KWAME NKRUMAH UNIVERSITYOF SCIENCE AND TECHNOLOGY

COLLEGE OF HEALTH SCIENCES

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

STABILITY STUDIES ON RECONSTITUTED AMOXYCILLIN-CLAVULANIC ACID ORAL SUSPENSION BY HPLC DEVELOPMENT AND QUANTIFICATION

BY

LAWRENCIA YEBOAH – AWUDZI

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by

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DECLARATION

The research work reported in this thesis was carried out at the Department of Pharmaceutical Chemistry, K.N.U.S.T. Any assistance obtained has been duly acknowledged.

This work has not been submitted for any other degree.

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(Supervisor) Signature Date

Certified by

PROF. R.K Adosraku ....

(Head of Department) Signature Date

DEDICATION

I dedicate this work to my parents Dr. Kwasi Yeboah-Awudzi and Georgina Yeboah-Awudzi, as well as my husband Rodney Enimil.

ACKNOWLEDGEMENT

It is the lord who gives wisdom, and from Him come knowledge and understanding. I am forever grateful to the almighty God who has been my best and closest companion through it all. I am thankful that He has brought me to an expected end.

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To God be the Glory, great things He has done.

ABSTRACT

Amoxycillin has a broad spectrum of activity against a wide number of organisms. However its usage as mono-therapy for certain infections such as respiratory tract infections became ineffective due to the emergence of beta lactamase producing bacteria. Clavulanic acid was thus introduced as a protective agent for amoxycillin against such organisms. The two also have a synergistic effect although clavulanic acid on its own has no significant antibacterial action. The formulation of amoxycillin and clavulanic acid (Co – amoxiclav) for paediatric use comes in the form of an oral powder, which has to be reconstituted before administration. Concerns have been raised with regards to the appropriateness of the use of certain kinds of water such as treated tap water or any portable drinking water for that matter, in the reconstitution of oral powders such as that of co – amoxiclav into suspensions. Thus, a stability study was carried out on co – amoxiclav oral powder reconstituted with distilled water (the recommended choice), treated tap water and a commercial mineral water, under the standard storage condition of 2 - 8ºC. From the study, it was found that the three kinds of water, irrespective of their mineral or ionic content, did not have any significant detrimental effect on the stability of amoxicillin and clavulanic acid, as long as the oral suspension was stored in a refrigerator (2 - 8 ºC) throughout the duration of therapy (7 days). Thus, the above mentioned types of water can be conveniently used for the reconstitution of co – amoxiclav in our part of the world. Further analysis was carried out to ascertain the stability of amoxicillin and clavulanic acid in their oral suspension (reconstituted with distilled water) when stored in the refrigerator (2 - 8ºC) only on alternate days during the duration of therapy. It was found that both amoxicillin and clavulanic acid remained stable in spite of the inconsistency in their storage conditions. However, the standard storage temperature should be adhered to stringently to guarantee maximum therapeutic benefit. A simulation was carried out where co

– amoxiclav oral suspension was kept in a bowl of water under normal room temperature and analysed. This also revealed that amoxicillin remained stable throughout the duration of therapy but clavulanic acid did not. To aid in the stability studies, a simple and cost effective High Performance Liquid Chromatographic method of assay was developed. The method made use of a reverse phase column of Phenomenex, Kromasil 5 (C_8), 250 X 4.60mm 5 micron column with size 305334. The flow rate was 1ml/min and a UV detector was used to detect at a wavelength of 220nm.The mobile phase system comprised of Water, methanol and sodium acetate buffer pH 4.4 in the ratio of 60:15:20. The LOD and LOQ for amoxicillin were 0.00614%w/v and 0.0186%w/v respectively and the LOD and LOQ for clavulanic acid were 0.00126%w/v and 0.003818%w/v for respectively. In all three brands of co-amoxiclav were assayed including the innovator brand.

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CHAPTER 1 - INTRODUCTION

1.1 General introduction

In 1875 Pasteur and Joubert found out that certain moulds could release toxic substances which killed bacteria. Unfortunately, these substances also happened to be detrimental to humans and hence of no clinical value. However, moulds were noted to be a prospective source of antibacterial agents. In 1928, Fleming noted that a bacterial culture which was abandoned several weeks in open air, had become contaminated with a fungal colony. What drew his attention, was an area encircling the fungal colony where the bacterial colonies had not thrived. He deduced then that the fungal colony must have been producing an antibacterial agent, extending into its immediate surroundings. He then set out to investigate the fungus and found it to be a relatively rare species of *Penicillium.* Although Penicillin had significant antibacterial properties as well as being non-toxic to humans, it was so unstable that Fleming was unable to isolate and purify the compound. Thus, seemed clinically unbeneficial. This limitation was overcome in 1938 by Florey and Chain through a freeze-drying process which allowed for isolation of the antibiotic under less harsh conditions by 1941. In 1945, Dorothy Hodgkins discovered the structure of penicillin by X-ray analysis. Although Penicillin was known to be a wonder drug, it still had some limitations. These limitations include it not being well absorbed in the intestinal tract, so much so that at least 70% of an oral dose of the drug is wasted. It is also short acting and thus, has half of the amount in circulation being removed from the body every half an hour. Penicillin is more active against gram positive bacteria than the gram negative ones. Staphylococci have also grown resistant to penicillin. Amoxycillin, a derivative of penicillin (specifically ampicillin) was synthesised to help offset the shortcomings of the latter. Amoxycillin only differs from ampicillin by the presence of a hydroxyl group. Thus, is better able to withstand damage from

stomach acid and so much less of an oral dose is wasted. Although it is still susceptible to destruction by Staphylococcal enzymes, it does have a much broader spectrum against the gram negative cell wall **(C.Wendy, 2012).** All penicillins including Amoxycillin have the beta-lactam ring as part of their structure. This β-lactam ring is very reactive but can be split open in either a neutral or basic medium, resulting in its inactivity. The ring can be also acted upon by β-lactamase, an enzyme produced by some bacteria which can degrade Penicillin antibiotics. Thus, Penicillin antibiotics are modified chemically in their structures to help increase their acid stability as well as heir β-lactamase resistance. The addition of an "electron withdrawing" group unto the 6-position of the amide group (located on the β-lactam ring) results in an increase in its acid stability due to the amide oxygen becoming less nucleophillic. This ensures that the amide oxygen does not attack the β-lactam ring's carbonyl group to open it up **(Beleh, 2006)**. Addition of a bulky substituent (such as a benzene ring) to this very position, shields the β-lactam ring's carbonyl group and thus increases the drug's β - lactamase resistance. To broaden the spectrum of activity of the penicillin, a polar group can be added to the 6-position amide group. This allows for activity of drug against gram negative bacteria as well as gram positive organisms. These polar groups enable access of drug into gram negative cell wall through porins. Amoxicillin has an electron withdrawing group with a polar hydroxyl group added to the 6 - position amide. This increases its spectrum of action and acid stability as compared to other Penicillins, such as Ampicillin. However, amoxycillin's substituent does not shield the β-lactam ring. Thus, still makes it vulnerable to β-lactamases and thus can be degraded by resistant bacteria **(Beleh, 2006).**

Mechanism of Action

Penicillins act on bacteria by preventing the cross linkage of peptidoglycan in the cell wall of bacteria. Peptidoglycan serves as the "backbone" of the bacterial cell hence giving the cell its

rigidity. Absence of peptidoglycan makes the cell wall unable to handle pressure from within resulting in the rupture and death of the cell. Amoxicillin binds to the penicillin-binding protein 1A (PBP-1A) located inside the bacterial cell well. It inhibits the last stage of bacterial cell wall synthesis by acylating the penicillin-sensitive transpeptidase C-terminal domain thereby stopping the cross-linking of peptidoglycan strands. Autolytic enzymes in the bacteria then lyse the cells. Penicillin antibiotics resemble the transition state of the cross linkage reaction and the Ala-Ala terminal of the peptidoglycan therefore, they are acted on by the transpeptidase enzyme **(Beleh, 2006)**.

Resistance

As stated earlier, resistance tends to occur if bacteria produce β-lactamase. This enzyme is found between the bacteria's outer membrane and peptidoglycan in gram negative bacteria. The enzyme attacks the carbonyl group of the β-lactam ring and causes degradation of the drug. This can however be prevented if a bulky substituent is attached to the 6-position to shield the reactive ring. Amoxicillin however does not have any such bulky group, and so is rather used in combination with a β-lactamase inhibitor such as Clavulanic acid. The latter is rather acted upon by the β-lactamase which is used up allowing the Amoxicillin to kill the bacteria without being degraded. Amoxycillin is usually prescribed with clavulanic acid as the potassium salt. Clavulanic acid is a naturally occurring , β-lactamase inhibitor , produced by fermentation of Streptomyces clavuligerus, for treatment of infection caused by β-lactamase producing bacteria that are resistant to amoxycillin alone **(Aghazadeh.A and G, 2001**).

1.2 Problem statement

Stability in relation to the drug dosage form refers to both the physical and chemical integrity of the former. A stable drug should also be able to guard against microbial contamination. The parameters that are peculiar to stability include; environmental conditions of storage such as temperature, light, air, humidity and the type of packaging. Pharmacopoeial articles must have the requisite storage conditions on their labeling. These are the conditions within which the expiration date is valid. Storage conditions specified on the labeling of the article must be adhered to, all through the supply of the article. It is however very uneasy to monitor and control storage conditions once product ends up with the consumer. Patient may not adhere stringently to instructions pertaining to the handling of drug (especially when it comes to oral powder for reconstitution) even though labeling might clearly spell out the appropriate storage conditions. **(USP 30-NF25, 2007).** Amoxicillin and clavulanic acid (Co – amoxiclav) oral powder for reconstitution can be stored under normal room temperature (25ºC), for as long as the expiry date will allow. After, reconstitution however, the suspension has to be strictly stored in a refrigerator (2 – 8ºC) to ensure stability during dosing regimen (5 or 7 days). Studies have indicated different 'drug in – home' storage practices such as the keeping of drugs on dining tables, on top of refrigerators, inside first aid boxes, in bags, in the car, within closed cabinets, suit cases and the like as well as in the kitchen and bathroom. These practices may result in degradation **(Nwokoye Peace, 2012)** Will non - adherence to the standard storage condition of 2 - 8ºC affect stability? Should reconstitution of the powder be done with a particular type of water, or the type of water can be varied? Will there be any significant effect on the stability of amoxicillin – clavulanic acid oral suspension?

1.3 General objective

The aim of this study is to conduct a stability assessment of different brands of reconstituted co-amoxiclav suspension.

1.4 Specific objectives

These are to;

(i) Ascertain whether or not there is variation in the type of water used for the reconstitution of amoxicillin – clavulanic acid oral powder for suspension.

(ii) Develop an HPLC method of assay for amoxicillin – clavulanic acid combination therapy.

(iii) Ascertain the effect of different qualities of water on the stability of reconstituted amoxicillin – clavulanic acid oral suspension.

(iv) Analyse the stability of reconstituted amoxicillin – clavulanic acid oral suspension under different storage conditions.

(v) Analyse the stability of reconstituted amoxicillin – clavulanic acid oral suspension under patient simulated conditions.

CHAPTER 2 – LITERATURE REVIEW

2.1 Amoxycillin

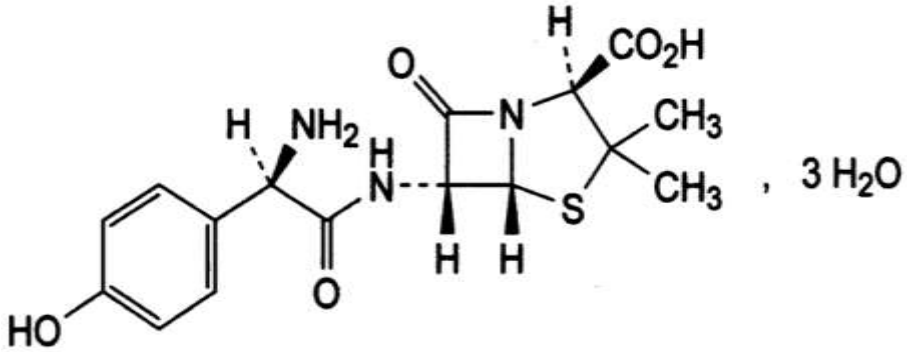

Figure 1: Chemical structure of amoxycillin trihydrate (Molecular weight = 419.4)

Amoxicillin is a beta-lactam antibiotic which is a cyclic amide. A 5-membered thiazolidine ring is fused to the beta-lactam ring and so the molecule contains a characteristic "V" shape which makes it more sensitive to hydrolysis. The mechanism of action of beta-lactam antibiotics involves inhibiting the enzymes that help to develop the peptidoglycan layer of the cell wall. Stage III of cell wall biosynthesis is inhibited and so there is no cross-linking of the peptidoglycan. If the bacteria are not able to form cell walls around them, they will not be protected from their environment and so they will stop multiplying. Amoxicillin has the essential substituents for activity including; the 1st position sulfur, two methyl groups at the 2nd position, a carboxylic acid at the 3rd position, a nitrogen at the 4th position and a carbonyl at the 7th position. It also does not have any substitutions on the 5th position which is critical. The 6th position allows for a variety of substitutions that in turn alter activity. Amoxicillin has a para - phenolic hydroxyl group which improves its blood levels in the body as compared to Ampicillin. Amoxicillin has a polar group which allows for a broader spectrum of activity, allowing passage through the porins of Gram negative bacteria. The primary amine that is attached to the C-6 substituent is electron withdrawing.

Thus, rendering the amide oxygen, less nucleophilic hence better acid stability. One (1) part of amoxycillin trihydrate dissolves in 400 parts of water, and 1 part of amoxicillin dissolves in a 1000 parts of ethanol whilst 1 part of amoxicillin dissolves in 200 parts of methanol. It is however insoluble in both chloroform and ether.

Structure of penicillin

Penicillin has a highly unstable bicyclic system consisting of a four- membered β-lactam ring fused to a five-membered thiazolidine ring. The skeletal structure of the molecule indicates a correlation with the amino acids cysteine and valine .The general shape of the molecule is likened to a half-opened book, as shown.

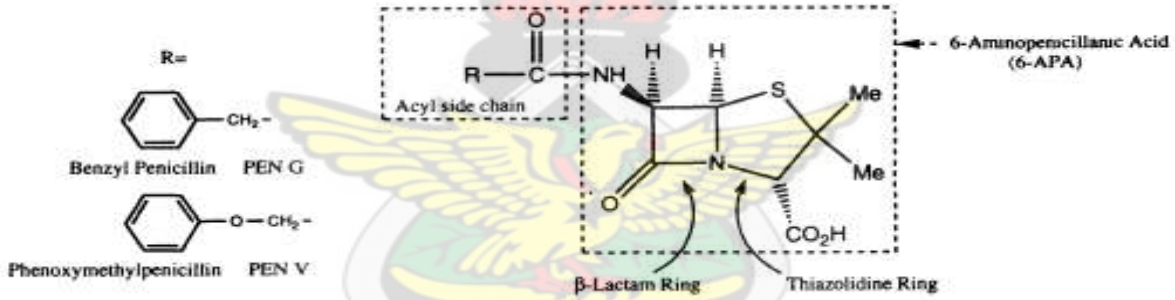

Figure 2: The structure of penicillin (The acyl side – chain (R) differs, with regards to the fermentation media)

Figure 3: Shape of penicillin and its derivitization from cysteine (CYS) and valine (VAL)

Penicillin analogues

One means of varying the side-chain is to include different carboxylic acids in the fermentation medium; for instance, adding phenoxyacetic acid ($PhOCH_2COOH$) results in penicillin V. There is however a restriction as to the type of carboxylic acid which can be added to the medium (thus, only acids of general formula RCH_2CO2H), and this in turn restricts the variety of analogues which can be derived. Another drawback in deriving analogues by this means is that it is tedious and time-consuming. In 1957, Sheehan was able to synthesize penicillin, and obtained a 1% yield of penicillin V by a multistep synthetic route. In 1958-60, however Beechams managed to isolate a biosynthetic intermediate of penicillin which was also present in Sheehan's synthetic intermediates. The compound was 6-Amino Penicillanic Acid (APA) and it resulted in the synthesis of a large number of analogues by a semi-synthetic method. Thus, fermentation resulted in 6-APA which could then be treated synthetically to give penicillin analogues. This was achieved by acylating the 6-APA with a range of acid chlorides .6-APA is now produced by hydrolysing penicillin G or penicillin V with an enzyme (penicillin acylase) or by chemical methods. These are more efficient methods than fermentation.

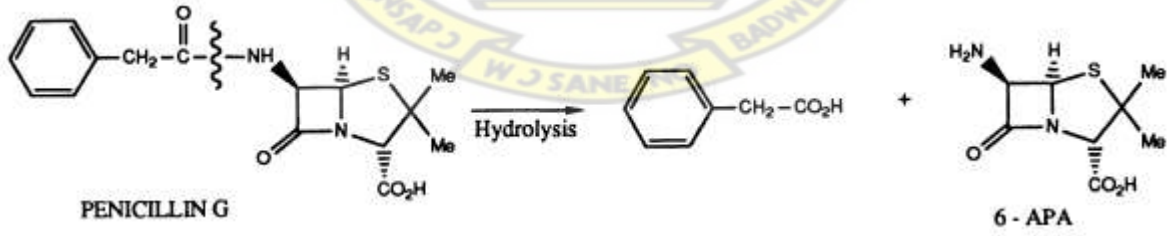

Figure 4: Production of 6-APA

The properties of benzyl penicillin are as follows; Active against Gram-positive bacilli (e.g. staphylococci, meningitis, and gonorrhoea) and many (but not all) Gram-negative cocci.

Non-toxic; The penicillins are amongst the safest drugs known in medicine. However, they are not active over a wide range (or spectrum) of bacteria. They are ineffective when taken orally. Penicillin G can only be administered by injection it is ineffective orally since it breaks down in the acid conditions of the stomach. They are sensitive to all known (β-lactamases) which are enzymes produced by penicillin resistant bacteria which catalyse the degradation of penicillins. Allergic reactions are experienced by some patients. It is obvious, there are a lot of problems associated with the use of penicillin G, with acid sensitivity being the predominant, sensitivity to penicillinase, and a narrow spectrum of activity. The essence of making semi-synthetic penicillin analogues is thus to find compounds which are free from these disadvantages. However, before any significant changes can be made, a structure-activity related study is necessary to help find out which portions of the penicillin molecule are crucial for its activity. These portions would then be maintained in all analogues which are made. Thus for structure - Activity Relationships of Penicillins, the strained β-lactam ring is crucial. The free carboxylic acid is also crucial. The bicyclic system is necessary (confers strain on the β - lactam ring, the greater the strain, the greater the activity and the greater the instability of the molecule to other factors).The acylamino side-chain is essential (except for thienamycin,). Sulfur is usual but not essential. The stereochemistry of the bicyclic ring with respect to the acylamino side-chain is necessary. Amoxycillin is given orally as the trihydrate and by injection as the sodium salt so as to aid in dissolution. Co – amoxiclav is given by mouth as a ratio of 2, 4, 7 or 14 parts to 1 part of clavulanic acid as the potassium salt. The stability of potassium clavulanate is very low, as such their formulation as an oral powder for reconstitution. This drug is however to be reserved only for bacterial infections likely to be caused by amoxicillin resistant beta-lactamase producing strains**(S.C, 2005)**. Findings from analysis carried out to showed that very little variation could be made on the penicillin nucleus. This variation is however limited to the acyl amino side - chain.

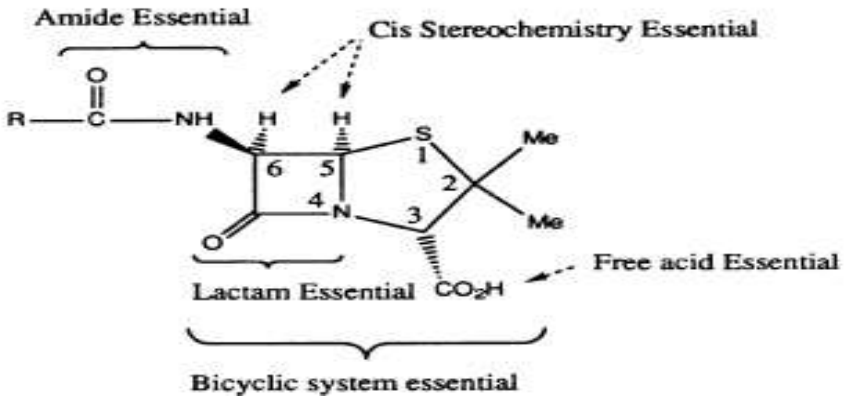

Figure 5: Structure Activity Relationships of Penicillins

Circumventing some of the limitations of penicillins.

Acid sensitivity of penicillins; There are three reasons for the acid sensitivity of penicillin G. These are;

(i) Ring strain; The bicyclic system in penicillin consists of a four-membered ring and a five - membered ring. As such, penicillin suffers large angle and torsional strains. Acid-catalysed ring opening tends to relieve these strains by breaking open the more highly strained four-membered lactam ring.

Figure 6: Ring opening

(ii) A highly reactive β-lactam carbonyl group. The carbonyl group in the β-lactam ring is very susceptible to nucleophiles and as such does not behave like a regular tertiary amide which happens to be quite resistant to nucleophilic attack. This difference in reactivity is

because of the fact that stabilization of the carbonyl is possible in the tertiary amide, but rather impossible in the β-lactam ring. The β-lactam nitrogen is unable to feed its lone pair of electrons into the carbonyl group since this would require the bicyclic rings to adopt an impossibly strained flat system. As such, the lone pair is localized on the nitrogen atom and the carbonyl group is far more electrophilic than should be for a tertiary amide. A normal tertiary amide is far less susceptible to nucleophiles since the resonance structures above reduce the electrophilic character of the carboxyl group.

(iii) Effect of the acyl side-chain (neighbouring group participation); There is a demonstration of how the neighbouring acyl group can actively play a role in a mechanism to open up the lactam ring. Thus, penicillin G has a self destruct mechanism built into its structure.

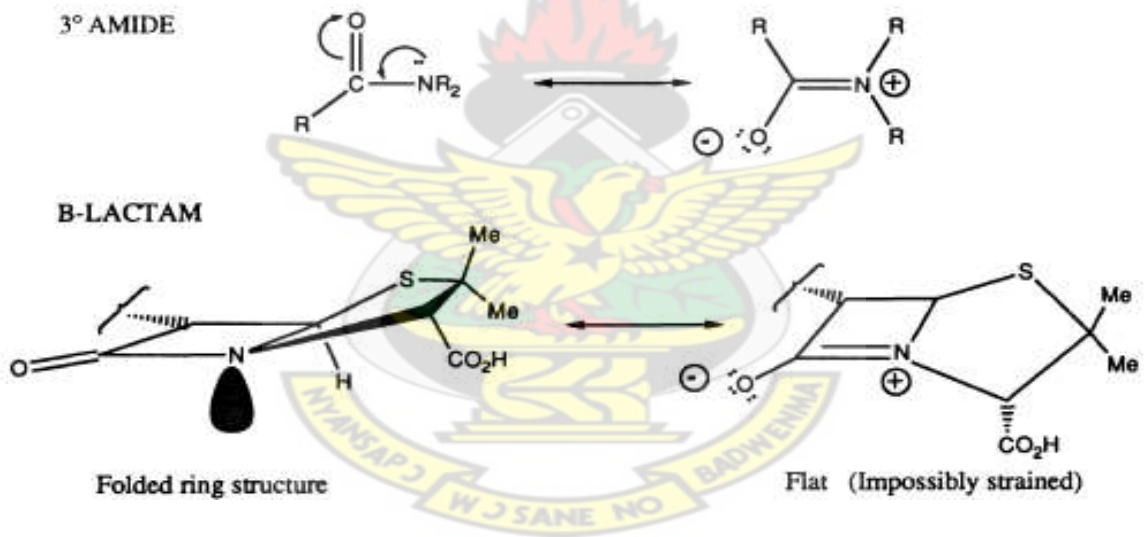

Figure 7: Highly reactive β-lactam carbonyl group

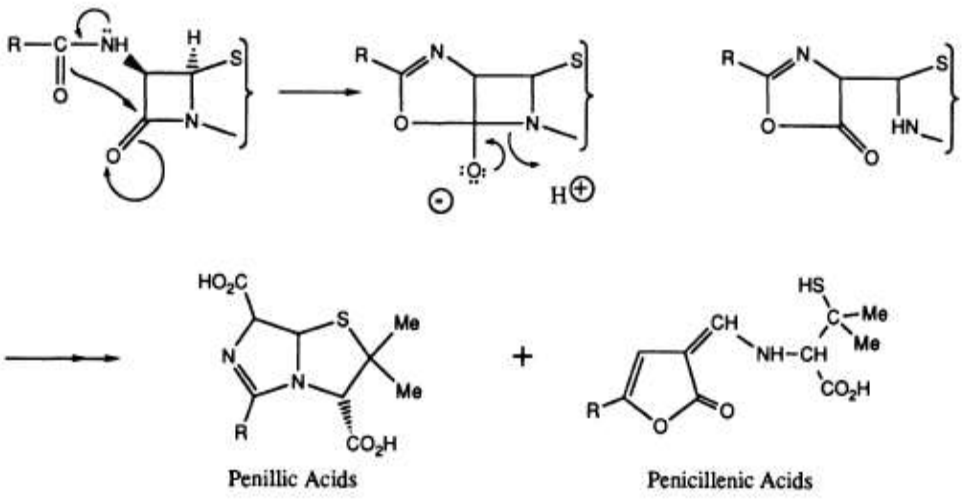

Figure 8: Influence of the acyl side chain on acid sensitivity

Tackling the problem of acid sensitivity

Not much can be done about the initial two factors since the β-lactam ring is vital for antibacterial activity. Without it, the molecule has no useful biological activity at all. Thus, just the third factor can be tackled. The agenda then becomes one of reducing the amount of neighbouring group involvement to make it uneasy, if not impossible, for the acyl carbonyl group to attack the β-lactam ring. Fortunately, such an objective is feasible. If an efficient electron withdrawing group is attached to the carbonyl group, then the inductive pulling effect would have to draw electrons away from the carbonyl oxygen and reduce the likelihood for it to act as a nucleophile. Penicillin V has an electronegative oxygen on the acyl side-chain with the electron withdrawing effect required.

