

# **CHAPTER ONE**

## **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 Hodgkin's 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. Amoxicillin, a derivative of penicillin (specifically ampicillin) was synthesized to help offset the shortcomings of the latter. Amoxicillin 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 (Wendy, 2012). All penicillin's including Amoxicillin have the beta-lactam ring as part of their structure. This  $\beta$ -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  $\beta$ -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 their  $\beta$ -lactamase resistance. The addition of an "electron withdrawing" group onto the 6-position of the amide group (located on the  $\beta$ -lactam ring) results in an increase in its acid stability due to the amide oxygen becoming less nucleophilic. This ensures that the amide oxygen does not attack the  $\beta$ -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  $\beta$ -lactam ring's carbonyl group and thus increases the drug's  $\beta$ -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 Penicillin's, such as Ampicillin. However, amoxicillin's substituent does not shield the  $\beta$ -lactam ring. Thus, still makes it vulnerable to  $\beta$ -lactamases and thus can be degraded by resistant bacteria (Beleh, 2006).

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 wall. It inhibits the last stage of bacterial cell wall synthesis by acylating the penicillin-sensitive Trans peptidase 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 Trans peptidase enzyme (Beleh, 2006). As stated earlier, resistance tends to occur if bacteria produce  $\beta$ -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  $\beta$ -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  $\beta$ -lactamase inhibitor such as Clavulanic acid. The latter is rather acted upon by the  $\beta$ -lactamase which is used up allowing the Amoxicillin to kill the bacteria without being degraded. Amoxicillin is usually prescribed with clavulanic acid as the potassium salt. Clavulanic acid is a naturally occurring,  $\beta$ -lactamase inhibitor, produced by fermentation of *Streptomyces clavuligerus*, for treatment of infection caused by  $\beta$ -lactamase producing bacteria that are resistant to amoxicillin alone (Aghazadeh.A and G, 2001).

Antibiotics for oral suspension are mainly available as dry powders for reconstitution. Many reconstituted antibiotic suspension is to be kept refrigerated in

order to get the optimal benefit from the drug. However, many patients do not keep to the specified storage conditions for different reasons like no refrigerator and irregular power supply resulting in various degrees of degradation of the product. In Africa power outage is common. Power supply is intermittent daily and outages can last for several hours to days at a stretch. This is not unique to Africa, for example a work carried out in Basrah Iraq showed that there is extended power outage, an average of 14 hrs/day (Jassim, 2010). Antibiotic was chosen for this work because more often than not reconstituted antibiotic requires refrigeration, a condition that may be difficult to meet in many resource limited environments as ours and also studies in Basrah Iraq and Sudan have shown that antibiotic is the most commonly encountered drug stored and consumed by patients in their homes and of the antibiotics stored, the beta-lactam antibiotics of penicillin and cephalosporin derivatives constituted the highest percentage at 26.43% and 22% respectively(Jassim, 2010; Yousif, 2002).

Instructing patients on storage of reconstituted antibiotics at home is a challenge for pharmacists in this situation. This study is important to ascertain effect of in-home storage conditions on stability of reconstituted suspensions of amoxicillin and give insight on appropriate pharmacists' instructions when adequate refrigeration is unachievable. Suspensions of amoxicillin acid are available for use in children and must be refrigerated (2-8°C) to maintain effectiveness once reconstituted. Liquid formulations generally tend to have much shorter shelf-lives than solid formulations and once opened should be used within 2 weeks to avoid any microbial contamination or reduction in activity (Obitte et al., 2009; Kiyong and Lauwo, 1993). The nature of syrup formulations in terms of added adjuncts such as sweetening, flavoring, and suspending, stabilizing and preserving agents makes the liquid formula complex one that is very prone to physical, chemical and

microbiological instability (Obitte et al., 2009). Stability is defined as the capacity of a drug substance or drug product to remain within the established specifications, to maintain its identity, strength, quality, and purity throughout the retest or until expiry date period. Stability testing of an active substance or finished product provides information on how the quality of drug substance or drug product varies with time, influenced by a variety of environmental factors such as temperature, humidity and light. Knowledge from stability studies enables understanding of the long-term effects of the environment on drugs. Stability testing also provides information about the degradation mechanisms, potential degradation products, possible degradation pathways of drug as well as interaction between the drug and the excipients in drug product (Lalitha et al., 2010; Uzunovic and Vranic, 2008). Climatic conditions can expose medications to dangerous temperatures that can potentially degrade the drug and often, unnoticed, example is Basrah where the summer heat can reach up to 50°C (Jassim, 2010), exceeding the U.S. Pharmacopeia's definition of room temperature (20-25 °C [68-77 °F]). High temperature and humidity accelerate deterioration, not only during transportation from overseas but also in warehouses and people's homes (Kiyong and Lauwo, 1993). An appropriate storage condition for reconstituted antibiotic is defined as keeping the medicines under refrigeration (2-8°C). Many homes in rural areas of developing nation may not have refrigerators or lack power supply, and even where there is refrigerator and power supply there may be erratic supply. Therefore medicines are stored at room temperature or kept in fridge's that has no power supply for several hours in a day thereby exposing these drugs to excessive temperatures far more than the room temperatures which ultimately may cause decomposition of both the excipients and active ingredient(s) (Yousif, (2002), in a Sudanese study reported that the rate of unsuitable storage conditions of drugs was 26.0%, compared to 31.8% in the Papua New Guinea study and that there was a higher rate of inappropriate storage in rural areas due to the Lack of refrigeration.

Drugs are chemicals that react to external stimuli such as, heat, humidity, light, microbial agents and dust. In many cases, such reactions can only lead to physical changes such as discoloration of the drug product. In many other cases, the reaction may affect the drug more seriously leading to the reduction or elimination of its effectiveness and/or potency. There are cases of drugs that, when affected, not only the failure of the drug to exert a therapeutic effect, but also cause adverse effects on the patient's health. Therefore storage conditions must not be taken lightly (Obitte et al., 2009). Studies have shown different drug in-home storage practices, some store or keep their drugs on the dining table, top of the refrigerator, first aid boxes, in their bags, in the car, closed cupboard or drawer, suit case, in the kitchen and even the bathroom and these practices may result to degradation (Obitte et al., 2009; Naidoo, 2006; Kiyangi and Lauwo, 1993). Instances of unsuitable storage often involve liquid preparations stored on open shelves, and reconstituted oral antibiotic powders stored for more than the recommended period after reconstitution or kept at freezing point (Kiyangi and Lauwo, 1993).

## **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 oral powder for reconstitution can be stored under normal room temperature (25°C), for as long as the expiry date will allow. After, reconstitution however, the suspension has to be strictly stored in a refrigerator (2 –8°C) 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 bath room. These practices may result in degradation (Nwokoye Peace, 2012). Will non - adherence to the standard storage conditions of affect stability? Will there be any significant effect on the stability of amoxicillin oral suspension.

### 1.3- Objectives:-

The aim of this study is to monitor the stability of reconstituted amoxicillin trihydrate oral suspension under different storage temperatures using HPLC technique.

The chosen storage conditions were:

- At 8°C kept in refrigerator.
- At 30°C kept in real stability chamber.
- At 40°C kept in accelerated stability chamber.

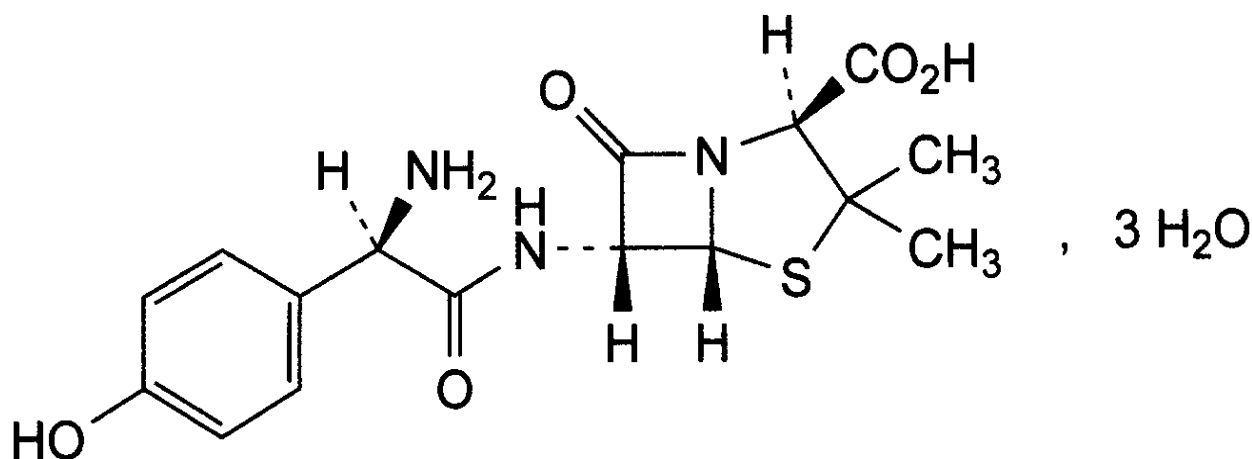


## CHAPTER TWO

### LITRATURE REVEIW

#### 2.1- Amoxicillin

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. Figure (1) shows the chemical structure of Amoxicillin trihydrate.



**Figure 1:** Chemical structure of amoxicillin trihydrate (Molecular weight = 419.4).

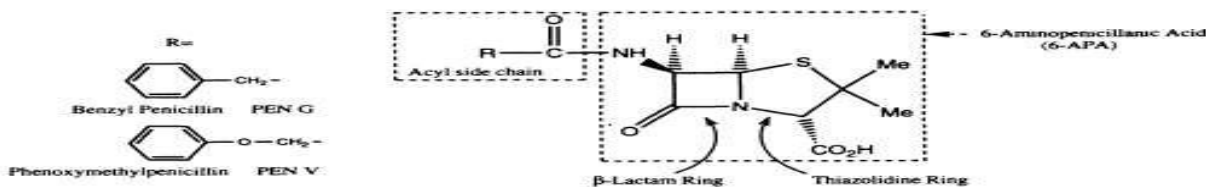
IUPAC name of Amoxicillin trihydrate: (2*S*, 5*R*, 6*R*)-6-[[ (2*R*)-2-Amino-2-(4-hydroxyphenyl) acetyl] amino]-3, 3-dimethyl-7-oxo-4- thia-1-azabicyclo [3.2.0] heptane-2-carboxylic acid trihydrate.

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.

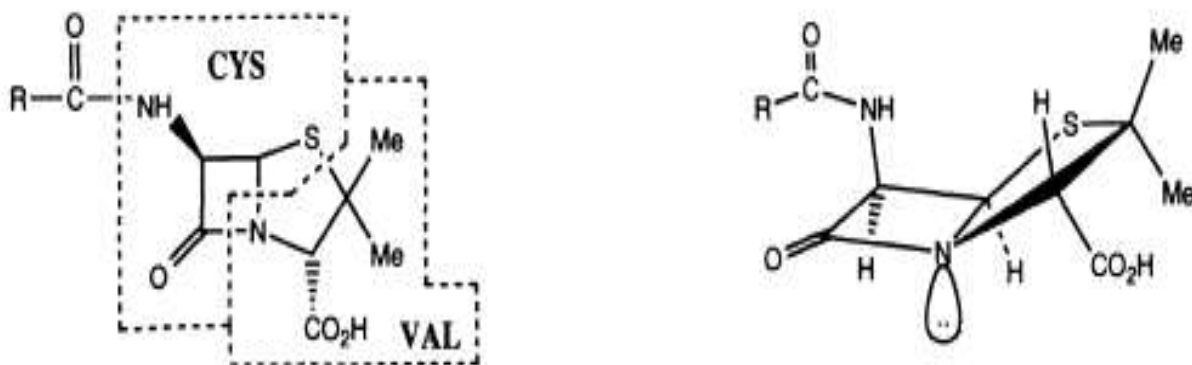
Amoxicillin has the essential substituents for activity including; the 1<sup>st</sup> position sulfur, two methyl groups at the 2<sup>nd</sup> position, a carboxylic acid at the 3<sup>rd</sup> position, a nitrogen at the 4<sup>th</sup> position and a carbonyl at the 7<sup>th</sup> position. It also does not have any substitutions on the 5<sup>th</sup> position which is critical. The 6<sup>th</sup> 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 amoxicillin 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. Its however insoluble in both chloroform and ether.

### 2.1.1- Structure of penicillin

Penicillin has a highly unstable bicyclic system consisting of a four membered  $\beta$ -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.



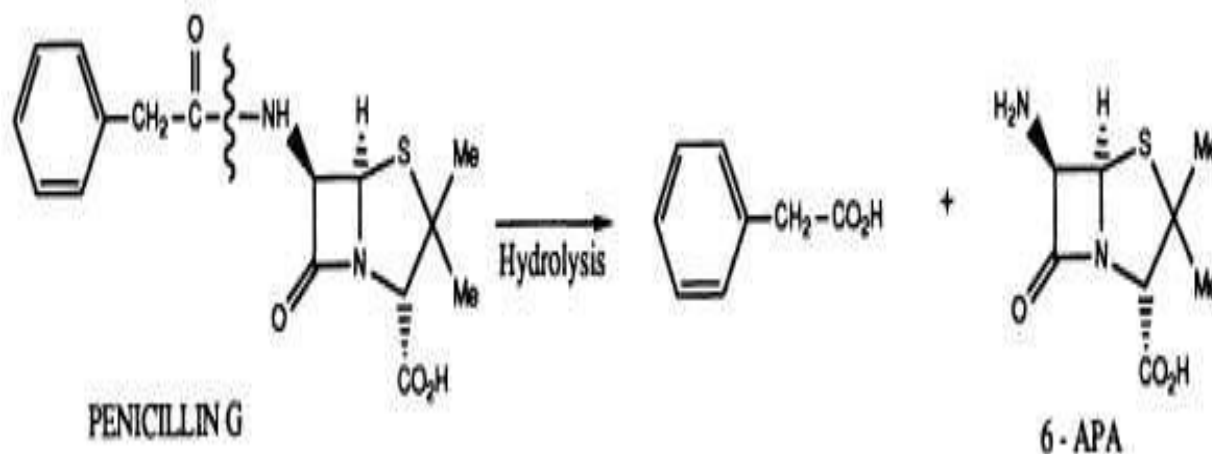
**Figure 2:** The structure of penicillin (The acyl side – chain (R) differs, with regards to the fermentation media).



