antibiotic. The plate was then incubated at 37°C for 24 hours and zones of growth inhibition recorded in millimeter (mm). The method used was repeated for all test samples in triplicate for *Bacillus subtillis, Escherichia coli* and *Pseudomonas aeruginosa*. Concentrations used were 0.125 to 1.0 µg/mL for amoxicillin samples and 1.25 to 10.0 mg/mL for flucloxacillin and cloxacillin samples.

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations of the various antibiotics were determined using the method described by Agyare *et al.* (2012). Sterile 96-well microtitre plates were labeled appropriately for *Staphylococcus aureus*. Total volumes of 200 µL were prepared by dispensing a fixed volume of 100 µL sterile double strength nutrient broth and 20 µL (10^5 cfu/mL) of 18-24 hour culture was aseptically added to the medium. Amoxicillin samples were evaluated within concentration range of 0.1 to 0.5 mg/mL. The MIC of flucloxacillin and cloxacillin samples was determined within a concentration range of 0.5 to 2.2 mg/mL.

Experiments were performed in triplicate under the same conditions for all samples. Reference samples were prepared and the MIC determined under the same conditions as described above.

The plates were incubated for at 37°C for 24 h. Microbial growth was determined by addition of 30 µL 3-(4,5-dimethylthiazole -2-yl)-2,5-diphenyltetrazolium bromide (MTT) after incubation and as growth of organism was indicated by purple to blue coloration and yellow coloration indicated no growth of organism. The well with least concentration of test sample without bacterial growth recorded as the MIC. The procedure above was repeated for all test samples using *E. coli*, *B. subtillis* and *P. aeruginosa* respectively.

2.2.2 Chemical analysis

Reference amoxicillin trihydrate sample was dissolved in 0.1M hydrochloric acid. Samples were analyzed at concentrations of 5.26, 10.52, 15.78, 21.04 and 26.3 µg/mL with an injection volume of 100 µL. Reference flucloxacillin and cloxacillin samples were dissolved in sterile distilled water. They were analyzed at concentrations of 25.35, 50.7, 101.4, 152.1 µg/mL and 11.72, 23.44, 35.16, 58.6 µg/mL for reference standard and the sample respectively, with an injection volume of 1 mL. All samples were analyzed under isocratic conditions with Shim-pac CLS ODS (M) C18 column for amoxicillin. Shim pac CLC-NH_2 C18 column was used in analysis of flucloxacillin and cloxacillin. An internal standard of 1025 µg/mL caffeine anhydrous was used in the development of HPLC method for amoxicillin and analysis of amoxicillin samples. Concentrations of 1.4156 µM and 1.3296 µM of acetaminophen (paracetamol) were used for the HPLC method development for flucloxacillin and cloxacillin respectively. The same concentrations were used for the analysis of flucloxacillin and cloxacillin samples.

Table 2.2 Chromatographic conditions under which samples were analyzed

	Amoxicillin	Flucloxacillin and cloxacillin
Detection	UV(230 nm)	UV (225 nm)
Flow rate	1.2 mL /min	1 mL /min
Mobile phase A	MeOH	MeCN
Mobile phase B	0.01 M KH_2PO_4	0.01 M KH_2PO_4
Gradient	65:35 (MeOH: 0.01 M KH_2PO_4)	60:40 (MeCN: 0.01 M KH_2PO_4)

AUC=area under curve; MeOH=methanol; MeCN=acetonitrile

Preparation of test sample solution

Concentrations of amoxicillin equivalent to 15.78 µg/mL were prepared. They were dissolved in 0.1M hydrochloric acid and mobile phase A (Table 2.2). Equivalent of 50.7 and 11.72 µg/mL of flucloxacillin and cloxacillin were prepared. Samples were dissolved in sterile distilled water and mobile phase A (Table 2.2).

Statistical analysis

All graphs were plotted with Excel version 2010 and graph pad prism (Graph Pad Prism 5 Software, San Diego, CA, USA) for all the statistical analysis. Data analysis was by one-way analysis of variance (ANOVA). There is not enough evidence at alpha = 0.05 and the model for the method development is not significant since F-value > F-crit and P < 0.05 (alpha). ChromQuest and Endnote X6 **(**Bld 6348**)** were used to generate HPLC analysis data and references respectively.

CHAPTER 3

RESULTS

3.1 MICROBIOLOGICAL EVALUATION

The antimicrobial evaluation was performed using modified method described by Agyare *et al*. (2012) and Girish and Satish (2008). The antibacterial activity and minimum inhibitory concentrations (MICs) of the samples of amoxicillin, flucloxacillin and cloxacillin were determined using the agar diffusion and micro-dilution methods respectively. The zones of inhibition of the various concentrations of the samples against the test bacteria were measured and the mean zones of inhibition determined. The MICs of the antibiotic samples were detected using MTT reagent to determine the lowest concentration of samples that showed no growth in the 96-well plates.

MICs of capsules were within the range of 200 to 800 µg/mL for amoxicillin test samples and $\geq$ 800 to 1900 for flucloxacillin and cloxacillin test samples. Reference samples showed lower MICs of 200 µg/mL against *E. coli*, 500 µg/mL against *P. aeruginosa*, 300 µg/mL against *B. subtilis* and 200 µg/mL against *S. aureus* as compared with the test samples for amoxicillin. MICs of flucloxacillin reference sample were 800 µg/mL against *E. coli*, 1500 µg/mL against *P. aeruginosa*, 1400 µg/mL against *B. subtilis* and 1400 µg/mL against *S. aureus*. MICs for cloxacillin reference sample were 800 µg/mL *against* E. *coli*, 1500 µg/mL against *P. aeruginosa*, 1500 µg/mL against *B. subtilis* and 1500 µg/mL for *S. aureus* (Table 3.1).

Table 3.1 MICs of Samples of amoxicillin, flucloxacillin and cloxacillin (capsules)

Sample	**Batches**	**Organisms/MIC(µg/mL)**			
		E. coli	***P. aeruginosa***	***B. subtilis***	***S. aureus***
Reference		200	500	300	200
01A	B7M1/11	200	500	400	400
01B	BO5M03/12	300	700	500	500
02A	3808L	300	700	400	200
02B	4209L	400	800	400	300
03A	AM092	200	600	300	300
03B	AM096	200	700	300	300
03C	AM105	200	600	300	300
04A	02185G	200	700	300	300
05A	AX76P	300	600	300	300
06A	1000961	400	800	400	400
06B	1000092	400	700	500	300
06C	1000372	300	700	500	400
07A	T7021044-06/11	200	500	300	200
07B	T71015Z	400	800	400	400
08A	103702	300	700	400	400
09A	MP11173	300	500	300	200
		FLUCLOXACILLIN			
Reference		800	1500	1400	1400
FLMG01	FLT5T	1300	1900	1500	1500
FLMG02	FL74S	1200	1700	1400	1500
FLMG02	FL77S	800	1500	1500	1500
FLLP04	0230170	1300	1800	1500	1500
FLLP05	0230122	1200	1600	1500	1500
FLLP06	0230121	1300	1700	1500	1500
FLAR07	FL029	800	1600	1500	1500
FLAR08	FL026	800	1600	1500	1500
		CLOXACILLIN			
Reference		800	1500	1500	1500
CLLP01	1110062	800	1500	1500	1500
CLLP02	1110102	900	1600	1500	1500
CLLP03	1110092	800	1500	1500	1500
CLAR04	CX022	900	1600	1500	1500
CLAR05	CX020	800	1500	1500	1500
CLMG06	CL25S	800	1400	1500	1400

MIC = minimum inhibitory concentration, µg/mL = microgram per millilitre

Table 3.2 represents results from the biological screening of suspensions. Evaluation of samples gave MICs within the range of 200 to 700 µg/mL for amoxicillin test samples, 800 to 1600 for flucloxacillin and 800 to 1700 cloxacillin test samples.

Table 3.2 MICs of suspension amoxicillin, flucloxacillin and cloxacillin samples

Sampls	*Batches*	*Organisms/MIC(µg/ mL)*			
		E. coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*
		AMOXICILLIN			
S01	565886	300	600	300	200
S02A	0270072	200	500	300	200
S02B	0271281	300	500	300	200
S02C	0270052	300	600	300	200
S03A	0110701407	200	500	300	200
S04A	D1105	300	600	300	200
S05A	2706L	300	500	300	200
S06A	AXS383	200	500	300	200
S06B	AXS383	300	700	400	300
S06C	AXS424	300	600	300	300
S07A	71	200	500	300	200
S08A	AS100	200	500	300	200
S08B	AS108	200	500	300	200
		FLUCLOXACILLIN			
FLSMG01	EMS-443	800	1500	1400	1400
FLSMG02	EMS-596	800	1600	1400	1400
FLSMG03	EMS-343	800	1500	1400	1500
FLSLP04	0390172	800	1600	1400	1600
FLSLP05	0390292	800	1600	1600	1600
FLSLP06	0390412	800	1500	1500	1400
FLSAR07	FS093	800	1500	1400	1400
FLSAR08	FS082	800	1500	1600	1400
		CLOXACILLIN			
CLSLP01	0280052	800	1500	1500	1600
CLSLP02	0280012	800	1700	1500	1500
CLSLP03	0280081	800	1600	500	1500
CLSMG04	CLS-183	800	1500	1600	1600
CLSMG05	CLS-184	800	1600	1600	1600

MIC = minimum inhibitory concentration, µg/mL = microgram per millilitre

Antimicrobial activity of samples (Agar diffusion method)

Table 3.3 Mean zones of inhibition ± SEM of test samples (capsules)

Samples	***ORGANISMS***				
	Concentrations(µg/ mL)	***Staphylococcus aureus***	***Escherichia coli***	***Bacillus subtillis***	***Pseudomonas aeruginosa***
		AMOXICILLIN			
01A	1000	22.33±0.82	16.00±0.63	20.50±0.55	21.67±0.52
	500	20.83±0.75	12.67±0.52	18.50±0.55	19.33±0.52
	250	25.00±0.00	12.00±0.00	18.17±0.41	17.83±0.75
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
01B	1000	25.83±0.41	26.66±0.52	24.67±0.82	21.67±0.52
	500	25.00±0.63	24.67±0.82	23.00±0.63	19.67±0.82
	250	22.67±0.52	22.67±0.52	21.00±0.89	18.33±1.37
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
02A	1000	25.67±1.03	24.00±0.9	19.00±00	23.50±0.55
	500	23.33±1.03	17.50±0.55	14.17±0.75	22.50±0.84
	250	22.17±0.41	16.17±0.75	17.00±0.00	21.50±0.55
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
02B	1000	25.33±0.52	23.33±1.21	25.33±0.51	23.00±0.89
	500	24.50±1.38	22.50±0.55	24.83±0.98	20.83±1.17
	250	22.50±1.05	18.50±1.05	22.67±0.52	18.50±0.84
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
03A	1000	24.8.±0.41	20.83±0.52	24.50±0.84	0.00±0.00
	500	21.83±0.41	23.83±0.75	24.00±0.89	0.00±0.00
	250	20.83±0.41	18.83±0.75	22.50±0.84	0.00±0.00
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
03B	1000	25.83±0.98	20.83±0.75	24.83±0.75	20.67±1.03
	500	22.67±1.21	18.00±0.63	23.83±0.41	17.83±0.75
	250	21.17±0.98	12.67±0.52	20.67±0.82	16.33±0.82
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
03C	1000	24.67±1.00	18.67±0.52	23.50±0.55	0.00±0.00
	500	22.17±1.17	16.83±0.98	21.33±1.37	0.00±0.00

	250	20.67±1.21	15.00±0.89	20.50±1.38	0.00±0.00
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
04A	1000	26.57±1.05	20.71±0.36	23.86±0.75	0.00±0.00
	500	22.14±0.84	18.43±0.52	21.57±0.52	0.00±0.00
	250	21.43±1.21	22.50±0.71	20.29±0.82	0.00±0.00
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
05A	1000	30.00±0.89	23.00±0.00	24.50±0.84	0.00±0.00
	500	27.67±1.03	26.00±0.00	21.33±1.03	0.00±0.00
	250	25.67±1.03	24.00±0.00	20.17±0.98	0.00±0.00
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
06A	1000	21.00±0.00	18.87±0.4	22.83±0.14	22.00±0.00
	500	20.00±0.00	22.00±0.00	22.30±0.18	21.67±0.18
	250	18.00±0.00	21.00±0.17	21.00±0.18	20.00±0.00
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
06B	1000	25.50±0.55	15.50±0.84	16.00±0.82	0.00±0.00
	500	24.50±0.84	12.67±0.52	12.00±0.82	0.00±0.00
	250	23.33±1.03	0.00±0.00	0.00±0.00	0.00±0.00
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
07A	1000	24.50±0.84	20.00±0.00	24.50±0.55	23.17±0.41
	500	21.83±1.17	19.83±1.17	22.83±0.75	22.50±1.05
	250	20.50±1.22	19.17±1.17	20.67±1.21	19.00±1.10
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
07B	1000	24.33±0.82	20.17±0.75	23.50±0.84	22.17±0.75
	500	21.67±0.52	19.67±1.03	23.00±1.10	0.00±0.00
	250	20.17±0.75	19.00±0.89	20.50±0.84	0.00±0.00
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
08B	1000	24.33±0.52	19.33±1.03	21.50±0.84	20.50±0.55
	500	22.17±0.75	17.5±0.55	18.67±0.82	16.33±0.82
	250	21.33±1.03	16.00±0.89	15.53±0.55	22.00±0.00
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
09A	1000	25.17±0.41	21.83±0.98	25.00±0.89	20.50±1.38
	500	23.50±0.55	21.17±0.98	24.17±0.75	18.83±0.98
	250	22.17±0.75	18.33±0.52	21.50±1.38	17.33±1.37