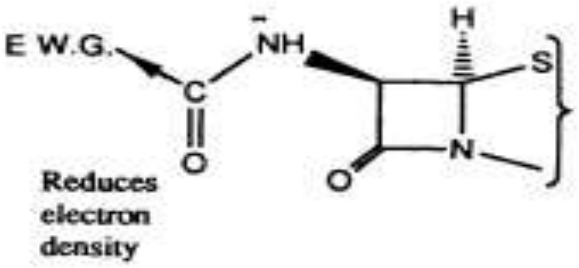

Figure 9: Reduction of Neighbouring Group's Participation with electron withdrawing group.

The molecule has better acid stability than penicillin G and is stable enough to survive the acid in the stomach. Thus, it can be given orally. However, Penicillin V is still sensitive to penicillinases and is slightly less active than penicillin G. It also has the problem of allergic sensitivity in some individuals just as penicillin G. A range of penicillin analogues which have been very successful are penicillins which are di-substituted on the alpha-carbon next to the carbonyl group. As long as one of the groups is electron withdrawing, these compounds are more resistant to acid hydrolysis and can be given orally (e.g. ampicillin) and oxacillin. Thus, the problem of acid sensitivity is duely minimised by having an electron withdrawing group on the acyl side-chain.

Penicillin sensitivity to β-lactamases

Beta-Lactamases are enzymes produced from penicillin-resistant bacteria which can catalyse the reaction of ring opening and deactivation of penicillin which occurred with acid hydrolysis.

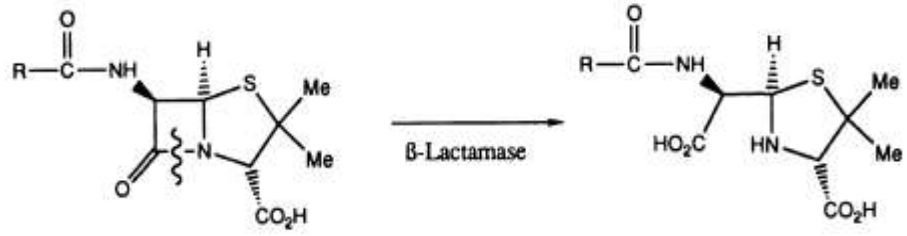

Figure 10: β-Lactamase deactivation of penicillin

The setback of β-lactamases became more evident in 1960 when the extensive use of penicillin G led to an frightening rise of *Staph. aureus* infections. These particular strains had gained the lactamase enzyme and had thus gained resistance to the drug. At some point in time, a larger percentage of all *Staph. aureus* infections in hospitals were due to virulent, penicillin-resistant strains. Surprisingly, these strains happened to be resistant to all other available antibiotics. Thankfully, a means of circumventing the problem was discovered. This was the introduction of Penicillinase - resistant penicillins. Through techniques in design that could counter the effects of the penicillinase enzyme.

Overcoming the problem of β-lactamase sensitivity

The approach was to hinder the penicillin from reaching the penicillinase active site. This could be done by placing a bulky group on the side-chain. This bulky group can then act as a 'shield' to ward off the penicillinase and therefore prevent binding. Several analogues were made and the strategy was found to work. There was however a limitation. If the side-chain happened to be too bulky, then the steric hinderance also prevented the penicillin from attacking the enzyme responsible for bacterial cell wall synthesis. Thus, a great deal of effort had to be put in find the ideal group which would be big enough to ward off the lactamase enzyme, and at the same time small enough to allow for anti-bacterial activity of penicillin. The vital role the β-lactam ring plays with regards to interaction with both enzymes accentuates the difficulty in discovering the ideal group. Fortunately, groups were found which could make that distinction. Methicillin was the first semi synthetic penicillin unaltered by penicillinase and was developed just in time to treat the problem created by *Staph. aureus.* The principle of the steric hinderance can be seen by the presence of two ortho - methoxy groups on the aromatic ring. Both of these are essential in protecting the lactam ring.

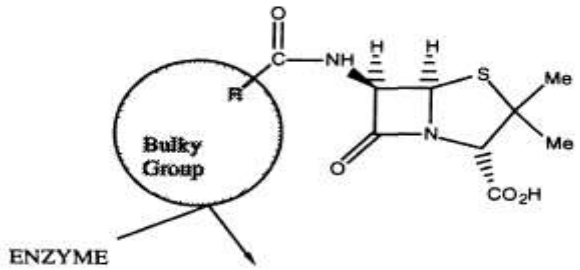

Figure 11: Blocking penicillin from reaching the Penicillase active site

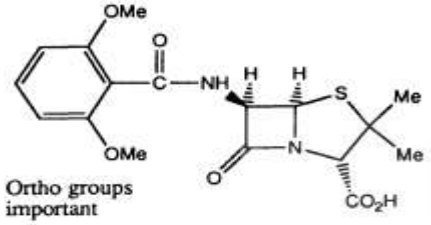

Figure 12: Structure of Methicillin

However, methicillin cannot be regarded as an ideal drug. Since it does not have an electron-withdrawing group on its side-chain, it is acid sensitive, and thus has to be injected. It is only one-fiftieth the activity of penicillin G against penicillin G sensitive organisms, it shows poor activity against some streptococci, and it is inactive against Gram negative bacteria. Further work carried out was able to circumvent the problem of acid sensitivity by incorporating into the side-chain, a five-membered heterocycle which was designed to act both as a steric hinderance and also as an electron withdrawing group.

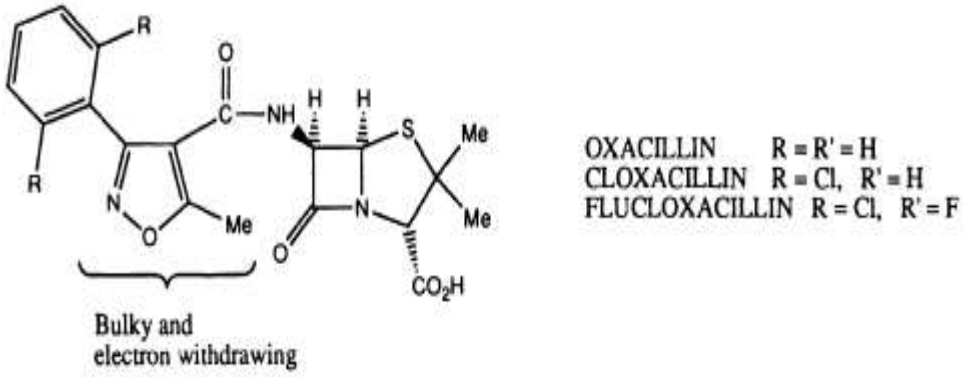

Figure 13: Incorporation of a five-membered heterocycle

These compounds (oxacillin, cloxacillin, and flucloxacillin) are acid-resistant and Penicillinase - resistant, and are also beneficial against *Staph. aureus* infections. The only variation between the above three compounds is the type of halogen substitution on the aromatic ring**(Patrick, 1995)**.

2.2 Clavulanic acid

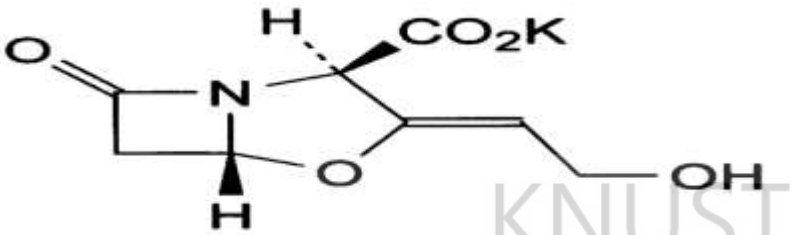

Figure 14: Chemical Structure of Clavulanic acid

Physical Appearance; Clavulanate potassium is a whitish crystalline powder. It is hygroscopic and has a percentage purity of 96.5 to 102.0 percent of the anhydrous powder. It has a pKa value of 2.7. Solubility; It is freely soluble in water, slightly soluble in alcohol and very slightly soluble in acetone. A solution of 0.400g of potassium clavulanate in 20ml of carbon dioxide free water gives a pH of 5.5 to 8.0.

2.3 Stability

Pharmaceutical stability in regards to a particular dosage form of drug is the ability of the former to maintain its physical and chemical integrity as well as offer protection against microbial contamination **(USP 29).**Generally, "significant change" for a pharmaceutical product can be said to occur when there is:

(i) A 5% change in assay from its initial value, or failure to meet the acceptance criteria for potency when using biological or immunological procedures.

(ii) Any degradation product above its acceptance criterion.

(iii) Failure to meet the acceptance criteria for appearance and physical attributes (e.g. colour, phase separation, re - suspendibility, caking, hardness).

(iv) Failure to meet the acceptance criterion for pH thus for liquid preparation **(ICH Q1A)**.

Factors that can affect the stability of a drug substance include; the chemistry of the substance, the presence of other materials such as excipients in the formulation,the environment within and outside the formulation as with regards to temperature, light, oxygen and humidity. Stability study on drugs is crucial because breakdown products could be detrimental to patients' health. Amoxicillin, amoxicilloates, amoxicillin oligomers and amoxicillin piperazine-2, 5-dione have been separated by the use of reversed-phase (C8) and gradient elution. Quantitative results have been gotten for a number of samples. Amoxicillin trihydrate samples mostly contain amoxicilloate as the main impurity. Samples of the sodium salt also contain the piperazine-2, 5-dione and the dimer. Higher oligomers such as the trimer and tetramer were not present in significant amounts**(De Pourcq et al., 1985)**. In order to ascertain some breakdown products from amoxicillin, the latter was stressed under acidic conditions. The degradation of amoxicillin (1) was induced by subjecting the pure drug substance to harsh acidic conditions, specifically a 0.1M HCl solution. The degradation of amoxicillin (1) in an acidic medium starts with the opening of the four-membered β-lactam ring and yields the product amoxicillin penicilloic acid (2), which contains a free carboxylic acid group and gives a higher polarity to this molecule. This leads to a shift towards an earlier retention time in the reverse phase liquid chromatography (RPLC) separation.

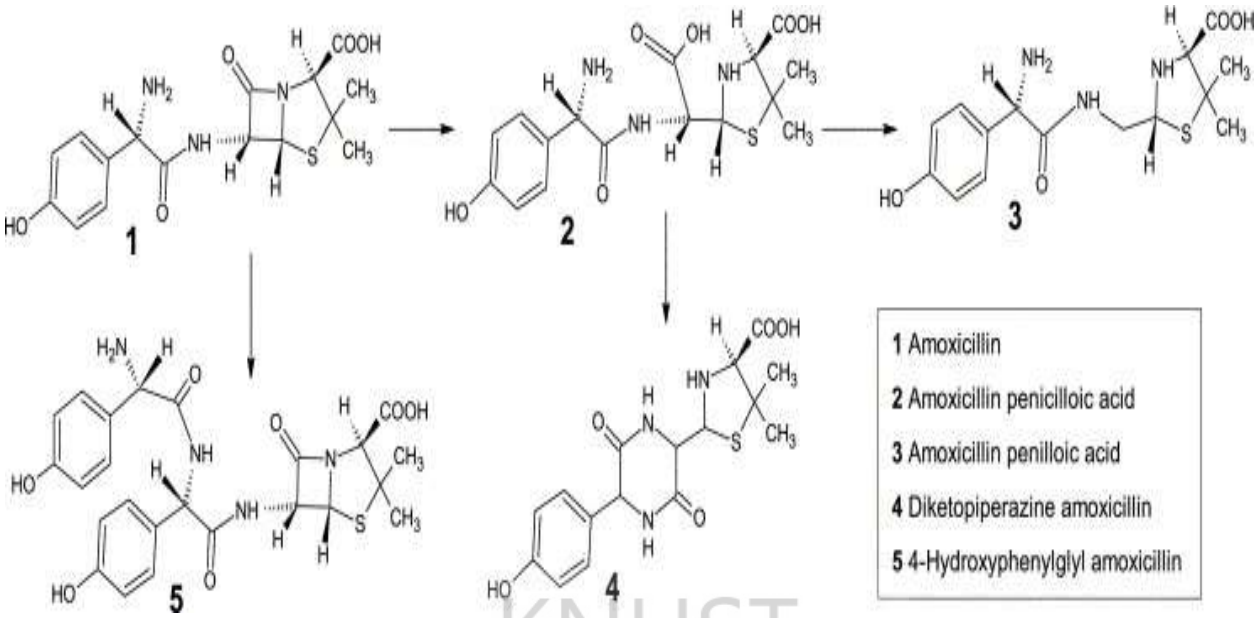

Figure 15: Degradation pathway of amoxycillin

Starting with Compound **(2)**, there are two possible pathways for further degradation. The first one is based on the decarboxylation of the free carboxylic acid and leads to the stereo isomeric compounds amoxicillin penilloic acid I and II **(3)**. The second possible degradation reaction of intermediate **(2)** is the formation of a new, stable, six-membered ring giving diketopiperazine amoxicillin **(4)**.The second reaction product derived from compound **(2)** the protonated diketopiperazine amoxicillin **(4)** the molecule undergoes fragmentation by cleavage of the bond between a six-membered diketopiperazine ring and a five-membered thiazolidine ring. In another reaction pathway, amoxicillin **(1)** undergoes a nucleophilic attack on itself, where the benzylic carbonyl group is attacked by the free amino group to form 4-hydroxyphenylglycyl amoxicillin **(5)**. The first degradation product of amoxicillin **(1)** obtained after breaking the four-membered β-lactam ring is amoxicillin penicilloic acid **(2)**.The subsequent degradation products obtained from amoxicillin penicilloic acid **(2)** by a decarboxylation of the free carboxylic acid group are the stereoisomeric amoxicillin penilloic acids I and II **(3)**. Beginning with amoxicillin penicilloic acid **(2)**, the degradation pathway

also leads to diketopiperazine amoxicillin **(4)** by the formation of a six-membered ring structure. Finally, the identity of the product obtained from a self-condensation reaction of amoxicillin **(1)** to 4-hydroxyphenylglycyl amoxicillin **(5)**.The base peak chromatogram (BPC) clearly shows the degradation of amoxicillin into various products**(Nägele and Moritz, 2005)**.

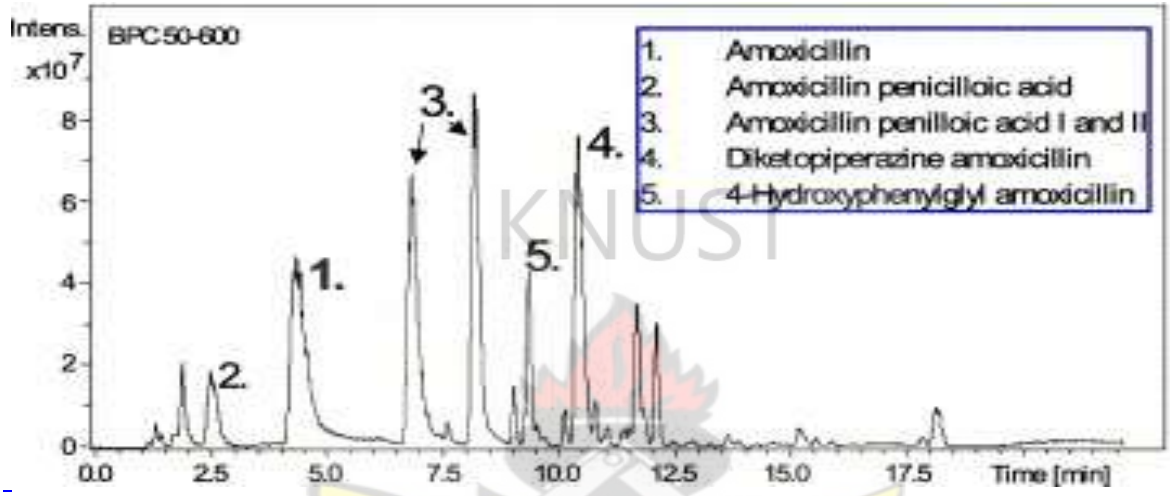

Figure 16: BPC of amoxicillin (1) and its degradation products after acid exposure.

STABILITY OF PROUCT AND KINETICS

The stability of a drug or any product can also be defined as the time from manufacture and packaging of the product to the time when its chemical activity is not lower than a predetermined level of labelled potency. Its physical characteristics should also be intact. Generally, 90% of labelled potency is generally regarded as the minimum acceptable potency level **(Patrick B O' Donell and Bokser., 2005)**.

Kinetics is the study of the rate at which processes take place. These changes could be chemical (as in the decomposition of a drug, radiochemical decay) or physical (transfer across a boundary, such as the intestinal lining or skin). Kinetic studies come in handy since

they give information on: the mechanisms of the changes involved, and allows for prediction of the degree of change which will take place after a given time has elapsed.

Order of reaction

This is the number of concentration terms which determine the rate. In a unimolecular process a molecule will only react if it has sufficiently high-energy. The number of high-energy molecules depends on how many molecules are present, thus their concentration in solution (or pressure as in the case of a gas). In a bimolecular process two molecules will have to collide to react, and the likelihood of collision is dependent on the concentrations of each species. The law of mass action states that the rate depends on the product of concentrations of the reactants. Thus, in the first step of the example reaction, $N_2O_5 = 2NO_2 + {}^1/_2\ O_2$ the rate of reaction = $k_1/ [N_2O_5]$, i.e. there is only one concentration term and the reaction is known as first order. In the second step, $½\ O_2 + ½O_2$, the rate of reaction = $k_2 [½\ O_2] [½\ O_2] = k_2 [½\ O_2]^2$,where k_1 and k_2 are the reaction rate constants. Thus, there are two concentration terms and the reaction is known to be a second order **(M.E). Aulton).**

Note should however be taken that the order of a chemical reaction cannot be determined from the chemical equation even if it has been balanced. The order of a reaction can only be found out experimentally from accurate measurements of the rate under varying conditions.

Reactions can be of the third order, zero order (this is often found in solid state reactions as occurs in the release of active ingredients from pharmaceutical suspensions) or even of a fractional order. The molecularity of a reaction is the total number of molecules involved in the slowest step of the elementary reaction. Most chemical reactions have two molecules colliding and reacting, as such the molecularity is two and reaction is termed bimolecular. Reactions in which just one molecule is involved are known (unimolecular) but often in the gaseous phase. Those with higher molecularity than two are rather rare **(Donald, 2008).**

Determination of the order of reaction

To determine the order of a reaction, one will have to ascertain the amount of drug decomposed after intervals and to substitute the data into the integrated equations for zero, first and second order reactions. The equation that results in the most consistent value of *k* for a series of time intervals is that corresponding most closely to the order of the reaction. Alternatively, the data may be displayed graphically according to the linear equations for the various orders of reactions until a straight-line plot is obtained. Thus, for example, if the data yield a linear graph when plotted as *t* against log(*a* - *x*) the reaction is then taken to be first-order. Fitting data to the standard rate equations may, however, produce misleading results if a fractional order of reaction applies**(Alexander and David, 2006).**

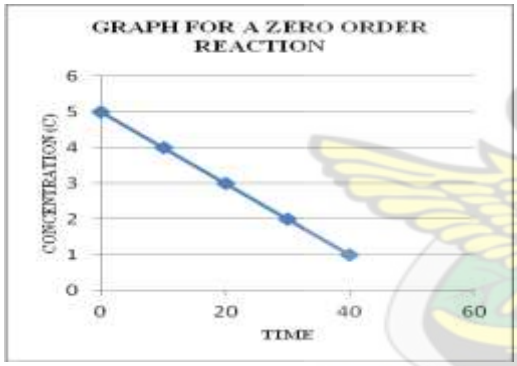

Figure 17: A zero order graph

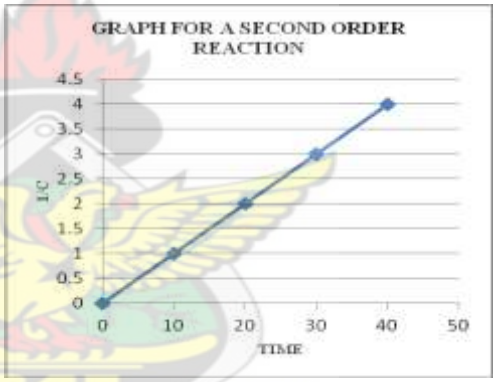

Figure 18: A second order graph

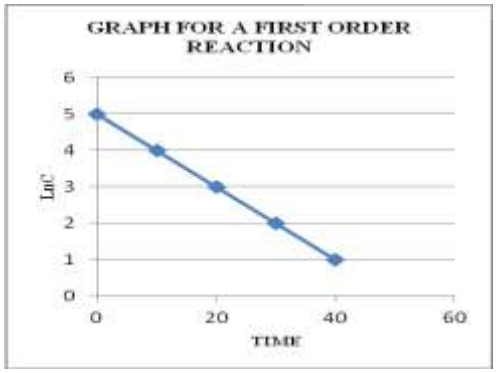

Figure 19: A first order graph

2.4 Analytical methods used in this project

Conductimetry;

This is the electrochemical means by which electrolyte solutions can be investigated. It is principally based on Ohm's Law; V = iR, where V, i and R represent the applied electromotive force (e.m.f), the current in amperes (A), and of resistance ohms. The conductance, G, of the solution is the parameter measured in all conductivity experiments. It is defined as the reciprocal of resistance and usually expressed in Siemens S. Thus, G = 1/R = k /(l/a) = 1/ p (l/a), where k, a and l are conductivity, cross-sectional area and length of conductor respectively. Conductivity is dependent on ionic concentration and tends to zero result upon dilution, whilst molar conductivity indicates the conductivity of a solution at a concentration of 1 mol/dm^3 attains its maximum value Λ^{o}, at infinite dilution due to total elimination of ionic interference. Conductivity = (1/Measured resistance) × Cell constant. A solution gets more concentrated as a result of more solute particles in a volume of the solution. This results in more ionic interference with each other and thus less ionic speed (G.I, 1972). Conductivity of water indicates its ability to conduct electrical current. This property is dependent on the presence and concentrations of ions, their oxidation states, and the mobility and temperature of the water. The basic unit of conductivity is the Siemens (S), formerly known as the mho. Since cell geometry affects conductivity values, standard measurements are expressed in specific conductivity units (S/cm) to compensate for variations in electrode dimensions. Specific conductivity (C) is the product of measured conductivity (G) and the electrode cell constant (L/A), where L is the length of the column of liquid between the electrode and A is the area of the electrodes. Thus, C = G × (L/A). If the cell constant is 1/cm, the specific conductivity is the same as the measured conductivity of the solution. Although an electrode can vary in terms of size, shape, position and condition, an electrode can always be represented by an equivalent theoretical cell.

Table **1:** Optimum conductivity ranges for cells of three different constants**.**

Cell constant	Optimum Conductivity Range (µS/cm)
0.1	0.5 – 400
1.0	10 – 2000
10.0	1000 – 200,000

Typical values for the cell constant are from 0.1 to 2.0. However, the cell constant can be practically determined by using the conductivity measure the resistance of a standard solution of 0.0100mol/dm^3 KCl. The conductivity of the solution (141.2 mS/m at 25ºC) multiplied by the measured resistance equals the cell constant value. The purity of water can be assessed by conductivity of which that of potable water must be below 2 micromhos/cm. Conductivity is the reciprocal of resistivity and expressed in the unit micromhos per centimeter (µmhos/cm) or mhos per centimeter (mho/cm). In SI units, conductivity is given as millisiemens per meter (mS/m) or microsiemens per centimeter (µS/cm). Thus, the conversion:1 mS/m = 10 *m*mhos/cm and 1 µS/m = 1 *m*mhos/cm. A 0.0100*M* potassium chloride solution (prepared by dissolving 745.6 mg of anhydrous KCl in reagent water to 1 L at 25°C and stored in CO_2-free atmosphere) can serve as a standard reference solution with a conductivity of 1412 micromhos/cm at 25°C **(Patnaick).**

Ultraviolet- Visible spectrophotometry

Ultraviolet-visible spectrophotometry is a widely used analytical method in pharmaceutical analysis. It involves the measurement of the amount of ultraviolet (190-380nm) or visible (380-800nm) radiation absorbed by an analyte in solution**(A.H and J.B, 1997)**.Ultraviolet-

visible spectrophotometers which are employed to measure the intensity of light absorbed often have both Deuterium and Tungsten lamps and selection of the appropriate lamp is made by moving either lamp mountings or mirrors **(Beckett and Stenlake, 1997)**. The use of the UV/Visible spectrophotometer for quantitative work follows the Beer-Lambert's law and it is given by the equation below: Log $_{10}$ (Io/I) = A = abc, here the name and value of 'a' depends on the units of concentration. When the concentration term c, is in moles per litre, the constant 'a' is known as molar absorptivity (formerly the molar extinction coefficient) and has the symbol ε. Thus, $\log_{10}$(Io/I) = A = εbc

Where; Io = intensity of incident light, I = intensity of transmitted light ε = molar absorptivity or molar extinction coefficient, c = concentration of solute in moles/ litre b = cell (path) length in cm**,** A = absorbance.

Another form of the Beer – Lambert proportionality constant is the specific absorbance. The most common form in pharmaceutical analysis is the A (1%, 1cm), which is the absorbance of a 1g /100ml (1% w/v) solution in a 1cm cell. Thus, the equation now becomes

$A = A^{1\%}_{1cm}$ bc. For analytical purposes, UV/Visible spectra are usually measured in solution. Optically pure solvents are used. The choice of solvent depends on the solvent's cut off point which is the wavelength below which the solvent is not transparent to ultraviolet radiation. An increase in the interaction between the compound being measured and the solvent leads to the loss of fine structure **(Manfred *et al.*, 2008)**.

Acid- Base Titrations

Several titrimetric methods exist but the choice of the method depends on the sensitivity required, presence of interfering substances and alternate methods of analysis**(Olaniyi, 2000)**. Acid-base titrations may involve a direct titration or a back titration. A direct titration

involves the accurate determination of the strength of a solution using a standard solution of known purity and strength. It usually results in the formation of salts which are not hydrolysed in aqueous solution. A back titration consists of the addition of a definite excess of a standard volumetric solution to a weighed amount of the sample and the determination of the excess not consumed by the sample **(A. Ajibola, 2000).**

Iodimetry**;**

This is the type of titration where a standardised solution of iodine is employed for the assay, whereas iodometry is a type of titration in which iodine is generated 'insitu' , thus within the reaction vessel **(A.Ajibola and Francis, 1998)**. Most penicillins have penicilloic acids as part of their breakdown products. Intact penicillins do react with iodine but the penicilloic acid moiety does react with the iodine. Thus, in the assay of amoxicillin by iodimetry, hydrolysis of the drug is effected by the addition of NaOH so as to ensure the presence of penicilloic acid moieties for the feasibility of the reaction.