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

### 2.1.2- Penicillin analogues

One means of varying the side-chain is to include different carboxylic acids in the fermentation medium; for instance, adding phenoxyacetic acid ( $\text{PhOCH}_2\text{COOH}$ ) 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  $\text{RCH}_2\text{CO}_2\text{H}$ ), 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 hydrolyzing penicillin G or penicillin V with an enzyme (penicillin acylase) or by chemical methods. These are more efficient methods than fermentation.

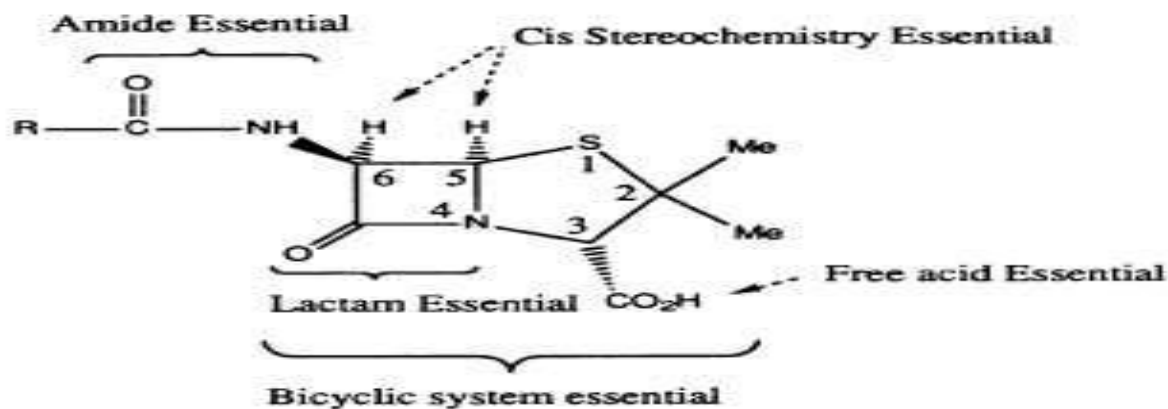


**Figure 4:** Production of 6-APA

The properties of benzyl penicillin are as follows; Active against Gram-positive bacilli (e.g. staphylococci, meningitis, and gonorrhea) 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 ( $\beta$ -lactamases) which are enzymes produced by penicillin resistant bacteria which catalyze 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 penicillin's, 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  $\beta$ -lactam ring is crucial. The free carboxylic acid is also crucial. The bicyclic system is necessary (confers strain on the  $\beta$  - 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. Amoxicillin is given orally as the trihydrate and by injection as the sodium salt so as to aid in dissolution. 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 show that very little variation could be made on the penicillin nucleus. This variation is however limited to the acyl amino side - chain.



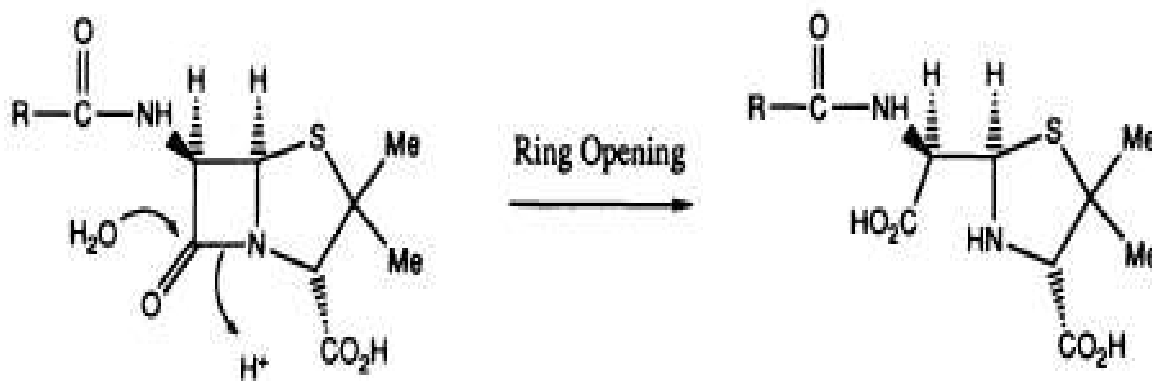
**Figure 5:** Structure Activity Relationships of Penicillins.

### 2.1.3- 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.

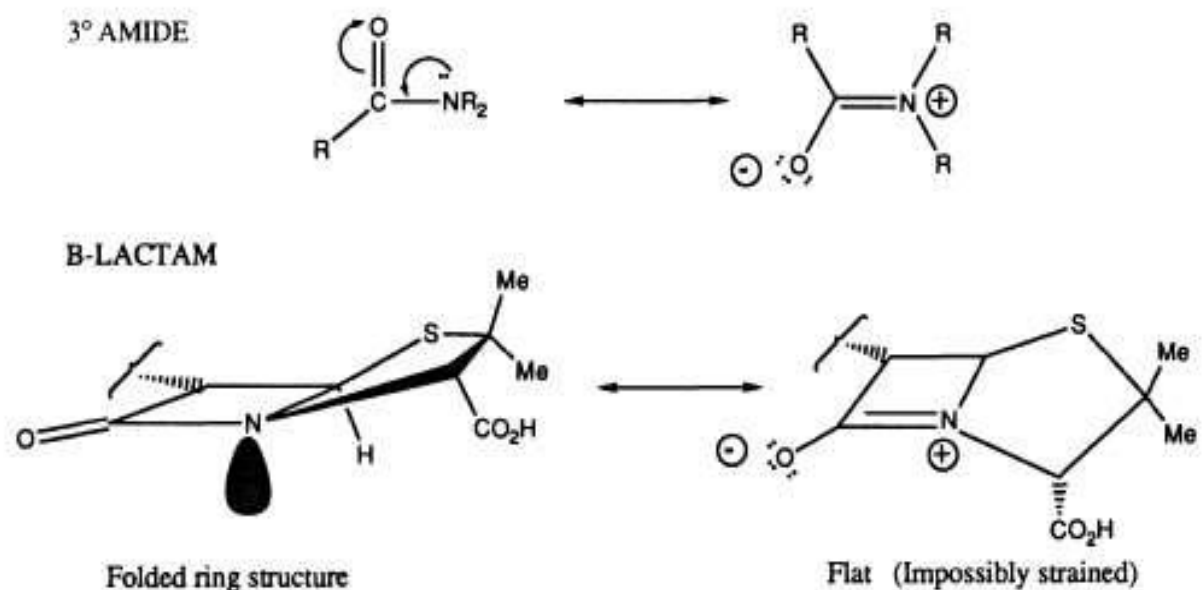
Acid-catalyzed ring opening tends to relieve these strains by breaking open the more highly strained four -membered lactam ring.



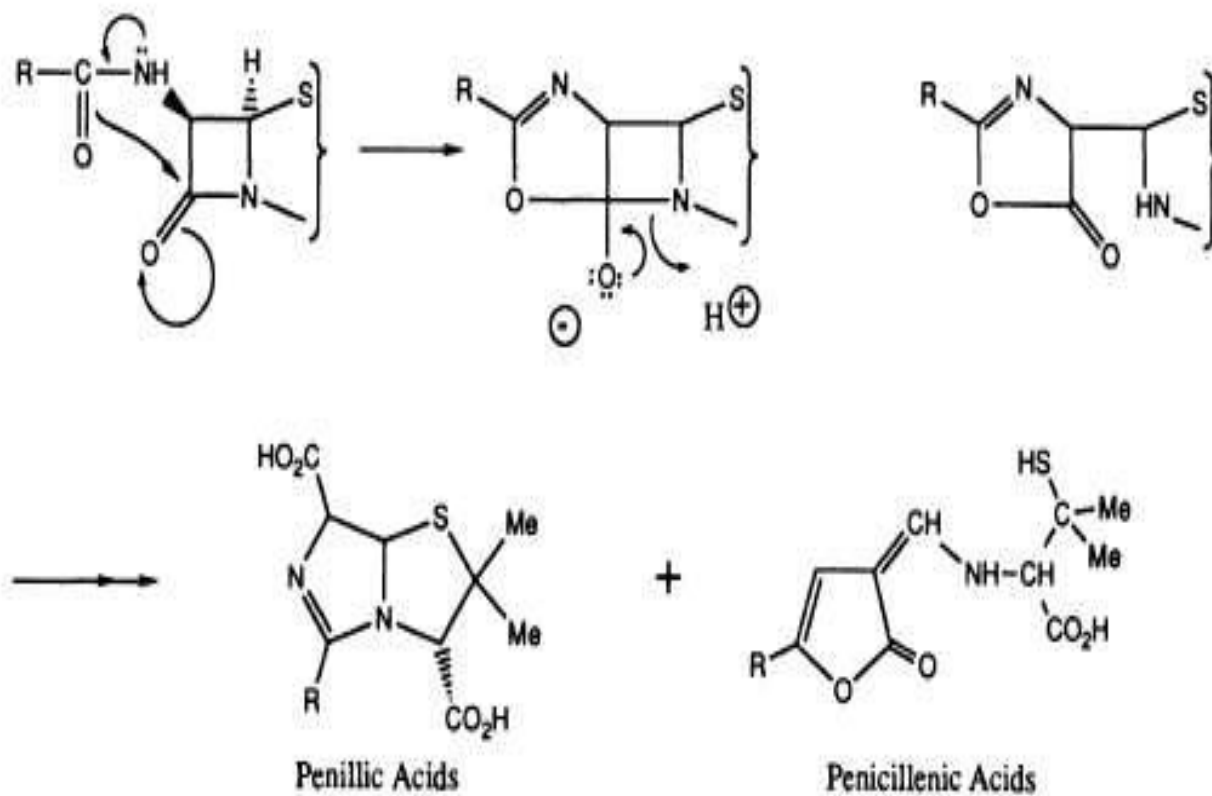
**Figure 6:** Ring opening

(ii) A highly reactive  $\beta$ -lactam carbonyl group. The carbonyl group in the  $\beta$ -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  $\beta$ -lactam ring. The  $\beta$ -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 carbonyl group.

(iii) Effect of the acyl side-chain (neighboring group participation); there is a demonstration of how the neighboring 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.



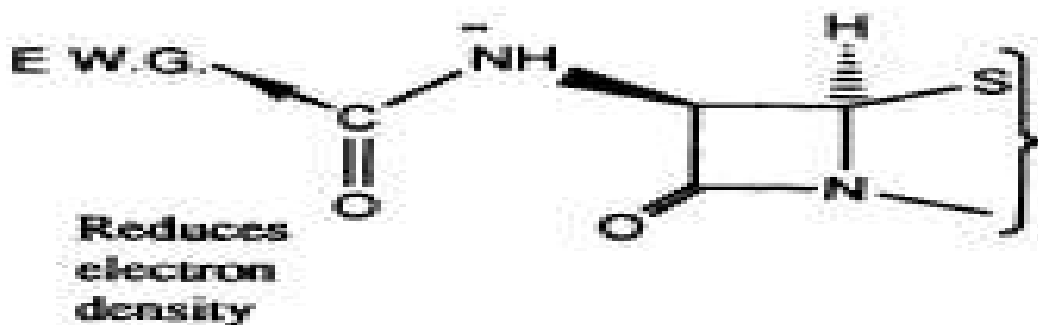
**Figure 7:** Highly reactive  $\beta$ -lactam carbonyl group



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

### 2.1.4- Tackling the problem of acid sensitivity

Not much can be done about the initial two factors since the  $\beta$ -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 neighboring group involvement to make it uneasy, if not impossible, for the acyl carbonyl group to attack the  $\beta$ -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 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.

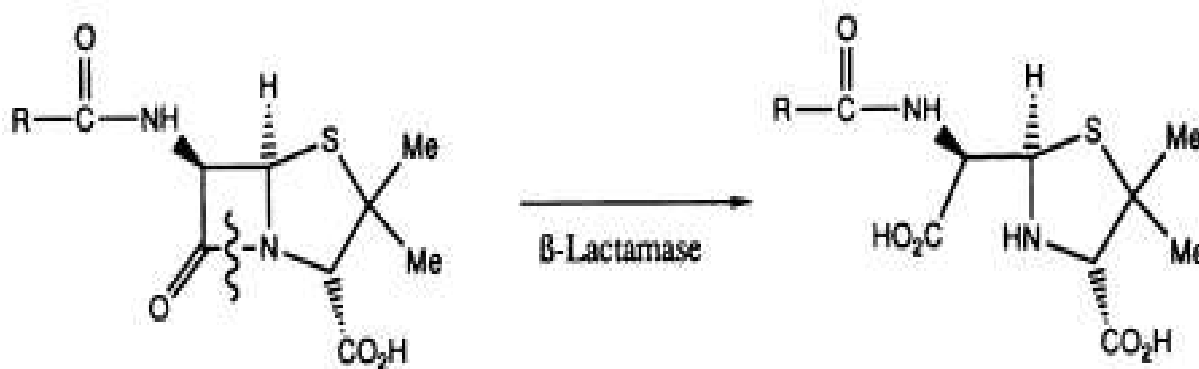
The molecule has better acid stability than penicillin G and is stable enough to survive the acid in the stomach. Thus, it can be given orally. However, Penicillin V is still sensitive to penicillinases and is slightly less active than penicillin G. It also has the problem of allergic sensitivity in 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 duly minimized by having an electron withdrawing group on the acyl side-chain.