	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
			FLUCLOXACILLIN		
FLMG01	10000	23.17±0.41	26.30±0.28	22.83±0.59	23.17±0.63
	5000	17.00±0.63	20.00±0.22	21.17±0.34	23.83±0.51
	2500	17.00±0.89	20.33±0.18	20.83±0.45	22.67±0.36
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
FLMG02	10000	25.67±1.21	21.33±1.03	28.67±1.03	19.83±0.98
	5000	22.50±1.38	19.50±1.22	27.67±1.21	17.00±0.52
	2500	20.67±0.81	18.50±0.55	24.50±0.84	16.00±0.00
	1250	17.67±1.37	17.00±0.00	19.00±0.69	14.75±0.50
FLMG03	10000	31.67±0.82	18.67±0.52	29.50±1.22	0.00±0.00
	5000	29.50±0.55	18.17±0.75	27.83±0.98	0.00±0.00
	2500	28.33±0.82	16.00±0.82	27.67±0.82	0.00±0.00
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
FLLP04	10000	30.67±1.10	24.17±0.41	30.50±0.55	0.00±0.00
	5000	27.33±0.52	19.67±0.51	27.83±0.41	0.00±0.00
	2500	26.83±0.41	0.00±0.00	27.00±0.63	0.00±0.00
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
FLLP05	10000	30.67±1.17	24.00±0.63	30.50±0.84	0.00±0.00
	5000	27.33±0.52	19.67±1.03	27.83±0.75	0.00±0.00
	2500	26.83±0.98	0.00±0.00	27.00±0.63	0.00±0.00
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
FLLP06	10000	26.17±0.41	25.50±0.55	24.00±0.63	22.17±0.98
	5000	24.33±0.52	23.00±0.63	23.50±0.55	21.17±0.75
	2500	22.67±0.52	21.67±0.51	21.00±0.08	16.83±0.75
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
FLAR07	10000	24.00±0.50	23.67±1.18	25.50±0.68	20.67±0.89
	5000	21.50±0.68	18.00±0.53	24.30±0.18	24.00±0.00
	2500	17.83±1.00	16.00±0.63	23.30±0.18	20.00±0.00
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
FLAR08	10000	20.00±0.89	22.67±0.82	25.83±0.41	0.00±0.00
	5000	18.50±0.55	19.33±0.52	22.50±1.05	0.00±0.00
	2500	0.00±0.00	16.33±0.07	0.00±0.00	0.00±0.00

	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
			CLOXACILLIN		
CLLP01	10000	29.83±0.41	21.83±0.41	29.50±0.84	25.17±0.41
	5000	27.83±0.98	19.33±0.52	26.67±0.52	25.33±0.82
	2500	26.17±0.40	20.17±0.41	25.67±0.52	23.50±0.55
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
CLLP02	10000	29.17±0.41	22.00±1.26	28.50±0.55	29.50±0.55
	5000	27.50±0.84	21.50±0.55	28.33±0.52	26.00±0.00
	2500	25.17±0.41	20.83±0.75	24.33±0.52	25.67±0.51
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
CLLP03	10000	29.00±0.89	26.33±1.03	27.33±0.82	22.67±0.82
	5000	28.17±1.33	23.67±0.82	26.00±0.89	16.67±0.52
	2500	26.33±1.03	22.50±0.84	24.00±0.89	15.17±0.75
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
CLAR03	10000	26.50±1.38	14.33±1.37	26.50±0.84	18.50±1.38
	5000	23.50±1.00	0.00±0.00	24.83±0.75	14.83±0.41
	2500	0.00±0.00	0.00±0.00	23.67±0.82	12.00±0.63
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
CLAR04	10000	26.17±0.98	21.00±0.89	24.17±1.17	17.50±0.55
	5000	23.00±0.89	23.17±0.75	25.67±0.52	11.50±0.55
	2500	23.33±0.52	23.17±1.17	20.30±0.52	0.00±0.00
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
CLMG	10000	27.50±0.84	20.83±0.98	23.67±0.82	27.67±1.21
	5000	25.17±0.41	25.33±0.52	23.50±0.55	24.83±0.41
	2500	23.17±0.41	22.33±0.52	22.50±0.55	24.17±0.47
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

± SEM = standard error mean

Table 3.4 Mean zones of inhibition ± SEM of test samples (suspensions)

		ORGANISMS			
Sample	**Concentration(µg / mL)**	***Staphylococcus aureus***	***Escherichia coli***	***Bacillus subtillis***	***Pseudomonas aeruginosa***
S01A	1000	21.83±1.22	18.00±0.68	22.50±0.81	0.00±0.00
	500	19.67±0.91	15.83±0.31	21.33±0.76	0.00±0.00
	250	18.67±0.91	15.00±0.00	18.83±0.42	0.00±0.00
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
S02A	1000	14.00±0.22	19.83±0.14	16.17±0.14	13.00±0.31
	500	15.50±0.19	19.50±0.29	15.00±0.22	11.50±0.19
	250	13.50±0.19	18.00±0.00	13.33±0.18	0.00±0.00
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
S02B	1000	19.33±0.18	19.50±0.29	19.83±0.26	0.00±0.00
	500	11.67±0.18	16.83±0.26	18.33±0.60	0.00±0.00
	250	15.17±0.14	15.67±0.28	15.38±0.34	0.00±0.00
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
S02C	1000	18.33±0.17	18.00±0.22	18.17±0.14	0.00±0.00
	500	15.80±0.29	16.33±0.18	15.17±0.14	0.00±0.00
	250	12.50±0.19	12.00±0.00	12.17±0.14	0.00±0.00
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
S03A	1000	20.00±0.00	17.67±0.28	20.33±0.56	22.67±0.28
	500	18.67±0.28	17.00±0.00	18.50±0.57	20.33±0.36
	250	19.17±0.45	14.67±0.28	18.00±0.38	19.67±0.18
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
S04A	1000	17.33±0.18	16.83±0.14	21.17±0.26	18.33±0.18
	500	15.83±0.14	14.67±0.17	20.17±0.14	17.33±0.28
	250	15.00±0.00	13.00±0.00	19.00±0.00	14.67±0.18
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
05A	1000	16.67±0.18	24.83±0.26	17.00±0.22	26.33±0.18
	500	16.00±0.22	23.00±0.53	15.33±0.28	24.50±0.19
	250	14.67±0.28	22.33±0.78	14.00±0.22	21.67±0.18
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
S06A	1000	20.00±0.00	16.83±0.14	17.83±0.14	28.67±0.18

	500	16.33±0.18	16.00±0.22	14.67±0.18	25.00±0.38
	250	14.00±0.00	12.67±0.18	12.67±0.18	22.17±0.34
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
S06B	1000	19.50±0.19	20.67±0.18	18.33±0.18	20.62±0.18
	500	17.50±0.29	20.50±0.29	16.83±0.14	17.00±0.00
	250	14.83±0.14	21.67±0.36	16.00±0.30	16.83±0.45
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
S06C	1000	19.83±0.14	19.83±0.40	15.17±0.34	22.50±0.42
	500	15.50±0.19	19.00±0.31	14.50±0.19	21.33±0.36
	250	16.67±0.18	17.17±0.14	13.83±0.14	16.33±0.18
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
S07A	1000	24.67±0.86	19.67±0.36	20.00±0.00	13.17±0.40
	500	19.50±0.36	19.0±0.22	18.67±0.18	17.17±0.63
	250	17.67±0.41	18.33±0.52	18.50±0.29	0.00±0.00
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
S08A	1000	20.33±0.18	19.33±0.18	20.00±0.00	24.67±0.36
	500	19.17±0.14	18.16±0.14	18.50±0.48	22.50±0.19
	250	18.50±0.29	16.00±0.22	17.17±0.40	20.33±0.18
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
S08B	1000	22.00±0.00	17.67±0.56	20.17±0.14	25.83±0.34
	500	20.33±0.18	16.50±0.19	18.67±0.35	24.17±0.14
	250	19.67±0.18	16.00±0.00	17.17±0.14	20.33±0.18
	125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
			FLUCLOXACILLIN		
FLSMG01	10000	27.17±0.41	20.17±0.41	32.83±0.75	29.67±0.52
	5000	22.23±0.52	18.00±0.63	31.17±1.32	28.83±0.41
	2500	11.17±0.41	0.00±0.00	30.17±0.40	27.83±0.41
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
FLSMG02	10000	25.50±0.55	18.83±0.75	25.60±1.05	25.00±0.89
	5000	21.17±0.75	17.67±0.82	25.33±1.03	25.5±0.55
	2500	0.00±0.00	0.00±0.00	25.00±0.63	24.17±0.41
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
FLSMG03	10000	24.67±1.03	19.50±0.84	27.33±0.51	18.50±0.55

	5000	20.17±0.41	15.50±0.55	26.00±0.63	15.67±0.52
	2500	14.83±0.98	0.00±0.00	24.17±0.75	0.00±0.00
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
FLSLP04	10000	34.67±0.52	21.67±0.82	30.50±0.55	20.33±0.52
	5000	29.83±0.41	18.50±0.55	26.00±0.63	16.67±0.52
	2500	29.33±0.52	0.00±0.00	25.00±0.00	11.00±0.00
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
FLSP05	10000	30.17±0.41	25.50±0.84	37.83±0.75	29.17±0.75
	5000	28.67±0.52	24.50±0.84	37.17±0.41	25.33±1.03
	2500	28.0.00±0.00	20.33±0.51	33.67±0.52	20.67±0.81
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
FLSP06	10000	32.33±0.41	20.50±1.23	30.33±0.52	20.33±0.52
	5000	29.17±0.75	17.83±0.98	26.50±0.55	0.00±0.00
	2500	29.17±0.75	0.00±0.00	25.00±0.63	0.00±0.00
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
FLSAR07	10000	27.83±0.75	20.83±0.41	29.50±0.55	0.00±0.00
	5000	25.50±0.55	16.33±0.52	28.00±0.63	0.00±0.00
	2500	24.00±0.63	0.00±0.00	26.33±0.52	0.00±0.00
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
FLSAR08	10000	25.67±0.52	28.83±0.75	29.67±1.03	0.00±0.00
	5000	24.17±0.98	26.67±0.52	27.50±1.05	0.00±0.00
	2500	21.83±0.41	25.17±0.41	25.50±0.55	0.00±0.00
	1250	19.83±0.75	22.83±0.41	24.50±1.22	0.00±0.00
			CLOXACILLIN		
CLSLP01	10000	33.83±0.41	14.17±0.75	20.67±0.82	15.67±0.52
	5000	32.00±0.00	11.17±0.41	16.17±0.41	14.33±0.52
	2500	31.00±0.00	0.00±0.00	11.00±0.00	0.00±0.00
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
CLSLP02	10000	20.33±0.52	19.17±0.41	20.33±0.52	20.17±0.41
	5000	0.00±0.00	14.83±0.41	14.67±0.52	12.00±0.00
	2500	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
CLSLP03	10000	19.83±0.41	14.83±0.41	30.17±0.41	20.33±0.52

	5000	16.17±0.41	13.83±0.41	26.00±0.00	13.83±0.41
	2500	14.67±0.52	0.00±0.00	24.83±0.41	0.00±0.00
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
CLMGS04	10000	33.33±0.82	20.83±0.98	31.17±0.75	20.00±0.63
	5000	30.17±0.41	11.17±0.41	30.17±0.41	14.83±0.75
	2500	27.67±0.52	0.00±0.00	29.50±0.55	0.00±0.00
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
CLMG	10000	17.00±0.00	21.17±0.75	17.33±0.52	24.00±0.63
	5000	15.83±0.75	23.00±00	15.17±0.41	20.50±0.55
	2500	15.00±0.89	22.50±0.58	14.17±0.41	22.00±0.00
	1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Diameter of cork borer = 10 mm

Table 3.5 Zones of inhibition ± SEM of reference samples

Concentration(µg/mL)	***Staphylococcus aureus***	***Escherichia coli***	***Bacillus subtillis***	***Pseudomonas aeruginosa***
	AMOXICILLIN			
5000	30.83±0.34	27.00±0.00	24.83±0.14	24.50±0.89
2500	27.17±0.14	24.67±0.28	24.00±0.31	21.67±0.18
1250	25.67±0.18	21.67±0.47	21.33±0.18	20.00±0.00
625	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	FLUCLOXACILLIN			
10000	35.17±0.14	26.33±0.28	38.00±0.31	29.67±0.36
5000	31.50±0.89	20.00±0.22	35.17±0.14	24.33±0.18
2500	29.67±0.18	20.33±0.18	32.83±0.14	22.67±0.36
1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	CLOXACILLIN			
10000	30.00±0.22	23.67±0.34	25.50±0.68	26.00±0.22
5000	28.00±0.26	18.00±0.53	24.33±0.18	28.33±0.28
2500	25.67±0.28	19.67±0.36	23.33±0.18	25.67±0.36
1250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

SEM = Standard error mean, Cork borer

3.2 CHEMICAL ANALYSIS OF SAMPLES

3.2.1 HPLC analysis of amoxicillin samples

The active pharmaceutical ingredients (APIs) in the samples were determined using the developed and validated HPLC method.