2.4.1 Chromatography

Chromatography is used to separate complex samples into individual components and allows for more variability, speed, as compared to other techniques. This separation is however dependent on the varying degrees to which they interact with two material phases. The analyte to be separated has to be dissolved in a little amount of the mobile phase or any other appropriate solvent. This is then injected and carried along by the mobile through the stationary phase either by gravity or any other kind of force. The components of the analyte tend to be attracted and slowed down by the stationary phase to varying degrees, and thus, they elute at different times and thus separated. The mobile phase may be a gas or a liquid, whereas the stationary phase too may be a liquid or solid. All techniques that employ a liquid mobile phase come under liquid chromatography. There is also gas–liquid chromatography

(GLC), gas–solid chromatography (GSC), liquid–liquid chromatography (LLC), and liquid–solid chromatography (LSC).

Types of Chromatography

Partition Chromatography;

In this type of chromatography, mobile phase is a liquid that moves through a liquid stationary phase as the mixture components partition or distribute themselves between the two phases and become separated. The separation means is thus, that of the dissolution of components of the mixtures to varying degrees in the two phases according to their respective solubility properties.

Adsorption Chromatography

As the name suggests, the separation mechanism is that of adsorption. The stationary phase comprises of finely divided solid particles packed into a tube. Here there is no liquid film around the particles, as occurs in partition chromatography. The components of the mixture, instead of dissolving in a liquid stationary phase, rather adsorb or stick to the surface of the solid packings.

Ion Exchange Chromatography

Ion exchange chromatography (IEC) comes in handy in the separation of ions, both inorganic and organic for that matter. The stationary phase comprises of very small polymer resin beads that have quite a number of ionic bonding sites on their surfaces. These sites exchange ions selectively with specific mobile phase compositions as the mobile phase travels along. Ions that connect to the charged site on the resin beads are thus detached from ions that do not bond hence separated.

Chromatography Configurations

Chromatography techniques can be still classified with regards to the configuration; thus how the stationary phase is held in place, how the mobile phase is configured with respect to the stationary phase in terms of the physical state (gas or liquid) and positioning, and how and in what direction the mobile phase travels, whether with gravity, capillary action, or by other forces. Thus the two main categories of configuration are the planar methods and the column methods. The planar methods make use of a thin sheet of stationary phase material and the mobile phase moves across this sheet, either in an upward, downward or horizontal direction.

Paper and Thin-Layer Chromatography

Paper chromatography and thin-layer chromatography (TLC) make up the planar methods. Paper chromatography has to do with the use of a sheet of paper having the consistency like that of cellulose filter paper to serve as the stationary phase. The paper tends to be hydrophilic, as such the stationary phase is actually a thin film of water unintentionally adsorbed on the surface of the paper. The mobile phase is always a liquid. With thin-layer chromatography, the stationary phase consists of a thin layer of material evenly spread over a plastic sheet or glass or metal plate. Such plates or sheets can be either purchased commercially already prepared or prepared in the laboratory. The most common method of configuring a paper or thin-layer experiment is the ascending configuration.

Instrumental Chromatography

GC and HPLC examples and they employ electronic sensors (detectors) for detecting mixture components as they elute from the column. These detectors generate electronic

signals which portray as a chromatogram. A chromatogram is the graphical representation of the separation, a plot of the electronic signal against time. The chromatogram is traced on a computer screen or other recording device as the experiment proceeds. The mobile phase serves as a blank such that the signal generated by the sensor is set at zero.

Retention time;

This is the time that elapses from the time the sample is first introduced into the flowing mobile phase until the apex of the peak is seen on the chromatogram is known as the retention time of that component, or the time that that mixture is retained by the column. Typically, retention times vary from a small fraction of a minute to about 20 minutes, although much longer retention times are possible. One major essence of the retention time information is in peak identification, or qualitative analysis.

Mobile Phase

The HPLC pump draws the mobile phase from a reservoir via vacuum action. In the process, air dissolved in the mobile phase may withdraw from the liquid and form bubbles in the flow stream unless such air is removed from the liquid in advance. Air in the flow stream is undesirable because it can cause a wide variety of problems, such as poor pump performance or poor detector response. Degassing the mobile phase is done either by helium sparging, ultrasonic agitation and drawing of vacuum.

Elution;

There are two mobile phase elution methods, isocratic and gradient that are used to elute mixture components from the stationary phase. Isocratic elution employs a single mobile phase composition for the entire separation experiment, whilst in gradient elution the mobile

phase composition is changed, often gradually, in the middle of the run. Gradient programmer is a hardware module used for gradient elution.

Normal Phase Columns;

Normal phase partition chromatography makes use of a polar liquid stationary phase chemically bonded to these polar particles, which consist of silica, Si–O–, bonding sites. Typical examples of normal phase bonded phases are those in which a cyano group (–CN), an amino group ($–NH_2$), or a diol group ($–CHOH–CH_2OH$) are part of the structure of the bonded phase. Typical mobile phases for normal phase HPLC are hexane, cyclohexane, carbon tetrachloride, chloroform, benzene, and toluene (John, 2003). In the separation of components in a mixture, relatively polar components will be retained more on the stationary phase than less polar ones which will be eluted much earlier.

Reverse Phase Columns

These have analytes that are relatively non – polar being retained on the column than more polar compounds **(McPolin, 2009).** Reverse phase bonded phases are non-polar groups which are bonded to the surface of the matrix by covalent bonding. Typical column names often have the carbon number designation which indicates the length of a carbon chain to which the non polar nature is attributed **(McPolin, 2009)**. Typical designations include C8, C18 (or ODS, octadecyl silane), etc. Common mobile phase liquids are water, methanol, acetonitrile (CH_3CN), and acetic acid buffered solutions **(kenkel, 2003).**

Detectors

HPLC detectors examine any solution that elutes from the column and portrays an electronic signal proportional to the concentrations of individual components present. The ultraviolet

(UV) absorption HPLC detector is basically a UV spectrophotometer that measures a flowing solution rather than a static solution. It has a light source, a wavelength selector, and a phototube like an ordinary spectrophotometer. The cuvette is a flow cell, through which the column effluent flows component that absorbs the wavelength elutes. As the mobile phase elutes, the chromatogram traces a line at zero absorbance, but when a component of the mixture elutes, the absorbance changes and a peak is traced on the chromatogram. With diode array detectors the light from the source passes through the flow cell and is then dispersed via a grating. The dispersed light then sprays across an array of photodiodes, each of which detects only a narrow wavelength band. With the help of the data system, the entire UV absorption spectrum can be immediately measured as each individual component elutes. Electrochemical detectors make use of electrical current or conductivity measurements for detecting eluting mixtures. Compounds that are either oxidised or reduced in the field of an electric potential can be detected even at very low concentrations by electrochemical measurements which are selective **(Satinder and Michael, 2005)**. Electrical conductivity detector is the most important of all electrochemical detection schemes. This detector is useful for ion exchange, or ion, chromatography where the analyte is in ionic form. Such ions elute from the column and need to be detected as peaks on the recorder trace. The presence of ions in any solution gives the solution a low electrical resistance and the ability to conduct an electrical current. The absence of ions means that the solution would not be conductive. Thus, solutions of ionic compounds and acids, especially strong acids, have a low electrical resistance and are conductive. This means that if a pair of conductive surfaces are immersed into the solution and connected to an electrical power source, such as a simple battery, a current can be detected flowing in the circuit. Alternatively, if the resistance of the solution between the electrodes were measured (with an ohmmeter), it would be low **(kenkel, 2003).**

2.5 Analytical method validation

Method validation is the process by which one ensures that a test procedure is accurate, reproducible, and robust within the specified analyte range for the intended purpose **(Michael, 2006**) . It can also be described as a systematic way to prove that the systems, facilities and processes are reliable **(Olaniyi, 2000**). Validation as per the ICH- guidelines focuses on parameters such as: Accuracy, Precision, Specificity, Limit of detection (LOD), Limit of quantification (LOQ), Linearity, Range, Robustness and system suitability testing.

Specificity**;**

Specificity for an assay ensures that the signal measured is from the substance of interest, and that there is no interference from excipient and/or degradation products and/ or impurities such as breakdown products, matrix, etc. Specificity is normally illustrated by measuring the response of the sample matrix and any expected or known species.

Linearity;

The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration of analyte in the sample. Linearity is normally expressed in terms of the variance around the slope of the regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte. R^2 should lie between 0.995and 1.

Accuracy;

The accuracy of an analytical procedure indicates 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. This is sometimes termed trueness. Accuracy is usually demonstrated by adding known amounts of analyte(s) to the sample matrix and determining the measured result using the analytical procedure. Accuracy may also be demonstrated by the method of standard additions, or by cross-correlation of results with a second, independent procedure. For an analytical method to be accurate percentage recovery should be between 98 and 102%.

Precision**;**

The precision of an analytical procedure indicates the closeness of agreement between a series of measurements obtained from multiple samplings of the same homogeneous sample under prescribed conditions. Precision is often expressed as the variance, standard deviation or relative standard deviation (co-efficient of variation)**(C.J and N.J, 2005)**. For precision of an analytical method to be established the Relative Standard Deviation (RSD) should not be more than 2%. Precision should be considered at different levels as follows;

Repeatability (Intra-assay precision);

Repeatability indicates the precision under the same operating conditions over a short period of time. A minimum of three determinations each of three concentrations across the intended range, or a minimum of six determinations of the test concentration is recommended.

Intermediate Precision**;**

Intermediate precision is expressed within-laboratory variations: different days, different analysts or equipment, etc.

Reproducibility**;**

Reproducibility indicates the precision between laboratories (collaborative studies, usually applied to standardisation of methodology). Reproducibility is usually demonstrated by way of an inter-laboratory trial.

Limit of Detection (LOD**)**;

The detection limit of an analytical procedure is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified as an exact value. The detection limit is usually expressed as the concentration of analyte in the sample.

Limit of Quantitation (LOQ**)**;

The quantitation limit of an analytical procedure is the lowest concentration of analyte in a sample that can be determined with suitable precision and accuracy under the stated experimental conditions. It is usually expressed as the concentration of analyte.

Robustness;

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness is demonstrated by making small deliberate changes to one of the operating parameters of the method, analysing samples and comparing the results to those obtained using the prescribed method.

Range;

The range of an analytical method is the interval between the upper and lower concentration of analyte for which it has been proven that the analytical procedure has a suitable level of precision, accuracy and linearity. Range is demonstrated by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range.

System Suitability Testing;

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analysed constitute an integral system that can be evaluated as such. Efficiency, capacity factor, resolution factor, and symmetry factor are parameters that are normally used in assessing the column performance **(ICH, 1996).**

CHAPTER 3 – EXPERIMENTAL WORK

3.1 Materials and equipment

- Stuart Melting point apparatus SMP10
- EC 215 Conductivity meter, Hanna instruments
- Gallenkamp regulator hotplate.
- Eutech instruments pH 510 pH meter
- Cecil CE 2041 2000 Series-UV Spectrophotometer
- Buchi R-210 water bath.
- Fisher scientific FS 28H sonicator.
- Adam-analytical weighing balance, WA 210 ; 210/0.0001g
- HPLC Chromatograph
    - Kontron instrument HPLC pump 422
    - Applied Biosystems 783 programmable Absorbance Detector
    - Powerchrome 280 software Integrator
    - Hp desktop
    - Kromasil 5 C_8 100A 250 × 4.60mm 5 micron size 305334
- Volumetric flasks (200ml, 1000ml, 50ml, 25ml)
- Conical flasks
- Measuring beakers (25ml)
- Measuring cylinders
- Transfer pipettes (0.5ml, 1ml, 2ml, 5ml, 10ml)
- Graduated pipettes (1ml, 5ml, 10ml)
- No. 1 sintered glass crucible
- No. 1 whatman filter paper

- Glass funnel
- Melting point capillary tubes
- Pre-coated TLC plates (Gf 254, 0.25mm Merck W.)

3.2 Reagents and samples

Sodium Acetate (99.6%) (BDH)

Glacial Acetic Acid Analar (99.8%)

Methanol (HPLC Grade)

0.1N Sodium Hydroxide

0.1N Hydrochloric Acid

KCl

0.01N Iodine VS

0.01N Sodium thiosulphate VS

Starch Iodide Paste Ts

Sodium Hydrogen Carbonate solution

Acetone R

Ammonium Acetate

Ferric Chloride

0.5M Hydrochloric Acid

Sodium Carbonate Solution

Dilute Ammonia R

Sodium Carbonate Solution R

Sodium Sulphide Solution

Tartaric acid

Table 2: Certified Reference Standard

Name of Standard	Source	Manufacturing Date	Expiry Date
Amoxycillin Trihydrate	Ernest Chemist lab	June, 2011	June, 2015
Clavulanic Acid Potassium	Shandong New Time PharmaceuticalCo. ltd	October, 2011	September, 2015

Table 3: Brands of reconstituted oral suspensions of amoxicillin-clavulanic acid

Brand	Code	Country of origin	Batch Number	Strength (mg)	Expiry Date
AUGMENTIN	A	United Kingdom	533121	457/5ml	May, 2013
AMOKSIKLAV	B	England	CA 0841	457/5ml	Sept, 2013
AMOVULIN	C	India	MB 812311	457/5ml	June, 2013

3.3 Methods

3.3.1 Identification tests

Identification tests for Amoxicillin Trihydrate (BP)

About 4.00mg of amoxicillin trihydrate was put in a test tube and a few drops of water was added to moisten it. 2.00ml of sulphuric acid-formaldehyde was added and this was put in a water bath for a minute.

b) about 4.00mg of amoxicillin was dissolved in water and 1-2drops of neutral ferric chloride solution. The colour was observed.

c) Determination of melting point; 5.00mg of Amoxicillin reference powder was packed into a sealed end capillary tube. It was then put in the melting point apparatus and the temperature range over which the sample melted was noted. This was repeated and the average temperature determined.

Identification test for Clavulanic Acid (BP)

a) About 8.00mg of potassium clavulanate was dissolved in 2.00ml of freshly boiled and cooled distilled water, 1.00ml of sodium carbonate solution was added and heated.0.10ml of sodium sulphide solution was added to the hot solution and cooled. 2.00ml of tartaric acid solution was added and observed

b) Determination of Melting point

10.00mg of Clavulanic acid reference was put into a sealed end capillary tube. It was put in the melting point apparatus and the temperature range over which the sample melted was noted. This was repeated and the average temperature determined

Thin layer chromatography (TLC)

Amoxicillin

The mobile phase comprised of 15.00ml of ethyl acetate, 5.00ml of glacial acetic acid and 5.00ml of water. The mixture was left in a chamber lined with filter paper and time allowed for the chamber to be saturated with solvent vapour. A parallel line to the bottom and 1.5cm from the bottom edge of the TLC plate was drawn with a pencil. 15.00mg of the amoxicillin reference standard was weighed and dissolved in 10.00ml of acidified aqueous acetone solution and shaken for five minutes. 1ml of this solution was pipetted and 7.00ml of the acidified aqueous acetone solution added. This was spotted on the TLC plate four times with a microcapillary tube. The TLC plate was then allowed to dry and placed in the chromatank with the mobile phase. After 30 minutes the TLC plate was removed. All residual solvent was allowed to dry and the chromatoplate observed under UV light.

Clavulanic Acid

A mobile phase consisting of 15.00ml of ethyl acetate and 5.00ml of methanol and 20.00ml of distilled water. The mobile phase was stored in a chamber lined with filter and allowed to be saturated with the solvent. 10.00mg of potassium clavulanate was dissolved in 15.00ml of distilled water. The solution was spotted on the plate four times and allowed enough time dry. The plate was developed in the mobile phase and observed under UV light after 30 minutes.

3.3.2 Assay of reference standards

Preparation and standardization of 0.01M Sodium thiosulphate solution.

0.6205g of Analar sodium thiosulphate crystals was accurately weighed, dissolved and quantitatively transferred into a 250.00ml flask and made up to volume with more distilled water. This was then standardised with 0.1787g of pure dried potassium iodate which was accurately weighed, dissolved with a little water and quantitatively transferred into a 250.00ml volumetric flask. This was made up to volume with more water. 20.00ml of this solution was pipetted into a conical flask. 2.000g of KI and 5.00ml of 2M HCl were added. The liberated iodine was titrated with 0.1M sodium thiosulphate solution with constant stirring. The liquid mixture was diluted to about 200ml with water when the colour had become pale yellow. 2ml of starch mucilage was subsequently added and titration continued until the colour changed from blue to colourless. The titration was repeated to obtain three replicate results.

Preparation and Standardization of 0.1M Sodium Hydroxide

2.0833g of analar sodium hydroxide was accurately weighed in a beaker, dissolved with a little water and quantitatively transferred into a 500.00ml volumetric flask. The solution was made up to volume with more distilled water. This was standardised against 0.9807g of sulphamic acid which was accurately weighed and dissolved in a little amount of water. The solution was quantitatively transferred into a 100.00ml volumetric flask and diluted to the mark with more distilled water and titrated against the 0.1M NaOH solution with methyl orange as indicator. Repeated determinations were carried out.

Assay of Amoxicillin reference standard by Iodimetry **(BP 1980).**

0.1000g of the amoxicillin was accurately weighed and dissolved in sufficient water to produce 100.00ml. 10.00ml of this solution was quantitatively transferred into a stoppered flask. 5.00ml of M $NaOH_{(aq)}$ was also added and allowed to stand for 20 minutes so as to ensure hydrolysis of the drug to its penicilloic acid forms. 20.00ml of a freshly prepared buffer solution, comprising of 5.44%w/v of Na acetate and 2.40%w/v of glacial acetic acid was also added. 5.00ml of M HCl and 25.00ml of 0.01M iodine volumetric solution was also added. The flask was closed with a wet stopper and allowed to stand for another 20 minutes, protected from light to ensure complete reaction between iodine and the penicilloic acids present. The excess of iodine was then titrated against 0.01M $Na_2S_2O_3$ volumetric solution, using starch as the indicator. To another 10.00ml of the initial solution, 20.00ml of the buffer solution and 25.00ml of the 0.01M iodine volumetric solution was added and allowed to stand for 20 minutes protected from light. This was titrated against 0.01M $Na_2S_2O_3$ volumetric solution with starch mucilage as indicator, added towards the end (when iodine has faded out to a very pale yellow colour). This second titration served as a blank and the difference between the titrations represented the volume of 0.01M iodine volumetric solution, equivalent to the total amount of penicillins.

Assay of Clavulanic acid reference standard (BP 1973, vol 1)

0.5000g of clavulanic acid was accurately weighed and dissolved in 25.00ml of carbon dioxide free water (prepared by boiling water vigorously and protecting from the atmosphere during cooling and storage) previously neutralised to phenolphthalein solution with 0.01*N* NaOH. 50.00ml of 0.1*N* NaOH was added and heated on a water-bath for twenty minutes with precautions against the absorption of carbon dioxide. The solution was allowed to cool

and the excess NaOH was titrated against 0.1*N* hydrochloric acid, using phenolphthalein solution as indicator. The process is repeated without the drug , thus the blank determination.

Calibration of Conductimeter and measurement of conductance of types of water.

Three standard solutions of KCl were prepared. They were made up of 0.7455g, 0.0746g and 0.0149g respectively of KCl per 1000ml of solution, using carbon-dioxide free water, prepared from distilled water. The conductivity for these solutions were taken and compared with standards. The conductimeter, after having been calibrated was then used to subsequently measure the conductivity of distilled water, treated tap water and mineral water.

Methodology for specific objectives

In order to ascertain whether or not there is variation in the type of water used for reconstitution of amoxicillin – clavulanic acid oral powder for suspension, a questionnaire was designed and distributed to health personnel who give out the drug to patients both at the hospital setting and the community pharmacies. These health personnel included the medical counter assistant, the dispensing technologist and the pharmacist. The questionnaire thus had the format as sampled on next page.

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QUESTIONNAIRE TO AID IN A STABILITY STUDY ON AMOXYCILLIN – CLAVULANIC ACID ORAL POWDER FOR SUSPENSION.

Please tick only the answer that applies to you in each question or write your answers where necessary.

1) Please indicate your role in the health facility.

   [ ] Medical Counter Assistant

   [ ] Dispensing Technologist

   [ ] Pharmacist

2) How will you grade your knowledge about co - amoxiclav oral powder for suspension?.

   [ ] low [ ] moderate [ ] high [ ] very high

3) What is the existing storage condition for co – amoxiclav oral powder for suspension in your health facility?

   (a) [ ] Normal Room Temperature

   (b) [ ] 24 hr Air conditioning

   (c) [ ] < 24 hr Air conditioning

   (d) [ ] other

   If you answered (b) or (c) in the above question, (3), go to (4)

4) What temperature range is normally set for the Air conditioner in the health facility?

Please specify, ...

5) Do you get ample time to counsel patient before giving out drug?

[ ] yes [ ] No

6) Do you enquire if patient has understood information given?

[ ] yes [ ] No [ ] Sometimes

7) In your view, Has the drug ever been prescribed inappropriately?

[ ] yes [ ] No

8) If yes, how? .....

9) Do you reconstitute drug personally for patient?

[ ] yes [ ] No [ ] Sometimes

10) If yes or sometimes, what kind of water do you normally use?

[ ] Distilled water

[ ] Treated tap water

[ ] Other, specify .....

11) Does the the kind of water you use vary from time to time?

[ ] yes [ ] No [ ] Sometimes

12) If yes or sometimes, why? .....

13) If No to question 9, why ?

[ ] Time not enough

[ ] Process is cumbersome

[ ] No water [ ] Other

14) If answer to question (9) is No, or where patient has to reconstitute subsequent bottles of drug after finishing first reconstituted drug, do you specify type of water to be used by patient?

[ ] yes [ ] No

15) If yes please specify, [ ] Distilled water

[ ] Treated Tap water

[ ] Other

3.4 Hplc method development

3.4.1 Chromatographic conditions

Column: phenomex Kromasil 5 C_8 100 A, 250 × 4.60mm 5 micron. Size 305334

Mobile phase: Water: methanol: Na acetate buffer (65:15:20)

Flow rate: 1.00ml/min

Wavelength of detection: 220nm

AUFS: 0.003

Mode of elution: Isocratic

Mode of HPLC: Reverse Phase Liquid Chromatography (RPLC)

Detection wavelength of Amoxicillin and Clavulanic acid;

The wavelength of detection of the two compounds, amoxicillin and clavulanic acid was 220nm gotten after an ultraviolet scan for the solution of the two in water was taken.

Selection of Stationary Phase

A reverse phased column, specifically phenomex Kromasil 5 C_8 100 A, 250 × 4.60mm 5 micron. Size 305334 was employed.

Flow rate of mobile phase

A flow rate of 1.00ml/minute for the mobile phase was found to be the optimum, after others such as 0.50ml/min, 1.50ml/min and 2.00ml/min, had been investigated.

3.4.2 Preparation of mobile phase and solutions of reference standards

Preparation of mobile phase

A sodium acetate buffer of pH 4.4 was first prepared by accurately weighing 4.1000g of sodium acetate. This was then completely dissolved in a 1000.00ml of distilled water. Exactly 5.00ml glacial acetic acid was added to the solution gradually and stirred well by means of a glass rod.500.00ml of the mobile phase was prepared by measuring 325.00ml of distilled water into a 500.00ml volumetric flask. 75.00ml of Hplc grade methanol was also added and finally 100.00ml of the Na acetate buffer solution was added and mixed effectively.

Preparation of solutions of reference standards

81.10mg of amoxicillin trihydrate and 11.40mg of clavulanic acid potassium were accurately weighed separately in two beakers and both were quantitatively transferred into one 100.00ml

volumetric flask with the aid of about 50.00ml of water. The solution was stoppered and sonicated at 26˚C for 5 minutes to aid in the complete dissolution of amoxicillin. The solution was then topped up to the 100.00ml mark on the flask with more distilled water.

3.4.3 Calibration curve for amoxicillin trihydrate and clavulanic acid potassium, preparation of stock solution.

A stock solution of both amoxicillin trihydrate and clavulanate potassium was prepared. 102.04mg of amoxicillin trihydrate reference powder and 14.40mg of clavulanate potassium were accurately weighed and quantitatively transferred into a 100.00ml volumetric flask with the aid of 50.00ml of water. The solution was then sonicated at a temperature of 26ºC for 5 minutes. This was to aid in the dissolution process. The solution was then topped up to the mark with more distilled water. This stock solution thus had a concentration of 0.1000%w/v of amoxicillin trihydrate and 0.0143%w/v of potassium clavulanate.

Calibration Curve for amoxicillin trihydrate and potassium clavulanate**.**

In order to obtain a calibration curve for both amoxicillin trihydrate and potassium clavulanate, the stock solution prepared was serially diluted to obtain concentrations of 0.0700%w/v, 0.0500%w/v, 0.0300%w/v and 0.0100%w/v for amoxicillin trihydrate and 0.0100%w/v, 0.00715%w/v, 0.00429%w/v, 0.00143%w/v simultaneously for potassium clavulanate. These solutions after being filtered with a membrane filter, were each injected three times unto the column. The resultant chromatogram was analysed and the average peak areas were calculated and plotted against their corresponding concentration to obtain the calibration curve.

3.5 Hplc method validation

3.5.1 Amoxicillin and clavulanic acid

Validation of analytical method

Linearity;

In order to indicate linearity, solutions of amoxicillin trihydrate of concentrations 0.1000%w/v, 0.0700%w/v, 0.0500%w/v, 0.0300%w/v and 0.0100%w/v together with potassium clavulanate of concentrations 0.0143%w/v, 0.0100%w/v, 0.00715%w/v, 0.00429%w/v, 0.00143%w/v simultaneously were prepared. These solutions after being filtered with a membrane filter, were each injected three times unto the column. The resultant chromatogram was analysed and the average peak areas were calculated and plotted against their corresponding concentration to obtain the calibration curve. The LOD and LOQ were also deduced from the graph plotted.

To demonstrate accuracy of the method, 50mg of Amoxycillin Trihydrate reference powder and 10mg of potassium clavulanate reference powder were accurately weighed and added to starch. The mixture was then transferred into a 100ml flask with the aid of about 50ml of water and sonicated to aid in dissolution of the drug. Sufficient water was added to top up to the mark. The resulting solution was filtered and injected and results recorded.