### 2.1.5- Penicillin sensitivity to $\beta$ -lactamases

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

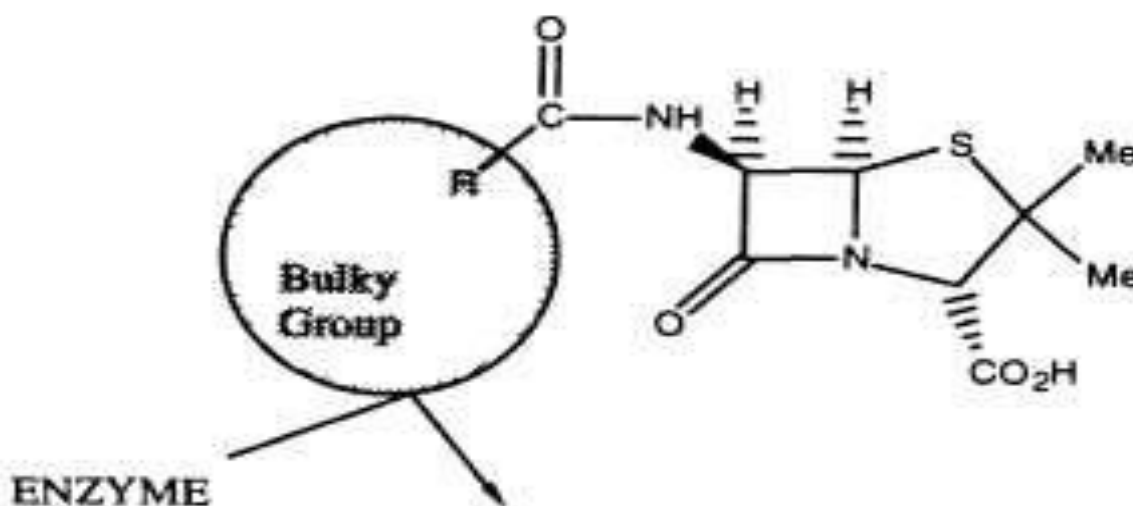


**Figure 10:**  $\beta$ -Lactamase deactivation of penicillin

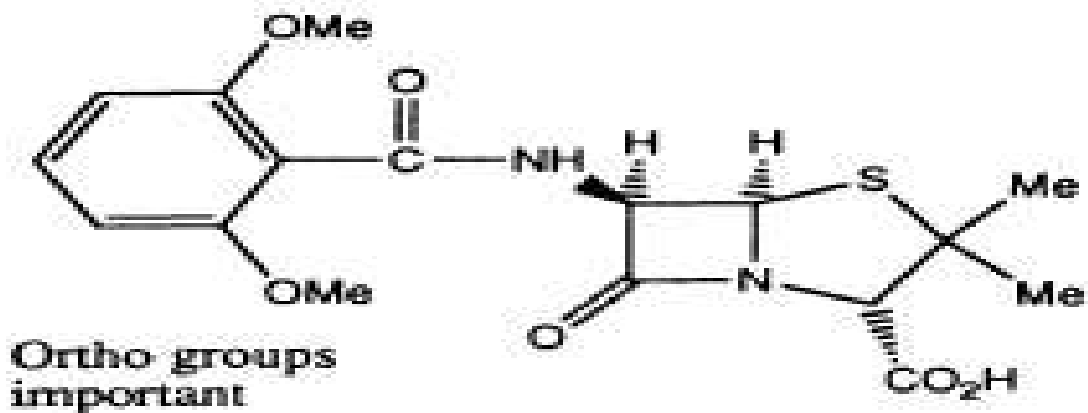
The setback of  $\beta$ -lactamases became more evident in 1960 when the extensive use of penicillin G led to a frightening rise of Staph. Aureus infections. These particular strains had gained the lactamase enzyme and had thus gained resistance to the drug. At some point in time, a larger percentage 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. 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 hindrance 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  $\beta$ -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 hindrance 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.

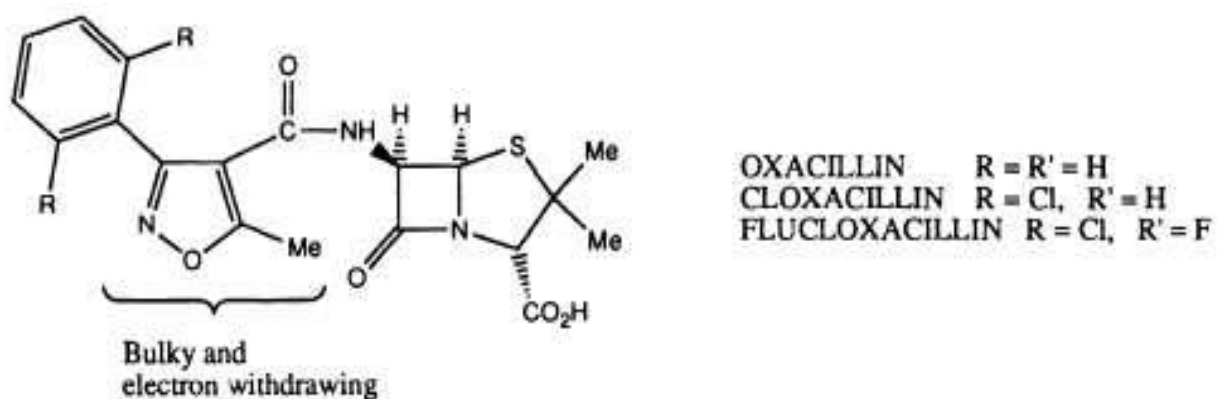


**Figure 11:** Blocking penicillin from reaching the Penicillase active site.



**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 hindrance 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- Stability**

The stability of a drug or any product is 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, 80% of labelled potency is generally regarded as the minimum acceptable potency level. 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 labelling. These are the conditions within which the expiration date is valid. Storage conditions specified on the labelling 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 labelling might clearly spell out the appropriate storage conditions. All penicillins including Amoxicillin have the beta-lactam ring as part of their structure. This  $\beta$ -lactam ring is very reactive but can be split open in either a neutral or basic medium, resulting in its inactivity. Although amoxicillin is still susceptible to destruction by Staphylococcal enzymes, it does have a much broader spectrum against the gram negative cell wall. Antibiotic susceptibility testing is essential to enable the identification of organisms that are resistant to penicillins such as amoxicillin and ampicillin. Amoxicillin is usually

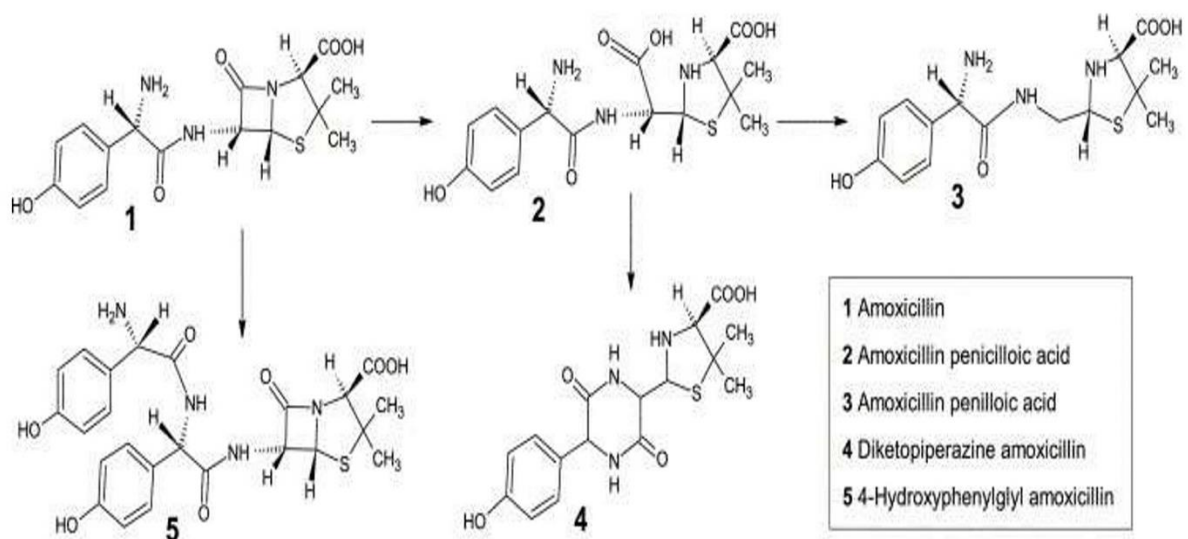
prescribed with clavulanic acid as the potassium salt. Clavulanic acid is a naturally occurring,  $\beta$ -lactamase inhibitor, produced by fermentation of *Streptomyces clavuligerus* for treatment of infection caused by  $\beta$ -lactamase producing bacteria that are resistant to amoxicillin alone.

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. color, phase separation, re - suspendibility, caking, hardness).
- (iv) Failure to meet the acceptance criterion for pH thus for liquid preparation (ICHQ1A).

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

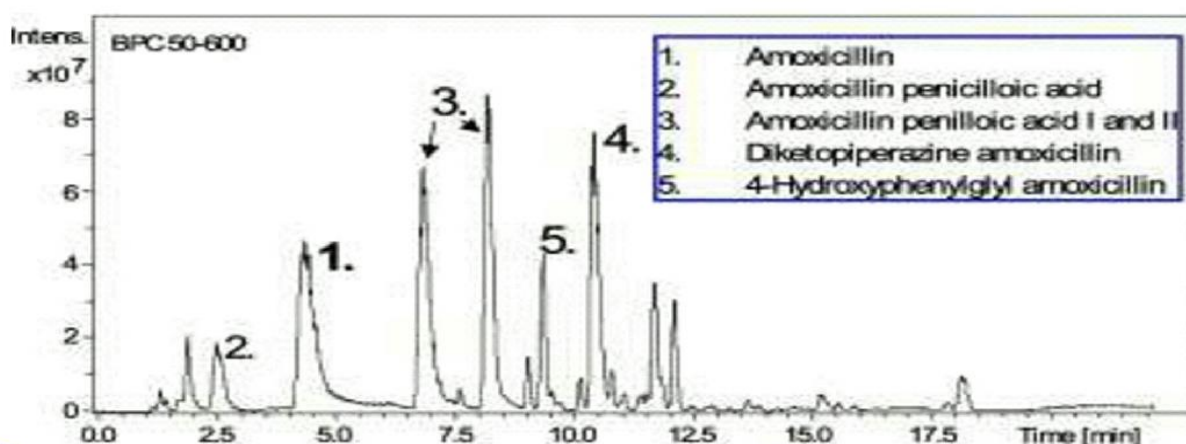
and tetramer were not present in significant amounts (De Pourcq et al., 1985). In order to ascertain some breakdown products from amoxicillin, the latter was stressed under acidic conditions. The degradation of amoxicillin (1) was induced by subjecting the pure drug substance to harsh acidic conditions, specifically a 0.1M HCl solution. The degradation of amoxicillin (1) in an acidic medium starts with the opening of the four-membered  $\beta$ -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 14:** Degradation pathway of amoxicillin

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 benzyl 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  $\beta$ -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 15:** BPC of amoxicillin (1) and its degradation products after acid exposure.

## **2.3- Analytical methods used in this project**

### **2.3.1- 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).

#### **2.3.1.1- Types of Chromatography**

##### **(i) 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.



## **(ii) 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 packing.

## **(iii) 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.

### **2.3.1.2- 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.

### **(i) 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.

### **(ii) 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 computer screen or other recording device as the experiment proceeds.

#### **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.

### **The 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 ( $-\text{CN}$ ), an amino group ( $-\text{NH}_2$ ), or a diol group ( $-\text{CHOH}-\text{CH}_2\text{OH}$ ) 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 <sup>[18]</sup>. 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 then on polar nature is attributed (McPolin, 2009). Typical designations include C8, C18 (or ODS, octadecylsilane), etc. Common mobile phase liquids are water, methanol, acetonitrile ( $\text{CH}_3\text{CN}$ ), 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 traded 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 oxidized 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 is 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).

# CHAPTER THREE

## MATERIALS AND METHODS

### 3.1- Materials

#### 3.1.1-Reagent and Samples

- ❖ Methanol (GR) - Sharlau
- ❖ Amoxicillin trihydrate working standard
- ❖ Amoxicillin trihydrate Reference Standard.
- ❖ Potassium dihydrogen orthophosphate.
- ❖ Acetonitrile –Sharlau
- ❖ Potassium hydroxide-carlo erba
- ❖ Potassium bromide
- ❖ Purified water.
- ❖ Karl fisher Reagent- Sharlau

**Table 1:** Certified Reference Standard

Name of Standard	Source	Manufacturing Date	Expiry Date
Amoxicillin Trihydrate	Pharmaland Pharmaceutical	June 2014	June 2018

**Table 2:** Brand of reconstituted oral suspensions of amoxicillin (Penamox 125 DS)

Code	Country of Origin	Batch Number	Strength mg/5ml	Expiry Date
A	Sudan	096	125	June 2017
B	Sudan	106	125	July 2017
C	Sudan	107	125	July 2017

### 3.2- Methods

#### 3.2.1- Identification tests for Amoxicillin working standard

##### a) Determination of melting point

10.00 mg of Amoxicillin trihydrate working standard was placed into a sealed end capillary tube. The melting point was determined using Stuart Melting point

apparatus SMP10 and the temperature range over which the sample melted was noted. This was repeated and the average temperature was determined.