The chromatographic conditions for the analysis of amoxicillin are as stated in Table 2.2 with mobile phase consisting of methanol: 0.01 M potassium dihydrogen phosphate (65:35, v/v) yielded maximum sensitivity and separation. Flow rates between 0.5 and 1.2 mL/min on a Shim-pack CLS-ODS C18 (M) 250 x 4.6 mm, 5 microns column were studied and a flow rate of 1.0 ml/min gave an optimal signal to noise ratio with a reasonable separation time of 1.42 min for amoxicillin when injected alone.

Figure 3.1 shows typical HPLC chromatogram of amoxicillin as reference sample and caffeine as internal standard (Figure 3.2.). The running time of the reference sample and the internal standard was less than 3 min. The major peak at 1.421 min is for amoxicillin whereas that for caffeine is 2.974 min.

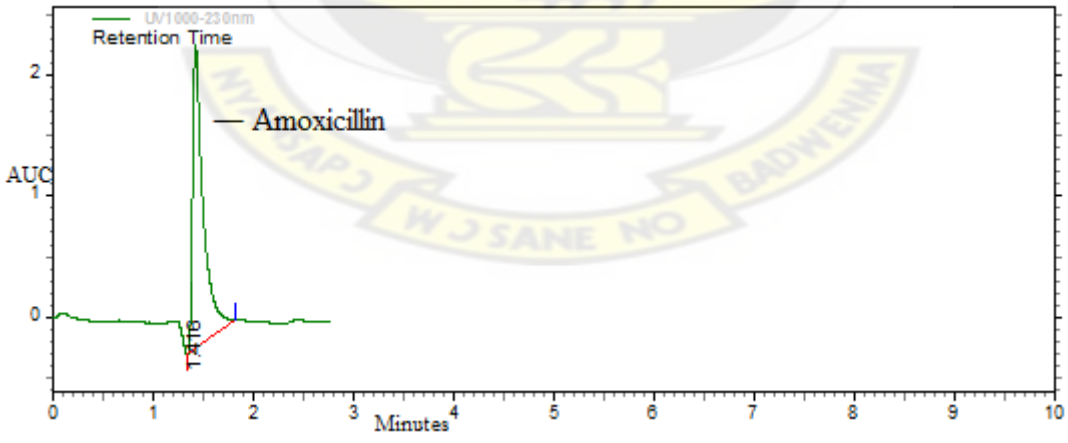

Figure 3.1 HPLC chromatogram of amoxicillin trihydrate as reference standard at wavelength (λ) 230 nm. AUC=Area under curve

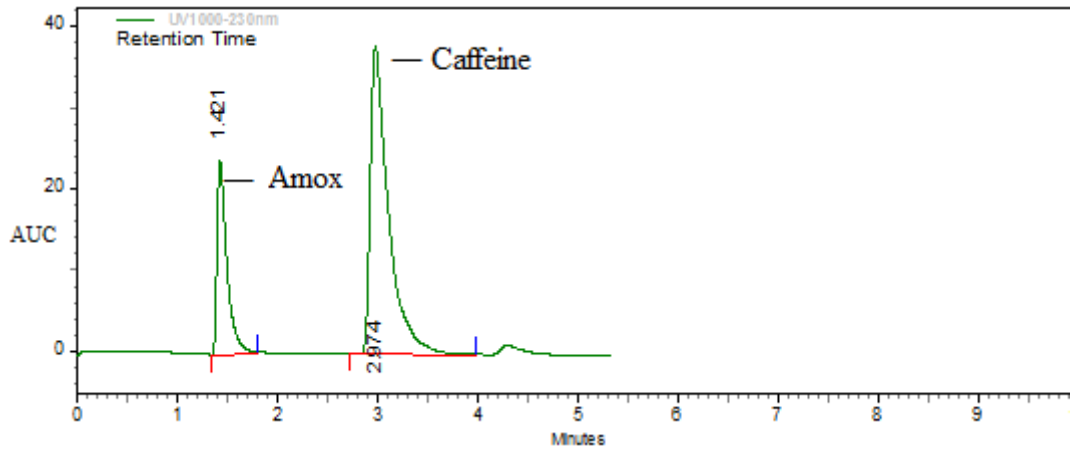

Figure 3.2 HPLC chromatogram of amoxicillin trihydrate as reference standard and caffeine anhydrous as internal standard at wavelength (λ) 230 nm

A five-point calibration curve was generated for amoxicillin in the concentrations range of 5.26 to 263.0 µg/mL. The calibration curve provided a linear relationship between the peak area (y-axis) and the concentrations of amoxicillin injected (x-axis) with the regression equation of y=194.41x + 0.004, R^2=0.9996 (Figure 3.3). The residual points of the calibration curve were well distributed within acceptable limits (Figure 3.4).

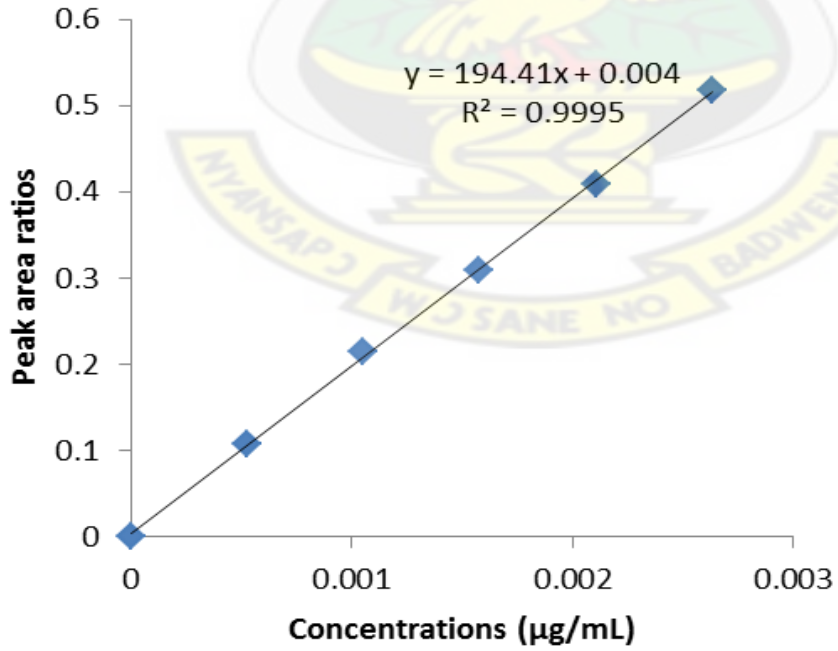

Figure 3.3 HPLC calibration curve of amoxicillin trihydrate

Regression analysis cannot minimize the distance for all points simultaneously but does it for most of the points. The residual plot of points shows maximum points closer to line for amoxicillin (Figure 3.4).

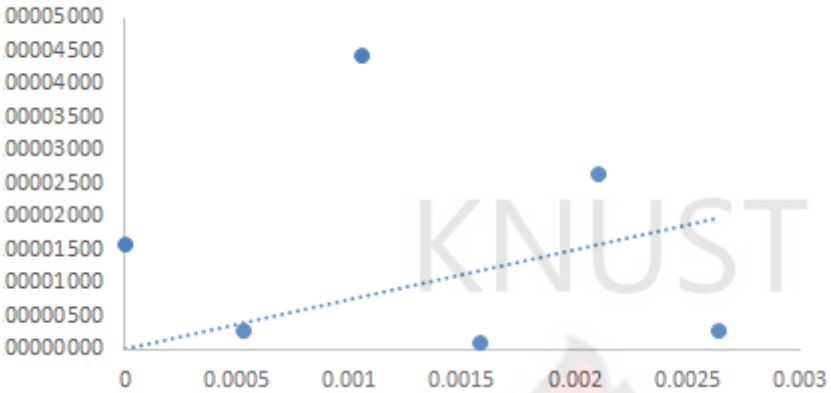

Figure 3.4 Residual plot of the HPLC calibration curve of amoxicillin

The developed HPLC methods were validated using the International Conference on Harmonization guidelines and the parameters therein (ICH, 2005a; 1995; 1996; 2005b). It was performed using a well-designed experiment and statistically relevant methods in accordance with International Conference on Harmonization (ICH) guidelines on validation of analytical procedures (ICH-Q2A, 1995; ICH-Q2B, 1996).

The linearity of the detector response for amoxicillin was confirmed from 5.26 to 263.0 µg/mL. The calibration curve (Figure 3.3) and the residuals (Figure 3.4) were inspected to asses linearity.

Table 3.6 Statistical validation of the calibration data for quantitative determination of amoxicillin

Parameter	Amoxicillin trihydrate
Concentration range	5.26 to 263.0 µg/Ml
Number	5
Average values	0.001315
Correlation coefficient	0.9995
Relative standard deviation (%)	0.7483
Calibration equation	y=194.41x + 0.004
Limit of detection (LOD)	1.6703×10^{-5}
Limit of quantification (LOQ)	5.0617×10^{-5}
System suitability	0.002
Method precision	0.58%

LOD=3.3 × σ/S, where σ= SDEV of the responses, S= slope of the regression line

LOQ=10 × σ/S, where σ= SDEV of the responses, S= slope of the regression line

The internal standard yielded accurate results as increase or decrease in peak area of analytes also affected area of internal standard. Peak ratios were directly proportional to concentrations (Table 4.7).

Table 3.7 Analysis of homogenous reference amoxicillin solution for system suitability and precision analysis.

IS (AUC)	RS (AUC)	IS:RS (AUC ratio)
165429	478918	0.3454
164384	472481	0.3478
166733	479600	0.3477
165828	474066	0.3498
166732	474678	0.3513
172047	493711	0.3484
		Mean=0.3484
		SDEV=0.00201
		%RSD=0.58%

IS= Internal Standard, RS= Reference Standard, AUC= Area under curve, SDEV= Standard deviation, %RSD = Percent relative standard deviation

3.2.2 HPLC analysis of flucloxacillin and cloxacillin samples

HPLC method was developed and validated for the evaluation of flucloxacillin and cloxacillin samples using the following conditions. Analysis were carried out in an ambient temperature (25°C) with Shim pack CLC-NH_2 C18 column 150 x 4.6 mm, 5 microns column and a Finnigan Spectra System HPLC instrument controlled by chromQuest software. A mobile phase consisting of Acetonitrile: 0.01 M potassium dihydrogen phosphate, KH_2PO_4, with a ratio of 60:40 (v/v) yielded maximum sensitivity and separation with sample detection at UV wavelength of 225 nm.

3.2.3 HPLC analysis of flucloxacillin

Figure 3.5 shows typical HPLC chromatogram of flucloxacillin as reference sample and acetaminophen (paracetamol) as internal standard (Figure 3.6). The running time for the reference sample and the internal standard were within four (4) min. The peak at 3.146 min is for flucloxicillin whereas that for acetaminophen is 1.953 min.

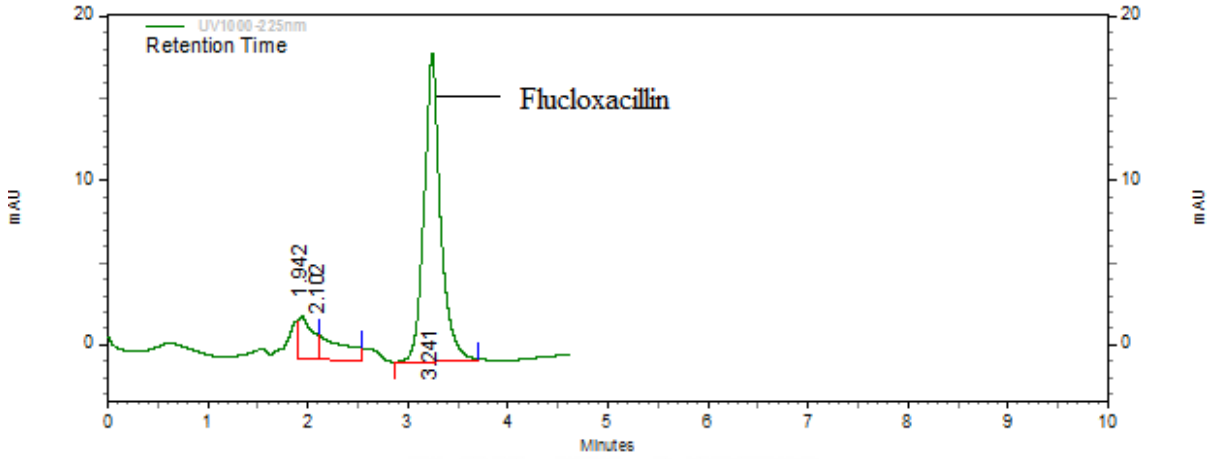

Figure 3.5 HPLC chromatogram of flucloxacillin as reference at wavelength (λ) 225 nm

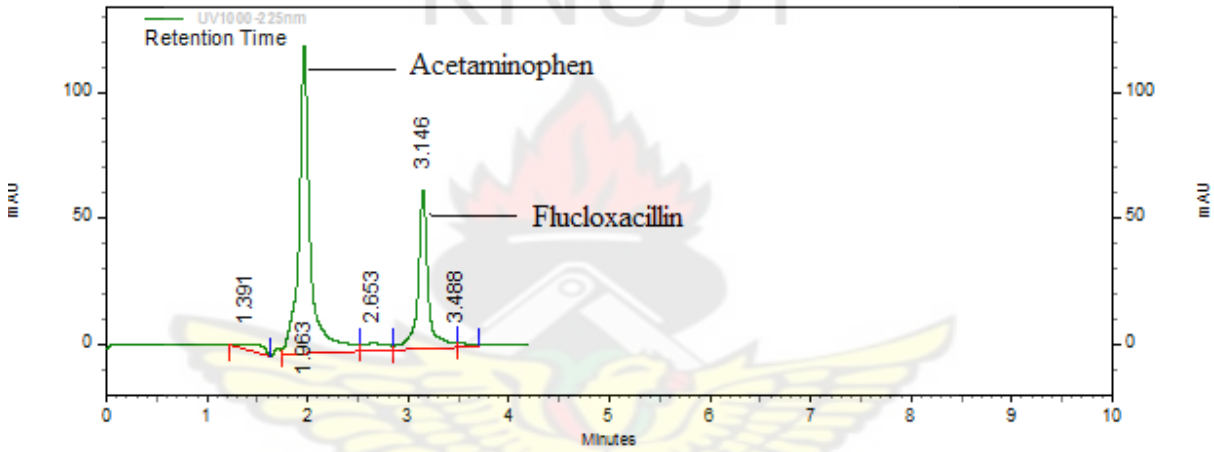

Figure 3.6 HPLC chromatogram of flucloxacillin as reference sample and acetaminophen as internal standard at wavelength (λ) 225 nm

A four-point calibration curve was generated for flucloxacillin in the concentrations range of 25.35 to 152.10 µg/mL. The calibration curve provided a linear relationship between the area under curve (y) and the concentrations of flucloxacillin injected (x) with the regression equation of y=156.94x + 0.0699 (R^2=0.995) (Figure 3.7). The residual points of the calibration curve were well distributed within acceptable limits (Figure 3.8).