Precision was determined by weighing 50mg of Amoxycillin Trihydrate reference powder and 10mg of potassium clavulanate reference powder was dissolved in sufficient water with the aid of sonication to obtain 100ml of solution. The solution was filtered and injected and results recorded. The procedure was repeated with the same concentration for six determinations.

For reproducibility, three different concentrations were weighed three times and injected and results recorded.

Precision-Repeatability (Intra-day precision)

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

Reproducibility (Inter-day precision)

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

Specificity

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

Robustness

To verify robustness, some chromatographic conditions for the method developed were varied, whilst others remained constant The conditions varied were the flow rate and the mobile phase composition.

3.6 Stability studies on reconstituted amoxicillin - clavulanic acid oral powder

3.6.1 Stability studies using different types of water for reconstitution.

Three brands of amoxicillin – clavulanic acid were to be used, these included the innovator brand (A), and two other brands (B and C) commonly used in two government hospitals, Tech Hospital and North Suntreso Government Hospital respectively. Three samples each of the three brands (A, B and C) of amoxicillin - clavulanic acid oral suspension were individually reconstituted with 62.00ml of distilled, tap and mineral water (specifically voltic). Thus in all, there were nine samples to be analysed. 1.00ml of each of the reconstituted suspension was pipetted and carefully transferred quantitatively into a 100.00ml volumetric flask respectively. About 50.00ml of distilled water added and vigorously shaken to ensure dissolution of the active ingredients. More distilled water was added and made up to the 100.00ml mark. The solutions were then filtered by means of filter paper as well as a membrane filter before injection. The concentration of final solution expected was 0.0800%w/v and 0.0114%w/v for amoxicillin and clavulanic acid potassium respectively. These concentrations to be analysed fell within the linear range of the calibration curve. An external standard, made up of the reference powders of amoxicillin – trihydrate and clavulanic acid potassium was employed. Concentrations of the external standard solution was prepared to have expected concentrations of the sample. The samples were injected alongside the same external standard. Each sample was injected three times and the average percentage content calculated for each brand and sample for that matter. All nine samples were kept in a refrigerator of temperature 2 – 8ºC which was ensured by means constant monitoring with a thermometer. There was no interruption in the power supply to the refrigerator. The in-use stability study was carried out for a period of seven days since this is the maximum number of days for which reconstituted products can be used as stated by the

World Health Organization (WHO) guideline for in-use stability studies of reconstituted products. The samples were analysed on day 1, day 2, day 3, day 4, day 5, day 6 and day 7. Thus, injections were made every 24 hours. The pH's of all nine samples were also monitored and recorded throughout the 7 – day period.

3.6.2 Stability studies on reconstituted amoxycillin – clavulanic acid oral suspension kept in and out of the refrigerator (2 - 8ºC, 25ºC).

The three brands of amoxycillin – clavulanic acid were used. Each was reconstituted with distilled water, the assumed standard water for reconstitution of drugs. The drugs had their pH's recorded and analysed by HPLC immediately after reconstitution, and then kept in the fridge of standard temperature (2 - 8ºC). This served as analysis for Day 1. The reconstituted drugs were kept in and out of the fridge on alternate days, throughout the seven day period. HPLC analysis and pH recordings were carried out on each of the seven days. When the drugs were taken out of the fridge, they were kept under normal room temperature (25ºC).

3.6.3 Stability study on reconstituted Amoxycillin – Clavulanic acid oral suspension under patient simulated conditions.

One brand (B) was used for this particular experiment. Two samples of brand B were obtained. They were both reconstituted with ordinary sachet water obtained from a sachet water seller. The pH of the sachet water was first recorded before being used for the reconstitution. The pH's of the two samples were also recorded after reconstitution. One sample was kept in the refrigerator for storage whilst the other was made to stand in a bowl of

water and inside a cupboard. Analysis for these two samples was carried out throughout the seven day period.

CHAPTER 4 – RESULTS AND CALCULATIONS

4.1 Identification tests for Amoxycillin and clavulanic acid

Table 4: Identification of Amoxicillin by colour reaction tests

Test	**Observation**	**Specification (BP)**
About 2.0mg of Amox was added to 1.50ml of distilled water and 2.00ml of Sulphuric acid-formaldehyde	Dark yellow colouration	Dark yellow colouration
About 5.0mg of Amox was added to1.00ml distilled water.1-2drops of neutral Ferric Chloride solution was added.	Blue colouration	Development of blue colouration

Table 5 Melting point Determination for Amoxycillin Trihydrate

Sample	**Melting point determination (°C)**			**Reference Range**
Amoxycillin	**1st Determination**	**2nd Determination**	**Average range**	
	192 - 194	194 - 196	193 - 195	192 - 196

Table 6 Identification of Clavulanic acid by colour reaction tests

Test	**Observation**	**Specification (BP)**
About 10.00mg of Clav was added to 1.00ml of distilled water and 2.00ml Na_2CO_3 solution + heat. 0.05ml of sodium sulphide was added and heated and cool. 2.00ml of tartaric acid was added and allowed to stand for 5minutes.	Appearance of white crystalline precipitate	Development of white crystalline precipitate

Table 7 Melting point determination of clavulanic acid potassium

Sample	**Melting point determination (ºC)**			**Reference Range (ºC)**
C.A potassium	**1st Determination**	**2nd Determination**	**Average range**	
	162 - 164	161 - 163	162 - 163	>160

Table 8 pH determinations of Amoxycillin and Clavulanic acid solutions

Sample	pH reading		Average pH	Reference Range
	1st determination	2nd determination		
Amoxicillin	5.20	5.40	5.30	3.50 – 5.50
Clavulanic acid	6.40	6.60	6.50	5.50 – 8.00

4.2 TLC of reference samples under uv light

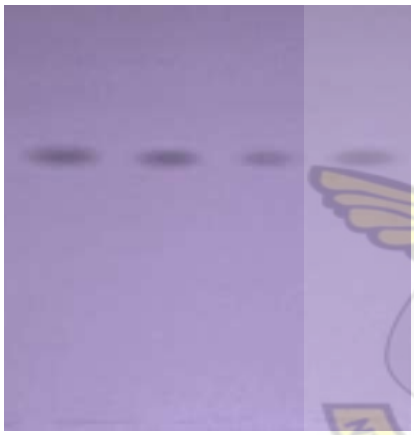


Figure 20 TLC for Amoxycillin

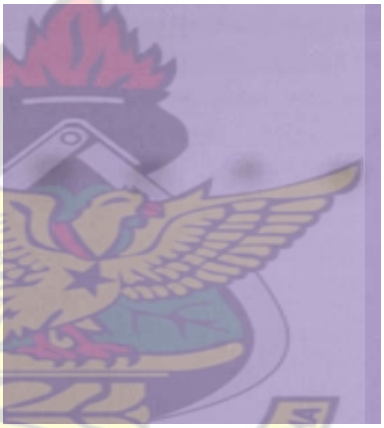


Figure 21 TLC for Clavulanic acid

4.3 Calculation of rf for amoxycillin and clavulanic acid

Retention factor (R_f) is given as = Distance moved by sample from baseline

Distance moved by solvent from baseline

For Amoxycillin,

Distance moved by sample from baseline = 4.60cm

Distance moved by solvent front from baseline = 6.00cm

R_f for one of the Amoxycillin spots = 4.60cm = 0.77

6.00cm

Similar calculations were done for the other Amoxycillin spots and an average was gotten.

Thus, average R_f value for Amoxycillin = (0.77 + 0.78 + 0.76 + 0.77) /4 = 0.77

For Clavulanic acid,

Distance moved by sample from baseline = 2.80cm

Distance moved by solvent front from baseline = 5.00cm

R_f for Clavulanic acid = $\frac{2.80cm}{5.00cm}$ = 0.56

4.4 Assay of amoxicillin trihydrate and clavulanic acid reference standard.

Preparation of 0.01M $Na_2S_2O_3$

248.1800g ($Na_2S_2O_3$) in 1000.00ml ≡≡ 1.00M $Na_2S_2O_3$

2.4818g ($Na_2S_2O_3$) in 1000.00ml ≡≡ 0.01M $Na_2S_2O_3$

0.6205g ($Na_2S_2O_3$) in 250.00ml ≡≡ 0.01M $Na_2S_2O_3$

Nominal weight = 0.6205g

Actual weight = 0.6203g

Standardization of $Na_2S_2O_3$

$KIO_3 + 5\ KI + 6\ HCl \rightarrow 3I_2 + 6\ KCl + 3\ H_2O$

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

Mole ratio of KIO_3 to $Na_2S_2O_3$ is 1: 6

→ (214.0000g) KIO_3 in 1000.00ml ≡ 6M $Na_2S_2O_3$

(35.6667g) KIO_3 in 1000.00ml ≡ 1M $Na_2S_2O_3$

(0.3567g) KIO_3 in 1000.00ml ≡ 0.01M $Na_2S_2O_3$

(0.17833g) KIO_3 in 500.00ml ≡ 0.01M $Na_2S_2O_3$

Nominal weight of KIO_3 = 0.1783g

Actual weight of KIO_3 = 0.1783g

Table 9 Standardization of 0.01M sodium thiosulphate with potassium iodate

Titration	**1**	**2**	**3**
Final volume (ml)	21.70	42.10	46.20
Initial volume(ml)	1.70	21.70	24.70
Titre volume (ml)	20.00	20.40	20.50

Average titre volume (ml) = $\frac{20.00 + 20.40 + 20.50}{3}$ = 20.45ml

Weight of iodine (I_2) = 2.5379g Factor (f) of KIO_3 = $\frac{\text{Actual weight}}{\text{Nominal weight}} = \frac{0.1783}{0.1783} = 1.0000$

$f(KIO_3) \times v(KIO_3) = f(Na_2S_2O_3) \times v(Na_2S_2O_3)$

$f(Na_2S_2O_3) = \frac{1.0000 \times 20.00ml}{20.45ml} = 0.9780$

Table 10 Main assay results of Amoxycillin Trihydrate after hydrolysis

Titration	**1**	**2**	**3**
Final volume (ml)	25.40	23.00	41.90
Initial volume(ml)	4.40	2.00	20.80
Titre volume (ml)	21.00	21.00	21.10

Average titre volume (ml) = (21.00 + 21.00 + 21.10) /3 = 21.03ml

Table 11 Results for blank determination (Unhydrolysed Amoxycillin)

Titration	**1**	**2**	**3**
Final volume (ml)	18.30	29.50	11.30
Initial volume(ml)	7.00	18.30	0.00
Titre volume (ml)	11.30	11.20	11.30

Average titre volume (ml) = $\frac{11.30 + 11.20 + 11.30}{3}$ = 11.30ml

Volume of $Na_2S_2O_3$ taken up by drug (Amoxycillin) = main assay titre – blank titre

= 21.00ml – 11.30ml = 9.70ml, thus volume ($Na_2S_2O_3$) taken up by drug.

But f ($Na_2S_2O_3$) = 0.9780, hence corrected volume = titre volume × f ($Na_2S_2O_3$)

= 9.70ml × 0.9780

= 9.49ml

Molar mass of Amoxycillin Trihydrate = 419.4000g/mol

419.4000g ($C_{16}H_{19}N_3O_5S.3H_2O$) in 1000.00ml ≡ 4M I_2

104.8500g ($C_{16}H_{19}N_3O_5S.3H_2O$) in 1000.00ml ≡ 1M I_2

1.0485g ($C_{16}H_{19}N_3O_5S.3H_2O$) in 1000.00ml ≡ 0.01M I_2

0.0010485g ($C_{16}H_{19}N_3O_5S.3H_2O$) in 1.00ml ≡ 0.01M I_2

1ml of 0.01M I_2 = 0.0010485g of Amoxycillin Trihydrate

→9.49ml of 0.01M I_2 = ?

$$= \frac{9.49ml \times 0.0010485g}{1.00ml \text{ of } 0.01M\ I_2}$$

= 0.009950265g of Amoxycillin Trihydrate.

Thus, actual weight of Amoxycillin Trihydrate that reacted = 0.009950265g

But from method,

→ 0.1004g dissolved and made up to 100.00ml

100.00ml = 0.1004g

10.00ml = ?

$$= \frac{10.00ml \times 0.1004}{100.00ml}$$

= 0.01004g (Nominal weight)

% purity = $\frac{\text{Actual weight}}{\text{Nominal weight}} \times 100\% = \frac{0.009950265\text{g}}{0.01004\text{g}} \times 100\% = 99.106\%\text{w/v} = 99.11\%\text{w/v}$

% Purity 2

Volume of $Na_2S_2O_3$ taken up by drug (Amoxycillin) = main assay titre – blank titre

= 21.00ml – 11.20ml = 9.80ml, thus volume ($Na_2S_2O_3$) taken up by drug.

But f ($Na_2S_2O_3$) = 0.9780, hence corrected volume = titre volume × f ($Na_2S_2O_3$)

= 9.80ml × 0.9780

= 9.58ml

Molar mass of Amoxycillin Trihydrate = 419.4000g/mol

419.4000g ($C_{16}H_{19}N_3O_5S.3H_2O$) in 1000.00ml ≡ 4M I_2

104.8500g ($C_{16}H_{19}N_3O_5S.3H_2O$) in 1000.00ml ≡ 1M I_2

1.0485g ($C_{16}H_{19}N_3O_5S.3H_2O$) in 1000.00ml ≡ 0.01M I_2

0.0010485g ($C_{16}H_{19}N_3O_5S.3H_2O$) in 1.00ml ≡ 0.01M I_2

1ml of 0.01M I_2 = 0.0010485g of Amoxycillin Trihydrate

→9.58ml of 0.01M I_2 = ?

$= \frac{9.58\text{ml} \times 0.0010485\text{g}}{1.00\text{ml of } 0.01\text{M } I_2}$

= 0.01004463g of Amoxycillin Trihydrate.

Thus, actual weight of Amoxycillin Trihydrate that reacted = 0.01004463g

But from method,

→ 0.1004g dissolved and made up to 100.00ml

100.00ml = 0.1004g

10.00ml = ?

$= \frac{10.00\text{ml} \times 0.1004}{}$

100.00ml

= 0.01004g (Nominal weight)

% purity = $\frac{\text{Actual weight}}{\text{Nominal weight}} \times 100\% = \frac{0.01004463\text{g}}{0.01004\text{g}} \times 100\%$

= 100.05%

% purity 3

Volume of $Na_2S_2O_3$ taken up by drug (Amoxycillin) = main assay titre – blank titre

= 21.00ml – 11.30ml = 9.70ml, thus volume ($Na_2S_2O_3$) taken up by drug.

But f ($Na_2S_2O_3$) = 0.9780, hence corrected volume = titre volume × f ($Na_2S_2O_3$)

= 9.70ml × 0.9780

= 9.49ml

Molar mass of Amoxycillin Trihydrate = 419.4000g/mol

419.4000g ($C_{16}H_{19}N_3O_5S.3H_2O$) in 1000.00ml ≡ 4M I_2

104.8500g ($C_{16}H_{19}N_3O_5S.3H_2O$) in 1000.00ml ≡ 1M I_2

1.0485g ($C_{16}H_{19}N_3O_5S.3H_2O$) in 1000.00ml ≡ 0.01M I_2

0.0010485g ($C_{16}H_{19}N_3O_5S.3H_2O$) in 1.00ml ≡ 0.01M I_2

1ml of 0.01M I_2 = 0.0010485g of Amoxycillin Trihydrate

→9.49ml of 0.01M I_2 = ?

= $\frac{9.49\text{ml} \times 0.0010485\text{g}}{1.00\text{ml of } 0.01\text{M } I_2}$

= 0.009950265g of Amoxycillin Trihydrate.

Thus, actual weight of Amoxycillin Trihydrate that reacted = 0.009950265g

But from method,

→ 0.1004g dissolved and made up to 100.00ml

100.00ml = 0.1004g

10.00ml = ?

$$= \frac{10.00\text{ml} \times 0.1004}{100.00\text{ml}}$$

= 0.01004g (Nominal weight)

$$\% \text{ purity} = \frac{\text{Actual weight}}{\text{Nominal weight}} \times 100\% = \frac{0.009950265\text{g}}{0.01004\text{g}} \times 100\%$$

= 99.106%w/v = 99.11%w/v

Average % purity of amoxicillin pure powder = $\frac{99.11\% + 100.05\% + 99.11\%}{3}$ = 99.42%w/v

Assay of clavulanic acid reference standard

Preparation and standardisation of 0.1M NaOH with sulphamic acid.

Preparation of 0.1M NaOH

40.0000g (NaOH) in 1000.00ml ≡ 1M NaOH

4.0000g (NaOH) in 1000.00ml ≡ 0.1M NaOH

2.0000g (NaOH) in 500.00ml ≡ 0.1M NaOH

But Assay = 96%

→ 96% = 2.0000g

Then, 100% = ? $= \frac{100\% \times 2.0000\text{g}}{96\%} = 2.0833\text{g}$

Nominal weight (NaOH) = 2.0833g

Actual weight (NaOH) = 2.0845g

Preparation of 0.1M NH_2SO_3H

97.0900g (NH_2SO_3H) in 1000.00ml ≡ 1M NaOH

9.7090g (NH_2SO_3H) in 1000.00ml ≡ 0.1M NaOH

0.9709g (NH_2SO_3H) in 100.00ml ≡ 0.1M NaOH

Assay = 99%

But 99% = 0.9709g

→ 100% = ? = $\frac{100}{99} \times 0.9709 = 0.9807$g

Table 12 Standardisation of 0.1M Sodium Hydroxide with Sulphamic acid

Titration	**1**	**2**	**3**
Final volume (ml)	26.00	29.10	1.00
Initial volume(ml)	0.00	3.00	27.20
Titre volume (ml)	26.00	26.10	26.20

Volume of pipette = 25.00ml = volume of sulphamic acid used for the titration.

Average titre = $\frac{26.00ml + 26.10ml + 26.20ml}{3} = 26.10ml$

1 mole of H_2NSO_3 reacts with = 1mole of NaOH

97.09g H_2NSO_3 in 1000.00ml ≡ 1M NaOH

9.709g H_2NSO_3 in 1000.00ml ≡ 0.1M NaOH

0.9709g H_2NSO_3 in 100.00ml ≡ 0.1M NaOH

0.009709g H_2NSO_3 in 1.00ml ≡ 0.1M NaOH

Factor (f_1) H_2NSO_3 = $\frac{\text{Actual weight}}{\text{Nominal weight}} = \frac{0.9807}{0.9807} = 1.0000$

Factor (f_2) of NaOH =? , Volume of H_2NSO_3 (v_1) = 25.00ml

Volume (NaOH) (v_2) = 26.10ml

Factor (f_2) of NaOH = $\frac{f_1(H_2NSO_3) \times v_1(H_2NSO_3)}{v_2(NaOH)} = \frac{1.0000 \times 25.00ml}{26.10ml}$

= 0.9579

Main assay results for clavulanic acid potassium

Preparation of 0.1M HCl

36.5000g (HCl) in 1000.00ml ≡ 1M HCl

3.6500g (HCl) in 1000.00ml ≡ 0.1M HCl

0.7300g (HCl) in 200.00ml ≡ 0.1M HCl

But Assay = 36% = 0.73g

→ for 100% = ? = $\frac{100}{36} \times 0.73g = 2.0278g = 2.0300g$

Density = $\frac{\text{mass}}{\text{volume}}$ = 1.18g/ml, volume = $\frac{\text{mass}}{\text{Density}} = \frac{2.0300g}{1.18g/ml} = 1.72ml$

Thus, 1.72ml of stock HCl was pipetted into a 200.00ml volumetric flask and topped to the mark with distilled water.

Table 14 Results for back titration of clavulanic acid reference powder

Titration	0.5011g	0.5021g	0.5003g	blank
	1	2	3	
Final volume (ml)	23.50	26.80	25.00	49.00
Initial volume(ml)	0.00	3.00	1.00	0.00
Titre volume (ml)	23.50	23.80	24.00	49.00

f (NaOH) × v (NaOH) = f (HCl) × v (HCl)

f (HCl) = $\frac{\text{f (NaOH)} \times \text{v (NaOH)}}{\text{v(HCl)}} = \frac{0.9579 \times 50.00ml}{49.00ml} = 0.9775$

for volume of NaOH taken up drug (clavulanic acid) = blank volume – titre volume

for % purity 1

199.3000g ($C_8H_9NO_5$) in 1000.00ml ≡ 1M NaOH

19.9300g ($C_8H_9NO_5$) in 1000.00ml ≡ 0.1M NaOH

0.01993g ($C_8H_9NO_5$) in 1.00ml ≡ 0.1M NaOH

for titre 1

Corrected titre volume = 23.50ml × 0.9775 = 22.97ml = 23.00ml

Corrected blank volume = 49.00ml × 0.9775 = 47.90ml

Volume taken up by clavulanic acid = 47.90ml – 23.0ml = 24.90ml

But 1.00ml = 0.01993g (clavulanic acid)

25.20ml = ?

$$= \frac{24.90\text{ml} \times 0.01993\text{g}}{1.00\text{ml}} = 0.4965\text{g}$$

→ % purity = $\frac{\text{Actual weight}}{\text{Nominal weight}} \times 100\% = \frac{0.4963\text{g}}{0.5011\text{g}} \times 100\% = 99.04\%$

% purity 2 (for titre 2)

Corrected titre volume = 23.80ml × 0.9775 = 23.26ml = 23.27ml

Corrected blank volume = 49.00ml × 0.9775 = 47.90ml

Volume taken up by clavulanic acid = 47.90ml – 23.27ml = 24.63ml

But 1.00ml = 0.01993g (clavulanic acid)

24.63ml = ?

$$= \frac{24.63\text{ml} \times 0.01993\text{g}}{1.00\text{ml}} = 0.4909\text{g}$$

→ % purity = $\frac{\text{Actual weight}}{\text{Nominal weight}} \times 100\% = \frac{0.4909\text{g}}{0.5021\text{g}} \times 100\% = 97.77\%$

% purity 3 (for titre 3)

Corrected titre volume = 24.00ml × 0.9775 = 23.46ml = 23.46ml

Corrected blank volume = 49.00ml × 0.9775 = 47.90ml

Volume taken up by clavulanic acid = 47.90ml – 23.46ml = 24.44ml

But 1.00ml = 0.01993g (clavulanic acid)

24.44ml = ?

$$= \frac{24.44\text{ml} \times 0.01993\text{g}}{1.00\text{ml}} = 0.4871\text{g}$$

$$\rightarrow \%\ \text{purity} = \frac{\text{Actual weight}}{\text{Nominal weight}} \times 100\% = \frac{0.4871\text{g}}{0.5003\text{g}} \times 100\% = 97.36\%$$

$$\text{Average \% purity for clavulanic acid} = \frac{99.40\% + 97.77\% + 97.36\%}{3} = 98.20\%$$

4.5 SURVEY RESULTS

Survey conducted in community and hospital pharmacies.

Over a 100 questionnaires were given out to pharmacists, dispensing technicians and medical counter assistants. Out of the questionnaires given out, there was a total of 82 respondents.