#### b) IR data

2 mg of Amoxicillin Trihydrate working standard was weighed and mixed with 400 mg of dried spectroscopic grade potassium bromide. 200 mg of the mixture was weighed and examined using FT-IR Shimadzu Tracer-100 Instrument.

### **3.2.2- Assay of Amoxicillin Trihydrate working standard**

#### **3.2.2.1-Chromatographic Conditions**

- Column: Thermo C18, 250 × 4.60mm 5 micron.
- Flow rate: 1.00 ml/min
- Wavelength of detection: 230 nm.
- Mode of elution: Isocratic.
- Mode of HPLC: Reverse Phase Liquid Chromatography (RPLC).
- Inject 20 µL of each solution.

#### **3.2.2.2- Preparation of mobile phase**

A Potassium dihydrogen orthophosphate buffer of pH 5.00 was first prepared by accurately weighing 6.8000 g of Potassium dihydrogen orthophosphate. This was then completely dissolved in a 1000 ml of distilled water. The solution stirred well by a magnetic stirrer and the pH was adjusted to 5.00 using 0.4 M potassium hydroxide solution. The mobile phase was prepared by measuring 960ml of Potassium dihydrogen buffer solution and 40 ml of HPLC grade Acetonitrile (BP 2013)

### **3.2.2.3- Preparation of Reference Standard**

30 mg of amoxicillin trihydrate reference standard was accurately weighed in 25.0 ml beaker using RAWAG-analytical weighing balance and was quantitatively transferred into 50.0 ml volumetric flask with the aid of about 30.0 ml of mobile phase solution. The solution was stoppered and sonicated at 30°C using SONOREX –SUPER RK103H Sonicator for 10 min to aid in the complete dissolution of amoxicillin. The solution was then topped up to the 50.0 ml mark on the flask with more mobile phase solution.

### **3.2.2.4- Preparation of Working Standard**

30 mg of amoxicillin trihydrate working standard was accurately weighed in 25.0 ml beaker using RAWAG-analytical weighing balance and was quantitatively transferred into 50.0 ml volumetric flask with the aid of about 30.0ml of mobile phase solution. The solution was stoppered and sonicated at 30°C using SONOREX –SUPER RK103H Sonicator for 10 min to aid in the complete dissolution of amoxicillin. The solution was then topped up to the 50.0 ml mark on the flask with more mobile phase solution (BP 2013).

### **3.2.2.5- Determination of the water content in Amoxicillin trihydrate working Standard**

25.0 ml of methanol was taken for water determination in a dried titration flask, 10 µl of distilled water was firstly titrated using Karl–Fisher Moisture Titrator MKS-520, the titration was repeated four times and the titer volume was recorded as water factor, the relative standard deviation and the average was also calculated. Then 0.1002 g of sample was accurately weighed using RAWAG-analytical weighing balance and transferred into the titration flask, and was dissolved by stirring. The end point was determined and recorded (BP 2013).



### **3.2.3- Stability studies of reconstituted Amoxicillin Trihydrate oral suspension kept in different Storage conditions (8°C, 30°C and 40°C).**

The three brands of amoxicillin were used, each was reconstituted with distilled water, the assumed standard water for reconstitution of drugs. The drugs had their pHs recorded and analyzed by HPLC Chromatograph Shimadzu LC2010C HT immediately after reconstitution, and then kept in three different conditions which were (Refrigerator- Ideal 2013 with 8°C, real Stability Chamber NEWTRONIC with 30°C and accelerated Stability Chamber NEWTRONIC with 40°C keeping the humidity constant at 65%. HPLC analysis and pH recording were carried out on initial, seven days and after fourteen Days in all conditions which were mentioned above. The conditions were monitor using Temperature and Humidity data logger instrument.

### **3.2.4- Determination of the pH for the Amoxicillin Trihydrate Suspension**

The pH was examined for all brands of amoxicillin trihydrate Suspension using Eutech instruments pH 510 pH meter.

### **3.2.5- Assay of Amoxicillin Trihydrate Suspension**

#### **3.2.5.1- Preparation of Mobile Phase**

A Potassium dihydrogen orthophosphate buffer of pH 5.00 was first prepared by accurately weighing 6.8000 g of Potassium dihydrogen orthophosphate. This was then completely dissolved in a 1000 ml of distilled water. The solution stirred well by a magnetic stirrer and the pH was adjusted to 5.00 using 0.4 M potassium hydroxide solution. The mobile phase was prepared by measuring 960 ml of Potassium dihydrogen buffer solution and 40 ml of HPLC grade Acetonitrile.

### **3.2.5.2- Preparation of Standard Solution**

130 mg of amoxicillin trihydrate was accurately weighed in 50.0 ml beaker using RAWAG-analytical weighing balance and was quantitatively transferred into 200.0 ml volumetric flask with the aid of about 150.0 ml of mobile phase solution. The solution was stoppered and sonicated at 30°C using SONOREX –SUPER RK103H Sonicator for 10 min to aid in the complete dissolution of amoxicillin. The solution was then topped up to the 200.0 ml mark on the flask with more mobile phase solution.

### **3.2.5.3- Preparation of Samples Solution**

5.0000 g of amoxicillin trihydrate oral suspension was accurately weighed in 50.0 ml beaker using RAWAG-analytical weighing balance and was quantitatively transferred into 200.0 ml volumetric flask with the aid of about 150.0ml of mobile phase solution. The solution was stoppered and sonicated at 30°C using SONOREX –SUPER RK103H Sonicator for 15 min to aid in the complete dissolution of amoxicillin. The solution was then topped up to the 200.0ml mark on the flask with more mobile phase solution.

### **3.2.5.4- Preparation of Placebo**

0.9055g of Placebo was accurately weighed in 50.0ml beaker using RAWAG-analytical weighing balance and was quantitatively transferred into 200.0ml volumetric flask with the aid of about 150.0ml of mobile phase solution. The solution was stoppered and sonicated at 30°C using SONOREX –SUPER RK103H Sonicator for 15 min to aid in the complete dissolution of amoxicillin. The solution was then topped up to the 200.0ml mark on the flask with more mobile phase solution.

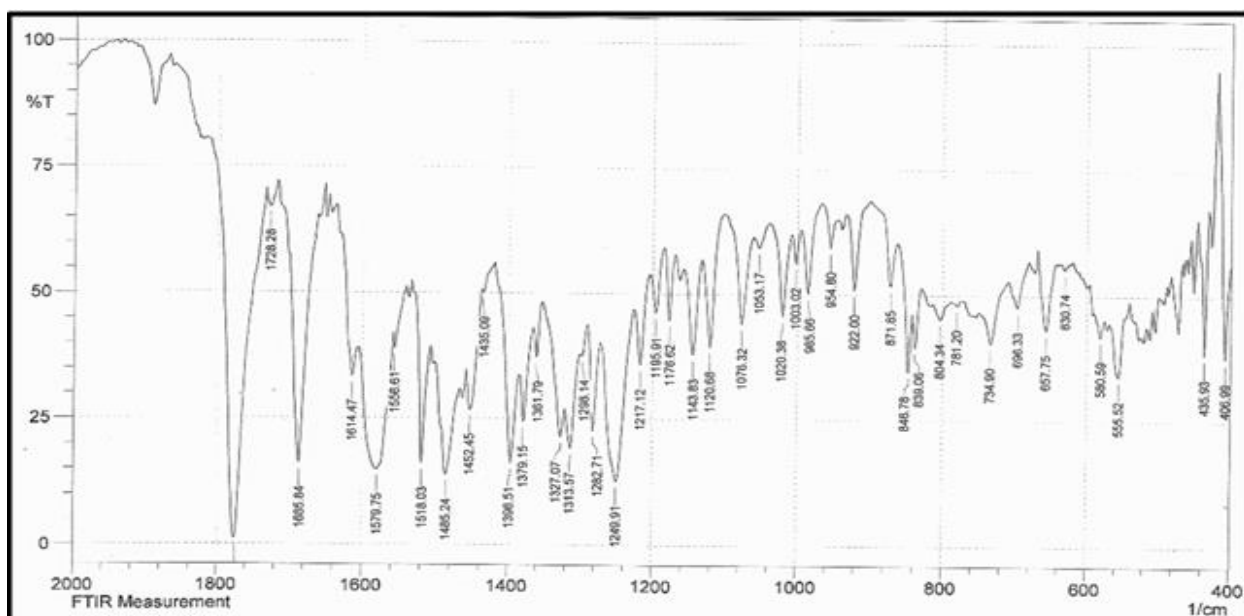
## CHAPTER FOUR

### RESULTS AND DISSCUSION

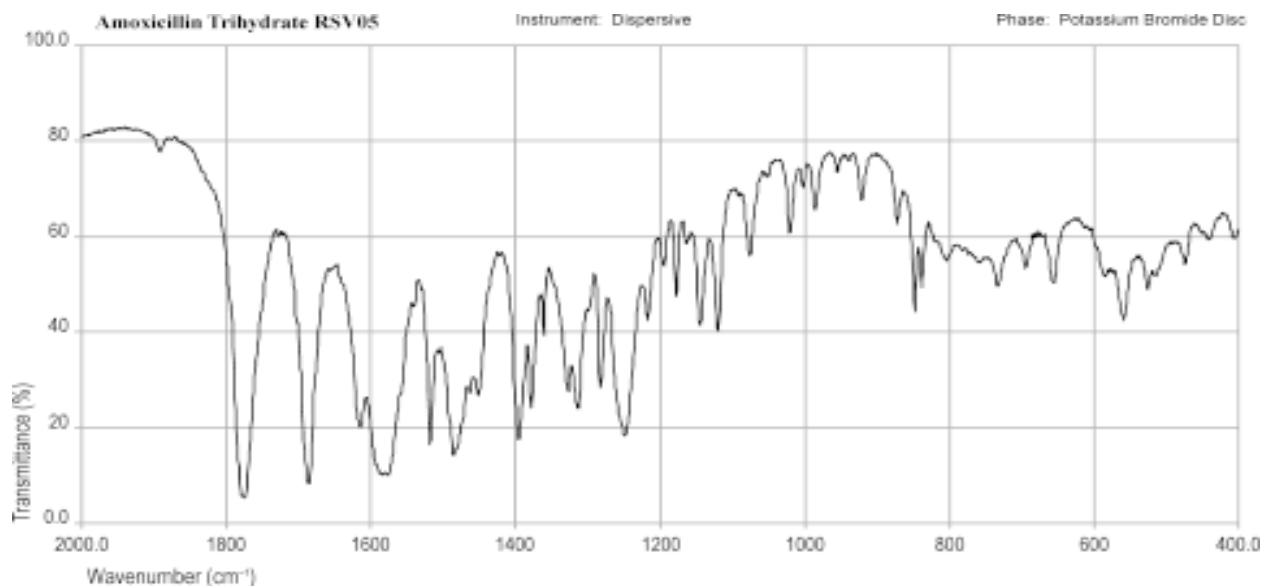
#### 4.1- Identification tests for Amoxicillin

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. Working standards as well, will have to be analyzed both qualitatively and quantitatively to validate their use as comparative standards for pharmaceutical analysis.

The identification processes involved could include simple physical observation of sample with regards to its nature, simple color reaction tests, determination of refractive index for liquids, melting point determination for solid samples, optical rotation determination through to spectral analysis of samples. In view of this, working standards were identified and assayed accordingly.



**Figure 16:** FT- IR Spectrum for Amoxicillin Trihydrate Working standard



**Figure 17:** Standard FT-IR Spectrum for Amoxicillin Trihydrate (BP 2013).

Identification of the Amoxicillin Trihydrate Working standard was carried out by FT-IR test which was compared with the standard FT-IR spectrum obtained from BP 2013 and it was identical.

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 BP range (192-196°C).

**Table 3:** Melting point Determination for Amoxicillin Trihydrate

Sample	Melting point determination (°C)			Reference Range
Amoxicillin	1st	2 <sup>nd</sup>	Average Range	192-196°C
	192-194°C	194-196°C	193-195°C	

## 4.2- pH for Reconstituted Amoxicillin trihydrate Suspension

**Table 4:** Determination of pH for Reconstituted Amoxicillin trihydrate Suspension

Storage Temperatures	Sample ID	pH Initial	pH after 7 Days	pH after 14 Days
08°C	A3	5.97	6.01	6.08
	B3	6.02	6.05	6.11
	C3	5.99	6.03	6.09

30°C	A1	6.02	6.21	6.48
	B1	5.99	6.24	6.53
	C1	5.97	6.27	6.59

40°C	A2	6.03	6.45	6.91
	B2	6.00	6.39	6.89
	C2	5.98	6.40	6.93

It is evident from Table 4 that the pH increased when the temperature was increased. All samples had the pH within the standard range of 4 to 7 (BP 2013).