The calibration curve and the residual plot for flucloxacillin are also shown in Figures 3.7 and 3.8 respectively.

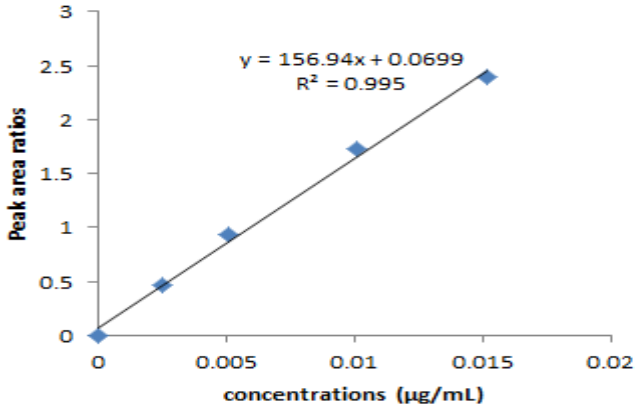

Figure 3.7 HPLC calibration curve of flucloxacillin reference standard

The residual plot for flucloxacillin (Figure 3.8) shows most of the points are closer to the regression line.

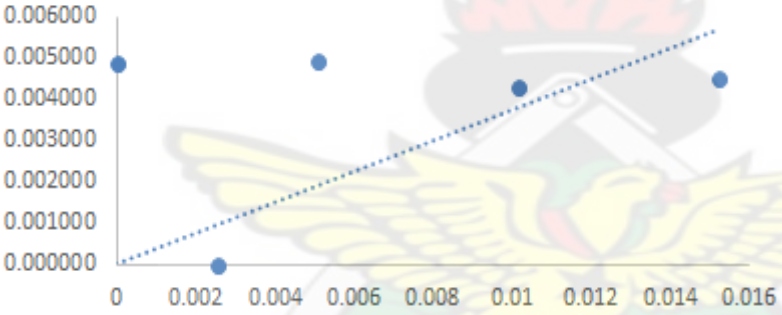

Figure 3.8 Residual plot of the HPLC calibration curve of flucloxacillin

The methods were validated using the International Conference on Harmonization guideline and the parameters therein. It was performed using a well-designed experiment and statistically relevant methods in accordance with International Conference on Harmonization (ICH) guidelines on validation of analytical procedures (ICH-Q2A, 1995; ICH-Q2B, 1996). The linearity of the detector response for flucloxacillin was confirmed to be 25.35 to 152.10 µg/mL.

Calibration curves were analyzed using a linear regression model and linear coefficients (Table 3.8). The limit of detection (LOD) and the limit of quantification (LOQ) were

calculated using the signal–to–noise ratio ICH-Q2B, 1996] and were found to be 1.2837 × 10^{-4} and 3.89 × 10^{-4} µg/mL (ICH-Q2B, 1996).

Table 3.8 Statistical validation of the calibration data for quantitative determination of flucloxacillin

Parameter	**Flucloxacillin**
Concentration range	25.35 – 152.10 µg/mL
Number	4
Average values	0.0066
Correlation coefficient (R^2)	0.9975
Relative standard deviation (%)	0.9262
Calibration equation	y=156.94x + 0.0699
Limit of Detection	1.2837 × 10^{-4} µg/mL
Limit of Quantification	3.89 × 10^{-4} µg/mL
System suitability	0.00253
Method precision	0.25%

LOD = Limit of detection, LOQ = Limit of quantification

LOD=3.3 × σ/S, where σ= SDEV of the responses, S= slope of the regression line

LOQ=10 × σ/S, where σ= SDEV of the responses, S= slope of the regression line

Areas under curve ratios were directly proportional to concentrations as increase or decrease in peak area of analytes also affected area of internal standard (Table 3.9).

Table 3.9 System suitability and precision parameters for flucloxacillin

IS (AUC)	RS (AUC)	IS:RS (AUC ratio)
780955	799289	1.0235
812336	830814	1.0227
801131	823499	1.0279
822182	843224	1.0256
797503	814643	1.0215
		Mean = 1.02424
		SDEV = 0.00253
		% RSD = 0.25%

AUC = Area under curve, IS = Internal standard, RS = Reference standard, SDEV= Standard deviation, %RSD = Percent relative standard deviation

Accuracy for flucloxacillin was determined by the mean and SDV of the percentage recovery studies (Table 3.10).

Table 3.10 Standard edition and internal standard recovery studies of flucloxacillin (n=4)

Number (n)	% Recovery
1	92.36
2	99.02
3	107.87
4	94.71
Mean	98.49
SDEV	6.834486

SDEV= Standard deviation

3.2.4 HPLC analysis of cloxacillin

Figure 3.9 shows the HPLC chromatograms of cloxacillin as reference sample and acetaminophen (paracetamol BP) as internal standard (Figure 3.10). The cloxacillin peak is at 2.874 min and that of acetaminophen is 1.933 min.

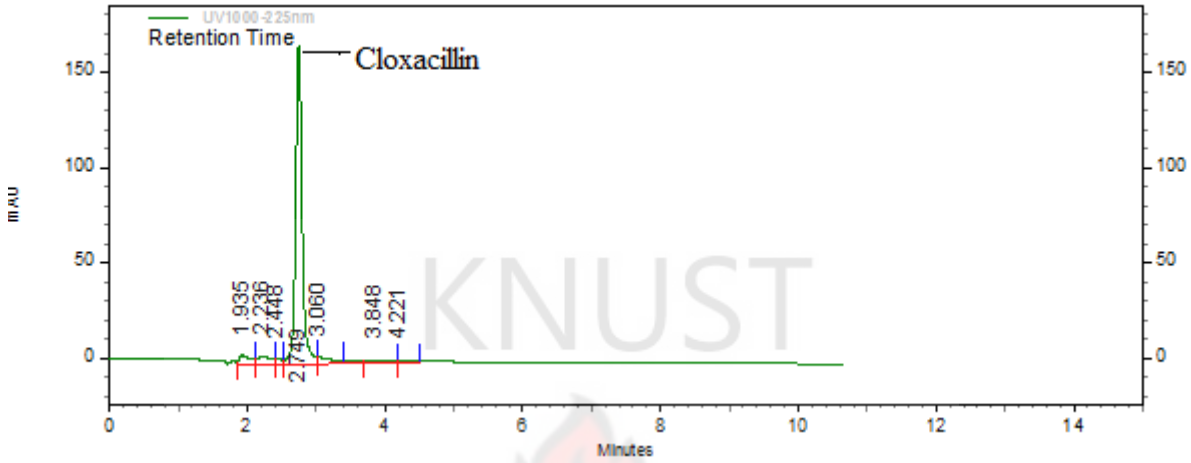

Figure 3.9 HPLC chromatogram of cloxacillin as reference at λ 225 nm

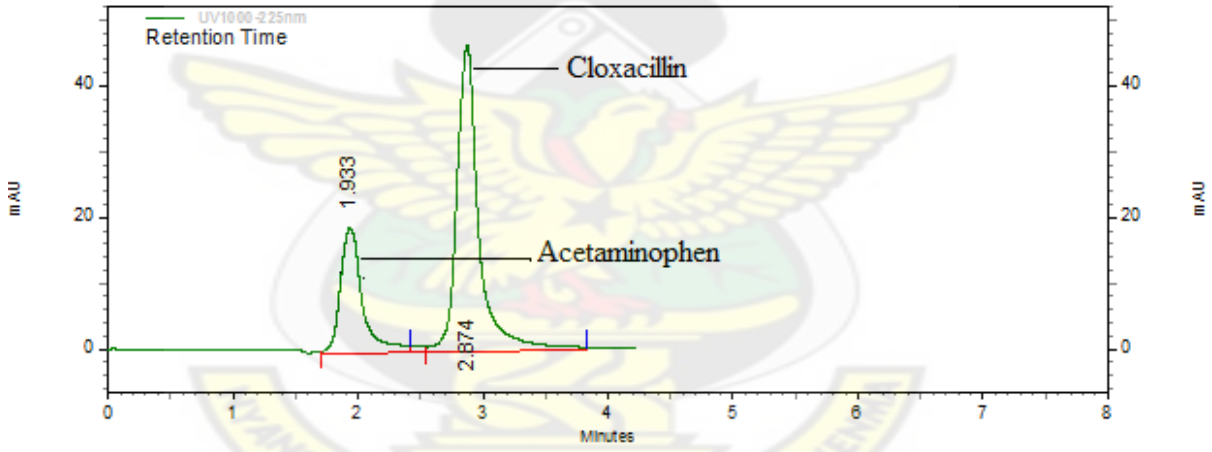

Figure 3.10 HPLC chromatogram of cloxacillin as reference and acetaminophen as internal standard at wavelength 225 nm

A four-point calibration curve was generated for cloxacillin in the concentration range of 11.72 to58.6 µg/mL. The calibration curve provided a linear relationship between the peak area (y) and the concentrations of amoxicillin injected (x) with the regression equation of $y=787.78x + 0.0839$ ($R^2=0.9986$) (Figure 3.11). The residual points of the calibration curve were well distributed within acceptable limits (Figure 3.12).

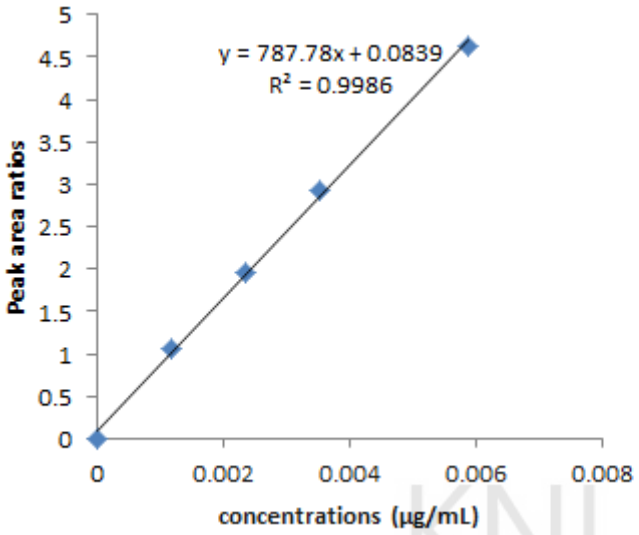

Figure 3.11 HPLC calibration curve of cloxacillin

The residual plot of points for cloxacillin (Figure 3.12) depicts the closeness of points to line.

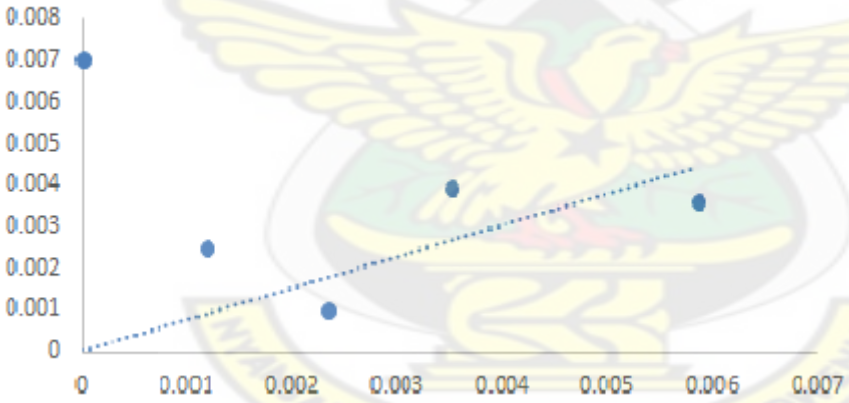

Figure 3.12 Residual plot of the HPLC calibration curve of cloxacillin

The methods were validated using the International Conference on Harmonization guidelines and the parameters therein. It was performed using a well-designed experiment and statistically relevant methods in accordance with International Conference on Harmonization (ICH) guidelines on validation of analytical procedures (Q2A and Q2B). The linearity of the detector response for cloxacillin was from 11.72 to 58.6 µg/mL. The

calibration curve (Figure 3.11) and the residuals (Figure 3.12) were inspected to asses linearity.

Calibration curves were analyzed using a linear regression model and linear coefficients (Table 3.11). The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the signal– to– noise ratio and were found to be 9.5246×10^{-6} µg/mL and 2.8861×10^{-5} µg/mL respectively.