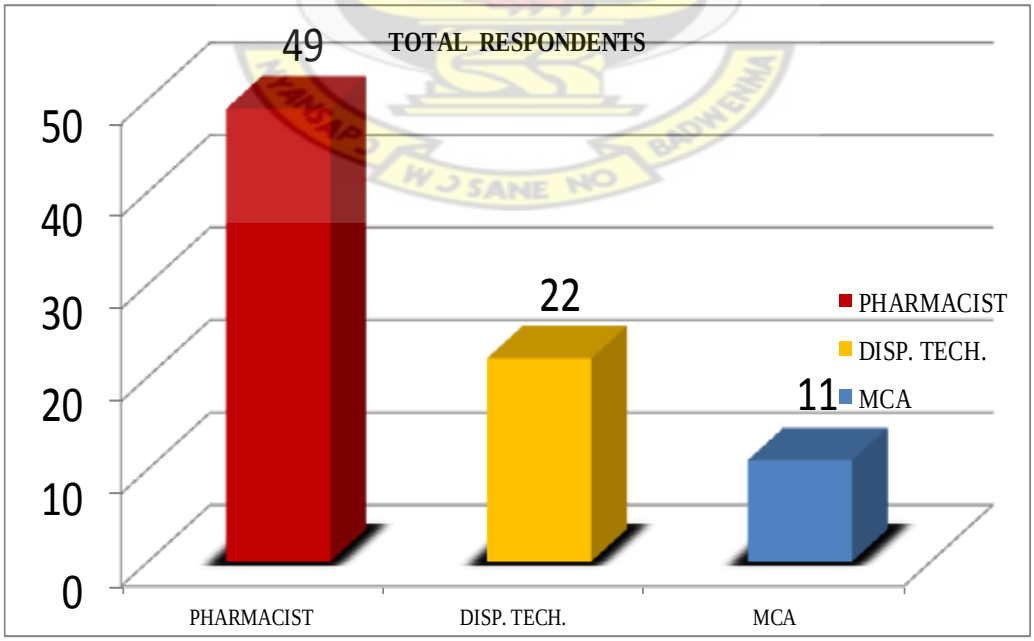


Figure 22 Total number of respondents for survey

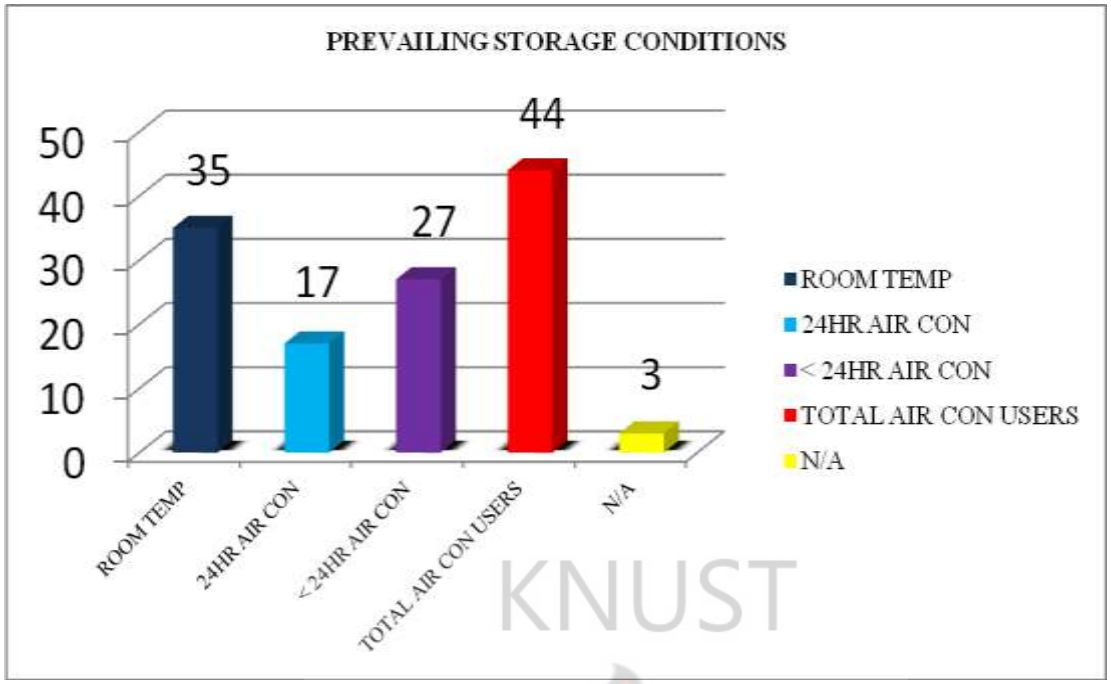


Figure 23 Prevailing storage conditions of pharmacies in selected suburbs of Kumasi

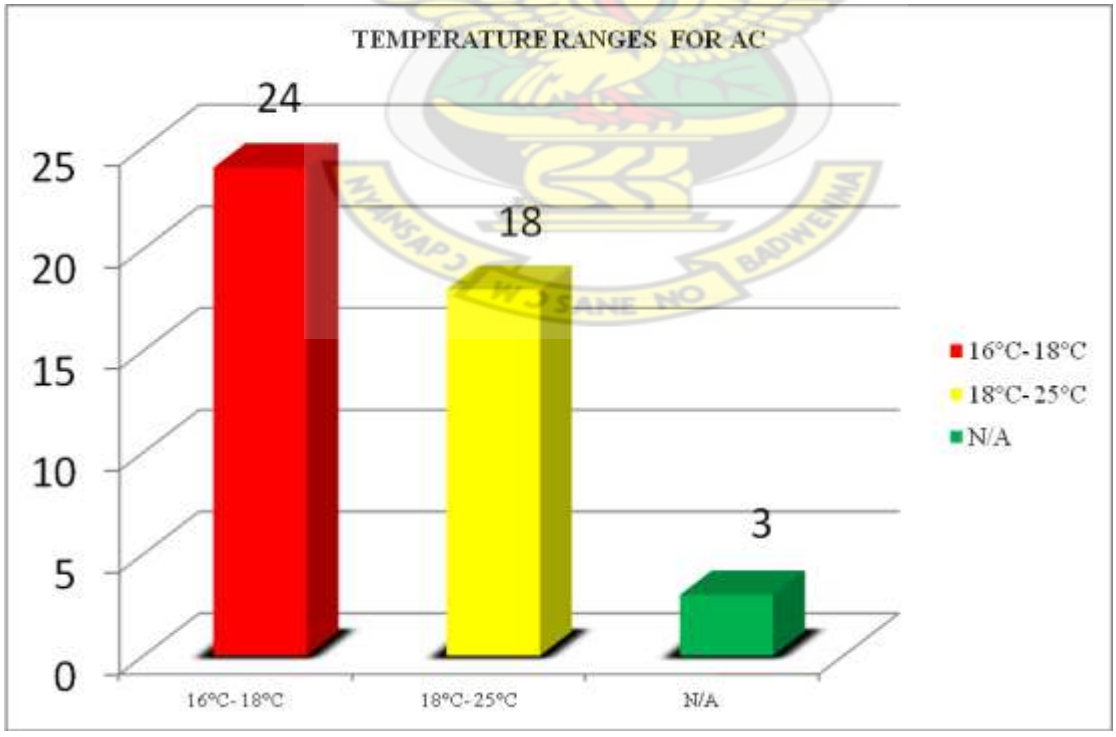


Figure 24Temperature ranges often set for Air conditioners present in the health facility

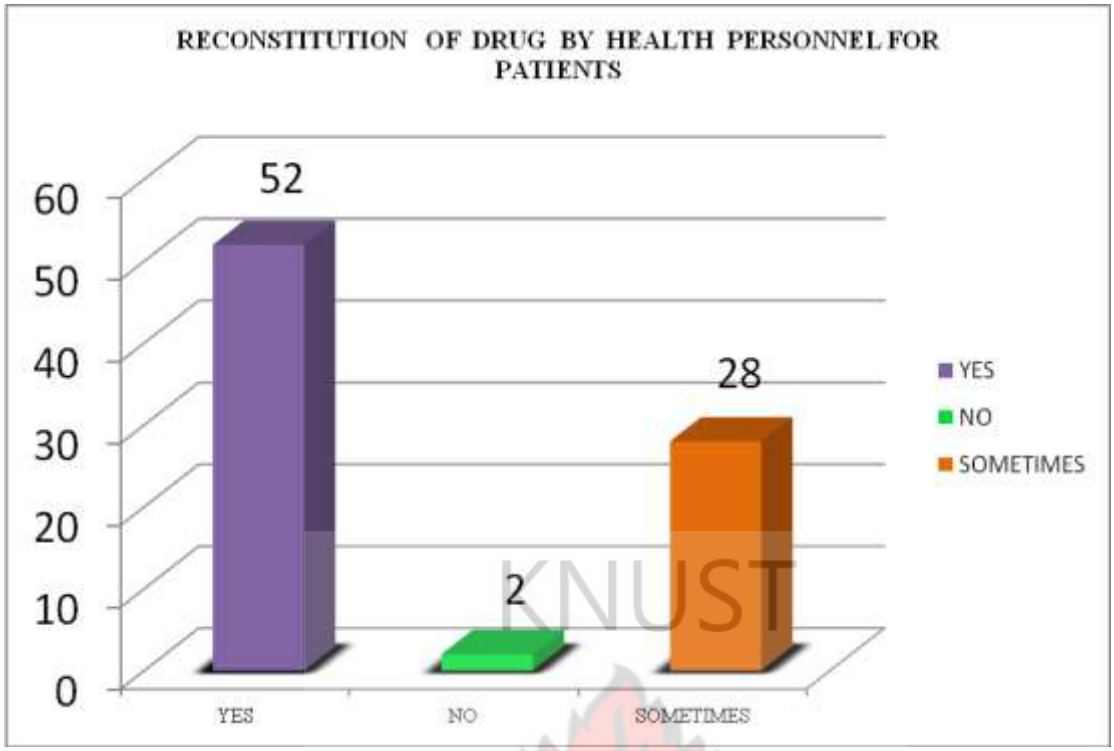


Figure 25 Reconstitution of drug by health personnel for patients.

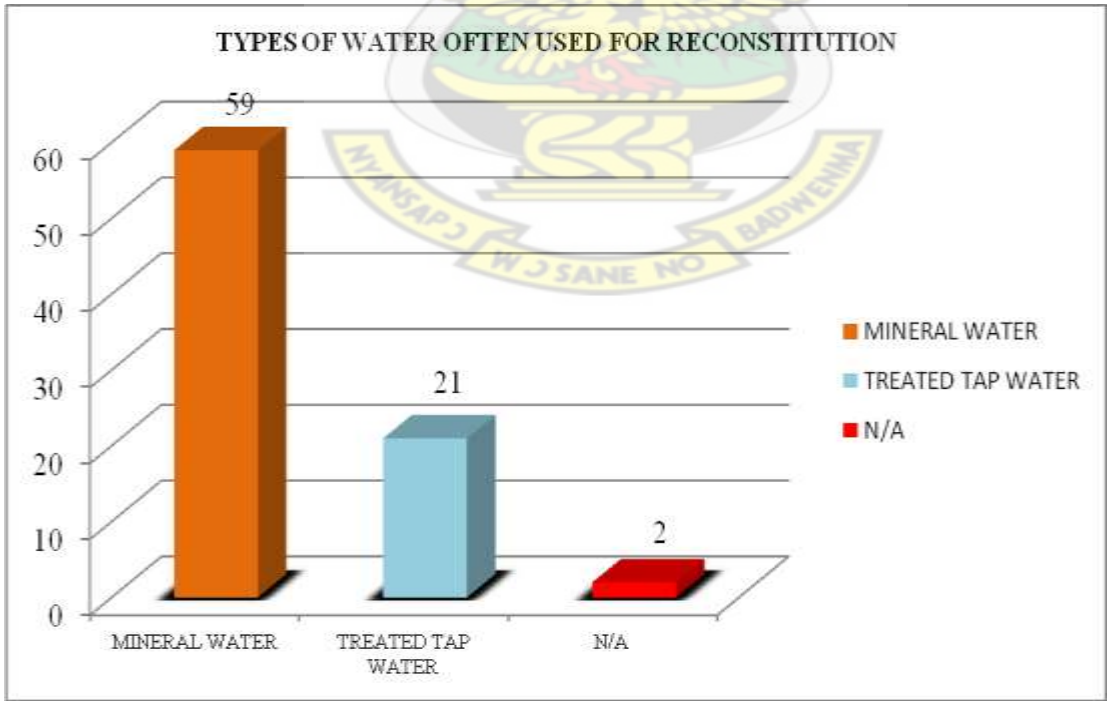


Figure 26 Common types of water used for reconstitution of co – amoxiclav oral suspension**.**

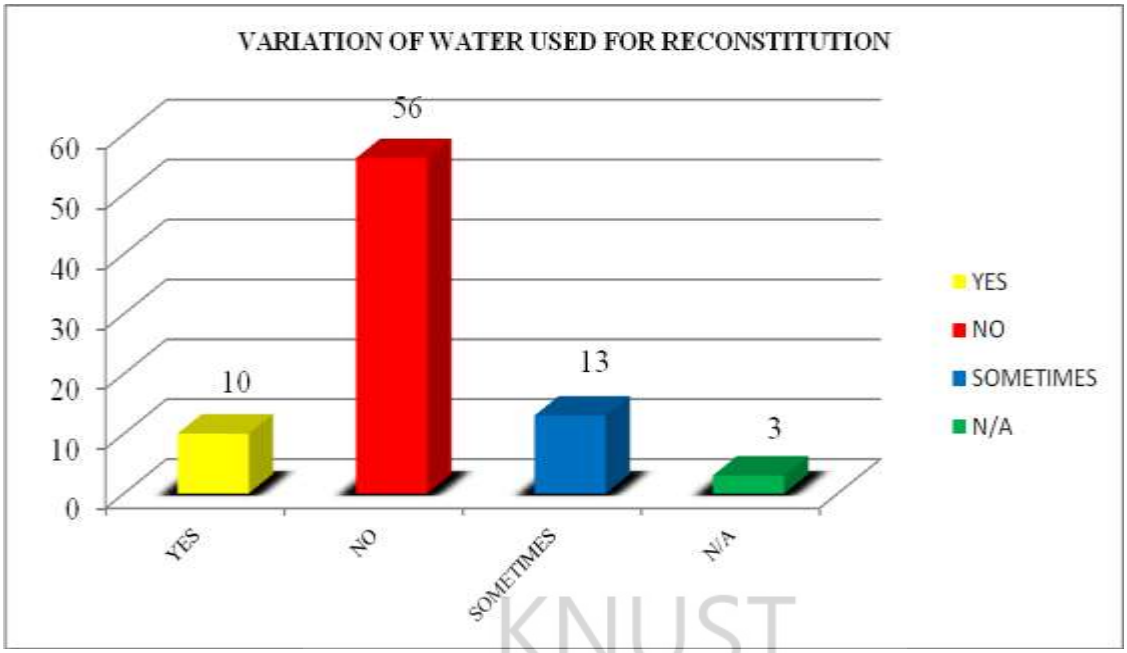


Figure 27 Variation in type of water used for reconstitution by health personnel**.**

UV Spectra of Amoxicillin and Clavulanic Acid

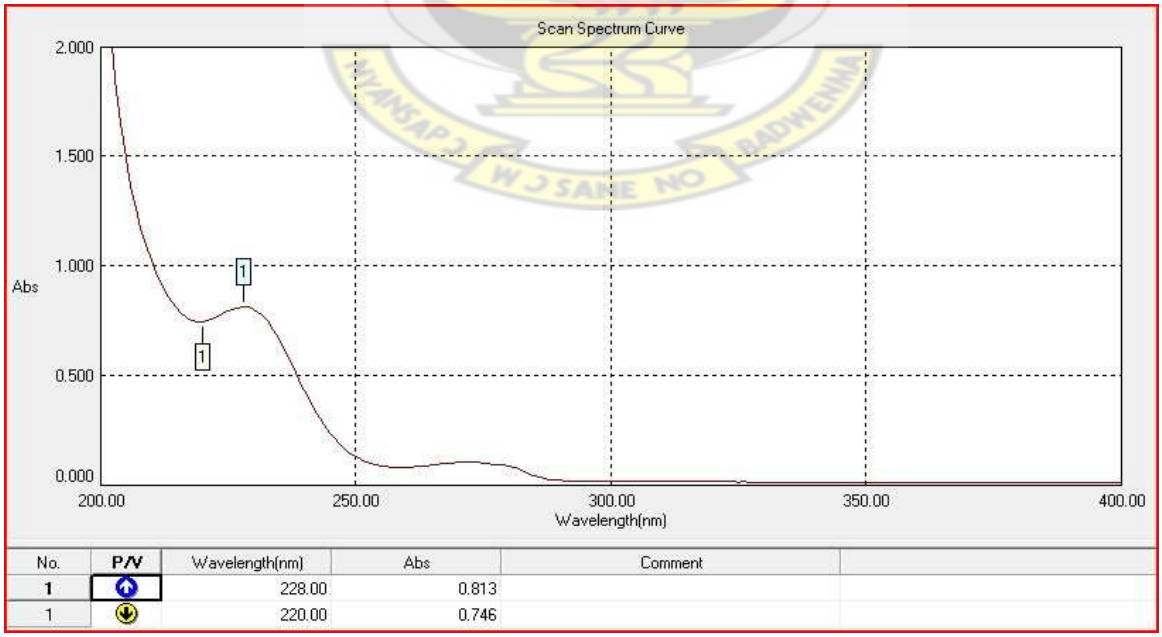
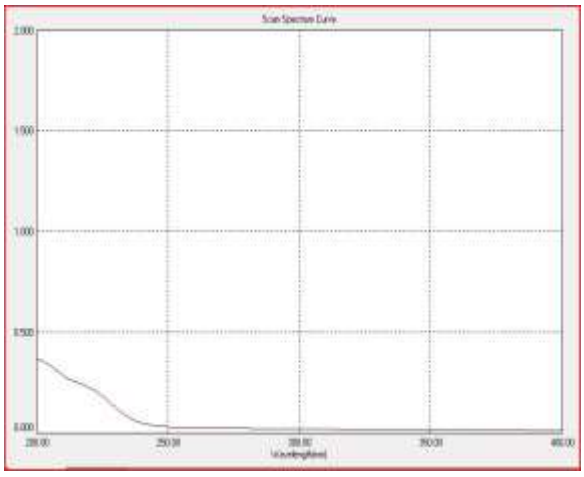


Figure 28 UV spectrum for Amoxycillin Trihydrate in water as solvent**.**



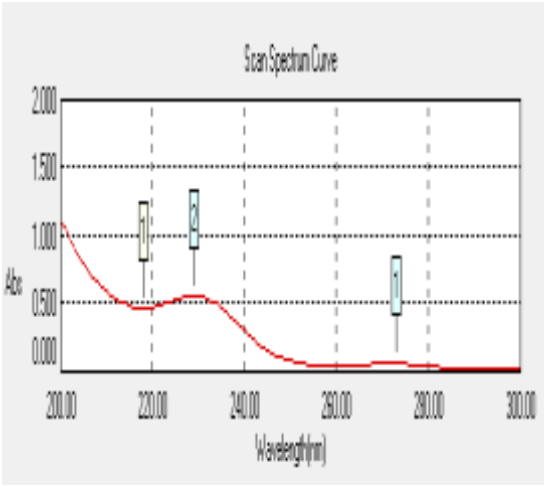


Figure 29UV spectrum of Clavulanic acid in in water (100%)

Figure 30UV spectrum of clavulanic acid water and methanol (90: 10)

CONDUCTIVITY RESULTS

Calibration of conductimeter

Table 14 calibration of conductimeter

Mass (g) of KCl / litre of solution	Measured Conductance (µs)	Conductivity(µs/cm)
0.7455	1394	1413
0.0746	175.1	147
0.0149	31.0	23.0

Calculation of cell constant

Conductivity (µS/cm) = cell constant(/cm) × measured conductance (µS)

→ for a 0.01M standard solution of KCl, cell constant = $\frac{\text{Conductivity (µS/cm)}}{\text{Measured conductance (µS)}}$

= $\frac{1413\ (\mu S/cm)}{1394\ (\mu S)}$ = 1.014 /cm ≈≈ 1.0 /cm

Table 15 Calibration standards for conductivity at 25°C

Concentration M KCl	Conductivity µS/cm	Upper limit µS/cm	Lower limit µS/cm
0.0500	6668	6801	6535
0.0200	2767	2822	2711
0.0100	1413	1441	1395
0.0050	717.8	735	700
0.0010	147.0	149	145
0.0005	73.9	77.8	70.2
0.0001	14.94	16.5	13.5

Table 16 Conductivity results for types of water used for reconstitution of amoxicillin-clavulanic acid oral powder for suspension

Type of water	pH	Measured Conductance (µs)	Conductivity(µs/cm)
Mineral water (voltic)	6.30	01.50	1.50
Distilled water	6.00	02.80	2.80
Treated Tap water	6.00	495.00	495.00

4.6 HPLC METHOD DEVELOPMENT AND VALIDATION

Optimal HPLC conditions established for Amoxicillin and Clavulanic acid

o Stationary phase: Phenomenex, Kromasil 5 C_8 ODS column, 250 X4.60mm 5 micron column, size 305334

o Mobile Phase: Water: Methanol: Na cetate Buffer (pH4.4) (65:15:20)

o Flow rate: 1.0ml/min

o Wavelength of detection: 220nm

4.6.1 Calibration curve for amoxicillin trihydrate

Table 17 Calibration values for Amoxicillin Trihydrate

CONCENTRATION (% w/v)	AVERAGE PEAK AREA
0.1000	6.78
0.0700	4.70
0.0500	3.27
0.0300	2.23
0.0100	0.74

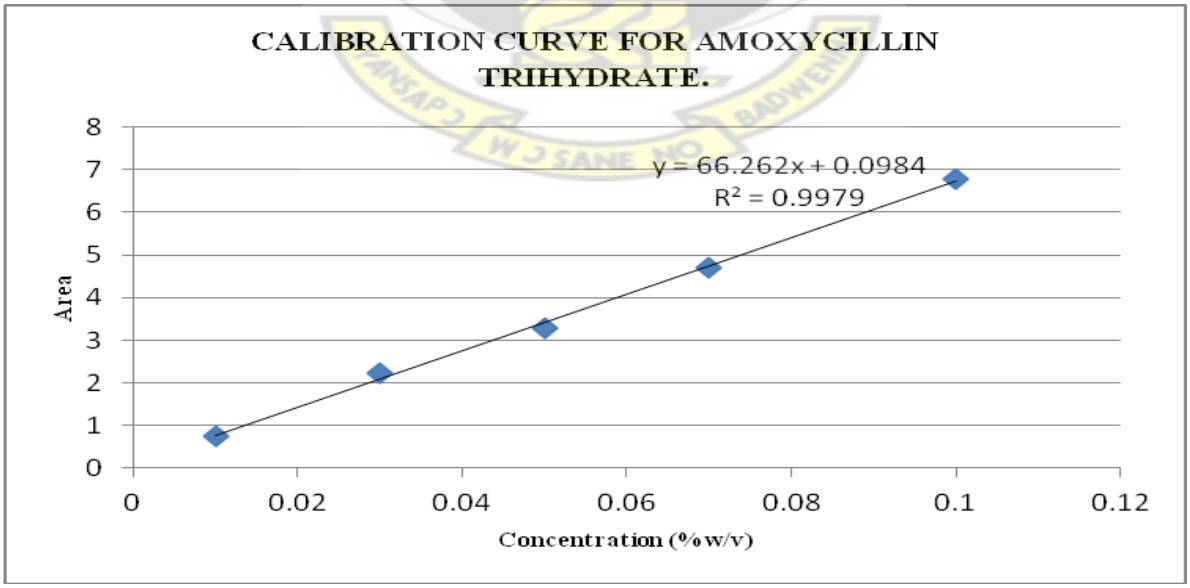


Figure 31Calibration curve of Amoxycillin Trihydrate

Table 18 Parameters of the calibration curve

Parameter	**Value**
R^2	0.9979
Intercept	0.0984
Slope	66.262
Range	0.0100%^{w}/v - 0.1000%^{w}/v

Range= 0.0100%^{w}/v - 0.1000%^{w}/v

Sample Calculation

The general equation of a line graph is represented by; y = mx + c,

Where;

y = Peak volume Ratio, m = Slope (gradient) of Calibration Curve, x = Concentration(%w/v),

c = y - intercept

From the graph,

Y = 66.262x + 0.0984

x = y - 0.0984 / 66.262

4.6.2 Limit of detection (LOD) for amoxycillin

LOD = (3.3 x Sy.x) / Slope (m), where Sy.x is the residual standard deviation gotten from the calibration curve and slope is that of the calibration curve. From the calibration curve the residual standard deviation of the calibration curve is 0.123213

Hence, LOD = (3.3× 0.123213) / 66.262 = 0.00614%w/v

LOD = 0.00614%w/v

4.6.3 Limit of quantification (LOQ) for amoxycillin

LOQ = (10× Sy.x) / Slope(m)

LOQ = (10 × 0.123213) / 66.262

LOQ = 0.0186%w/v

Precision

4.6.4 Repeatability (intraday precision) amoxycillin

Table 19 Repeatability for HPLC method for Amoxycillin

Sample	Nominal Concentration(%w/v)	Peak Area	Actual Concentration(%w/v)	% Recovery
1	0.0600	4.061	0.0599	99.83
2	0.0600	4.000	0.0584	97.33
3	0.0600	4.034	0.0595	99.17
4	0.0600	4.048	0.0597	99.50
5	0.0600	4.061	0.0599	99.83
6	0.0600	3.990	0.0589	98.17

Mean = 4.032333 Standard deviation = 0.030755

Relative Standard Deviation (RSD) = (100*S) /mean = 0.76%

4.6.5 Inter-day precision for amoxycillin

Table 20 Inter -day precision for Amoxycillin(HPLC method)

Sample no.	Nominal Concentration (%w/v)	Actual Concentration (%w/v)	Peak Area	RSD (%)
1	0.10	0.0982	6.70	1.66
2	0.10	0.0987	6.50	
3	0.10	0.0985	6.68	
4	0.10	0.0993	6.73	1.74
5	0.10	0.0959	6.50	
6	0.10	0.0976	6.62	
7	0.10	0.0992	6.73	0.65
8	0.10	0.0981	6.65	
9	0.10	0.0982	6.66	

RSD = 1.03%

4.6.6 Accuracy for amoxycillin

Table 21 Accuracy for HPLC method

DETERMINATION	**% RECOVERED**
1	99.83%
2	99.50%
3	99.20%
Average	**99.51%**

4.6.7 Robustness

Figure 32 Robustness of Amoxycillin and clavulanic acid

Table 22 Robustness for Amoxycillin

		%Recovery
Flow rate (ml/min)	1.50	100.5
Wavelength (nm)	225	96.08

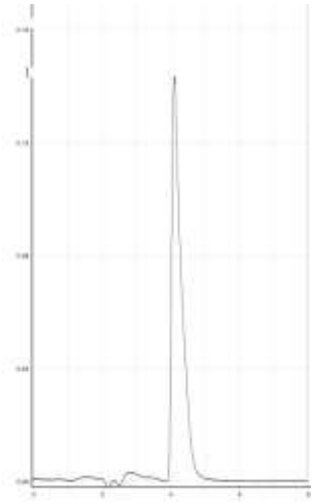


Figure 33 Chromatogram of Pure Amoxycillin Trihydrate.