## 4.3- Assay of Amoxicillin Trihydrate working standard

Water Content of working standard:

Water factor (four readings were taken):

1) 5.8554; 2) 5.8399; 3) 5.8789; 4) 5.8493

Average = 5.8559                      RSD = 0.03 %

$$\text{Water content \%} = \frac{\text{Factor} \times \text{T.V} \times 100}{\text{Sample Wt.} \times 1000}$$

T.V = Titer volume

RSD = Relative Standard Deviation

T.V = 2.231 ml

Water Factor = 5.8559

Sample Wt. = 0.1002 g

$$\text{Water content\%} = \frac{5.8559 \times 2.231 \times 100}{0.1002 \times 1000} = 13.0\%$$

$$\text{Assay \%} = \frac{\text{Sample area} \times \text{STDWt} \times 50 \times (100 - \text{Water content of STD})}{\text{STD area} \times 50 \times \text{Sample Wt} \times (100 - \text{Water content of Sample})} \times \text{Potency}$$

STD weight = 0.0306 g

Sample weight = 0.0302 g

Water content of Reference STD = 12.7%

Water content of Sample = 13.0%

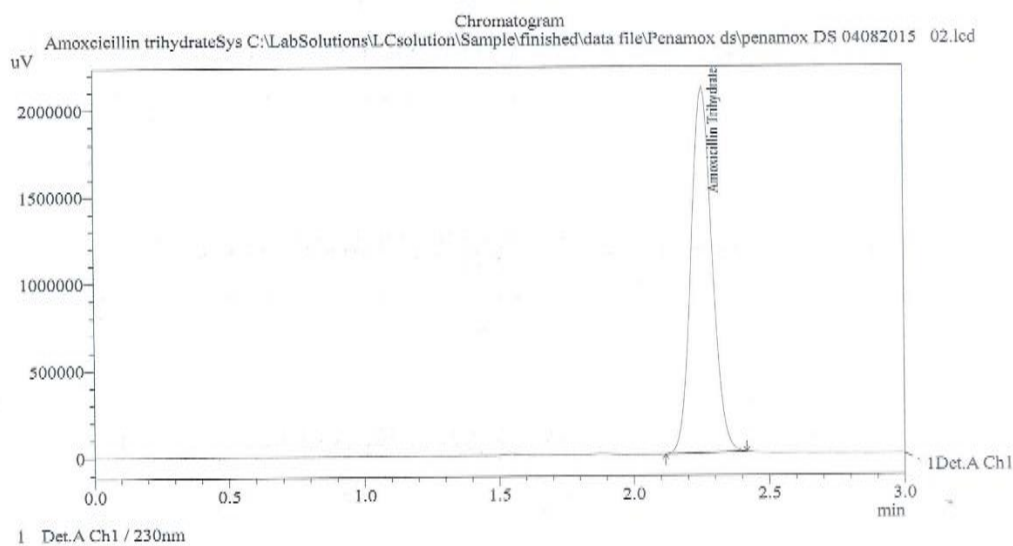
**Table 5:** Assay for Amoxicillin Trihydrate working standard

Reference Standard Area	Sample Area	Assay
1660198 1660078 1660523 1661627 1660940	1634391   1635086	100.1%   100.1%
Average = 1660673	Average = 1634739	100.1%

Amoxicillin working standard sample was assayed by means of HPLC. The results showed that the average percentage purity of the amoxicillin reference standard was 100.1%. This value falls well within the range of acceptable BP specification of 95 – 102% (BP 2013).

**Pharmaland pharmaceuticals  
shimadzu LC-2010 CHT  
HP-QC07**

Acquired by : Admin  
 Sample Name : Amoxicillin trihydrateSys  
 Sample ID : SDT002  
 Tray# : 1  
 Vial# : 1  
 Injection Volume : 20 ul.  
 Data Filename : penamox DS 04082015 02.lcd  
 Method Filename : Penamox DS.lcm  
 Batch Filename : Penamox DS 01.lcb  
 Report Filename : report format 1.lcr  
 Date Acquired : 8/4/2015 3:27:15 AM  
 Data Processed : 8/4/2015 3:30:19 AM



PeakTable

Detector A Ch1 230nm				
Name	Ret. Time	Area	Theoretical Plate#	Tailing Factor
Amoxicillin Trihydrate	2.254	11382939	3323.666	1.063
		11382939		

**Figure18:** HPLC Chromatogram for Amoxicillin Trihydrate working Standard

#### **4.4-Assay of Reconstituted Amoxicillin Trihydrate Suspension by HPLC**

A reversed – phase high-performance liquid chromatography (RP-HPLC) was employed in the analysis of amoxicillin. The analysis was carried out by means of a Thermo (C18),  $250 \times 4.60$  mm 5 micron column. The mobile phase consisted of acetonitrile, and potassium dihydrogen phosphate buffer (pH 5.00) in the ratio (40:960). The injection volume was 20 $\mu$ l 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 230 nm. Chemically, amoxicillin is carboxylic acid. Amoxicillin experiences the Zwitterion effect at pH's less than 9 hence its solubility is greatly reduced. Being a lactam, it is susceptible to acid- and base-catalyzed hydrolysis of the four-membered lactam ring. It has been observed that amoxicillin tends to be more stable at lower pH conditions because at these pH 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 (lactam). The simultaneous analysis of amoxicillin by the United States employ the use of phosphate buffers in their mobile phase system. The use of inorganic buffers such as that 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).

Acetonitrile and phosphate buffer 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 40:960 for acetonitrile and phosphate buffer (pH 5.00) respectively. The retention times for amoxicillin was 2.3 minutes with standard deviations of  $\pm 0.2$ . The extent to which a drug is ionized in a solution is highly



dependent on the pH. Complete ionization 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. Thus since the mobile phase has a greater proportion than water, the elution of the more water soluble compound is probable. Furthermore, amoxicillin has ionizable groups in their structures. It has three ionizable groups of –OH, –COOH, and –NH<sub>2</sub> which have pK<sub>a</sub> values of 7.4, 2.4 and 9.6 respectively (Martin A.N, 1969). Most compounds get ionized at a pH within 1-2 units of their pK<sub>a</sub> values (Yuri and Rosario, 2007). Amoxicillin is completely ionized if at least two of its ionizable groups are ionized. However, when found in a mobile phase with pH 5.00, the –COOH becomes ionized resulting in partial ionization of drug hence decreasing the affinity for the mobile phase.

$$\text{Assay \%} = \frac{\text{Sample area} \times \text{STDWt} \times 200 \times (\text{Wt/ml}) \times 5 \times 0.87}{\text{STD area} \times 200 \times \text{Sample Wt} \times 0.125} \times \text{Potency}$$

Potency of Working Standard = 100.1%

Standard Weights:

Initial = 0.1301g              7days = 0.1299 g              14 days = 0.1300 g

The results of the study are summarized in tables 6, 7 and 8 which show the concentrations of amoxicillin as percentage assay.

**Table 6:** Assay of Reconstituted Amoxicillin Trihydrate Suspension (initial)

<b>Standard Area</b>	<b>Sample ID</b>	<b>Sample Area</b>	<b>Sample Wt</b>	<b>Wt/ml (Density)</b>	<b>Assay %</b>
11374795	A1 30 °C	12210139	5.0001 g	1.070 g/ml	104.1%
11391723					
11355907	B1 30 °C	12666654	5.0000 g	1.070 g/ml	108.0%
11340631					
11409071	C1 30 °C	11861170	5.0000 g	1.070 g/ml	101.2%
Average=					
11374425	A2 40 °C	1219020	5.0002 g	1.070 g/ml	104.0%
	B2 40 °C	11701042	5.0000 g	1.070 g/ml	99.8%
	C2 40 °C	12633785	5.0001 g	1.070 g/ml	107.7%
	A3 8 °C	12303716	5.0000 g	1.070 g/ml	104.9%
	B3 8 °C	12123099	5.0001 g	1.070 g/ml	103.4%
	C3 8 °C	12341773	5.0000 g	1.070 g/ml	105.3%

**Table 7:** Assay of Reconstituted Amoxicillin Trihydrate Suspension (After 7 Days)

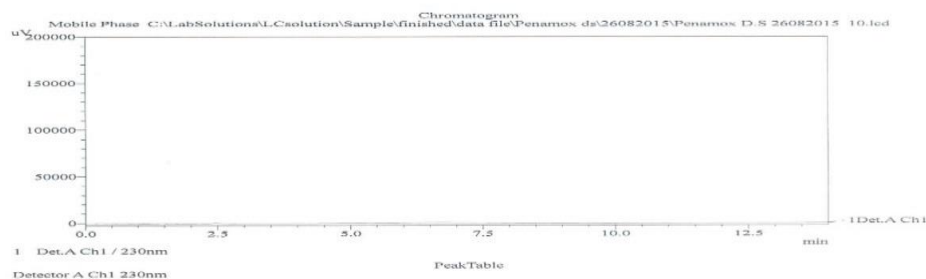
Standard Area	Sample ID	Sample Area	Sample Wt	Wt/ml (Density)	Assay %
11433913	A1 30 °C	11577743	5.0023 g	1.069 g/ml	98.3%
11382939					
11371265	B1 30 °C	11591417	5.0015 g	1.069 g/ml	98.4%
11416919					
11366071	C1 30 °C	11532376	5.0004 g	1.069 g/ml	97.9%
Average =					
11394222	A2 40 °C	11126641	5.1245 g	1.069 g/ml	92.2%
	B2 40 °C	11618776	5.1426 g	1.069 g/ml	96.0%
	C2 40 °C	11476069	5.0891 g	1.069 g/ml	95.8%
	A3 8 °C	12358989	5.0605 g	1.069 g/ml	103.7%
	B3 8 °C	12358989	5.1145 g	1.069 g/ml	101.8%
	C3 8 °C	12421725	5.0771 g	1.069 g/ml	103.9%

**Table 8:** Assay of Reconstituted Amoxicillin Trihydrate Suspension (After 14 Days)

Standard Area	Sample ID	Sample Area	Sample Wt	Wt/ml (Density)	Assay %
11468100	A1 30 °C	10803545	5.0001 g	1.069 g/ml	91.2%
11476820					
11432515	B1 30 °C	10491952	4.9711 g	1.069 g/ml	89.1%
11501689					
11492997	C1 30 °C	10850205	5.0006 g	1.069 g/ml	91.6%
Average=					
11474424	A2 40 °C	7979309	5.0002 g	1.069 g/ml	67.4%
	B2 40 °C	8604871	5.0012 g	1.069 g/ml	72.6%
	C2 40 °C	9942069	5.0001	1.069 g/ml	83.9%
	A3 8 °C	11631417	5.0000 g	1.069 g/ml	98.2%
	B3 8 °C	11913529	5.0062 g	1.069 g/ml	100.4%
	C3 8 °C	11046906	4.8125 g	1.069 g/ml	96.9%

**Pharmaland pharmaceuticals  
shimadzu LC-2010 CHT  
HP-QC07**

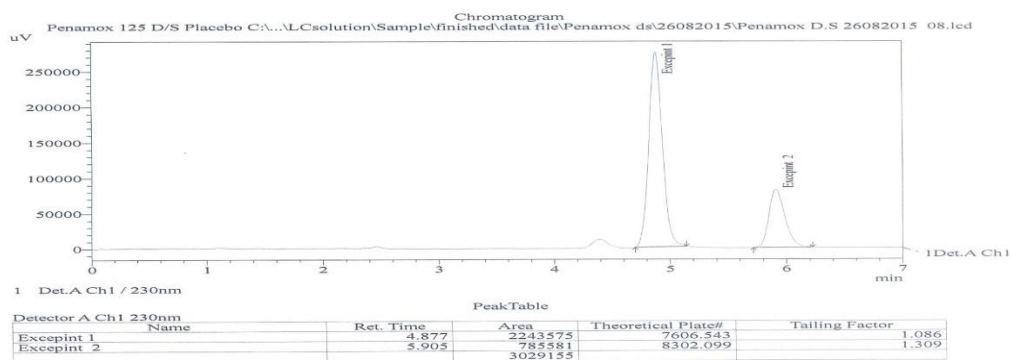
Acquired by : Admin  
Sample Name : Mobile Phase  
Sample ID : Unk001  
Tray# : 1  
Vial# : 4  
Injection Volume : 20 uL  
Data Filename : Penamox D.S. 26082015 10.1cd  
Method Filename : Penamox D.S.1cm  
Batch Filename : Penamox DS 001.1cb  
Report Filename : report format 1.1cr  
Date Acquired : 8/26/2015 4:36:44 PM  
Data Processed : 8/29/2015 9:45:04 AM



**Figure19: HPLC Chromatogram for mobile phase**

**Pharmaland pharmaceuticals  
shimadzu LC-2010 CHT  
HP-QC07**

Acquired by : Admin  
Sample Name : Penamox 125 D/S Placebo  
Sample ID : Unk001  
Tray# : 1  
Vial# : 3  
Injection Volume : 20 uL  
Data Filename : Penamox D.S. 26082015 08.1cd  
Method Filename : Penamox D.S.1cm  
Batch Filename : Penamox DS 001.1cb  
Report Filename : report format 1.1cr  
Date Acquired : 8/26/2015 4:19:27 PM  
Data Processed : 9/9/2015 3:45:46 PM



**Figure20: HPLC Chromatogram for Placebo**

**Table 9:** Degradation of reconstituted Amoxicillin trihydrate Suspension

<u>Storage</u> <u>Temperatures</u>	<u>Sample</u> <u>ID</u>	<u>Assay initial</u>	<u>Assay after</u> <u>7day</u>	<u>Assay after 14</u> <u>days</u>
<b>08°C</b>	<b>A3</b>	104.9%	103.7%	98.2%
	<b>B3</b>	103.4%	101.8%	100.4%
	<b>C3</b>	105.3%	103.9%	96.9%

<b>30°C</b>	<b>A1</b>	104.1%	98.3%	91.2%
	<b>B1</b>	108.0%	98.4%	89.1%
	<b>C1</b>	101.2%	97.9%	91.6%