Table 3.11 Statistical validation of the calibration data for quantitative determination of cloxacillin

Parameter	**Cloxacillin**
Concentration range	µg/mL
Number	4
Average values	0.0025784
Correlation coefficient	0.9993
Relative standard deviation (%)	1.1340
Calibration equation	y=787.78x + 0.0839
Limit of detection (LOD)	9.5246×10^{-6} µg/mL
Limit of quantification (LOQ)	2.8861×10^{-5} µg/mL
System suitability	0.00275
Method precision	0.0336%

LOD=3.3 × σ/S, where σ= SDEV of the responses, S= slope of the regression line LOQ=10 × σ/S, where σ= SDEV of the responses, S= slope of the regression line

Peak ratios were directly proportional to concentrations as increase or decrease in peak area of analytes also affected area of internal standard (Table 3.12).

Table 3.12 Internal standard, system suitability and precision parameters for cloxacillin

IS (AUC)	RS (AUC)	IS:RS (AUC ratio)
232461	195259	0.8391
237534	200609	0.8391
238890	185172	0.8445
230526	187178	0.7751
232653	190099	0.8171
Mean=0.81754, SDEV = 0.0275, % RSD = 0.0336		

SDEV = Standard deviation, %RSD = Percent relative standard deviation, IS = Internal standard, AUC = Area under curve

Accuracy for cloxacillin was determined by the mean and SDV of the percentage recovery studies (Table 3.13).

Table 3.13 Standard and internal standard recovery studies of cloxacillin (n=4)

Number	% Recovery
1	91.17
2	91.51
3	96.46
4	113.41
Mean	98.1375
SDV	10.46475

Results from HPLC analysis show that 75% amoxicillin capsules and 92.3% of suspension fell within USP specification with % assay of 93.2 to 104.3 and 81.0 to 104.1. Sample of flucloxacillin capsules had 62.5% within specification with % assay of 96 to 120.5, all suspension samples were below the required USP specification. None of cloxacillin capsule samples were within the USP specification. All the suspension samples, however, were within USP specification of 114.4 to 120.0%. The USP

specifications for amxicillin and flucloxacillin are 92.5 to 110% and 80 to 120% of stated amount for capsules and suspensions, respectively. Cloxacillin samples had 90 to 120% of API for both capsules and suspensions.

Table 3.14 HPLC analysis of amoxicillin, flucloxacillin and cloxacillin test samples (capsules).

Sample / assay / % ssay								
92.5 to 110 (USP, 2011)			**92.5 to 110 (USP, 2011)**			**90 to 120 (USP, 2011)**		
Amoxicillin capsules 250 mg			**Flucloxacillin capsules 250 mg**			**Cloxacillin capsules 250 mg**		
Sample code	**Assay (mg)**	**% assay**	**Sample code**	**Assay (mg)**	**% assay**	**Sample code**	**Assay (mg)**	**% assay**
01A	260.85	104.34	FLMG01	276.10	110.44	CLLP01	156.00	62.40
01B	227.80	91.12	FLMG02	161.63	64.65	CLLP02	177.75	71.10
02A	255.95	102.38	FLMG03	111.85	44.74	CLLP03	145.18	58.07
02B	244.83	97.93	FLLP04	269.08	107.63	CLAR04	139.60	55.84
03A	203.83	81.53	FLLP05	250.98	100.39	CLAR05	201.95	80.78
03B	240.15	96.06	FLLP06	239.90	95.96	CLAR06		
03C	244.53	97.81	FLAR07	301.13	120.45	CLMG		
04A	230.07	92.03	FLAR08	147.65	59.06			
05A	237.45	94.98						
06A	217.20	86.88						
06B	253.48	101.39						
06C	238.58	95.43						
08A	232.97	93.19						
Amoxicillin capsules 500mg								
07A	480.00	96.00						
07B	481.85	96.37						
09A	493.15	98.63						

Table 3.15 HPLC analysis of amoxicillin, flucloxacillin and cloxacillin test samples (suspensions)

Sample code / assay / % assay								
80 to 120 (USP, 2011)			**80 to 120 (USP, 2011)**			**90 to 120 (USP, 2011)**		
Amoxicillin (125 mg / 5 mL)			**Flucloxacillin (125 mg / 5 mL)**			**Cloxacillin (125 mg / 5 mL)**		
Sample code	**Assay**	**% assay**	**Sample code**	**Assay**	**% assay**	**Sample code**	**Assay**	**% assay**
S01	117.56	94.05	FLMG01	52.66	42.13	CLSLP01	140.28	112.22
S02A	101.29	81.03	FLMG02	47.51	38.01	CLSP02	149.96	119.97
S02B	114.15	91.32	FLMG03	48.03	38.42	CLSLP03	143.05	114.44
S02C	101.66	81.33	FLLP04	48.91	39.13	CLSMG04	143.86	115.09
S03A	120.56	96.45	FLLP05	58.21	46.57	CLSMG05		
S04A	117.30	93.84	FLLP06	62.59	50.07			
S05A	98.38	78.70	FLAR07	45.05	36.04			
S06A	125.53	100.42	FLAR08	45.35	36.28			
S06B	126.75	101.40						
S06C	127.23	101.79						
S07A	130.14	104.11						
S08A	121.20	96.96						
S08B	110.53	88.42						

CHAPTER 4

DISCUSSION

4.0 GENERAL DISCUSSION

The samples of the three penicillins evaluated varied slightly from the standard reference samples in the microbiological evaluation. Suspensions had lower MICs as compared to capsules. All samples in general showed higher MIC compared to the reference standards. The developed and validated HPLC methods were suitable for the intended purpose. HPLC analysis of the samples showed some of the test samples containing the right amount of active pharmaceutical ingredients as stated in the USP, 2011 and BP, 2010 but they had higher MICs against the test bacteria.

4.1 MICROBIOLOGICAL EVALUATION, HPLC METHOD DEVELOPMENT, VALIDATION AND ANALYSIS OF SAMPLES

4.1.1Microbiological evaluation of samples

Most of the penicillin samples were active against all the organisms but the mean zones of inhibition varied with different bacteria and sample as well as different concentrations. The diffusion method, however, helped in the choice of concentrations to be used in the dilution method. Some of the limitations with the diffusion method such as effect of concentration and diffusion rate on the zone size were observed.

The pattern of zones of inhibition were not consistent as, in some cases, lower concentrations of the same sample had bigger or same sizes of zones of inhibition as compared to higher concentrations. This could be attributed to the fact that the antibiotic had to diffuse through the solid medium and the more concentrated they are, the higher the viscosity, hence, less diffusion rate. Consequently, the micro-dilution method was selected

and used in the determination of the MIC as the test organisms are in direct contact with the antibiotic (Agyare *et al*., 2013).

Helegbe *et al*. (2009) in their study reported that some selected antibiotics were active against some bacteria and recommended further studies on a larger scale. The current study, however, revealed higher MIC for the samples and this may be due to insufficient amount in the penicillin samples analyzed. A typical example is the report by Rahman *et al.* (2008) that had zones of inhibition of amoxicillin tested against standard selected bacteria at 100 µg/mL to be 19.5 mm for *E. coli*, 15.3 mm for *B. subtillis* and 17.0 mm for *S. aureus*. The current study on the other hand had no zones of inhibition at concentration below 250 µg/mL. The amoxicillin samples had MIC of 125, 180 and 220 µg/mL against *E. coli, S. aureus* and *B. subtillis* respectively and the current study, amoxicillin had MICs of 200, 200 and 300 µg/mL against *E. coli, S. aureus* and *B. subtillis* respectively.

There are differences between the literature values and that obtained from this study, but samples showed some level of sensitivity towards the test bacteria. Generally, there were differences in the sensitivity of Gram-negative and Gram-positive bacteria which could be due to the composition of the cell wall of two types of bacteria (Butaye *et al*., 2003; Gupte, 2007; Mirghaed and Yadollahi, 2013).

Some samples exhibited variations in the MIC. The antibacterial activity and MICs of samples varied from bacteria to bacteria which were similar to that of the reference sample. It was observed that, there were also variations among various brands and even batches within the same brand but variations were not significant ($p<0.05$).

Other reason that could account for differences in literature values and that of present study is the inoculum size of test organisms. Gbedema *et al*. (2010) reported MIC of 0.46, 640, 0.29 and 0.26 mg/mL against *E. coli, P. aeruginosa S. aureus and B. subtillis* with inoculum size of 0.5 McFarland's, equivalent to 10^5 cfu/mL using the agar diffusion

method. The inoculum size used in the present study was 10^6 cfu/mL and it is higher than the inoculums size used by Gbedema *et al*. (2010). This might have resulted in the higher MICs recorded for the samples compared to the values reported in literature (Gbedema *et al.*, 2010; Rahman *et al.*, 2008). Besides that, the micro-dilution method used in the determination of the MICs is reported to be better approach than the agar diffusion technique (Agyare *et al*., 2012; Girish and Satish, 2008).

Beta-lactams are inhibited by the beta lactamases produced by bacteria and the size of inoculum will have direct influence on the performance of the antibacterial agent. The inoculum size will determine the amount of beta-lactamase present to deactivate the beta lactam ring (Lancini *et al.*, 1995).

Comparison between results from the biological and chemical method revealed that some of the samples passed the chemical assay but had higher MIC values. For this reason higher doses of these samples of amoxicillin are required for the treatment of infections due to these bacteria. Amoxicillin has enantiomers with its mirror image having the same chemical structure. A compound and its enantiomer show different activity with only one of its enantiomers usually biologically active (Nandanwar *et al.*, 2005).

Antibacterial activity of samples were similar but not the same as those of the reference standard. In general, flucloxacillin and cloxacillin samples were much active against *S. aureus* and *B. subtilis* compared to *E. coli* and *P. aeruginosa*. This could be due to the simple reason that isoxazolyl antibiotics are not very active against Gram-negative bacteria (Helegbe *et al.*, 2009). Samples in suspension forms showed higher activity as compared to the capsules against Gram-negative and Gram-positive bacteria. The possible reason could be due to the nature of formulation and the type of experimental design (*in vitro*) used.

Capsules are to be swallowed and an acidic environment is required to enhance dissolution and release of API.

The isoxazolyl antibiotics such as flucloxacillin are not sensitive to penicillinase enzymes secreted by many penicillin-resistant bacteria, but able to bind to penicillin-binding proteins (PBPs) and inhibit peptidoglycan cross-linkage. This is made possible due to the presence of the isoxazolyl group on the side-chain of the penicillin nucleus which facilitates the β-lactamase resistance, since they are relatively intolerant of side-chain steric hindrance but it is not inactivated by β-lactamases. They are acid stable and have proven to be effective against *S. aureus* (Smith et al., 1962; Sutherland et al., 1970).

4.1.2 Development of HPLC method for analysis of samples

There are some antibiotics that have been found to be substandard and counterfeited (Newton *et al.*, 2006; Reidenberg and Conner, 2001). Substandard and counterfeit antibiotics are also noted to be one of the main cause of bacterial resistance of to antibiotics (Okeke *et al.*, 1999). Reports on substandard and/or counterfeit antibiotics on various markets have triggered investigations into their quality and activity. Different approaches, both biological and chemical analysis are used in the evaluations. The unavailability of specific materials such as the type of column and solvent systems to be used in chemical analysis in some laboratories in some developing countries and comparison of the results with specifications in standard reference books such as United State Pharmacopoeia (USP) and the British pharmacopoeia (BP) has made it necessary for the modification and validation of the existing methods with materials readily available to suit the type of analysis being performed especially in resource restrain areas or settings.

4.1.3 HPLC analysis of samples

The internal standard (IS), caffeine, was selected based on the fact that caffeine did not interact with the sample and absorbs at the same wavelength as the sample but it did not have the same retention time as the sample.

HPLC method with a good linearity depicts the direct proportionality between concentration of analytes and the area under curve of the peaks. With correlation coefficient (r) of 0.9997 and R^2 of 0.9995 from the regression analysis of the calibration curve shows the direct proportional relationship between concentrations and peak area ratios. This represents an excellent linearity between them and how precise the HPLC method is. The method was shown to be linear. Observation of the calibration curve also confirms the linearity of the method developed (Figure 3.3).

The ability for the analyte of interest as far as this study is concerned, to elute in the presence of other compounds was ensured. A specific method is able to distinguish analytes even in the presence of other similar compounds. The ability of the amoxicillin to elute at the same retention time when spiked with the internal standard (Figure 3.2) attests to the fact that the method was specific for the samples. The method can be used in the assessment of caffeine the analyte of interest. The internal standard was able to achieve the purpose for which it was intended (Table 3.12). Changes that could not be or difficult to control such as variations from run to run temperature and pressure during the run time were monitored by the internal standard. Relationship between the area under curve for the internal standard and area under curve for the reference standard yielded consistent area ratios (Table 3.13). The internal standard method is therefore considered the ideal as it yields accurate and precise results (Kavittha *et al.*, 2012).