Table 23 Retention time of Amoxycillin Trihydrate

PARAMETER	VALUE (min)
Retention time	4.09 ± 0.02

4.6.8 Calibration curve for clavulanic acid

Table 24 Calibration values for Clavulanic acid

CONCENTRATION (% w/v)	AVERAGE PEAK AREA
0.01425	0.97
0.00998	0.67
0.004989	0.38
0.0015	0.20
0.00015	0.08

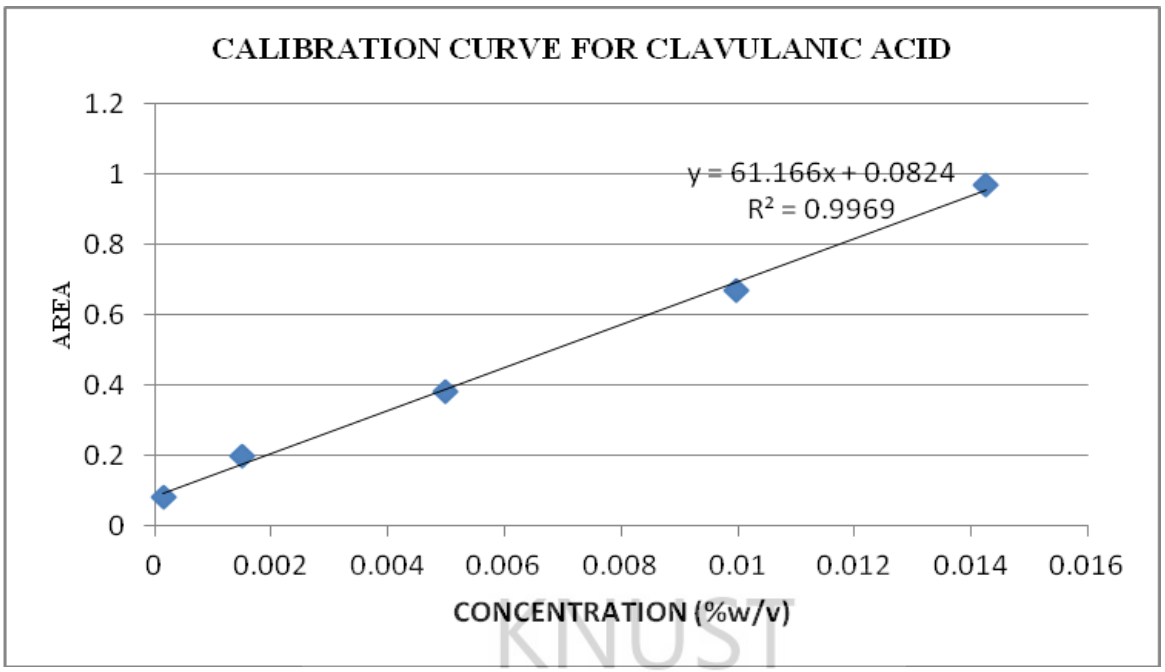


Figure 34 Calibration curve for Clavulanic acid

4.6.9 Limit of detection (LOD) for clavulanic acid

LOD = (3.3 x Sy.x) / Slope (m), where Sy.x is the residual standard deviation gotten from the calibration curve and slope is that of the calibration curve. From the calibration curve the residual standard deviation of the calibration curve is 0.023355

Hence, LOD = (3.3× 0.023355) / 61.166 = 0.00126%w/v

LOD = 0.00126%w/v

4.7.0 Limit of quantification (loq) for clavulanic acid

LOQ = (10× Sy.x) / Slope(m)

LOQ = (10 × 0.023355) / 61.166

LOQ = 0.003818%w/v

4.7.1 Repeatability (intra-day precision) for clavulanic acid

Table 25 Repeatability for HPLC method for clavulanic acid

Sample	Nominal Concentration (%w/v)	Peak Area	Actual Concentration(%w/v)	% Recovery
1	0.0500	3.3453	0.0499	99.80
2	0.0500	3.3460	0.0499	99.80
3	0.0500	3.3501	0.0500	100.00
4	0.0500	3.3623	0.0502	100.40
5	0.0500	3.3480	0.0500	100.00
6	0.0500	3.3477	0.0518	103.60

Mean = 3.3499 Standard deviation = 0.006302698

Relative Standard Deviation (RSD) = 0.18814585 = 0.20%

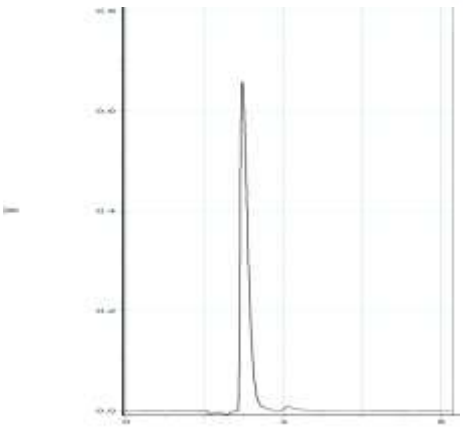


Figure 35; Chromatogram of Pure Clavulanic acid.

Table 26 Retention time of Clavulanic acid

PARAMETER	VALUE (min)
Retention time	2.96 ± 0.01

Robustness

Figure 36 Robustness of clavulanic acid

Table 27 Robustness for Clavulanic acid

		%Recovery
Flow rate (ml/min)	1.50	98.50
Wavelength (nm)	225	99.30

Reconstitution of amoxicillin – clavulanic acid with different types of water.

Table 28 pH's of reconstituted amoxycillin – clavulanic acid oral powder (Day 1)

TYPE OF WATER	A	B	**C**
Distilled water	5.05	5.38	5.22
Mineral water (voltic)	5.18	5.42	5.33
Treated Tap water	5.03	5.39	5.32

Table 29 pH's of reconstituted amoxycillin – clavulanic acid oral powder (Day 7)

TYPE OF WATER	A	B	**C**
Distilled water	5.50	5.72	5.66
Mineral water (voltic)	5.58	5.75	5.72
Treated Tap water	5.48	5.73	5.64

4.7.2 Assay of three brands of co-amoxiclav oral suspension

Percentage content calculation of assayed brands (A, B and C)

Percentage content calculation for amoxycillin

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

$Y = 66.262x + 0.0984$

Where, y is = Mean peak area,

x is = concentration of sample and

66.262 is = slope of the curve.

Sample A, When y = 5.1120, → x = ?

$X = (5.1120 – 0.0984) / 66.262 = 0.07566$

X = 0.07566 Amount of amoxicillin in undiluted sample is 80mg/ml.

→ 1.00ml of undiluted syrup is expected to contain 80.00mg of amoxicillin.

80.00mg of amoxicillin was diluted to a 100.00ml of solution, → 0.0800g of amoxicillin in a 100.00ml of solution.

Thus, expected concentration of solution = 0.0800%w/v

If x = 0.08%w/v, then what will be the expected area y,

$Y = 66.262x + 0.0984$, $y = (66.262)(0.08) + 0.0984 = 5.3994$.

Thus, % content = $\frac{\text{Actual concentration}}{\text{Expected concentration}} \times 100\%$ or $\frac{\text{Actual Peak Area}}{\text{Expected Peak Area}} \times 100\%$

Thus, % content (sample A) = $\frac{0.0757\%w/v}{0.0800\%w/v} \times 100\% = 94.625\% \approx 95\%$

Or % content (sample A) = $\frac{5.1120}{5.3994} \times 100\% = 94.677\% \approx 95\%$

The subsequent brands were calculated as such on the first day after reconstitution and the following were obtained: (**Brand A**) 98%, 97%, 94.58% and 100.38%, (**Brand B**) 96.88%, 103.93%, 104%, 100%, 104% and 100% and (**Brand C**) 107.5%, 100.5%, 100.5% and 99.63%

Percentage content calculation for clavulanic acid

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

Y = 61.166x + 0.0824

Where, y is = Mean peak area,

x is = concentration of sample and

61.166 is = slope of the curve.

Sample C, When y = 0.9600, → x = ?

X = (0.9600 – 0.0824) / 61.166 = 0.01435

X = 0.01435

Amount of clavulanic acid in undiluted sample is 11.40mg/ml.

→ 1.00ml of undiluted syrup is expected to contain 11.40mg of clavulanic acid.

11.40mg of clavulanic acid was diluted to a 100.00ml of solution, → 0.0114g of clavulanic acid in a 100.00ml of solution.

Thus, expected concentration of solution = 0.0114%w/v

If x = 0.0114%w/v, then what will be the expected area y,

Y = 61.166x + 0.0824, y = (61.166) (0.0114) + 0.0824 = 0.7797.

Thus, % content = $\frac{\text{Actual concentration}}{\text{Expected concentration}} \times 100\%$ or $\frac{\text{Actual Peak Area}}{\text{Expected Peak Area}} \times 100\%$

Thus, % content (sample C) = $\frac{0.01435\%w/v}{0.0114\%w/v} \times 100\% = 125.877\% \approx 126\%$

The subsequent brands were calculated as such on the first day after reconstitution and the following were obtained: (**Brand A**) 100%, 101.75%, 101.23% and 99.12%, (**Brand B**) 105.26%, 105.26%, 96.50%, 98.25%, 98.25% and 99.12% and (**Brand C**) 126.3%, 116.7%, 111.4% and 99.12%.

4.7.3 Stability studies on co-amoxiclav suspension

Results for assay of oral suspension C in treated tap water (2 - 8ºC)

Chromatographs for brand C oral suspension in treated tap water

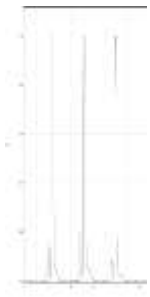


Figure 37 Day 1 (C in Tap water)

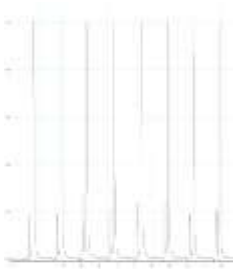


Figure 38 Day 5 (C in Tap water)

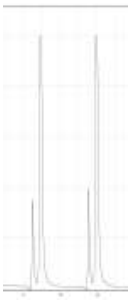


Figure 39 Day 7 (C in Tap water)

Table 31 HPLC results for brand C oral suspension in treated tap water (2 -8ºC)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Area (C.A)	0.9650	0.9643	0.9637	0.9630	0.9610	0.9603	0.9603
(%w/v) C.A	0.0144	0.01439	0.01438	0.01437	0.01434	0.01433	0.01433
% content (C.A)	126.3	126.2	126.1	126	125.8	125;7	125.7
Area (Amox)	5.7969	5.5982	5.4325	5.2205	5.0681	4.9223	4.7831
%w/v (Amox)	0.0860	0.0859	0.0858	0.0855	0.0854	0.0852	0.0850
% content (Amox)	107.5	107.4	107.3	106.9	106.7	106.5	106.3

Results for assay of oral suspension C in mineral water (2 - 8ºC)

Chromatographs for brand C oral suspension in voltic (mineral) water.

Figure 40 Day 1 (C in mineral water)

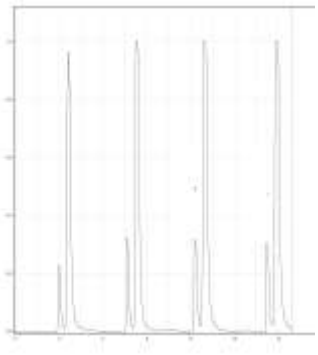


Figure 41 Day 7 (C in mineral water)

Table 32 HPLC results for brand C oral suspension in mineral water (2 -8ºC)

	Day 1	Day2	Day 3	Day 4	Day 5	Day 6	Day 7
Area (C.A)	0.8980	0.8940	0.8905	0.8897	0.8880	0.8440	0.8000
%w/v (C.A)	0.0133	0.0133	0.0133	0.01328	0.01327	0.01325	0.01324
% content (C.A)	116.7	116.7	116.6	116.5	116.4	116.3	116.2
Area (Amox)	5.4259	5.4126	5.3994	5.3794	5.3596	5.3464	5.3265
%w/v of (Amox)	0.0804	0.0803	0.0802	0.0800	0.0799	0.0798	0.0797
% content (Amox)	100.5	100.4	100.3	100.1	99.9	99.7	99.6

Results for assay of oral suspension C in distilled water (2 - 8ºC)

Chromatograph for brand C oral suspension in distilled water

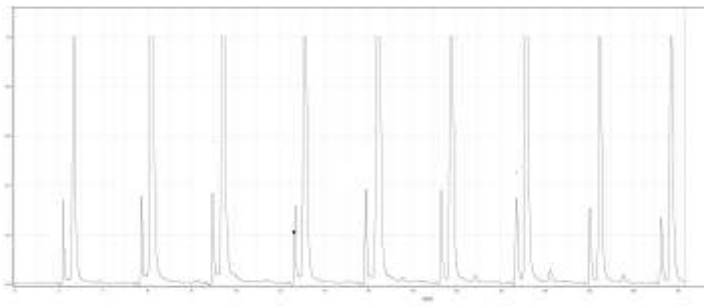


Figure 42 Day 6 (C in distilled water)

Table 32 HPLC results for brand C oral suspension in distilled water (2 -8ºC)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Area (C. A)	0.8600	0.8593	0.8586	0.8580	0.8566	0.8559	0.8553
%w/v (C.A)	0.0127	0.01269	0.01268	0.01267	0.01265	0.01264	0.01263
% content (C.A)	111.4	111.3	111.2	111.1	111	110.9	110.8
Area (Amox)	5.4259	5.3729	5.3198	5.2536	5.1608	5.1078	5.0548
(%w/v) Amox	0.0804	0.0803	0.0802	0.0800	0.0798	0.07976	0.07976
% content (Amox)	100.5	100.4	100.2	100	99.8	99.7	99.7

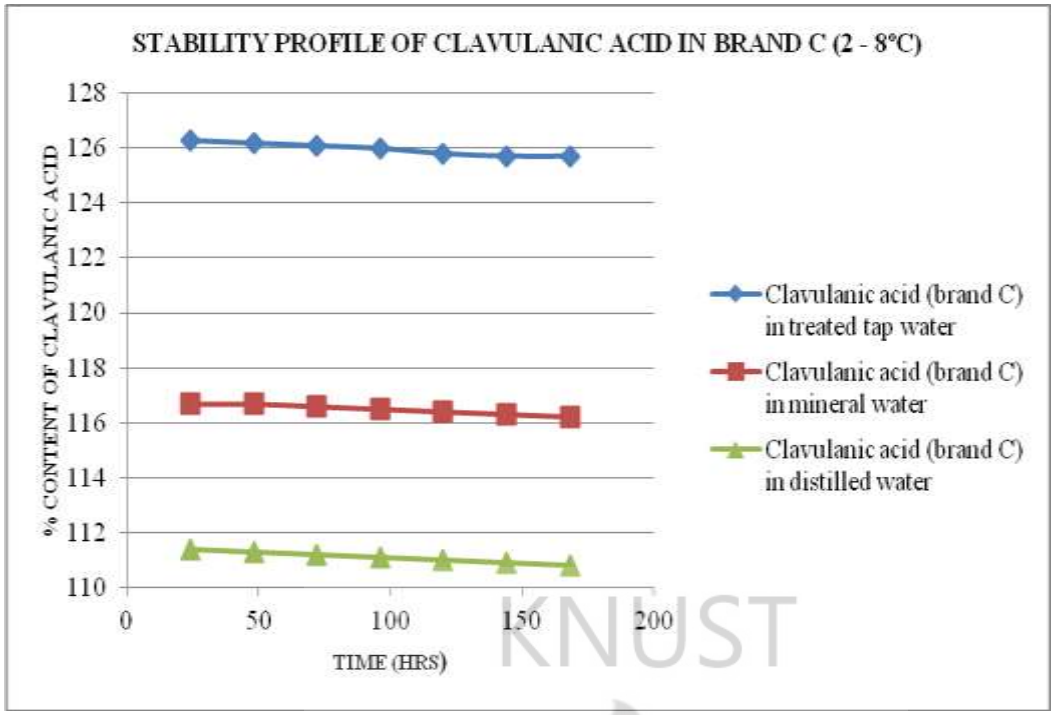


Figure 43; Stability profile of clavulanic acid in brand C (2 - 8ºC)

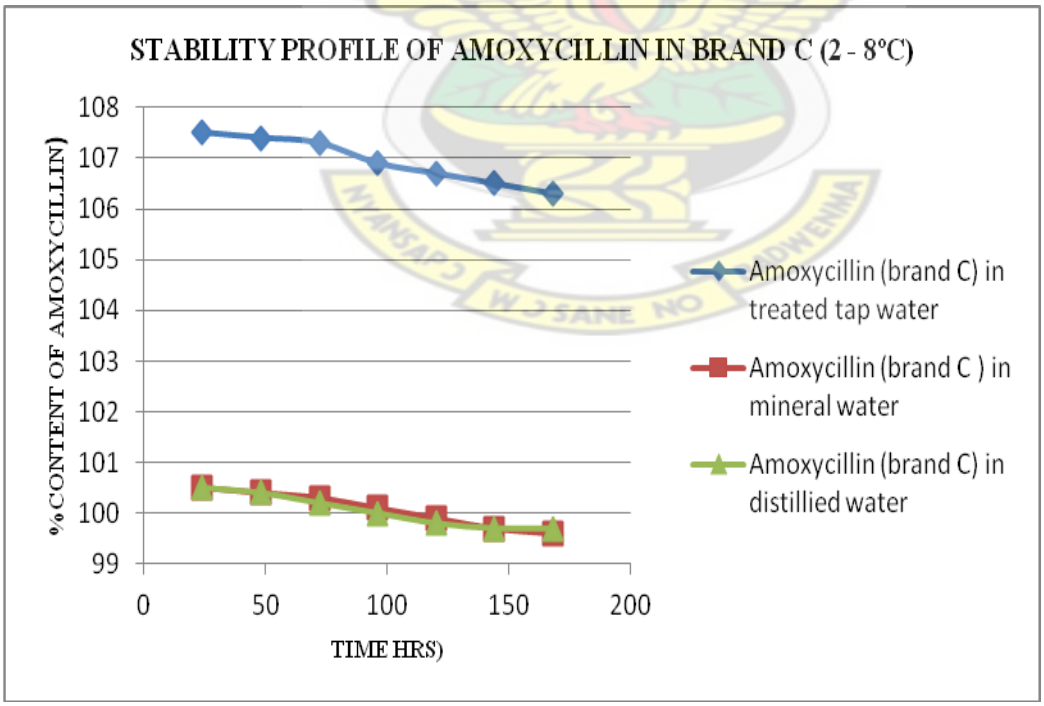


Figure 44; Stability profile of amoxicillin in brand C (2 - 8ºC)

Results for assay of oral suspension B in treated tap water (2 - 8ºC)

Chromatographs for brand B oral suspension in treated tap water

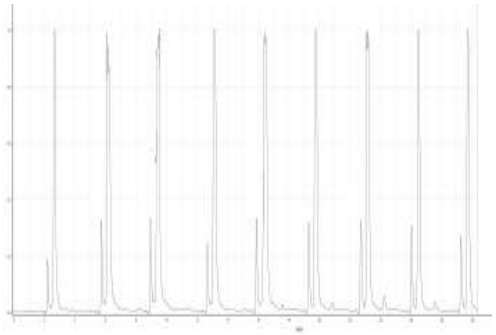


Figure 45; Day4 B(treated tap water)

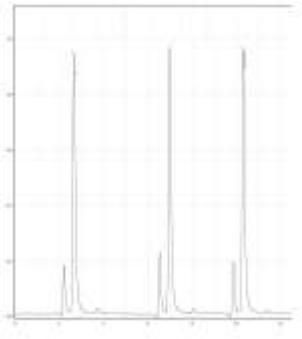


Figure 46; Day 6 B(treated tap water)

Table 33 HPLC results for brand B oral suspension in treated tap water (2 - 8ºC)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Area (C.A)	0.8160	0.8160	0.8160	0.8160	0.8160	0.8092	0.8092
(%w/v) C.A	0.0120	0.0120	0.0120	0.0120	0.0120	0.0119	0.0119
% content (C.A)	105.3	105.2	105.1	104.9	104.7	104.6	104.4
Area (Amox)	5.2320	5.2320	5.2252	5.2185	5.2185	5.2118	5.2118
%w/v (Amoxy)	0.0775	0.0775	0.0774	0.0773	0.07726	0.0772	0.0772
% content (Amox)	96.9	96.9	96.8	96.7	96.6	96.5	96.5

Results for assay of oral suspension B in mineral water (2 - 8ºC)

Chromatographs for brand B oral suspension in (voltic) mineral water

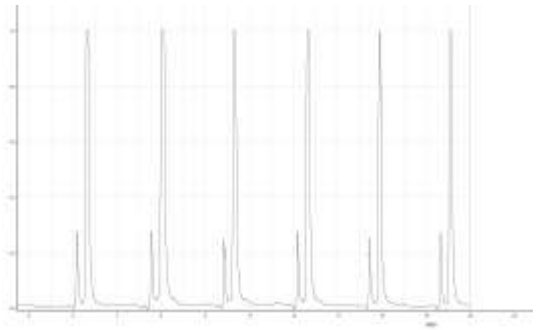


Figure 47 Day 4 B (mineral water)

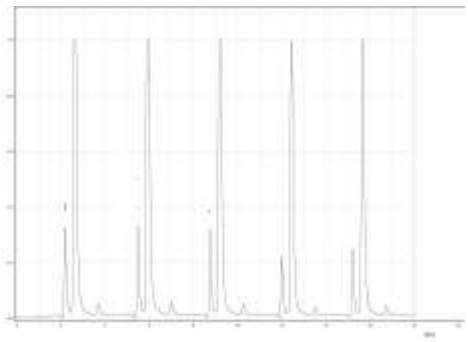


Figure 48 Day 6 B (mineral water)

Table 34 HPLC results for brand B oral suspension in mineral water (2 - 8ºC)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Area (C.A)	0.8160	0.8160	0.8160	0.8092	0.8092	0.8092	0.8092
%w/v (C.A)	0.0120	0.0120	0.0120	0.0119	0.0119	0.0119	0.0119
% content (C.A)	105.3	105.2	105.1	104.8	104.6	104.5	104.5
Area (Amox)	5.6075	5.6074	5.6074	5.6072	5.6068	5.6055	5.6050
%w/v (Amox)	0.08314	0.08314	0.0830	0.0829	0.0828	0.0827	0.0827
% content (Amox)	103.9	103.9	103.7	103.6	103.5	103.4	103.4

Results for assay of oral suspension B in distilled water (2 - 8ºC)

Chromatograph for brand B oral suspension in distilled water

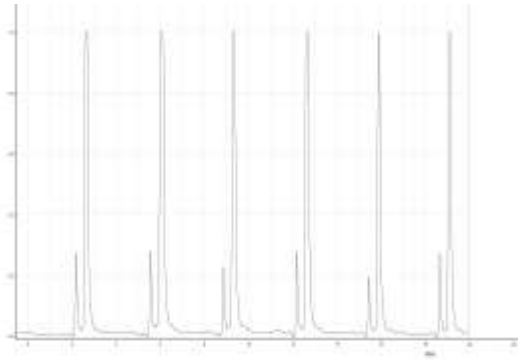


Figure 49 Day 4 B (distilled water)**.**

Table 35 HPLC results for brand B oral suspension in distilled water (2 - 8ºC)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Area (C.A)	0.7560	0.7560	0.7560	0.7560	0.7491	0.7491	0.7491
%w/v (C.A)	0.0110	0.0110	0.0110	0.0110	0.0109	0.0109	0.0109
% content (C.A)	96.5	96.4	96.3	96.1	96	95.8	95.7
Area (Amox)	5.6082	5.6080	5.6080	5.6078	5.6076	5.6074	5.6070
(%w/v) Amox	0.0832	0.0832	0.0831	0.0830	0.0830	0.0830	0.0829
% content (Amoxy)	104	104	103.9	103.8	103.7	103.7	103.6

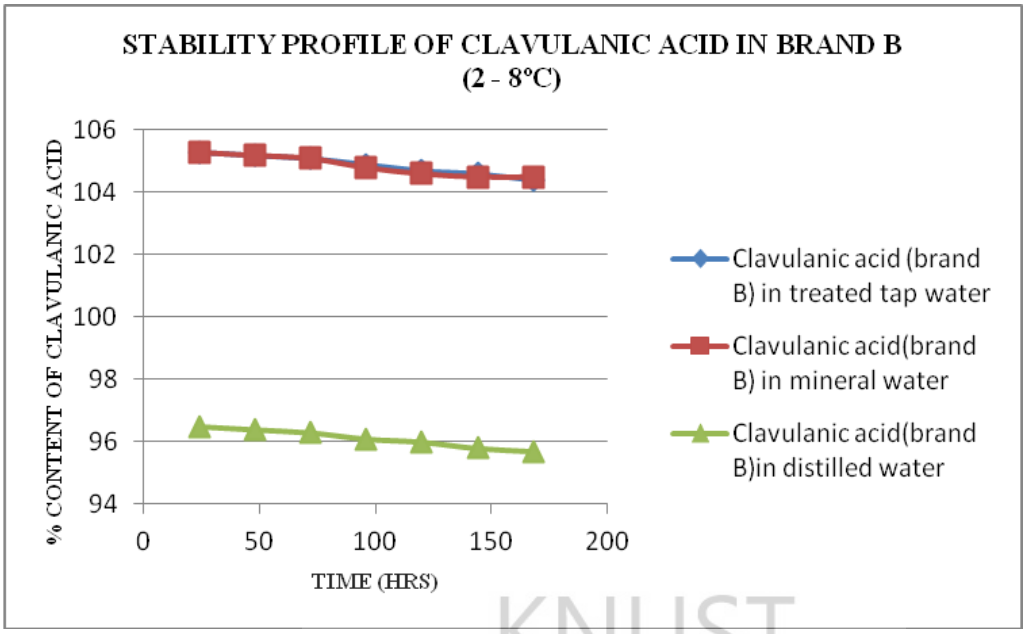


Figure 50; Stability profile of clavulanic acid in brand B (2 - 8ºC)

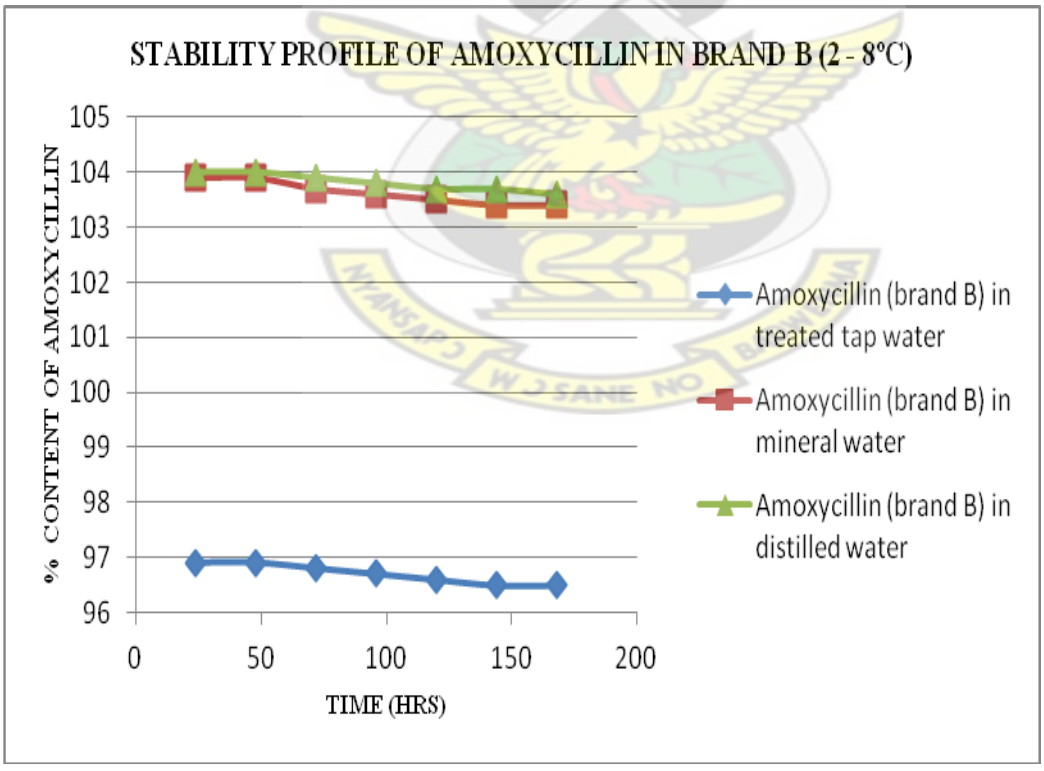


Figure 51; Stability profile of amoxicillin in brand B (2 - 8ºC)

Results for assay of oral suspension A in treated tap water (2 - 8ºC)