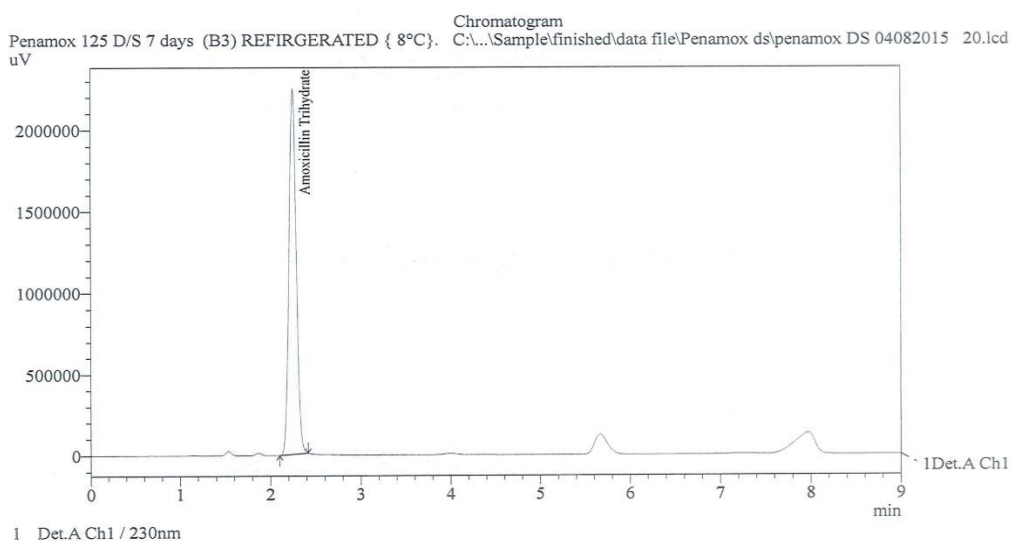
<b>40°C</b>	<b>A2</b>	104.0%	92.2%	67.4%
	<b>B2</b>	99.8%	96.0%	72.6%
	<b>C2</b>	107.7%	95.8%	83.9%

The BP states that amoxicillin oral suspension should contain the equivalent of not less than 80.0 percent and not more than 120.0 percent of the labelled amount of amoxicillin. The results of the HPLC analysis of all samples of the three different conditions for initial, seven days and fourteen days were as follows. Thus, all samples had the percentage content of amoxicillin well within the standard range, except two samples of accelerated Stability Chamber (40°C) which had 67.4%, 72.6% and that after fourteen days. The two that fell outside the range was lower than the minimum limit 80%-120%. Thus, all samples had the required therapeutic amount of amoxicillin trihydrate. From the results obtained for the analysis of all samples under different conditions which were all reconstituted with distilled water and kept in the refrigerator (8°C), real stability chamber (30°C) and accelerated stability chamber (40°C), on initial, seven and fourteen days. The rate for breakdown of amoxicillin was increased when the suspensions were left under accelerated stability chamber (40°C) conditions as compared the suspensions were kept in the

real stability chamber (30°C) condition and refrigerator (8°C) conditions. The storage of the suspensions in the refrigerator 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 in relation to their baseline concentrations for all samples were all greater than 80% but that of accelerated stability chamber (40°C) had its amoxicillin content 67.4%, 72.6% after fourteen days below the recommended 80%.

**Pharmaland pharmaceuticals  
shimadzu LC-2010 CHT  
HP-QC07**

Acquired by : Admin  
 Sample Name : Penamox 125 D/S 7 days (B3) REFRIGERATED { 8°C}.  
 Sample ID : Unk001  
 Tray# : 1  
 Vial# : 9  
 Injection Volume : 20 uL  
 Data Filename : penamox DS 04082015 20.lcd  
 Method Filename : Penamox DS.lcm  
 Batch Filename : Penamox DS 01.lcb  
 Report Filename : report format 1.lcr  
 Date Acquired : 8/4/2015 6:50:49 AM  
 Data Processed : 8/4/2015 6:59:54 AM



PeakTable

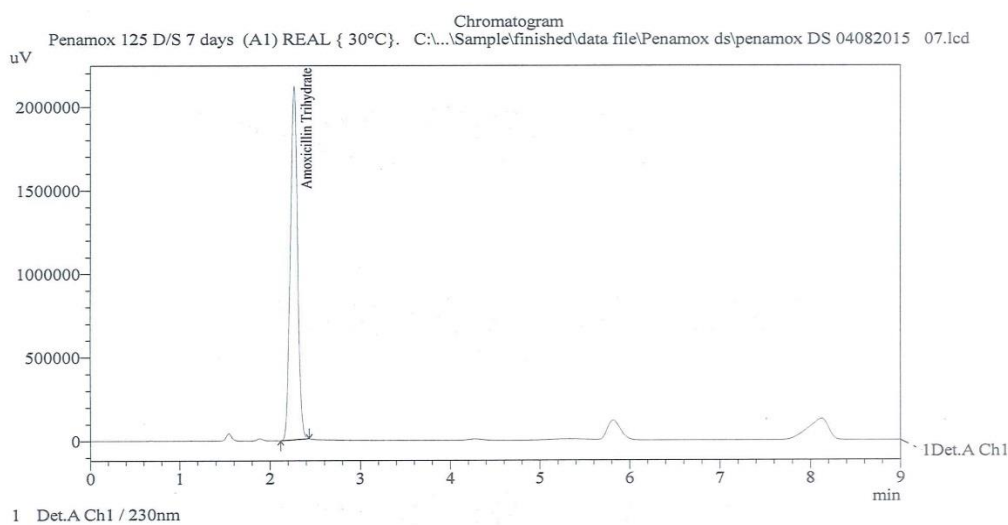
Detector A Ch1 230nm				
Name	Ret. Time	Area	Theoretical Plate#	Tailing Factor
Amoxicillin Trihydrate	2.247	12435941	3169.052	1.066
		12435941		

**Figure21:** HPLC Chromatogram for Reconstituted Amoxicillin Trihydrate Suspension (sample B3 at 8°C)



**Pharmaland pharmaceuticals  
shimadzu LC-2010 CHT  
HP-QC07**

Acquired by : Admin  
 Sample Name : Penamox 125 D/S 7 days (A1) REAL { 30°C}.  
 Sample ID : Unk002  
 Tray# : 1  
 Vial# : 2  
 Injection Volume : 20 uL  
 Data Filename : penamox DS 04082015 07.lcd  
 Method Filename : Penamox DS.lcm  
 Batch Filename : Penamox DS 01.lcb  
 Report Filename : report format 1.lcr  
 Date Acquired : 8/4/2015 4:00:26 AM  
 Data Processed : 8/4/2015 4:09:30 AM



PeakTable

Detector A Ch1 230nm				
Name	Ret. Time	Area	Theoretical Plate#	Tailing Factor
Amoxicillin Trihydrate	2.264	11617152	3267.615	1.067
		11617152		

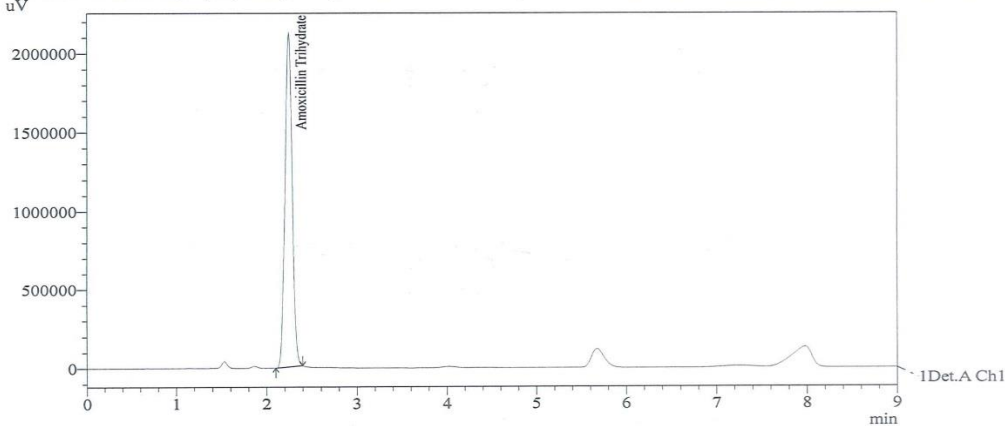
**Figure22:** HPLC Chromatogram for Reconstituted Amoxicillin Trihydrate Suspension (sample A1 at 30°C)

**Pharmaland pharmaceuticals  
shimadzu LC-2010 CHT  
HP-QC07**

Acquired by : Admin  
 Sample Name : Penamox 125 D/S 7 days (B2) ACC{ 40°C}.  
 Sample ID : Unk002  
 Tray# : 1  
 Vial# : 6  
 Injection Volume : 20 uL  
 Data Filename : penamox DS 04082015 15.lcd  
 Method Filename : Penamox DS.lcm  
 Batch Filename : Penamox DS 01.lcb  
 Report Filename : report format 1.lcr  
 Date Acquired : 8/4/2015 5:48:59 AM  
 Data Processed : 8/4/2015 5:58:05 AM

Chromatogram

Penamox 125 D/S 7 days (B2) ACC{ 40°C}. C:\...LCsolution\Sample\finished\data file\Penamox ds\penamox DS 04082015 15.lcd  
 uV



PeakTable

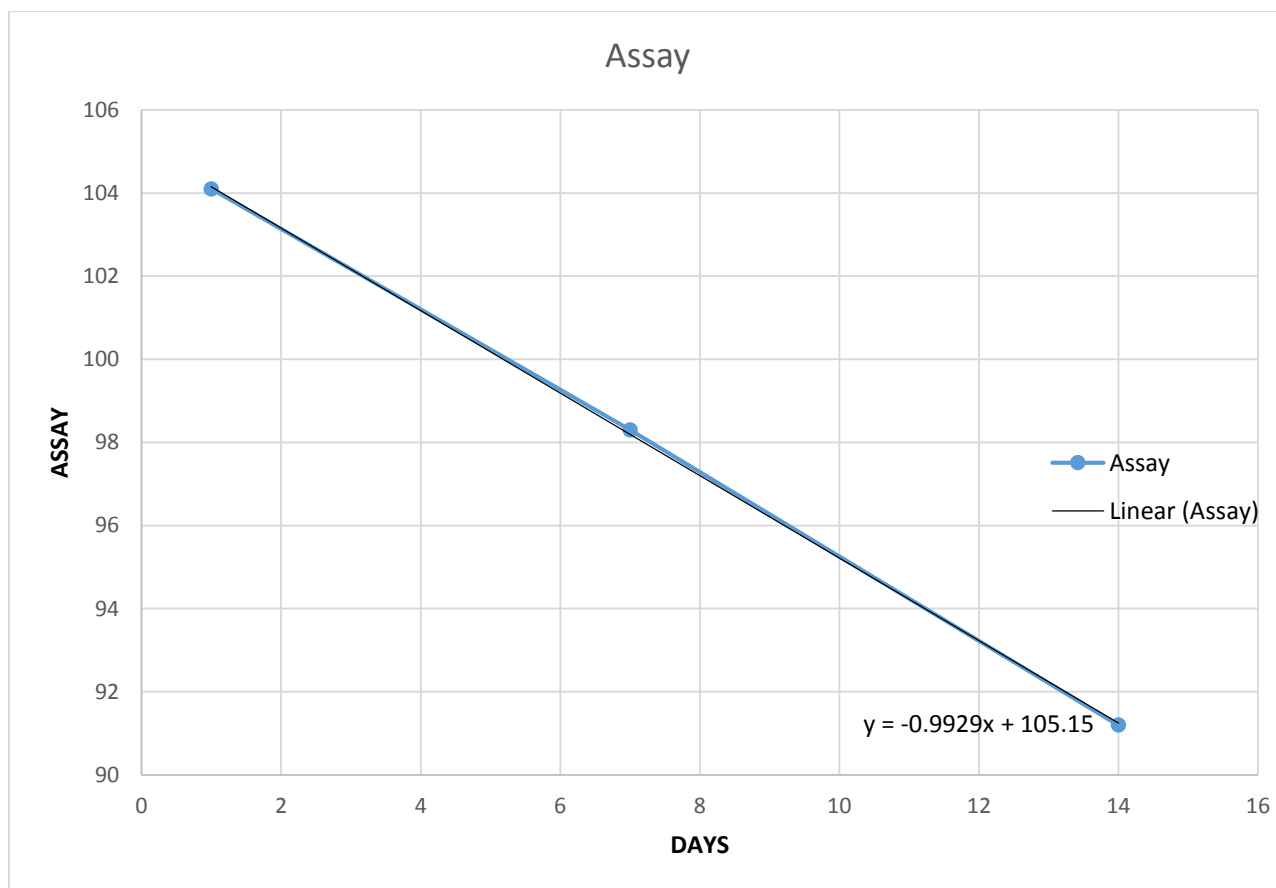
Detector A Ch1 230nm				
Name	Ret. Time	Area	Theoretical Plate#	Tailing Factor
Amoxicillin Trihydrate	2.244	11612345	3210.785	1.070
		11612345		