With respect to the suitability of a method, the USP (2011) states that the percent relative standard deviation (%RSD) from a six replicates runs of homogenous samples must not be

more than 2. The current method developed yielded %RSD of 0.58 which is less than 2% and this is an indication of the suitability and precision of the method. The limits of detection and quantification values (Table 3.6) were indicative of how sensitive the method is. The attributes of the validation parameters considered shows that the method could be used to analyze amoxicillin samples within a considerable time using the readily available materials. The retention time of caffeine (internal standard) was 2.97 min whereas that of amoxicillin was 1.42 min at wavelength of 230 nm. The maximum absorptions of the two compounds were detected at the same wavelength. Penicillins have no specific chromophore (Foulstone and Reading, 1982) and eluent must be maintained at wavelength less than 230 nm to obtain a meaningful detection limits. In this study, however amoxicillin was detected at wavelength of 230 nm. The reason for the possible difference in retention time could be due to the different types of columns used and flow rates used. This was the method used by Ashnager and Naseri (2007) to analyze amoxicillin samples at wavelength of 230 nm using Spherimage-80, ODS, 2-5 μm C18 column. A similar study of amoxicillin gave a retention time of 10 min for amoxicillin using the same buffer system and temperature whereas retention time of 1.42 min was recorded for amoxicillin in this current study. Abreu and Ortiz (2003) also had a retention time of 5.2 min for amoxicillin using the C18 column at wavelength (λ) of 229 nm with mobile phase of phosphate buffer and acetonitrile. The limits of detection and quantification values as (Table 3.6) were indicative of how sensitive the method was. The specificity of the method was confirmed when the internal standard and reference standard were spiked with different concentrations of the same samples and they gave distinctive peaks of the two compounds at their respective retention times (Figure 3.2).

Analysis of the samples revealed that the content of all 16 different samples of the capsules were in the range of 81.53 to 104.34% (Table 3.14). Twelve samples had their content

falling within the USP (2011) specification of 92.5 to 110.0%. The sample with API of 93.2% was analyzed just 2 years before its expiry and few months after manufacturing and this means that the probability of the product failing later analysis before its expiry may be high.

Assay of the samples in suspension form showed 92.3% of them having their content falling within the acceptance limit (USP, 2011). Percentages of active ingredient range of the suspension samples were from 81.03 to 104.1%. Two batches from sample S02 were found to contain 81.0 and 81.33% active ingredient respectively and these samples have their API fall below the USP (2011) specification. The fact that they were analyzed few months after their manufacture may indicate the samples may breakdown before expiry or did not contain the right amount of API. Almost 8% of the samples had their APIs below the USP (2011) range.

After observing flow rates between 0.5 and 1 mL/min, the later was found to give an optimal signal-to-noise ratio with a reasonable separation and retention. In the quest of finding internal standard, various reference standards were used including amoxicillin cloxacillin and flucloxacillin. Injection of flucloxacillin and cloxacillin gave peaks with almost the same retention time and hence could not be used as the internal standard. Acetaminophen gave a retention time different from that of cloxacillin and flucloxacillin. Hence, it was used as internal standard for the analysis of cloxacillin and flucloxacillin samples. Environmental changes that could not be or difficult to control such as variations from run to run, temperature, pressure and power fluctuations during the run time were also monitored by the use of the internal standard in the analysis of the samples (Table 3.9 and Table 3.12).

The limit of detection and limit of quantitative of the analysis indicate the sensitivity of the method. The direct proportional relationship between concentrations and peak area ratios

with correlation coefficient R^2 of 0.995 for flucloxacillin and 0.9986 for cloxacillin from the regression analysis of the calibration curves (Figure 3.7 and Figure 3.11) and these indicate the level of linearity. For five runs of the same homogenous reference solution (Table 3.9, Table 3.12) the suitability and precision of the method were in the acceptable limit as stated in USP, 2011 with SDEV of 0.0025 and %RSD of 0.25 for flucloxacillin and standard deviation of 0.028 and %RSD of 0.034 for cloxacillin. All these values were less than 2% and fall within USP (2011) specification.

The range of recovery for flucloxacillin and cloxacillin were 92.4 to 107.9% and 91.2 to 113.4% respectively with an average percentage recovery of 98.5% for flucloxacillin and 98.1% for cloxacillin. These represent a high level of accuracy of the methods.

In the evaluation of flucloxacillin samples (capsules) using the acceptance limit of 92.5 to 110 % as stated in USP (2011), 5 out of 8 samples evaluated were within the specification of USP (2011) with percentage assay of 95.96 to 120.45 representing 62.5% of samples. The remaining samples had API of 44.7 to 64.7% which did not meet the specification in USP (2011).

All the samples of flucloxacillin suspension analyzed were in the range of 36.0 to 50.1%. These content are outside the USP range of acceptance limit of 80 to 120% specified in the BP (2010). These low amounts of APIs may be due to insufficient active ingredients and/or poor storage conditions of the samples.

Antibiotics of this quality are threat to patients, the nation, and the world at large. Patients receiving such antibiotics would obviously not respond to minimum doses and would have to resort to higher doses. The activity of these antibiotic samples that failed the various evaluations may lead to antibiotic resistance in previously susceptible organisms.

Ensuring the quality, efficacy and safety of antibiotics would go a long way to prevent the problems associated with substandard and/or counterfeit antibiotics. The regulatory authorities that have the mandate to regulate medicines must intensify their effort to monitor the quality and conditions of storage conditions of these antibiotics.

CHAPTER 5

CONCLUSION

All penicillin samples (amoxicillin, flucloxacillin and cloxacillin) evaluated showed activity against test bacteria (*E. coli*, *P. aeruginosa*, *S. aureus* and *B. subtilis*). The level of activity and concentrations of penicillin samples gave different zones of inhibitions against these bacteria. Amoxicillin was observed to have broad spectrum activity showing activity against all bacteria used in the evaluation. Flucloxacillin and cloxacillin samples were observed to have better activity against Gram-positive bacteria as compared to Gram-negative bacteria. *P. aeruginosa* was found to be most resistant bacteria to the penicillin samples. Suspension samples exhibited higher activity compared to capsule formulations. The MICs of 200 to 800 µg/mL were recorded for amoxicillin samples whereas flucloxacillin and cloxacillin samples had MIC of 800 to 1900 µg/mL. Only *B. subtilis* showed significant variation ($p<0.05$) in the MIC determinations.

The results generated by this study have provided proven alternative chemical methods for the analysis of samples of amoxicillin, cloxacillin and flucloxacillin. All samples of flucloxacillin suspensions and cloxacillin capsules had their API below the USP specification. Variation in % assay content within brands were not significant in capsules but variation in samples S02 and S06 was significant ($p<0.05$). Almost eighty three percent of amoxicillin samples contained the right amount of API compared to 32.1 % of flucloxacillin and 44.4% cloxacillin samples.

RECOMMENDATIONS

- Further studies on the real time and accelerated stability studies as well as the rate of dissolution of the test samples must be considered.
- More samples from other parts of the country should be evaluated and analyzsed in order to find out the extent of the samples of penicillins which had their API below the official required specifications.
- There should be regular and consistent monitoring and surveillance of the activity and content (API) of antibiotics on the market by the regulatory bodies such as Food and Drug Authority.
- Conditions of storage of these antibiotics including the penicillins by pharmacies and pharmaceutical wholesalers should be checked and monitored and they must comply with specified conditions for each group of antibiotic.

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APPENDICES

APPENDIX I : STANDARDIZATION OF MICROBIAL SUSPENSIONS

Standardization of organisms

The inoculum size of 10^5cfu/mL was standardized using ultraviolent spectrophotometric absorbance. A 24 h broth culture was diluted to 10 in 10, 1 in 10^2, 1 in 10^3 and 10^{14} and their absorbance determined at wavelength 420 nm. Each dilution was incubated at 37 for 24 hours on plate count agar. The colony forming units (cfu) were counted by the help of colony counter. A graph of log 10 cfu/mL plotted against absorbance.

Table 0.1 Absorbance of 24 hour broth culture

P. aeruginosa		***B. subtilis***		***S. aereus***		***E. coli***	
Mean Ab	**Log cfu/mL**	**Mean Ab**	**Log cfu/mL**	**Mean Ab**	**Log cfu/mL**	**Mean Ab**	**Log cfu/mL**
0.781	10.34044	0.358	2.454845	0.357	2.570543	0.822	10.25527
0.671	9.340444	0.331	2.283301	0.34	2.127105	0.502	9.255273
0.576	8.34044	0.329	1.50515	0.335	1.643453	0.497	8.255273
0.494	7.340444					0.4976	7.255273