Chromatograph for brand A oral suspension in treated tap water

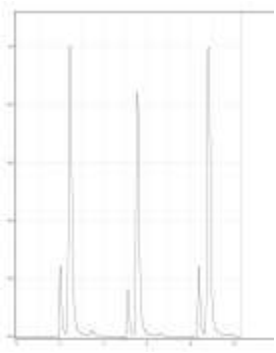


Figure 52; Day 7 A (tap water)

Table 36 HPLC results for brand A oral suspension in treated tap water (2 - 8ºC)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Area (C. A)	0.7780	0.7780	0.7780	0.7760	0.7746	0.7739	0.7739
%w/v (C.A)	0.0114	0.0114	0.0114	0.01137	0.01135	0.01134	0.01134
% content (C.A)	100	100	99.8	99.7	99.6	99.5	99.5
Area (Amoxy)	5.2920	5.2915	5.2913	5.2905	5.2880	5.2872	5.2861
%w/v (Amoxy)	0.0784	0.0784	0.0783	0.0724	0.07816	0.07808	0.078
% content (Amoxy)	98	98	97.9	97.8	97.7	97.6	97.5

Results for assay of oral suspension A in mineral water (2 - 8ºC)

Chromatographs for brand A oral suspension in mineral water

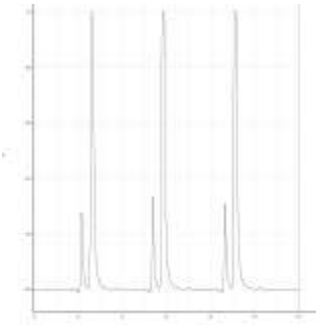


Figure 53; Day 5 A (mineral water)

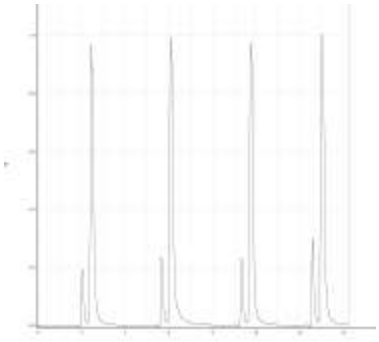


Figure 54 Day 7 A (mineral water)

Table 37 HPLC results for brand A oral suspension in mineral water (2 - 8ºC)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Area (C.A)	0.7890	0.7890	0.7890	0.7869	0.7849	0.7849	0.7849
%w/v (C.A)	0.0116	0.0116	0.0116	0.01157	0.01154	0.01154	0.01154
% content (C.A)	101.8	101.8	101.7	101.5	101.4	101.3	101.3
Area (Amoxy)	5.2385	5.2382	5.2380	5.2375	5.2370	5.2365	5.2360
%w/v (Amoxy)	0.0776	0.0776	0.0775	0.0774	0.07736	0.0772	0.0771
% content (Amoxy)	97	97	96.9	96.8	96.7	96.5	96.4

Results for assay of oral suspension A in distilled water (2 - 8ºC)

Chromatographs for brand A oral suspension in distilled water

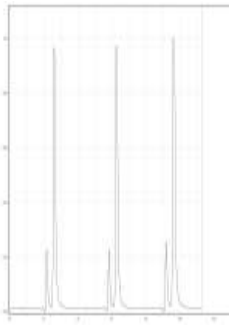


Figure 55; Day 6 A (distilled water)

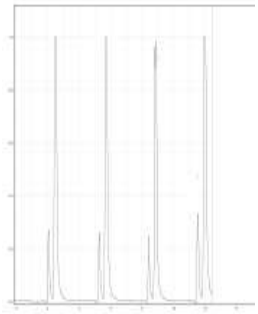


Figure 56; Day 7 A (distilled water)

Table 38 HPLC results for brand A oral suspension in distilled water (2 - 8ºC)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Area (C.A)	0.7883	0.7883	0.7876	0.7863	0.7849	0.7842	0.7842
%w/v (C.A)	0.01154	0.01154	0.01153	0.01151	0.01149	0.01148	0.01148
% content (C.A)	101.2	101.2	101.1	100.9	100.8	100.7	100.7
Area (Amoxy)	5.1120	5.1120	5.1115	5.1105	5.1080	5.0980	5.0520
%w/v (Amoxy)	0.07566	0.07566	0.07558	0.0755	0.0754	0.07534	0.0753
% content (Amoxy)	94.6	94.6	94.5	94.4	94.3	94.2	94.1

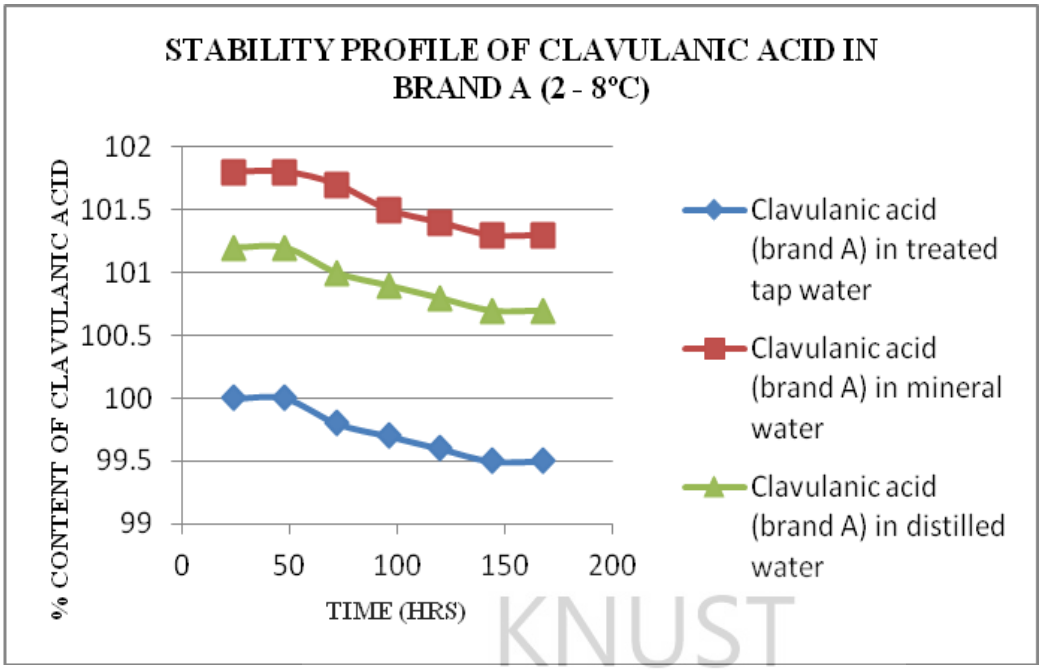


Figure 57; Stability profile of clavulanic acid in brand A (2 - 8ºC)

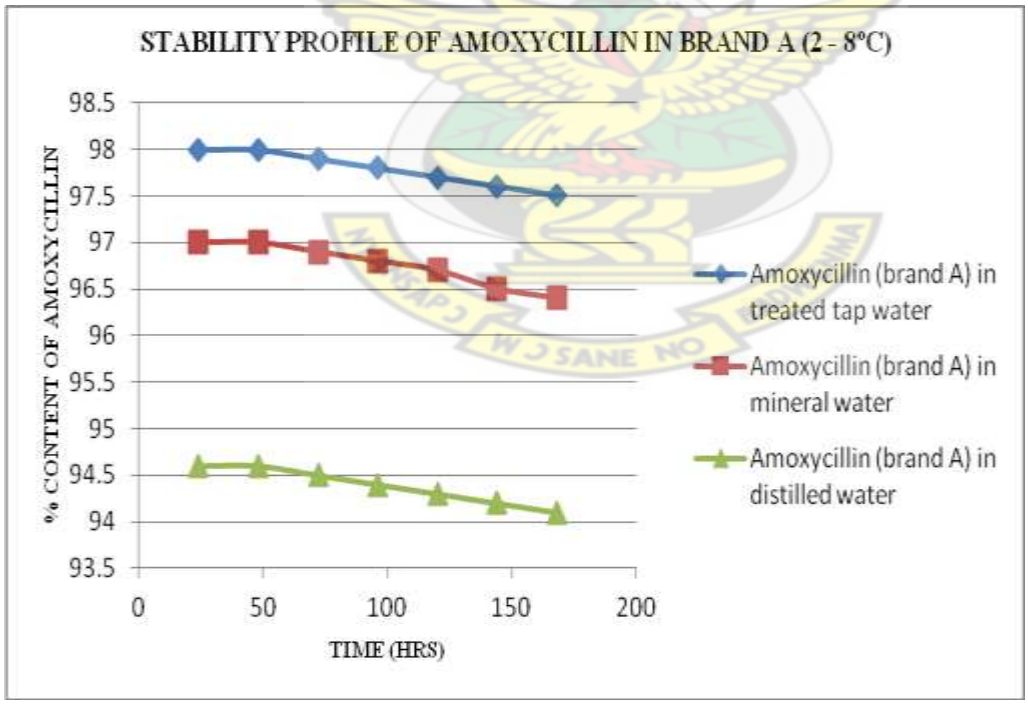


Figure 58; Stability profile of amoxycillin in brand A (2 - 8ºC)

Results for assay of oral suspension B in sachet water (simulation) (2 - 8ºC)

Chromatographs for Brand B suspension in sampled sachet water

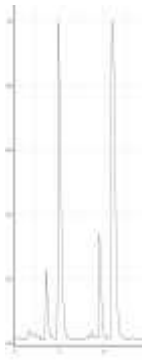


Figure 59; Day1 B(sachet water, fridge)

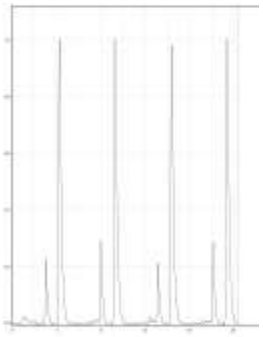


Figure 60; Day 2 B(sachet water, fridge)

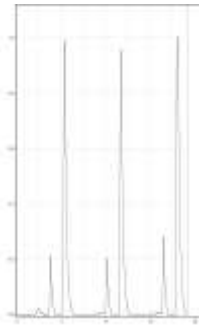


Figure 61;Day 3 B(sachet water, fridge)

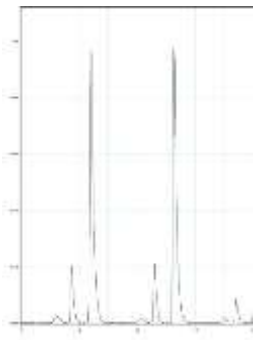


Figure 62; Day 4 B(sachet water, fridge)

Figure 63; Day 5 B(sachet water, fridge)

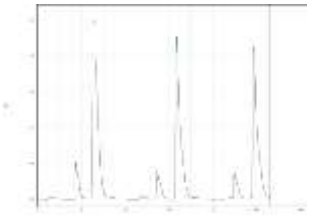


Figure 64; Day 6 B(sachet water, fridge)

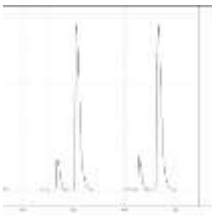


Figure 65; Day 7 B(sachet water, fridge)

Table 39 HPLC results for brand B oral suspension in sachet water (2 - 8ºC)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Area (C.A)	0.7660	0.7620	0.7600	0.7500	0.7400	0.7320	0.7260
%w/v (C.A)	0.0112	0.0111	0.01107	0.0109	0.0108	0.0106	0.0105
Area (Amoxy)	5.4000	5.3960	5.3920	5.3260	5.3200	5.2900	5.2900
%w/v (Amoxy)	0.0800	0.7995	0.07989	0.07890	0.0788	0.07835	0.07834

Table. 40 pH's of Brand B reconstituted in sachet water and stored in a fridge and bowl of water.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Brand B, stored in Fridge	4.60	4.70	5.50	5.50	5.50	5.50	5.50
Brand B, kept in bowl of water	4.60	5.50	6.00	6.30	6.40	6.60	6.60

Results for assay of oral suspension B in sachet water (simulation in bowl of water) (25ºC)

Chromatographs for Brand B suspension in sampled sachet water

Figure 66; Day 1 B(sachet water, bowl)

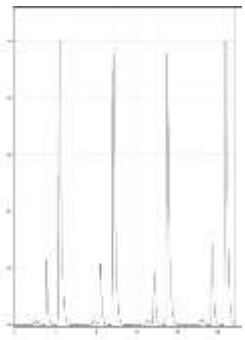


Figure 67; Day 2 B(sachet water, bowl)

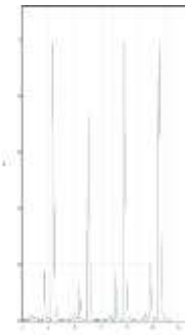


Figure 68; Day 4 B(sachet water, bowl)

Figure 69; Day 5 B(sachet water, bowl)

Figure 70; Day 6 B(sachet water, bowl)

Figure 71; Day 7 B(sachet water, bowl)

Table 41 HPLC simulation results of brand B (sachet water, in bowl of water)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Area (C.A)	0.7660	0.7500	0.7260	0.6520	0.5060	0.4480	0.3300
%w/v (C.A)	0.0112	0.0109	0.0105	0.0093	0.0069	0.0060	0.0041
% content (C.A)	98.3	95.6	92.1	81.6	60.5	42.6	36
Area (Amoxy)	5.6100	5.6000	5.5900	5.5400	5.5320	5.5280	5.2780
%w/v (Amoxy)	0.0832	0.0830	0.0829	0.0821	0.0820	0.0819	0.0782
% content (Amoxy)	104	103.7	102.6	101.6	100.3	99	97.8

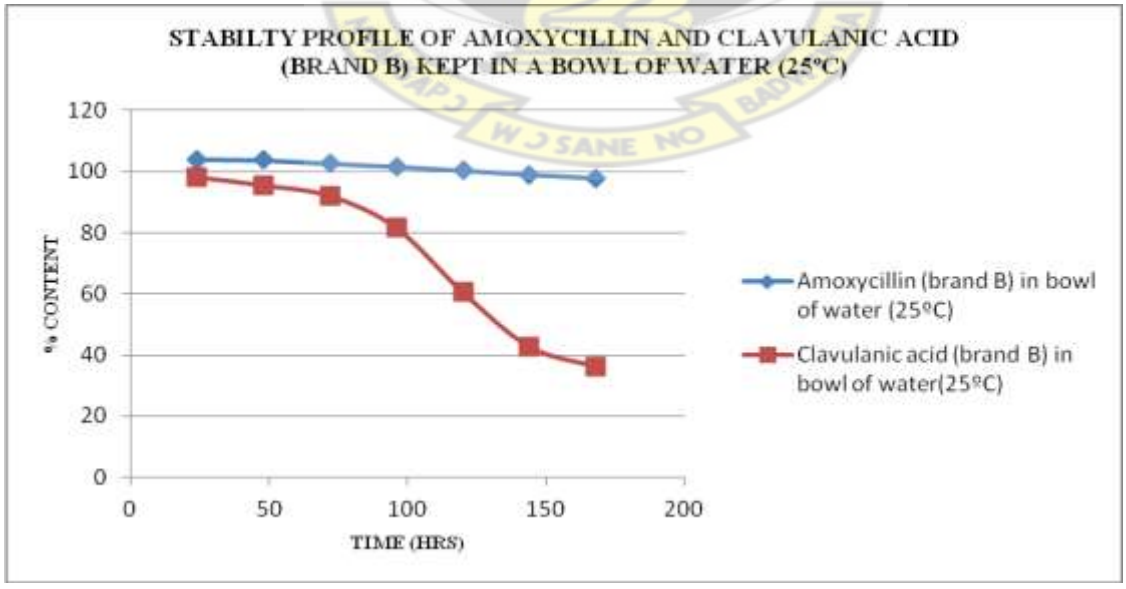


Figure 72; Stability profile of amoxicillin and clavulanic acid (brand B) kept at 25ºC

pH of sampled sachet water = 5.50 – 5.70

Table. 42 pH's of Brands A, B and C oral suspensions kept in and out of fridge (2-8ºC, 25ºC)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Brand C	4.50	4.90	5.50	6.00	6.30	6.50	6.30
Brand A	4.40	4.90	5.20	5.70	6.30	6.50	6.30
Brand B	4.90	4.90	5.20	5.50	6.30	6.50	6.60

Results for brand C oral suspension kept in and out of fridge during 7 day period.

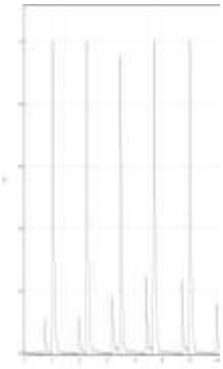


Figure 73; Day1 (2-8ºC,25ºC)

Figure 74; Day 2 (2-8ºC,25ºC)

Figure 75; Day 4(C) (2-8ºC,25ºC)

Figure 76; Day 6 (C) (2-8ºC,25ºC)

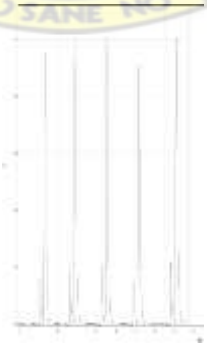


Figure 77; Day 7 (C) (2-8ºC,25ºC)

Table 43 HPLC results for brand C oral suspension (2 - 8ºC, 25ºC)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Area (C.A)	0.7720	0.7600	0.7560	0.7200	0.6100	0.4940	0.4040
%w/v) (C.A)	0.0113	0.0109	0.0108	0.0105	0.0105	0.0101	0.0101
% content (C.A)	99.1	95.2	95	92.4	92.1	88.5	88.2
Area (Amoxy)	5.3800	5.3400	5.3392	4.7420	4.4280	4.1720	3.1620
%w/v (Amoxy)	0.0797	0.0788	0.0787	0.0775	0.0772	0.0745	0.0742
% content (Amoxy)	99.6	98.5	98.3	96.8	96.5	93.1	92.7

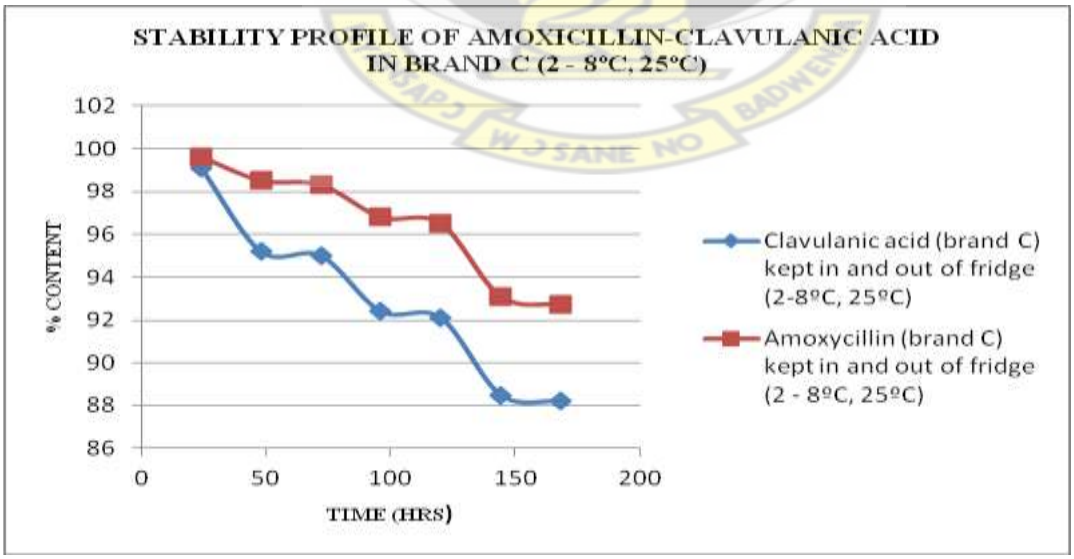


Figure 78; Stability profile of amoxicillin and clavulanic acid in brand C (2-8ºC, 25ºC)

Results for brand B oral suspension kept in and out of fridge during 7 day period

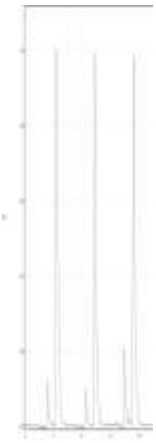


Figure 79; Day1(B) (2-8ºC, 25ºC)

Figure 80; Day 2 (2-8ºC, 25ºC)

Figure 81;Day 3 (2-8ºC, 25ºC)

Figure 82: Day 4(B) (2-8ºC, 25ºC)

Figure 83; Day 5(B) (2-8ºC, 25ºC)

Figure 84; Day 6(B) (2-8ºC, 25ºC)

Table 44 HPLC results for brand B oral suspension (2 - 8ºC, 25ºC)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Area (C.A)	0.7720	0.7515	0.7447	0.7241	0.7241	0.7036	0.6969
%w/v (C.A)	0.0113	0.0110	0.0109	0.0106	0.0106	0.0103	0.0102
% content (C.A)	99.1	96.1	95.8	93.2	92.8	90.3	89.5
Area (Amoxy)	5.4020	5.3970	5.3960	5.2062	5.1927	5.0576	5.0374
%w/v (Amoxy)	0.0800	0.0788	0.0786	0.0771	0.0769	0.0749	0.0746
% content (Amoxy)	100	98.5	98.3	96.4	96.1	93.6	93.2

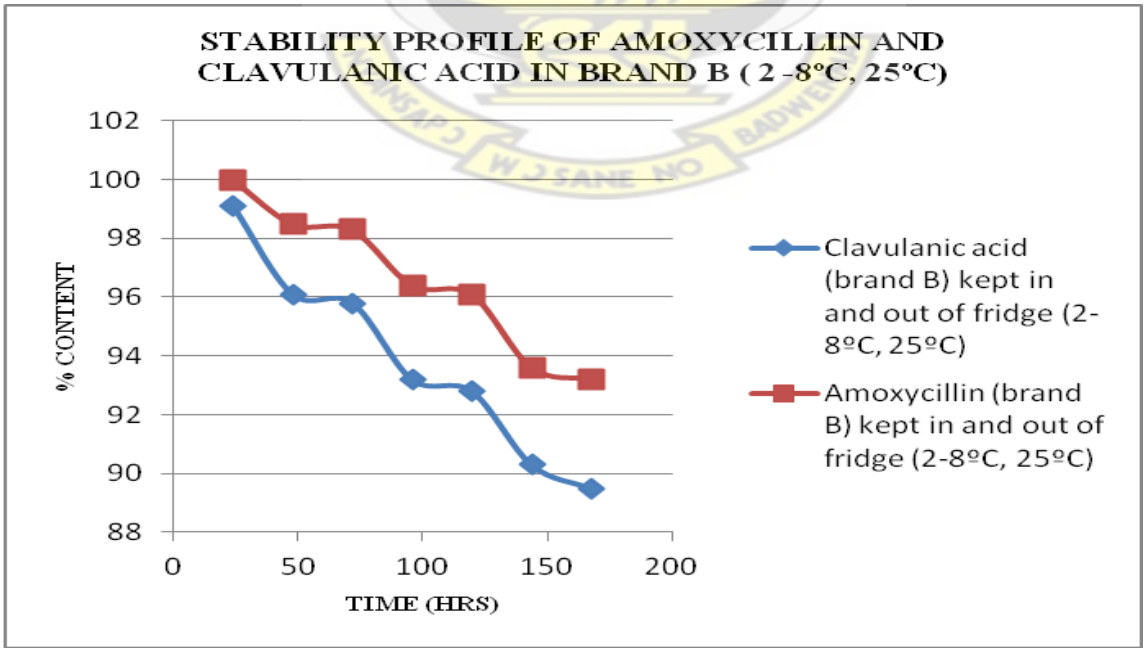


Figure 85; Stability profile of amoxicillin and clavulanic acid in brand B (2 -8ºC, 25ºC)

Results for brand A oral suspension kept in and out of fridge during 7 day period

Figure 86; Day 1(A)

(2 -8ºC, 25ºC)

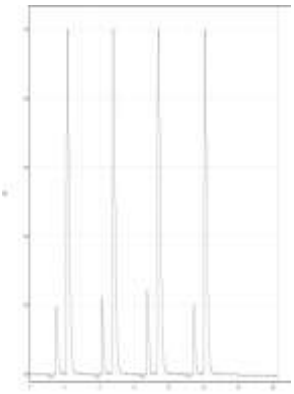


Figure 87; Day 2(A)

(2 -8ºC, 25ºC)

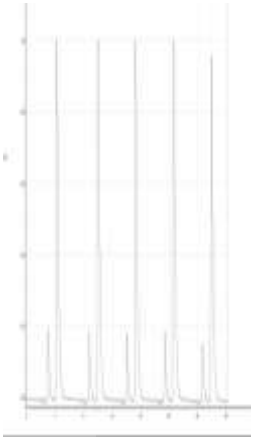


Figure 88; Day 3(A)

(2 -8ºC, 25ºC)

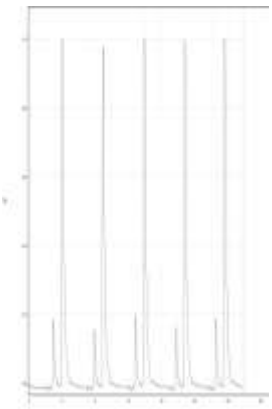


Figure 89; Day 4 (A)

(2 -8ºC, 25ºC)

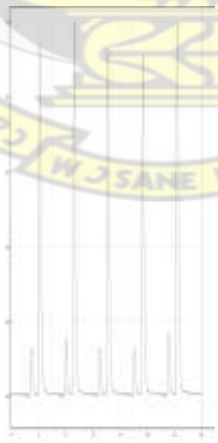


Figure 90; Day 6 (A)

(2 -8ºC, 25ºC)

Table 45 HPLC results for brand A oral suspension (2 - 8ºC, 25ºC)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Area (C. A)	0.7740	0.7535	0.7466	0.7261	0.7261	0.7055	0.7055
%w/v (C.A)	0.0113	0.0110	0.0109	0.0106	0.0106	0.0103	0.0103
% content (C.A)	99.2	96.2	96	93	92.8	90.8	90.6
Area (Amoxy)	5.4200	5.4000	5.3960	5.3800	5.1000	4.7000	4.3000
%w/v (Amoxy)	0.0803	0.0790	0.0799	0.0789	0.0772	0.0763	0.0761
% content (Amoxy)	100.3	98.7	98.6	96.5	96.4	95.3	95.1