**Figure23: HPLC Chromatogram for Reconstituted Amoxicillin Trihydrate Suspension (sample B2 at 40°C)**

#### 4.5-Degradation of amoxicillin versus time period under different Temperature

##### 1-Sample A1 at 30 °C

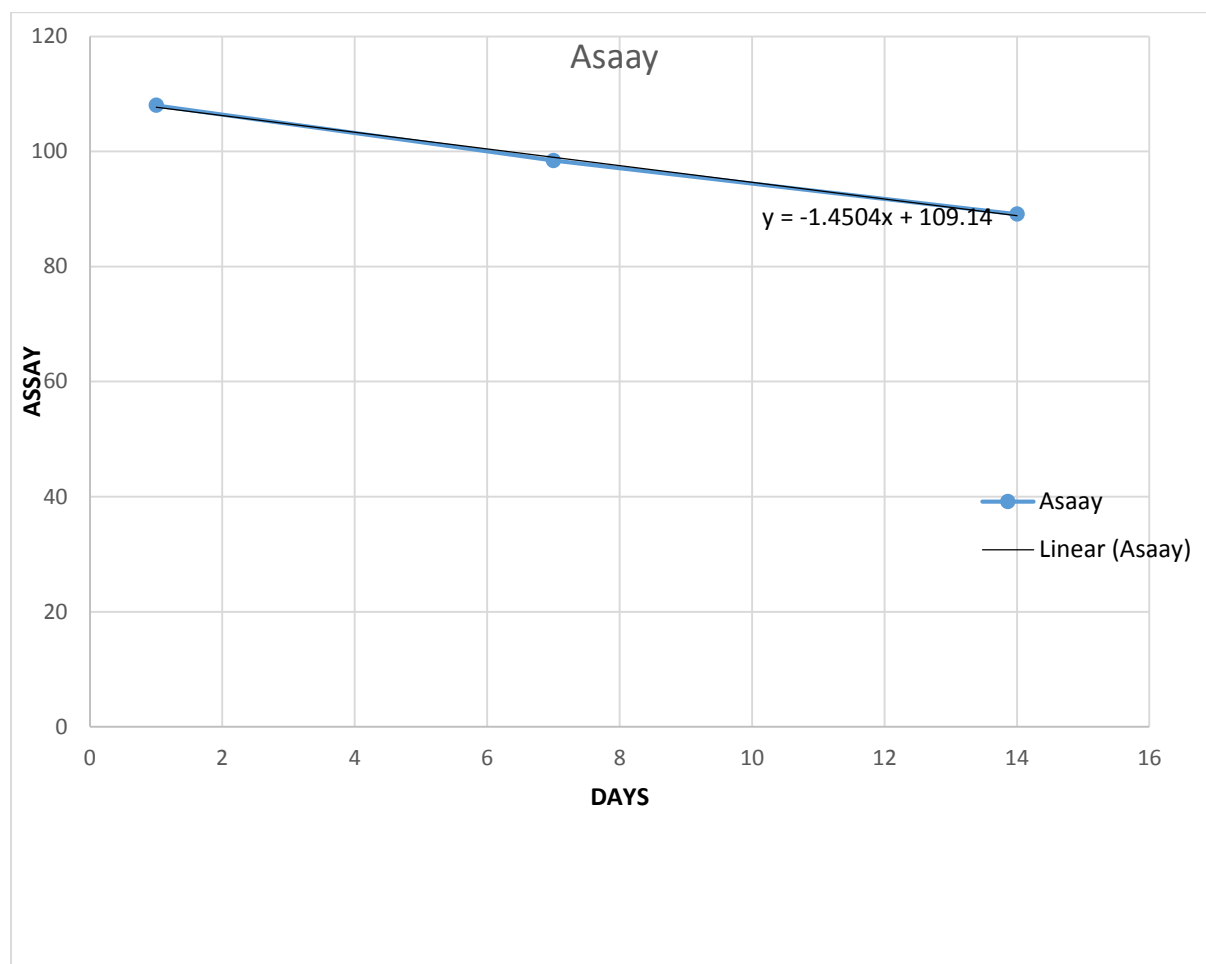
Days	Assay
1	104.1
7	98.3
14	91.2



**Figure24:** Degradation of Reconstituted amoxicillin Trihydrate Suspension  
Sample (A1) versus time under 30 °C

## 2- Sample B1 at 30 °C

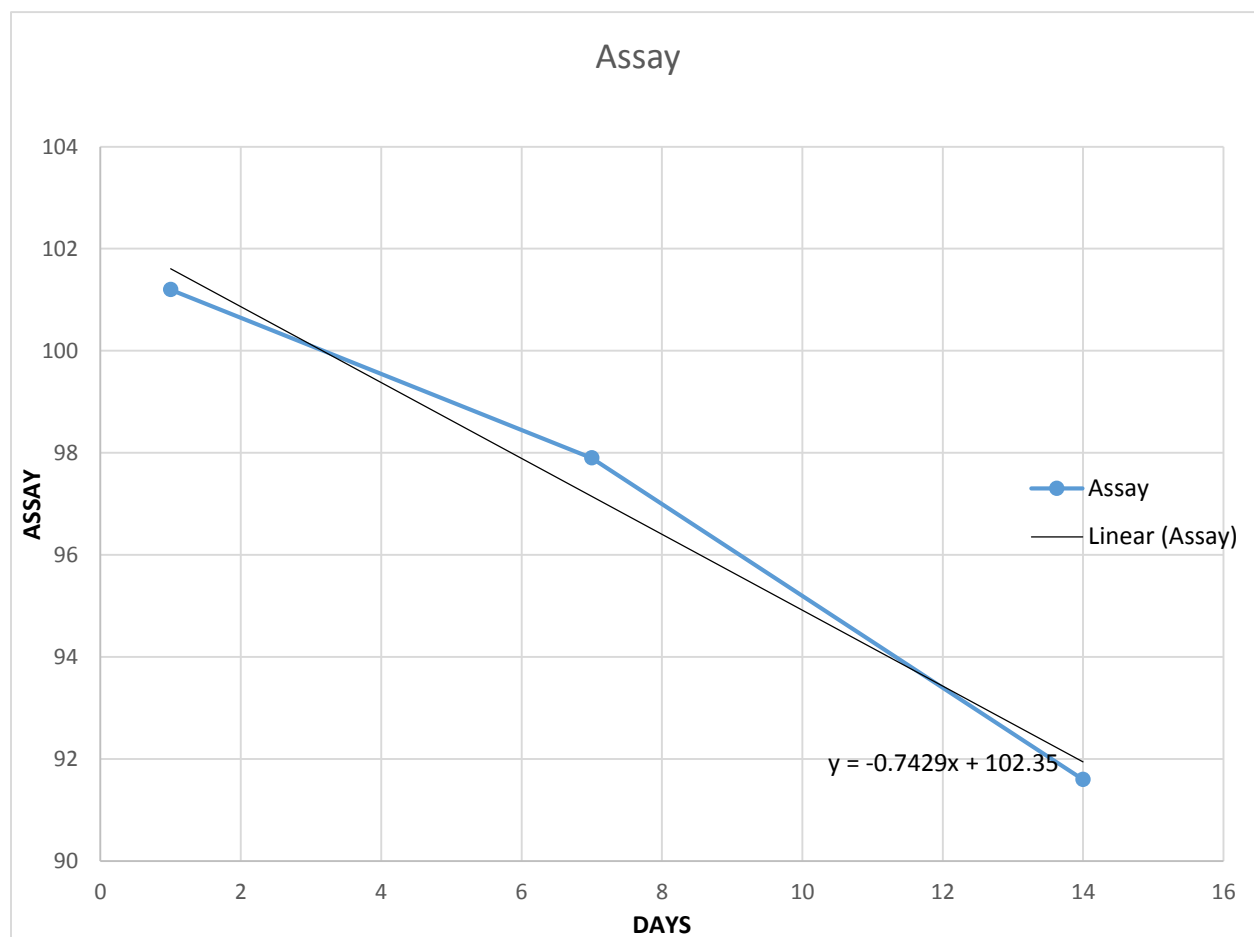
Days	Assay
1	108.0
7	98.4
14	89.1



**Figure25:** Degradation of Reconstituted amoxicillin Trihydrate Suspension  
Sample (B1) versus time under 30 °C

### 3-Sample C1 at 30 °C

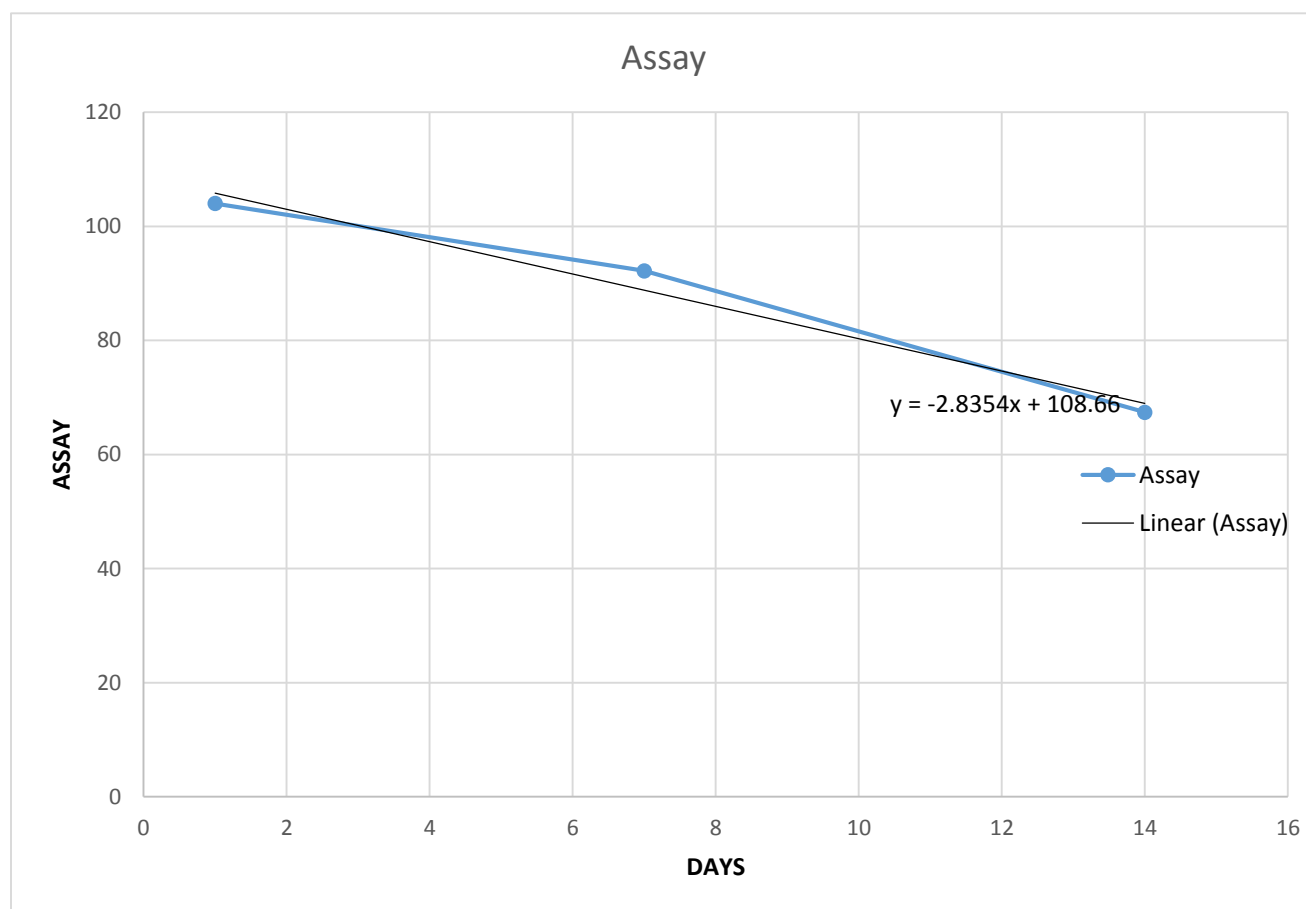
Days	Assay
1	101.2
7	97.9
14	91.6



**Figure26:** Degradation of Reconstituted amoxicillin Trihydrate Suspension  
Sample (C1) versus time under 30 °C