Ab = Absorbance

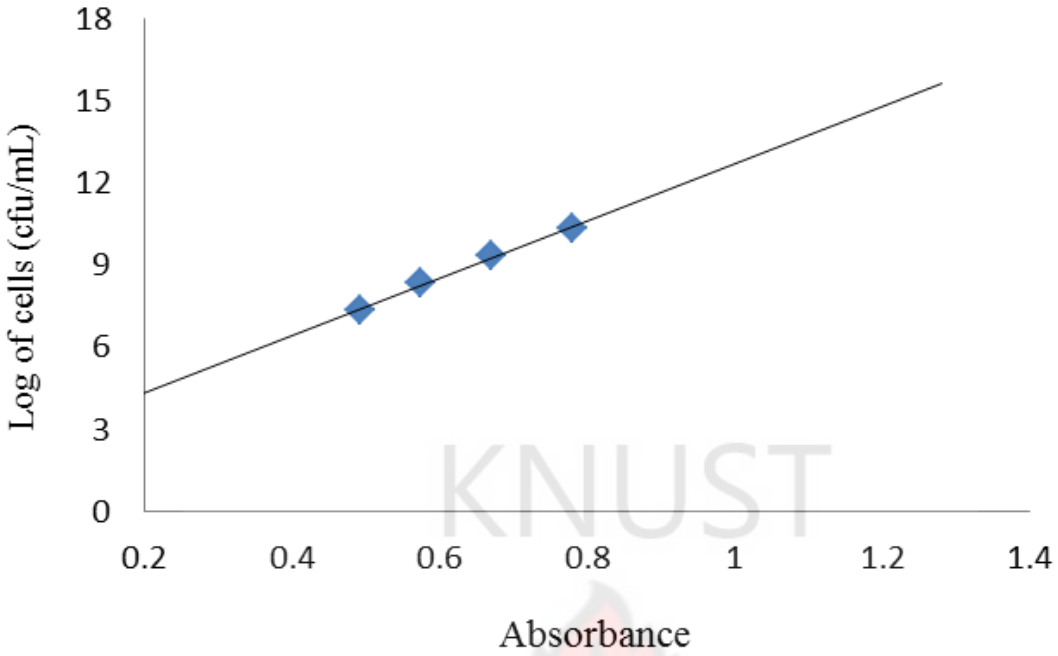

Figure 0.1 Standardization of *Pseudomonas aeruginosa* suspension

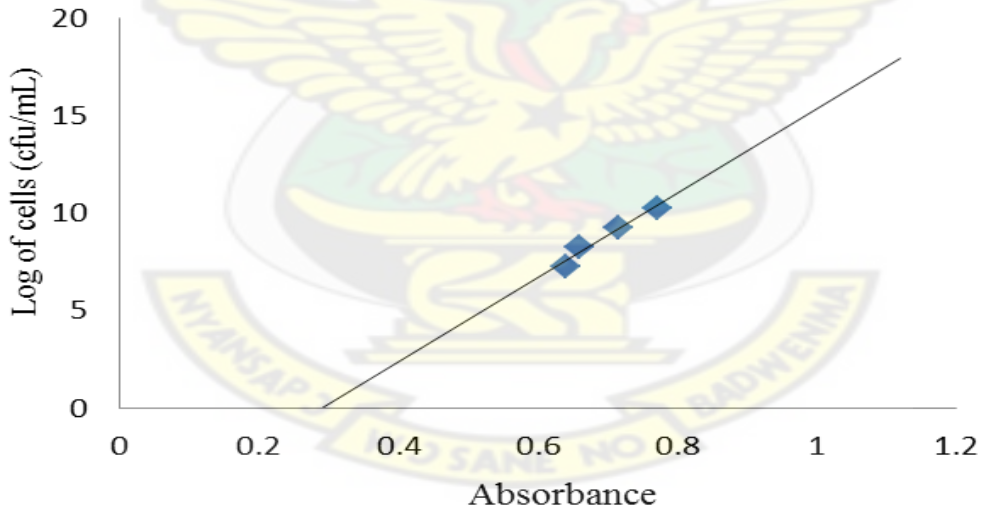

Figure 0.2 Standardization of *Bacillus subtillis* suspension

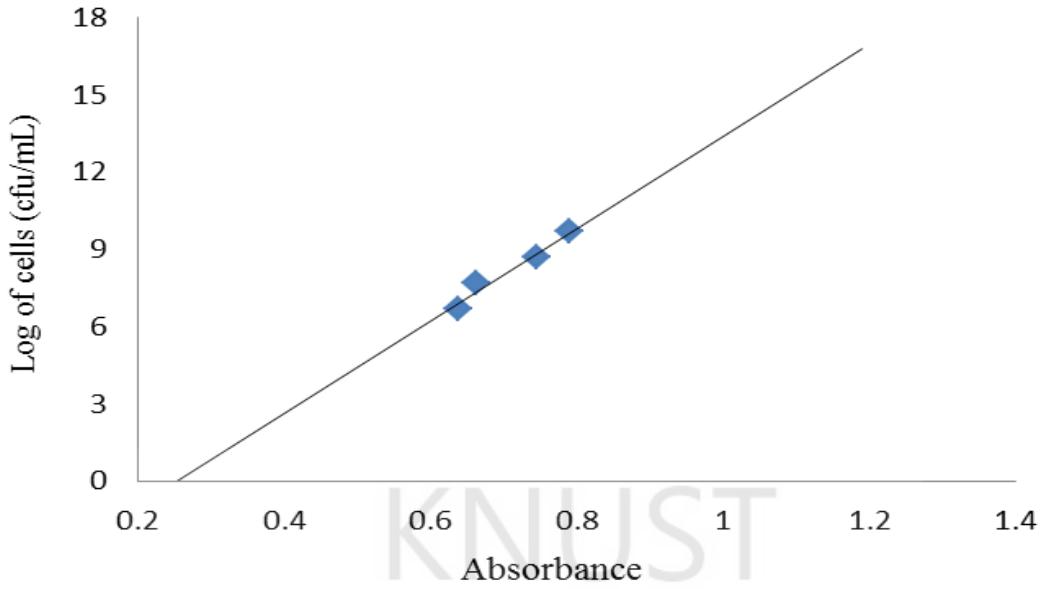

Figure 0.3 Standardization of *Staphylococcus aureus* suspension

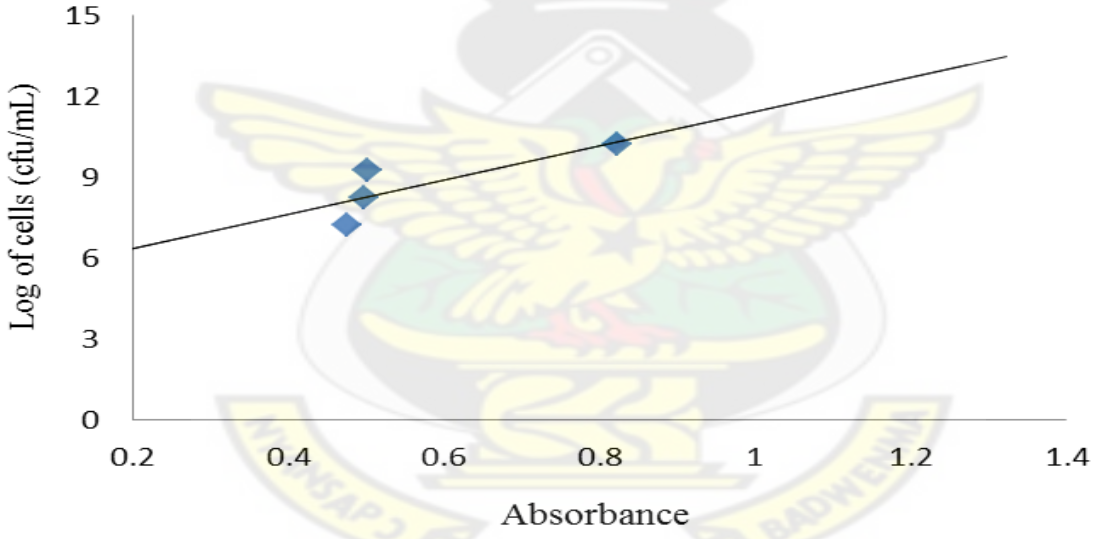

Figure 0.4 Standardization of *Escherichia coli* suspension

APPENDIX II: PROCEDURE, HPLC METHOD DEVELOPMENT, VALIDATION AND SAMPLE ANALYSIS

Preparation of 0.01M KH_2PO_4

An amount of 136.67 mg of potassium dihydrogen orthophosphate was accurately weighed and quantitatively transferred into a 1L volumetric flask. It was dissolved with 200 mL distilled water; and further made to the mark with distilled water. The solution was then filtered with Whatman cellulose membrane filter paper of pore size, 0.45 millipore.

Preparation of reference standard solutions

Amoxicillin reference standardd solution

An amount of 5.26 mg amoxicillin trihydrate was accurately weighed into a 100 ml volumetric flask. 10 mL 0.1 M HCl was added and shaken. It was then made to the mark with the mobile phase. Further dilution of 3 mL standard solution to 100 mL was prepared as final stock.

Preparation of internal standardd solution (IS)

Exactly 10.25 mg caffeine anhydrous reference powder was weighed into a 100 mL volumetric flask. More mobile phase was added to the 100 mL mark.

Calibration curve for Amoxicillin

Volumes of 1, 2, 3, 4 and 5 ml reference amoxicillin solution were pipetted into five different 100 mL volumetric flasks. To each flask, 5 ml of the IS was added and topped up to 100 mL mark with mobile phase. These were injected in to the HPLC at concentrations 5.26, 10.52, 15.78, 21.04 and 26.30 µg/m L and the area under curve (AUC) of internal standard and reference sample, as well as the ration between them.

Table 0.1 Retention time and percentage area under curve of reference standards (Amoxicillin, Flucloxacillin and Cloxacillin)

Reference sample(RS)/Internal standard (IS)	Retention time	AUC	% AUC
Amoxicillin(RS)	1.424	47731	9.643
Caffeine(IS)	2.942	447262	90.357
Flucloxacillin (RS)	3.146	458868	29.548
Paracetamol (Acetaminophen)	1.953	977785	62.962
Cloxacillin (RS)	2.874	566922	71.166
Paracetamol (Acetaminophen) (IS)	1.933	229699	28.834

AUC = Area under curve, IS = Internal standard, RS = Reference standard

Table 0.2 Analysis of flucloxacillin reference sample and paracetamol (acetaminophen) for the generation of calibration curve

Volume of stock pipetted (mL)	Concentration(µg/mL)	AUC RS	AUC IS	AUC ratio	Average ration AUC
1	5.26	50426	452326	0.1115	
		46563	439503	0.1067	0.108
		47240	446070	0.1059	
2	10.52	92031	42103	0.21585	
		89504	420773	0.21271	0.2152
		88847	414666	0.21426	
3	15.78	139671	450619	0.30995	
		139247	448677	0.31035	0.3097
		137287	444400	0.3089	
4	21.04	55067	136041	0.40478	
		183727	446974	0.411	0.40789
5	26.3	235762	460784	0.5117	
		240226	459850	0.5224	0.517

AUC = Area under curve, IS = Internal standard, RS = Reference standard

Table 0.3 Represent the results of some of the test samples showing area under curve of reference standard and internal standard, their ratios, as well as average ratios

Test sample	AUC RS	AUC IS	AUC ratio	Average AUC ratio
02B	158444	504342	0.3142	
	155339	4988042	0.3114	0.3123
	157100	504775	0.3112	
04A	200370	514347	0.3896	
	207210	531409	0.3899	0.3907
	203988	519512	0.3926	
05A	155562	510712	0.3046	
	158509	516002	0.3072	0.3056
	160522	526373	0.3049	
03A	159856	509065	0.3140	
	165547	533291	0.3104	0.3115
	165823	534670	0.3101	
06A	199656	519724	0.3842	
	204394	539514	0.3788	0.3809
	202215	532478	0.3798	
01A	193191	544789	0.3546	
	191498	542763	0.3528	0.3539
	192448	543296	0.3542	
07A	47873	212297	0.2255	
	112722	513339	0.2196	0.2197
	112047	523804	0.2139	
08A	215109	534493	0.4026	
	215781	535000	0.4033	0.4042
	213839	525746	0.4067	
07B	108850	505021	0.2153	0.2154
	113696	527655	0.2155	
	113694	527853	2154	
09A	104742	472858	0.2215	
	112512	512975	0.2139	0.2204
	111131	504336	0.2204	
SOIA	387129	492431	0.7862	
	403493	519740	0.7808	0.7832
	414275	529342	0.7826	

AUC = Area under curve, IS = Internal standard, RS = Reference standard

Preparation of test sample solutions

Amoxicillin capsules

20 filled capsules of amoxicillin were weighed. The capsules were opened and the contents emptied into a container, emptied shells were the weighed and the weight noted. An amount equivalent to 51.0 mg was weighed and 5 ml 0.1M hydrochloric acid (HPLC grade) was added. It was then made up to 100 mL. with mobile phase. The solution was filtered and 3 mL of the filtrate and 5ml of the internal standard was pipetted into 100 mL. the solution was then made up to 100 ml with mobile phase. It was the injected into the HPLC

Suspensions

An equivalent of 250 mg (5 mL) was pipetted and 5 ml of 0.1M hydrochloric acid added. Solution was topped with mobile phase and filtered. A volume of 3 ml of the sample solution and 5 ml of the internal standard was pipetted into 100 mL. the solution was then made up to 100 ml with mobile phase. It was then injected into the HPLC.

Methods for cloxacillin and flcucloxacillin

Preparation of reference standard solutions

Flucloxacillin reference standard solution

An amount of 50.7 mg of reference flucloxacillin powder was weighed into a 100 mL volumetric flask and dissolved with distilled water. It was then diluted with mobile phase to 100 mL.

Cloxacillin reference standard solution (RS)

Exactly 117.2 mg reference cloxacillin powder was weighed and transferred into a 100 mL volumetric flask. This was dissolved with 5 mL methanol and topped with mobile phase to mark. Solution was filtered with whatman filter paper.

Preparation of internal standard solutions (IS)

IS for flucloxacillin

An amount of 21.4 mg of acetaminophen was weighed into a 100 mL volumetric flask and 5 mL methanol was added to dissolve particles completely. Mixture was brought to the 100 mL with mobile phase to and shaken well for a uniform mixture.

Internal Standard for cloxacillin

An amount of 20.1 mg of acetaminophen (paracetamol) was weighed into a 100 mL volumetric flask and 5 mL methanol was added to dissolve particles completely. Mixture was topped up with mobile phase to the 100 mL mark and shaken well for a uniform mixture.

Calibration curve for flucloxacillin

Different volumes of 5 10, 20, and 3 mL of the RS were pipetted into four different 100 mL volumetric flasks. A volume of 2 mL IS was added to each flask. Each was topped up with mobile phase to the 100 mL mark. These were injected in to the HPLC and the average peak area ratio of the samples is shown in table 5.4. A four-point calibration curve was drawn from the peak area ratio obtained (Figure 3.7).

Table 0.4 Analysis of cloxacillin reference sample and acetaminophen (paracetamol) for the generation of calibration curve

volume of stock pipetted (mL)	Concentration (µg/mL)	AUC IS PARA	AUC RS	AUC ratio	Average ration AUC
5	2.535	997682	4664680	0.4676	
		976369	461188	0.4724	0.4693
		977605	4574488	0.468	
10	5.07	947591	86009	0.916	
		954509	901785	0.9448	0.9358
		926053	876506	0.9465	
20	10.14	942685	1624593	1.7234	
		940576	1627681	1.7305	1.7268
		946506	1634068	1.7264	
30	15.21	990661	236976	2.392	
		1003343	2362472	2.3546	2.3897
		980252	2374686	2.4225	

Table 0.5 Recovery studies of flucloxacillin

	AUC IS	**AUC RS**	**AUC ratio**	**Average AUC ratio**	**SDEV**	**%RSD**
1 ml RS + 2 ml IS	780955	799289	1.0235	1.02424	0.00253	0.25
	812336	830814	1.0227			
	801131	823499	1.0279			
	822182	843224	1.0256			
	797503	814643	1.0215			
1 ml SFLLP +2 ml IS	867978	660695	0.7612			
	854775	659188	0.7712	0.7612		
	852916	660738	0.7747			
1 ml SFLLP + 1 ml RS + 2 ml IS	284876	490727	1.7226			
	787520	1382553	1.7556	1.7391		
	848356	1475416	1.7391			
SFLAR	850184	407274	0.4790	0.4759		
	856841	405201	0.4729			
SFLAR + RS	850163	1206557	1.4192	1.4219		
	852469	1214477	1.4247			
CFLMG	853944	533174	0.6244	1.2439		
	869147	538448	0.6195			
CFLMG + RS	848884	1387659	1.6347	3.2855		
	838979	1385002	1.6508			
FLP CAPS	802244	1087373	1.3554			
	820509	1104010	1.3455	1.3502		
	801895	1082399	1.3498			
FLP + STD	772029	1885842	2.4427			
	790768	1927687	2.4377	2.4551		
	781560	1942187	2.4850			

AUC = Area under curve, IS = Internal standard, RS = Reference standard

Calibration curve for cloxacillin

Volumes of 1, 2, 3 and 5 mL of the RS were pipetted into four different 100 mL volumetric flasks. To each flask 5 mL of IS was added and solution topped to the 100 mL mark with mobile phase. A four-point calibration curve was drawn from the peak area ratio obtained Figure 3.11. Results are shown in Appendix/Table

Table 0.6 Analysis of cloxacillin reference sample and acetaminophen (paracetamol) for the generation of calibration curve

VOLUME OF STOCK USED	CONCENTRATION (µg/mL)	AUC IS	AUC RS	AUC RATIO (IS:RS)	AVERAGE RATIO
1	0.001172	99017	104914	1.0596	
		138911	131263	0.9449	
		112930	116243	1.0293	
		73476	77776	1.0585	1.05697
		127800	139125	1.0886	
		171394	180044	1.0505	
		37020	39067	1.0553	
2	0.002344	176550	352281	1.9954	
		196686	379825	1.8874	1.9622
		144645	289825	2.0037	
3	0.003516	62606	179069	2.8603	
		108770	325111	2.989	2.9166
		179292	520031	2.9005	
5	0.00586	163572	774346	4.733397	4.64
		1966192	894784	4.5608	

AUC = Area under curve, IS = Internal standard, RS = Reference standard

Table 0.6 Determination of amount of active pharmaceutical ingredient (API) in the samples