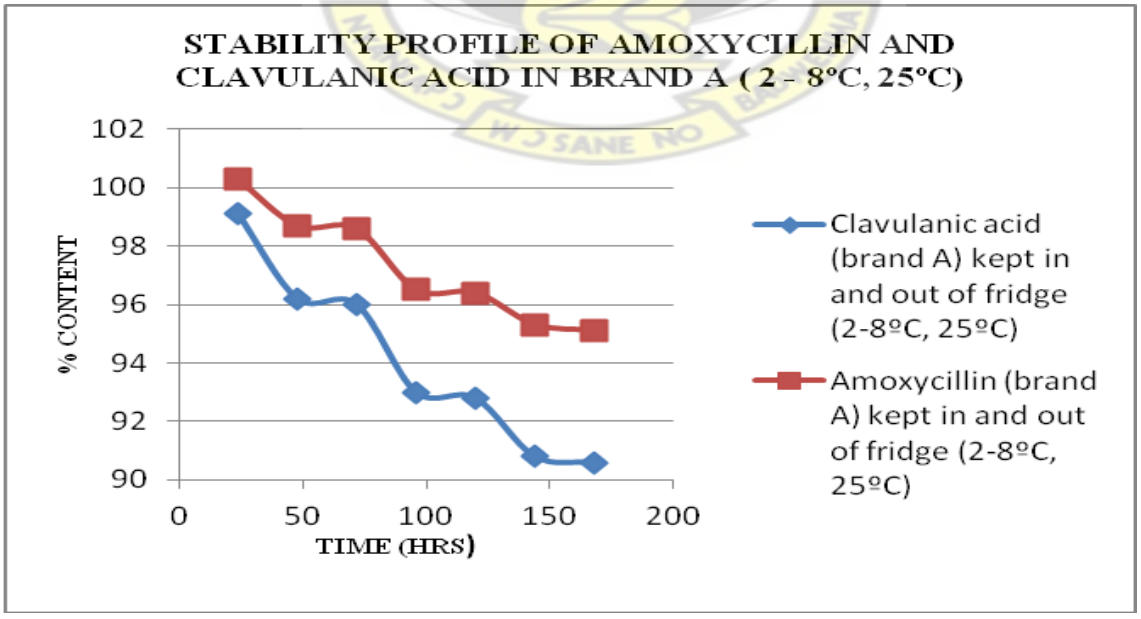


Figure 91; Stability profile of amoxicillin and clavulanic acid in brand A (2 - 8ºC, 25ºC)

Table 45 HPLC results for brand A oral suspension (2 - 8ºC, 25ºC)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Area (C. A)	0.7740	0.7535	0.7466	0.7261	0.7261	0.7055	0.7055
%w/v (C.A)	0.0113	0.0110	0.0109	0.0106	0.0106	0.0103	0.0103
% content (C.A)	99.2	96.2	96	93	92.8	90.8	90.6
Area (Amoxy)	5.4200	5.4000	5.3960	5.3800	5.1000	4.7000	4.3000
%w/v (Amoxy)	0.0803	0.0790	0.0799	0.0789	0.0772	0.0763	0.0761
% content (Amoxy)	100.3	98.7	98.6	96.5	96.4	95.3	95.1

PHYSICAL OBSERVATION OF SAMPLES OF BRANDS A, B AND C AFTER BEING KEPT IN AND OUT OF FRIDGE (2 - 8ºC, 25ºC)

Figure 92; Creamy white colour (A and B) Day 5

Figure 93; Creamy white colour (A and B) Day 6

Figure 94; Creamy white colour (A and B) Day 7

Figure 95; Brownish Layer (C) Day 4

Figure 96; Brownish layer (C) Day 5

Figure 97; Brownish layer (C) Day 6

Figure 98; Brownish) layer (Day 7)

CHAPTER 5 – DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 DISCUSSION

5.1.1 Identification and assay of reference standard

Contamination of pharmaceutical products or the occurrence of mix-ups can result in very dire consequences such as serious health hazards or chemical accidents in the laboratory. In view of this, it is mandatory to carry out identification tests on samples before their use in laboratory experiments. Reference standards as well, will have to be analysed both qualitatively and quantitatively to validate their use as comparative standards for pharmaceutical analysis. The use of identification processes such as thin layer chromatography brings to light the occurrence of degradation and consequently the degradation products of samples. The identification processes involved could include simple physical observation of sample with regards to its nature, simple colour reaction tests, determination of refractive indices for liquids, melting point determination for solid samples, optical rotation determination through to spectral analysis of samples. In view of this, reference standards were identified and assayed accordingly.

AMOXYCILLIN

Identification of the Amoxicillin reference standard was carried out by means of colour reaction tests and melting point determination. From the results, Amoxicillin solution showed a dark yellow colouration with sulphuric-acid formaldehyde. The addition of two drops of a neutral solution of Ferric Chloride also resulted in a blue colouration [Table 4]. The test results conforms with the specification of BP with regards to the identification test for amoxycillin. The blue coloration is as a result of a reaction between the phenolic –OH of amoxicillin and ferric chloride. The identity of Amoxicillin was further confirmed with the melting point determination which is also an index of purity of the compound. The melting

point range of 193-195˚C obtained for amoxicillin falls within the literature range of 192-196˚C.

CLAVULANIC ACID

Identification of the clavulanic acid reference standard was ascertained by also colour reaction tests and melting point determination. The colour reaction test results indicated that a solution of clavulanic acid gave a white crystalline precipitate upon the addition and heating with a solution of sodium carbonate and sodium sulphide [Table 6]. The melting point of clavulanic acid was found to be of 162 - 163˚C which complies to the > 160ºC literature value [Table 7]. Thus, the identity of clavulanic acid was confirmed.

THIN LAYER CHROMATOGRAPHY

The purity of the Amoxicillin and Clavulanic acid reference standards were further investigated by running a thin layer chromatography. From the results obtained, there were only single spots on plates, thus no impurities or breakdown products for both amoxicillin and clavulanic acid (Fig.21 and Fig.22).

5.1.2 Assay of amoxicillin and clavulanic acid reference standard

Amoxicillin reference standard sample was assayed by means of Iodimetric titration where a standardised solution of iodine was made to indirectly react with amoxicillin through penicilloic acid derivatives. From the results of the assay obtained the average percentage purity of the amoxicillin reference standard was found to be 99.42%. This value falls well within the range of acceptable BP specification of 95 – 102% **(BP 2007)**. Clavulanic acid was also assayed by an indirect titration to determine its percentage purity. The titration was an acid - base reaction between clavulanic acid and sodium hydroxide which had been previously standardised with sulphamic acid. The average percentage purity from the assay was found to be 98.20%, which falls within the acceptable range of the BP specification of

96.5 – 102% for clavulanic acid reference standard. Both the amoxicillin and clavulanic acid reference standard passed the assay and thus eligible for the study.

5.1.3 Survey

The survey was conducted to find out the practical conditions prevailing with regards to the reconstitution and storage of amoxicillin - clavulanic acid oral powder for suspension. One hundred (100) questionnaires were given out to specific health personnel in the health facilities, out of which 82 respondents were recorded. From the total respondents, there were 49 pharmacists, 22 dispensing technicians and 11 medical counter assistants. The prevailing storage conditions on ground for amoxicillin – clavulanic acid oral powder for suspension were ambient room temperature, 24 hour air conditioning system and less than (<) 24 hour air conditioning system. There were 35 respondents who stored the drug in normal room temperature, 17 who stored drug under 24 hour air conditioning surrounding and 27 who partially stored the drug under air conditioning. Out of the air condition users, 24 respondents set their air conditioners between 16 - 18ºC, 18 between 18 - 25ºC and 3 respondents did not specify the temperature range. 52 of the respondents consistently reconstitute the drug personally for patients, 28 of the respondents sometimes reconstitute drug for patients but not on a consistent basis. 2 respondents however do not reconstitute drug for patients. Types of water often used for the reconstitution of amoxicillin – clavulanic acid oral powder include mineral water and treated tap water. Distilled water was not readily available in majority of the health facilities. 2 of the respondents however did not indicate accordingly. 59 respondents often used. 59 respondents often used mineral water for reconstitution and 21 respondents used treated tap water for reconstitution. 10 of the respondents however indicated that they could vary the type of water, thus use any available type of water for the

reconstitution. 56 of the respondents however did not vary the type of water used for reconstitution, whereas 13 respondents indicated that they sometimes did.

UV SPECTRA FOR AMOXYCILLIN AND CLAVULANIC ACID

A uv analysis was conducted on Amoxycillin with water (100%) as the solvent. This resulted in a well defined spectrum which indicated a maximum absorbance of 0.813 at a wavelength of 228nm (Fig 29). The high maximum absorbance of amoxicillin could be due its conjugate double bond system, coupled with its high electron density as well as the presence of an auxochrome on its structure. Another uv analysis was carried out on clavulanic acid with water as the solvent, but this did not depict a well defined spectrum (Fig 30). However, when the same uv analysis was carried out on clavulanic acid again, but this time with a mixture of water and methanol (90:10) as solvent, a much better and well defined spectrum was observed with a maximum absorbance of 0.520 occurring at a wavelength of 230nm. However, for a concurrent uv analysis of amoxicillin and clavulanic acid, a wavelength of 220nm was arrived at as the optimum wavelength of detection.

CONDUCTIVITY

The conductimeter was first calibrated by measuring the conductance of standard solutions of KCl to ascertain its efficiency. From the results obtained, mineral water had a conductivity of 1.50 µs/cm, distilled water 2.80 µs/cm and that of treated tap water being 495 µs/cm. Thus, treated tap water had the highest amount of ions relative to that of commercial mineral water and distilled water. The pH's of the three types of water was measured. Treated tap water and distilled water both had a pH of 6.00 whilst that of commercial mineral water was 6.30, slightly higher. The similarity in the pH's of the type of water implies that the amount of ions in water does not necessarily cause a change in pH but rather the type of ions, e.g. H^+ ions and OH^- ions. It can also be deduced that all three types of water were slightly acidic and not neutral.

5.1.4 Hplc method development

A reversed – phase high-performance liquid chromatography (RP-HPLC) was employed in the analysis of amoxicillin and clavulanic acid. The analysis was carried out by means of a Phenomenex, Kromasil 5 (C_8), 250 X 4.60mm 5 micron column with size 305334. The mobile phase consisted of water, methanol and Na acetate Buffer (pH 4.4) in the ratio (65:15:20).The injection volume was 100 µl and the flow rate was set at 1.00 ml/minute. The concentrations of the active substances were detected by a UV-spectrophotometer at a wavelength of 220 nm. Chemically, amoxicillin and clavulanic acid are both carboxylic acids. Amoxycillin experiences the zwitterion effect at pH's less than 9 hence it's solubility is greatly reduced. Since amoxicillin and clavulanic acid are both β-lactams, they are susceptible to acid- and base-catalyzed hydrolysis of the four-membered β-lactam ring. It has been observed that amoxicillin tends to be more stable at lower pH conditions because at these pHs the amine group is protonated, thereby utilizing the free electron pair of the nitrogen. When the amine is deprotonated at higher pH (pH > 8), a pair of free electrons is available for nucleophilic attack of the cyclic amide (β-lactam. The simultaneous analysis of amoxycillin and clavulanic acids by the United States Pharmacopoiea (USP) 27 and other Hplc methods in literature often employ the use of phosphate buffers in their mobile phase system. The use of inorganic buffers such as one of phosphate, though having the general drawback of all buffers, which is the deposition of salt crystals on column when not painstakingly washed, also has the added disadvantage of not being easily washed away and thus long and repeated washing of the column is required **(Barbara et (al, 1982)** . Since in our part of the world (developing countries), one does not have the luxury of extra brand new columns, extreme care of the few available ones is critical. In the quest for developing a suitable method for the analysis of the two drugs, the use of an organic buffer was settled upon. Sodium acetate was specifically chosen since it was readily available and easily went

into solution. Methanol and water were a part of the mobile phase system due to their availability and cost effectiveness. The ratio of components of the mobile phase system was 65:15:20 for water, methanol and Na acetate Buffer (pH4.4) respectively. The retention times for clavulanic acid and amoxycillin were 2.96 and 4.09 minutes respectively with standard deviations of ±0.01 and ±0.02 respectively. This indicates that clavulanic acid is eluted earlier than amoxicillin. The extent to which a drug is ionised in a solution is highly dependent on the pH. Complete ionisation of a compound renders it polar and thus will enable it have much affinity for a polar mobile phase but less affinity for a stationary phase and thus, elute faster. As such, clavulanic acid which is more polar is readily soluble in water as compared to amoxicillin. Thus since the mobile phase has a greater proportion being that of water, the elution of the more water soluble compound is probable. Furthermore, both compounds have ionisable groups in their structures. Amoxicillin has three ionisable groups of –OH, -COOH, and $–NH_2$ which have pKa values of 7.4, 2.4 and 9.6 respectively**(Martin A.N, 1969)**. Most (Yuri and Rosario, 2007compounds get ionised at a pH within 1-2 units of their pKa values from literature **(Yuri and Rosario, 2007)**. Amoxicillin is completely ionised if at least two of its ionisable groups are ionised. However, when found in a mobile phase with pH 4.38 - 4.41 the –COOH becomes ionised and resulting in partial ionisation of drug hence decreasing the affinity for the mobile phase. Clavulanic acid however, just has one ionisable group with a pKa of 2.7 is completely ionised at a mobile phase pH of 4.38 - 4.41 and thus has a greater affinity for the mobile phase than amoxicillin, as such is eluted faster.

5.1.5 Hplc method validation

Amoxicillin

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

was validated as per the ICH guidelines on analytical method validation. The method demonstrated linearity over a concentration range of 0.0100 - 0.1000%w/v of amoxicillin. From the calibration curve the R^2 value gotten was found to be 0.9979. This shows linearity between the concentrations of amoxicillin and the mean peak areas over the range specified. From the results, the intra-day precision gave an RSD of 0.76% whilst that of inter-day precision was 1.03%.Thus both the intra-day and inter-day precision RSD fell below the maximum RSD limit of 2% as per the ICH standard for analytical method validation. This indicates that the method was precise. A different column from a different manufacturer was used as well as a change in the flow rate to indicate robustness of the method. The method can be said to be specific since excipients present in the formulation did not interfere with the analysis of amoxicillin and clavulanic acid when injected. From the results and calculations the Limit of Detection (LOD) and the Limit of Quantification (LOQ) of the method for amoxicillin were found to be 0.00614%w/v and 0.0186%w/v respectively.

Clavulanic Acid

Linearity was established for concentrations of clavulanic acid over a range of 0.01425%w/v to 0.00015%w/v and their corresponding mean peak areas. The R^2 value over this range of concentration from the calibration curve of clavulanic acid was found to be 0.9969 which is not far from 1.0 which is the ideal. The intra-day precision of the method for clavulanic acid gave RSD values of 0.2%. This RSD value was well below the maximum limit of 2% and thus the method has good precision. The flow rate of the mobile phase was changed and a column from a different manufacturer was also used to indicate the robustness of the method. The method proved to be specific since the excipients in the formulation did not interfere with the analysis of amoxicillin and clavulanic acid after injection. From the results and calculations the Limit of Detection (LOD) and the Limit of Quantification (LOQ) of the method for clavulanic were found to be 0.00126%w/v and 0.003818%w/v respectively.

5.1.6 Assay of brands A, B and C of amoxycillin – clavulanic oral suspension.

The USP-30 states that amoxicillin and potassium clavulanate oral suspension should contain the equivalent of not less than 90.0 percent and not more than 120.0 percent of the labelled amount of amoxicillin and the equivalent of not less than 90.0 percent and not more than 125.0 percent of the labelled amount of clavulanic acid. The results of the HPLC analysis of all samples of the three brands of amoxicillin- clavulanic acid oral powder for suspension were as follows. For amoxicillin, **Brand A** had 98%, 97%, 94.58% and 100.38%, (**Brand B**) 96.88%, 103.93%, 104%, 100%, 104% and 100% and (**Brand C**) 107.5%, 100.5%, 100.5% and 99.63%. For clavulanic acid, **Brand A** had 100%, 101.75%, 101.23% and 99.12%, (**Brand B**) 105.26%, 105.26%, 96.50%, 98.25%, 98.25% and 99.12% and (**Brand C**) 126.3%, 116.7%, 111.4% and 99.12%. Thus, all samples of the brands had the percentage content of amoxicillin well within the standard range. All but one sample of brand C had their clavulanic acid content fell within the standard range of 90 – 125%. The one that fell outside the range was slightly higher (126.30%) than the maximum limit of 125%. Thus, all samples had the required therapeutic amount of amoxicillin and clavulanic acid.

5.1.7 Stability studies

5.1.7.1 Different types of water used in reconstitution of brands A, B and C (2 - 8ºC)

For the stability studies of amoxicillin – clavulanic acid oral suspension, three brands were selected. These brands included the innovator brand (A) and two others (B and C) which were often used in two major hospitals in the Kumasi metropolis. The brands were each reconstituted with distilled water, mineral water and treated tap water. The pH's of the drugs were monitored on the first day of reconstitution through to the 7th day of storage between 2 - 8º C. All three brands had their initial pH's around 5.00. Thus, there were just some slight variations with respect to individual samples whose pH's were slightly above 5. By the 7th day, all samples had slight increases in their initial pH values. Thus, none of the samples had

a 1 unit change in pH value. This implies that the buffers present (as a result of their capacities) were able to prevent drastic changes in pH. The storage temperature also helped to slow down if not halt any form of chemical degradation which could result in changes in pH. Both compounds, that is amoxicillin and clavulanic acid were considered to be stable if they retained 90% or more of their baseline (initial) drug concentration**(Mehta A.C *et al.*, 1994)**. By the 7th day, **Brand C** reconstituted with treated tap water, mineral water and distilled water had its clavulanic acid content (relative to baseline concentration) being 99.5%, 99.6% and 99.5% respectively, whilst that of its amoxycillin content was 98.8%, 99.13% and 99.2%. By the 7th day **Brand B** reconstituted with treated tap water, mineral water and distilled water had its clavulanic acid content (relative to baseline concentration) being 90.3%, 99.2% and 99.1% respectively, whilst that of its amoxycillin content was 93.3%, 99.6% and 99.5%. By the 7th day **Brand A,** reconstituted with treated tap water, mineral water and distilled water had its clavulanic acid content (relative to baseline concentration) being 96.49%, 95.69% and 98.70% respectively, whilst that of its amoxycillin content was 99.87%, 99.87% and 98.81%. The result indicates that all the samples were stable and still had their therapeutic dose. This could be attributed to the integrity of the excipients employed in some samples of brand C. The pH's of the three kinds of water employed for reconstitution were 6 for distilled water and treated tap water whilst that of mineral water was 6.3. Amoxycillin and clavulanic acid are both sensitive to moisture with regards to hydrolysis. As such the addition of water to such compounds to form a suspension should be accompanied with the needed temperature control. Thus, from the above results, the storage temperature of 2 - 8ºC was effective in slowing down if not halting any form of degradative process. Thus, there no intense breakdown of active drug throughout the duration of therapy. The differences in the mineral composition of the three kinds of water did not have any degradable effect on the active drug, especially under the proposed standard storage condition of 2 - 8ºC. The buffers most likely

to be present in all three brands can be said to be effective in maintaining the rate of hydrolysis to a minimal.

5.1.7.2 Simulation of brand B

Two samples of Brand B were used for simulation, where a randomly selected commercial water (sachet) with a pH of range of 5.5 – 5.7 was used to reconstitute the drugs. One of the reconstituted drugs was kept in a refrigerator (2 - 8 ºC) whilst the other was kept in a bowl of water and in a cupboard for the 7 day period. For the sample stored in the fridge throughout the 7 day period, its clavulanic acid content was 93.75% of the initial concentration by the 7th day. Its amoxicillin content was by the 7th day was also 97.93% of its initial concentration. However, for the sample of brand B that was kept in the bowl of water under normal room temperature, it had its clavulanic acid content reducing drastically to 36.61% of its initial concentration. Its amoxicillin content was however stable since it had 93.99% of its initial concentration. From the results it can be deduced that amoxicillin is more stable as compared to clavulanic acid, under the same storage conditions. It can also be observed that the rate of hydrolysis is greatly increased at room temperature (25ºC) as compared to 2 - 8ºC storage condition. The pH (5.6) of the sachet water was quite low, hence acidic. This could be attributed to the infiltration of atmospheric carbon dioxide into the water during its production. The two samples had an initial pH of 4.6 after being freshly reconstituted with the sachet water. This was a much lower pH for the 1st day as compared to samples that had been reconstituted with distilled water, treated tap water and mineral water which was around 5.The sample of brand B which was kept in the refrigerator still had its pH rise gradually to 4.7 on the second day and drastically to a pH of 5.5 on the third day at which point the pH remained constant through to the 7th day. The sample of brand B which was kept in the bowl of water had a sharp change of pH from 4.7 to 5.5 by the 2nd day. The pH rose quite sharply again to a pH of 6.00 on the 3rd day and much gradually to a pH of 6.6 on the 6th and7th day.

From the above results it can be deduced that temperature has an effect on the change of pH. Amoxycillin was more stable at the pH of 5.5 than at 6.6 and clavulanic acid was highly stable at the pH of 5.5 than at 6.6. Note however should be taken that the effectives of the drug is dependent on the synergistic action of the two. Thus, both should have an appreciable amount or ideally the required percentage contents in order to provide the needed therapeutic effect.

5.1.7.3 Brands A, B and C oral suspensions kept in and out of fridge.

From the results obtained for the analysis of brands A, B and C which were all reconstituted with distilled water but kept in the fridge (standard storage condition of (2 – 8ºC) on alternate days. The rate or breakdown of both clavulanic acid and amoxicillin were increased when the suspensions were left under room temperature as compared to when the suspensions were kept in the fridge. The storage of the suspensions in the fridge on the alternate days was however able to completely slow down breakdown processes that had been initiated by the surrounding room temperature, as such by the end of the duration of therapy, the percentage contents of amoxicillin and clavulanic acid in relation to their baseline concentrations for brands A, B were all greater than 90% but that of brand C had its clavulanic content (89.38%) slightly below the recommended 90%. The pH's of all three brands (A, B and C) were 5.2, 5.2 and 5.5 respectively. By the 6th day however, the pH's had risen to 6.5 for each sample. Amoxycillin and clavulanic acid have been indicated earlier to be less stable at a pH of 6.5 It is evident now that higher temperatures especially room temperature (25ºC) facilitates pH change. Brands A, B and C were physically observed throughout the 7 day period of intermittent storage in the refrigerator. From the observation, there was no visible evidence of precipitation or gas formation throughout the storage period in all three samples. Brands A and B still maintained their original colour of creamy white (off white) throughout the 7 day period. Brand C however by the 4th day had started discolouring (browning) and partitioning

into two distinct phases, a lower creamy yellow portion and an upper brownish layer**.** The partitioning and discolouring increased in volume by the day. From this observation, brand C can be said to have degraded physically. This could be due to incompatibilities of the excipients used in this brand especially at higher temperatures. This result buttresses the fact that the standard storage condition of 2 - 8ºC is the ideal condition of storage for amoxicillin - clavulanic acid oral suspension and if available to patient, will have to be adhered to stringently.

5.2 CONCLUSION

5.2.1 Identification test

The identification tests carried out by the BP specification indicated that the reference samples employed in the assay were pure. The samples passed the identification tests by colour reactions. The melting ranges for clavulanic acid and amoxicillin were 162 - 163ºC and 193 - 195ºC respectively.

5.2.2 Method development and validation

A Reverse - Phase HPLC method was developed in this study with a mobile phase system of Water, methanol and sodium acetate buffer pH 4.4 in the ratio of 60:15:20) and a stationary phase made of Phenomenex, Kromasil 5 (C_8), 250 X 4.60mm 5 micron column with size 305334. The method could separate and quantify amoxicillin and clavulanic acid in reconstituted oral suspensions of amoxicillin and clavulanic acid.The HPLC method was validated against all the parameters such as precision, accuracy, specificity, linearity, and robustness as required by ICH guidelines on analytical method validation. The method was accurate, precise and linear within the range of 0.0100 - 0.1000%w/v for amoxicillin and0.01425%w/v to 0.00015%w/v for clavulanic acid. The LOD and LOQ for amoxicillin were 0.00614%w/v and 0.0186%w/v respectively and the LOD and LOQ for clavulanic acid were 0.00126%w/v and 0.003818%w/v for clavulanic acid respectively. The method demonstrated specificity in the presence of excipients of the oral suspension. The method was robust under conditions of a change in mobile phase composition and a different column from a different manufacturer.

5.2.3 Assay of amoxycillin – clavulanic acid oral suspension

The USP states that amoxycillin and clavulanic acid for oral suspension should contain an equivalent of not less than 90.0 percent and not more than 120.0 percent of the labelled

amount of amoxycillin and the equivalent of not less than 90.0 percent and not more than 125.0 percent of the labelled amount of clavulanic acid. All but for one sample of one of the three brands of oral suspensions of amoxicillin and clavulanic acid analysed passed the assay with an average percentage content of a 104% of the labelled amount of amoxicillin and 90% of the labelled amount of clavulanic acid. The sample of brand C that failed the assay had a mean percentage content of 126.3% for clavulanic acid.

5.2.4 Stability studies

The HPLC method developed could separate the degradation products of amoxicillin and clavulanic acid from the active drugs. Amoxycillin and clavulanic acid are stable when oral powder for paediatric suspension is reconstituted with distilled water, treated tap water or mineral water and stored under a standard storage condition of 2 to 8˚C over a period of seven days. Distilled water and treated tap water had a pH of 6 whilst that of mineral water was 6.3. Amoxycillin is more stable under acidic conditions than clavulanic acid. Amoxycillin is stable under ambient room temperature (25˚C) throughout the seven day period of therapy whilst clavulanic acid is not. The two compounds are not stable throughout the seven day period when stored in the refrigerator (2- 8˚C) only on alternate days.

5.3 RECOMMENDATIONS

Further stability studies should be carried out on the reconstitution of amoxicillin - clavulanic acid oral powder with different kinds of water but kept under non standard storage conditions. Also in order to prevent antibiotic resistance and achieve maximum therapeutic effect of amoxicillin – clavulanic acid oral suspension, the first line of storage which is refrigeration of drug between 2and 8˚C should be emphasised and encouraged. In rural areas

where the only available water is that from water bodies, boiling of the water should be mandatory before being used for the reconstitution of amoxicillin - clavulanic acid oral powder. This will help prevent the use of acidic water by the elimination of atmospheric carbon dioxide as well as the destruction of some microorganisms.

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