Amoxicillin capsule samples	Amoxicillin suspension samples	Recovery studies
Example: Sample 01A contains 250 mg amoxicillin trihydrate Using the equation from the calibration curve y=194.41x + 0.004, where y= peak area ratio and x= concentration of sample weight of 20 capsules = 9.01632 Weight of empty shells= 1.50272 Content = 7.5136 Therefore average weight of a capsule=0.37568 g From (table 0.3) Area under curve ratio of sample 01A (y) = 0.3539 0.3539 – 0.004/194.41 = x, Therefore x=1.79980 × 10^{-3} mg in 3 mL of sample Therefore in 100 mL will be 1.79980 × 10^{-3} × 100 / 3 x = 0.0599935 g. Weight of sample taken = 0.0761 g. If 0.37568 g ≡ 0.25 g, Then 0.0761=0.0761 × 0.25/0.37568 = 0.0506 g Therefore % active in sample = 0.0599935 g ×100/0.0506 g = 118.56 % × 12% moisture = 104.34 % active in test sample Actual amount in mg: If 100 % = 250 mg, 104.34 % =104.34 × 250 / 100 = 260.85 mg	Example: Sample S01 (125 mg/5 mL) Volume of sample pipetted = 5 mL ≡ 0.125 g of amoxicillin trihydrate Using the equation of the curve y=194.41x + 0.004, where y= area under curve ratio and x= concentration of sample Implies x = y-0.004/194.41 From table 0.3 area under curve ratio of sample 01A is (y) = 0.7832 Putting y=0.7832 into the equation above 0.7832 – 0.004/194.41 = x = 4.00802×10^{-3} in 3 mL Therefore in 100 mL = 4.00802×10^{-3} × 100 / 3= 0.1336 g Therefore % active in sample = 0.1336 g ×100/0.125 g = 106.88 % × 12% moisture = 94.05 Actual amount in mg If 100% = 125 mg/5 mL, then 94.05% =94.05 × 250 / 100 = 117.56 mg/5 mL	Using Sample CLAR 03 as an example: Area under curve ratio of Sample + standard=AB Area under curve ratio of sample =B Area under curve ratio of reference standard =X Therefore area under curve ratio of standard =AB-B=A % Recovery=A/X × 100 Area under curve ratio of Sample + standard=2.5472 Area under curve ratio of sample =0.9762 Therefore peak ratio of standard=2.5472 – 0.9762 = 1.571 % Recovery=1.571/1.6286 × 100= 96.46%

Table 0.7 Anova: Single Factor calibration for amoxicillin (A)

SUMMARY				
Groups	***Count***	***Sum***	***Average***	***Variance***
Concentration	6	0.00789	0.001315	9.68366E-07
Area under curve	6	1.55779	0.259632	0.036620154

Table 0.8 Anova: Single Factor calibration for amoxicillin (B)

ANOVA						
Source of Variation	***SS***	***Df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	0.200182501	1	0.200183	10.93262522	0.007926661	4.964602744
Within Groups	0.18310561	10	0.018311			
Total	0.383288111	11				

Table 0.9 Anova: Single Factor calibration for flucloxacillin (A)

SUMMARY				
Groups	***Count***	***Sum***	***Average***	***Variance***
Concentration (µg/mL)	5	0.032955	0.006591	3.72721E-05
Area under curve	5	5.5216	1.10432	0.922713787

Table 0.10 Anova: Single Factor calibration for flucloxacillin (B)

ANOVA						
Source of Variation	***SS***	***Df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	3.012522394	1	3.012522	6.52943687	0.033895198	5.317655072
Within Groups	3.691004236	8	0.461376			
Total	6.70352663	9				

Table 0.11 Anova: Single Factor calibration for cloxacillin (A)

SUMMARY				
Groups	***Count***	***Sum***	***Average***	***Variance***
Concentration (µg/mL)	5	0.012892	0.002578	5.08226E-06
Area under curve	5	10.57577	2.115154	3.158546941

Table 0.12 Anova: Single Factor calibration for cloxacillin (B)

ANOVA						
Source of Variation	***SS***	***Df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	11.15743916	1	11.15744	7.064907644	0.028895447	5.317655072
Within Groups	12.63420809	8	1.579276			
Total	23.79164726	9				

Table 0.13 **Residual analysis of amoxicillin**

ACTUAL *y*	**PREDICTED *y***	**X**	**RESIDUALS**
0.108	0.10625966	0.000526	0.00000303
0.2152	0.20851932	0.001052	0.00004463
0.3097	0.31077898	0.001578	0.00000116
0.40789	0.41303864	0.002104	0.00002651
0.517	0.5152983	0.00263	0.00000290
0	0.004	0	0.00001600

Table 0.14 **Residual analysis of flucloxacillin**

ACTUAL *y*	**PREDICTED *y***	**X**	**RESIDUALS**
0.4693	0.4677429	0.002535	0.000002
0.9358	0.8655858	0.00507	0.004930
1.7268	1.6612716	0.01014	0.004294
2.3897	2.4569574	0.01521	0.004524
0	0.0699	0	0.004886

Table 0.15 **Residual analysis of cloxaicillin**

ACTUAL *y*	PREDICTED *y*	X	RESIDUALS
1.05697	1.00717816	0.001172	0.002479227
1.9622	1.93045632	0.002344	0.001007661
2.9166	2.85373448	0.003516	0.003952074
4.64	4.7002908	0.00586	0.003634981
0	0.0839	0	0.00703921

Preparation of test sample solution

Flucloxacillin capsules

Twenty filled capsules of flucloxicillin sodium were weighed and the weight noted. Contents of capsules were emptied into a container. Weight of emptied shells was also noted. An amount equivalent to 51.0 mg was weighed into a 100 mL volumetric flask and dissolved with distilled water. It was then topped with mobile phase to the 100 mL mark. The solution was filtered with whatman cellulose membrane filter paper of pore size, 0.45 Millipore. Volumes of 10 mL of the filtrate and 20 mL of the internal standard were pipetted into a 100 mL volumetric flask made up to 10 mL mark with mobile phase. It was the injected into the HPLC.

Suspensions

An equivalent of 250 mg (5 mL) flucloxacillin sodium was pipetted into a 100 mL volumetric flask and dissolved with distilled water. Solution was topped with mobile phase to the 100 mL mark and filtered. Volumes of 10 mL of the filtrate and 20 mL of the internal standard were pipetted into a 100 mL volumetric flask made up to 100 mL mark with mobile phase. It was the injected into the HPLC.

Cloxacillin capsules

Twenty filled capsules of cloxicillin sodium were weighed and weight noted. Contents of capsules were emptied into a container and weight of emptied shells also noted. An amount equivalent of 250 mg was weighed into a 100 mL volumetric flask and dissolved with distilled water. It was then topped with mobile phase to the 100 mL mark. The solution was filtered. Volumes of 2 mL of the filtrate and 2 mL of the internal standard were pipetted into a 100 mL volumetric flask made up to 100 mL mark with mobile phase. It was then injected into the HPLC.

Suspensions

An equivalent of 250 mg (5 mL) was pipetted into a 100 mL volumetric flask and dissolved with distilled water. Solution was topped with mobile phase to the 100 mL mark and filtered. Volumes of 1 mL of the filtrate and 2 mL of the internal standard were pipetted into a 100 mL volumetric flask made up to 100 mL mark with mobile phase. It was the injected into the HPLC.

APPENDIX III: PREPARATION OF MICROBIAL MEDIA

1.0 NUTRIENT AGAR (OXOID)

Composition	Quantity (g)
Lab-lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0
Distilled water to	1 L

A quantity of 28 g nutrient agar powder was weighed into a beaker and dissolved with distilled water to 1 L. The mixture was then heated on a water bath to boil and poured into glass test tubes. The mixture was then sterilized in an autoclave at 121^{o}C for 15 min.

2.0 NUTRIENT BROTH (OXOID)

Composition	Quantity (g)
Beef extract	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Distilled water to	1 L

A quantity of 13 g nutrient broth powder was weighed into a beaker and dissolved with distilled water to 1 L. The mixture was then poured into glass tubes and sterilized at 121^{o}C for 15 min in an autoclave

.3.0 PLATE COUNT AGAR (OXOID)

Composition	Quantity (g)
Tryptone	5.0
Yeast extract	2.5
Dextrose	1.0
Agar	9.0
Distilled water to	1 L

A quantity of 17.5 g of plate count agar powder was weighed into a beaker and dissolved with distilled water to 1 L. the mixture was then heated on a water bath to boil and poured into glass test tubes. The mixture was then sterilized in an autoclave at 121^{o}C for 15 min.

4.0 MACONKAY AGAR (OXOID)

Composition	**Quantity (g)**
Lactose	10.0
Bile salts	1.5
Peptone	20.0
Sodium chloride	5.0
Neutral red	0.03
Crystal violet	0.001
Agar	15.0
Distilled water to	1 L

A quantity of 51.5 g maconkay agar powder was weighed into a beaker and dissolved with distilled water to 1 L. The mixture was boiled and sterilized in an autoclave at 121^{o}C for 15 min.

5.0 MANNITOL SALT AGAR (OXOID)

Composition	**Quantity (g)**
Lab-lemco powder	1.0
Mannitol	10.0
Peptone	10.0
Sodium chloride	75.0
Agar	15.0
Phenol red	0.025
Distilled water	1 L

A quantity of 111 g mannitol salt agar powder was weighed into a beaker and dissolved with distilled water to 1 L. The mixture was boiled and sterilized in an autoclave at 121^{o}C for 15 min.

6.0 CETRIMIDE AGAR (OXOID)

Composition	Quantity (g)
Gelatin peptone	20.0
Potassium sulphate	10.0
Magnesium chloride	1.4
Cetrimide	0.3
Agar	13.6
Distilled water to	1 L

A quantity of 45.3 g cetrimide agar powder was weighed into a beaker and dissolved with distilled water to 1 L. The mixture was boiled and sterilized in an autoclave at 121^{o}C for 15 min.

7.0 POTATO DEXTROSE AGAR (OXOID)

Composition	Quantity (g)
Potato extract	4.0
Dextrose	20.0
Agar	15.0
Distilled water to	1 L

A quantity of 39 g of potato dextrose agar powder was weighed into a beaker and dissolved with distilled water to 1 L. The mixture was boiled and sterilized in an autoclave at 121^{o}C for 15 min.

8.0 KOSER CITRATE MEDIUM (OXOID)

Composition	Quantity (g)
Sodium Ammonium Phosphate	1.5
Potassium Dihydrogen Phospate	1.0
Magnesium sulphate	0.2
Bromothymol blue	0.016
Distilled water to	1 L

A quantity of 5.2 g of the powder was weighed into a beaker and dissolved with of distilled water to 1 L. Ten milliliter of the mixture was then distributed into glass test tubes and sterilized at 121^{o}C for 15 min in an autoclave.

9.0 TRYPTONE BROTH (OXOID)

Composition	Quantity (g)
Tryptone	10.0
Sodium chloride	5.0
Distilled water to	1 l

A quantity of 15 g of the powder was weighed into a beaker and dissolved with distilled water to 1 L. Ten milliliter of the mixture was then distributed into glass test tubes and sterilized at 121^{o}C for 15 min in an autoclave.

10.0 MRVP MEDIUM (OXOID)

Composition	Quantity (g)
Peptone	7.0
Glucose	5.0
Phosphate buffer	5.0
Distilled water to	1 L

A quantity of 17 g of MRVP powder was weighed into a beaker and dissolved with distilled water to 1 L. Ten milliliter of the mixture was then distributed into glass test tubes and sterilized at 121^{o}C for 15 minutes in an autoclave.

APPENDIX IV: IDENTIFICATION AND CONFIRMATION OF MICROORGANISMS BY SELECTIVE MEDIA AND BIOCHEMICAL REACTIONS

- ***Pseudomonas aeruginosa***

This microorganism was identified by cultivating on cetrimide agar. All greenish colonies observed on the surface of the agar after 24 h incubation at 37 ºC was confirmed as *P. aeruginosa.* Some of the identified colonies were sub-cultured in test tubes containing 10 mL Koser's citrate medium. The appearance of deep blue colouration confirmed the identity of *P. aeruginosa.*

- ***Staphylococcus aureus***

This microorganism was identified by culturing on mannitol salt agar. The appearance of yellow colonies on the surface of the agar after 24 h incubation at 37 ºC indicates the presence of *S. aureus.*

The identity of the organism was further confirmed by the coagulase test was used confirm. A quantity of 0.1 mL of a 24 h broth culture of isolated colonies from the mannitol salt agar was inoculated into nutrient agar containing 10% $^{v}/_{v}$ rabbit blood plasma. This was incubated for 24 h at 37 ºC. The gelling of the plasma confirms *S. aureus.*

- ***Escherichia coli***

E. coli was identified by culturing on Macconkay agar. The appearance of red-violet colonies on the surface of the agar after 24 h incubation at 37 ºC indicates the presence of *E. coli.* The organism was further confirmed by performing the indole and MRVP tests. The indole test was performed by inoculating the organism in tryptone broth and

incubating at 37 ºC for 24 h. The appearance of a pink/red colour after the addition of Kovac's reagent to the overnight culture indicates the presence of *E. coli.* The MRVP test was performed by inoculating a 24 h broth culture of the organisms in MRVP broth. The culture after incubation was divided into two; one part for the MR test and the other for the VP test. The MR test was performed by the addition of methyl red to the culture; the appearance of pink/red colouration indicates that the organism is MR positive. The VP test was performed by the addition of α-naphthol and KOH solutions; the absence of a cherry red colouration indicates that the organism was VP negative.

Bacillus subtilis

The organism was identified by starch and casein hydrolysis. A 24 h broth culture of the organism was streaked centrally on the surface nutrient agar containing 10% $^{w}/_{v}$ starch and incubated at 37 ºC for 24 h. The culture was then sprayed with iodine solution after incubation; the appearance of clear region due to the hydrolysis of starch amidst a blue-black surrounding indicates the presence of *B. subtilis.*