#### 4-Sample A2 at 40 °C

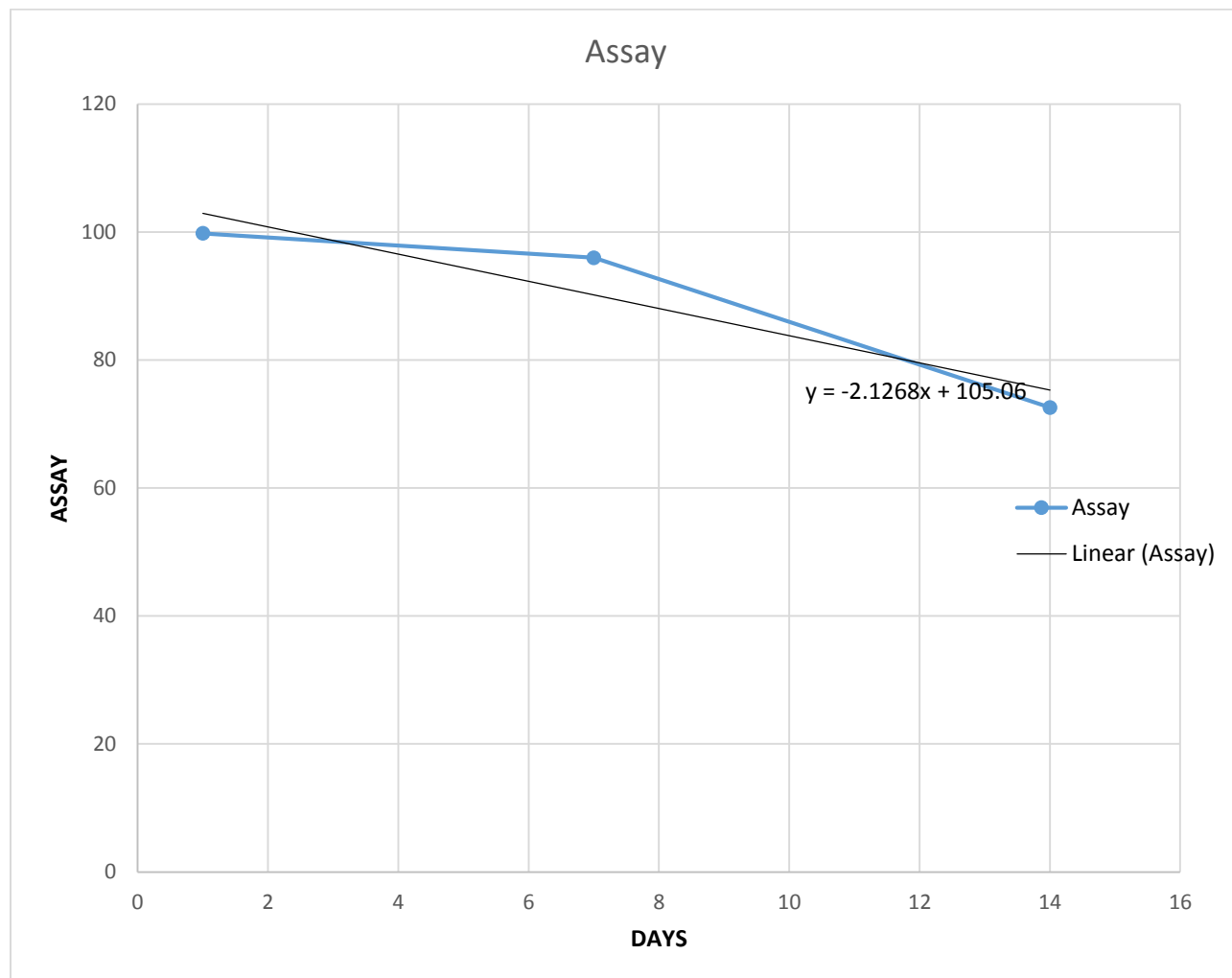
Days	Assay
1	104
7	92.2
14	67.4



**Figure27:** Degradation of Reconstituted amoxicillin Trihydrate Suspension  
Sample (A2) versus time under 40 °C

### 5-Sample B2 at 40 °C

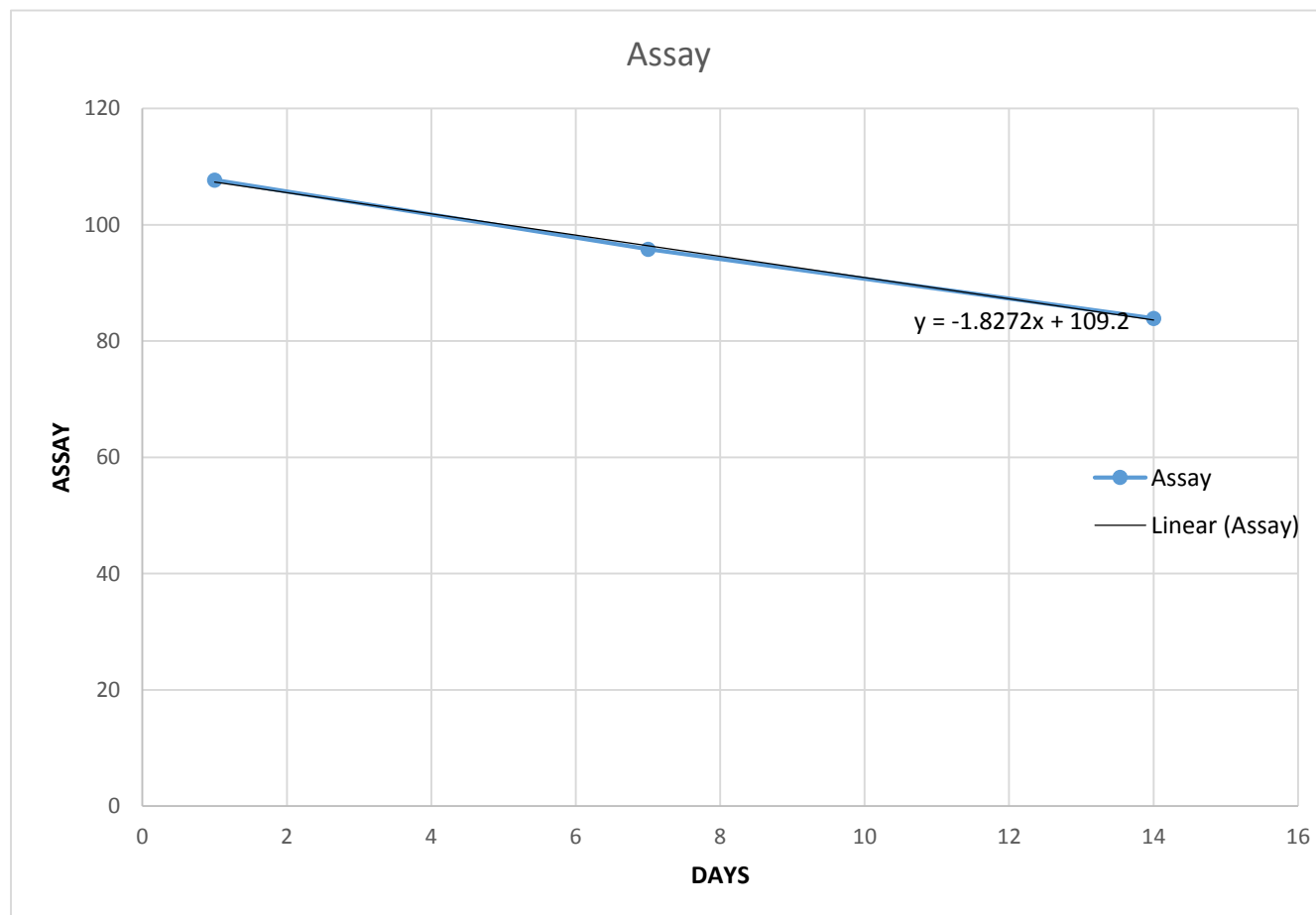
Days	Assay
1	99.8
7	96.0
14	72.6



**Figure28:** Degradation of Reconstituted amoxicillin Trihydrate Suspension  
Sample (B<sub>2</sub>) versus time under 40 °C

## 6- Sample C 2 at 40 °C

Days	Assay
1	107.7
7	95.8
14	83.9

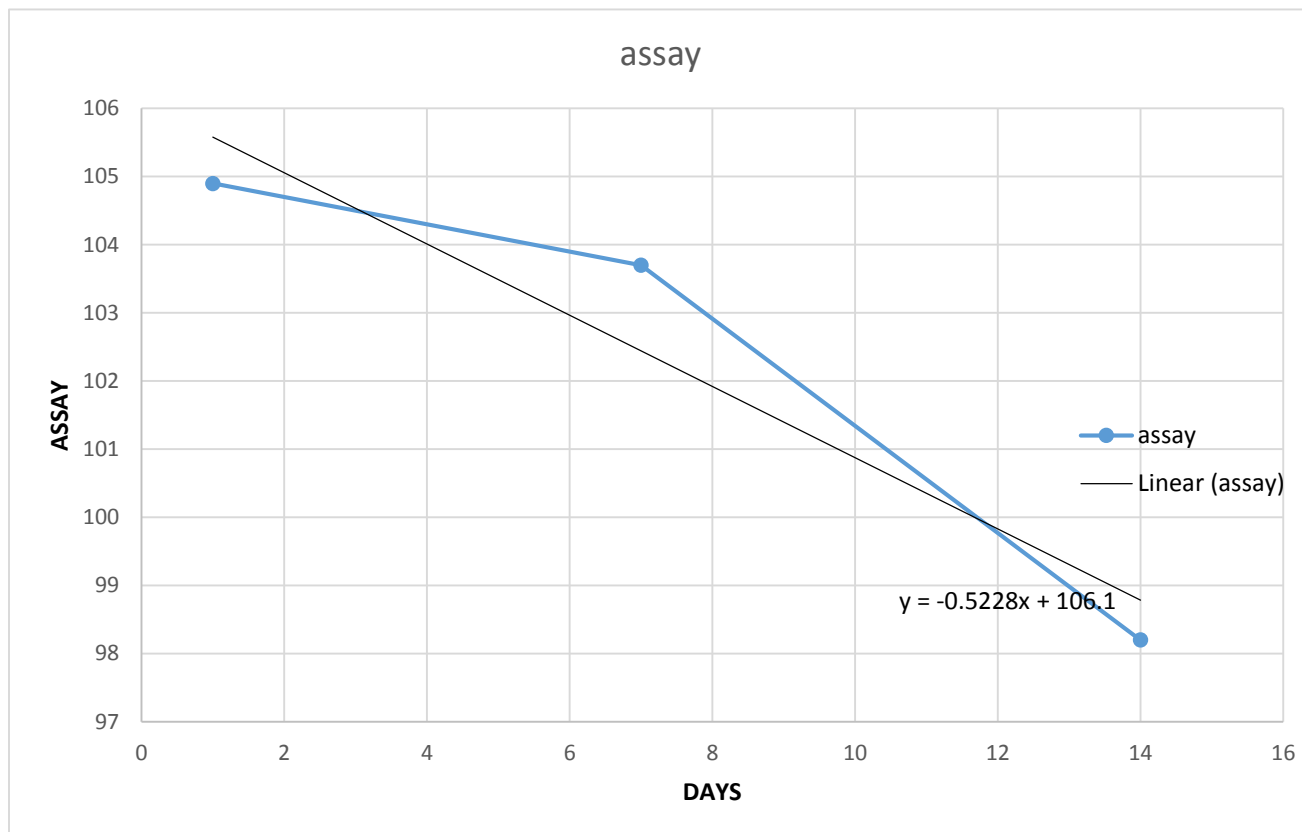


**Figure29:** Degradation of Reconstituted amoxicillin Trihydrate Suspension  
Sample (C2) versus time under 40 °C



### 7-Sample A3 at 8 °C

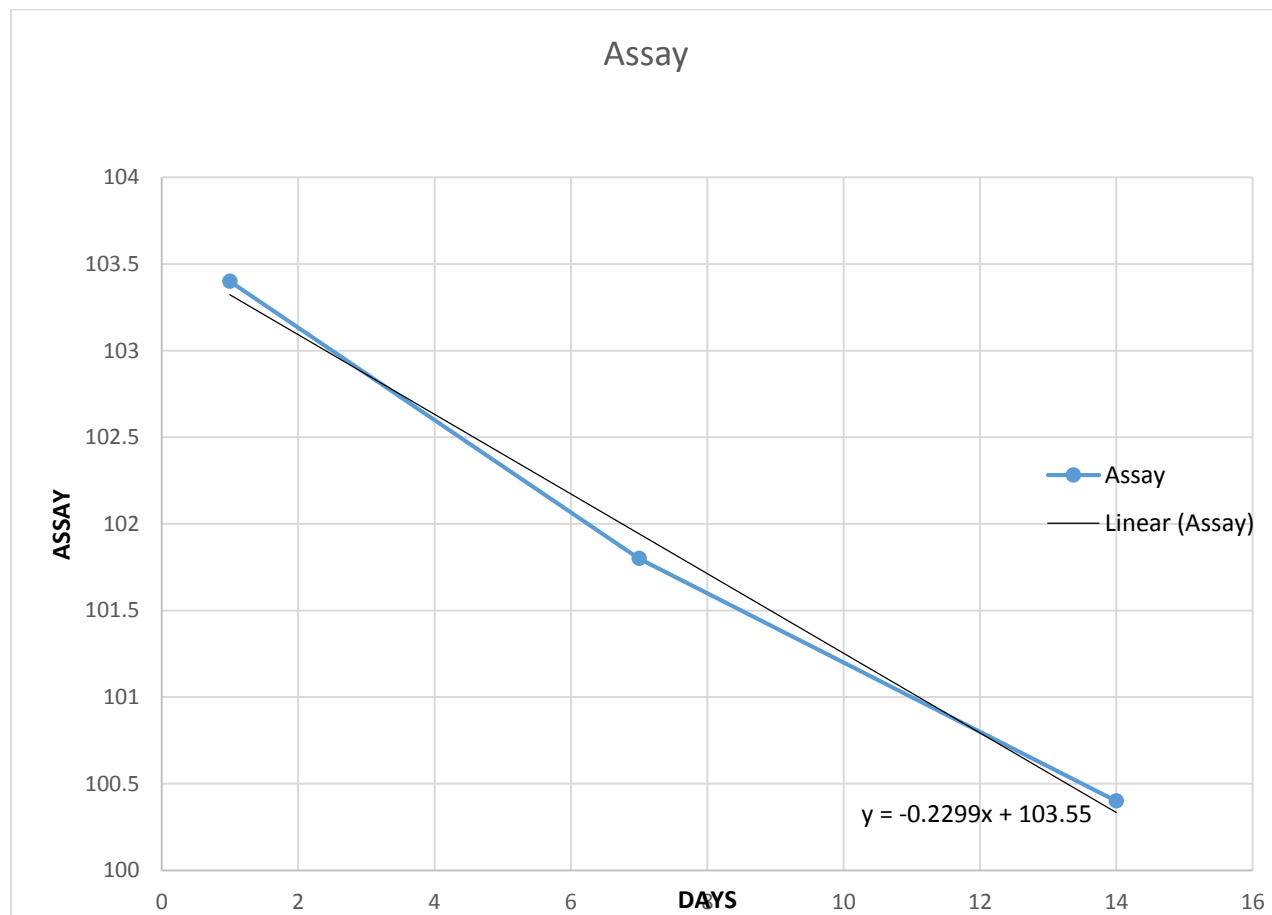
Days	assay
1	104.9
7	103.7
14	98.2



**Figure30:** Degradation of Reconstituted amoxicillin Trihydrate Suspension  
Sample (A3) versus time under 8 °C

### 8- Sample B3 at 8 °C

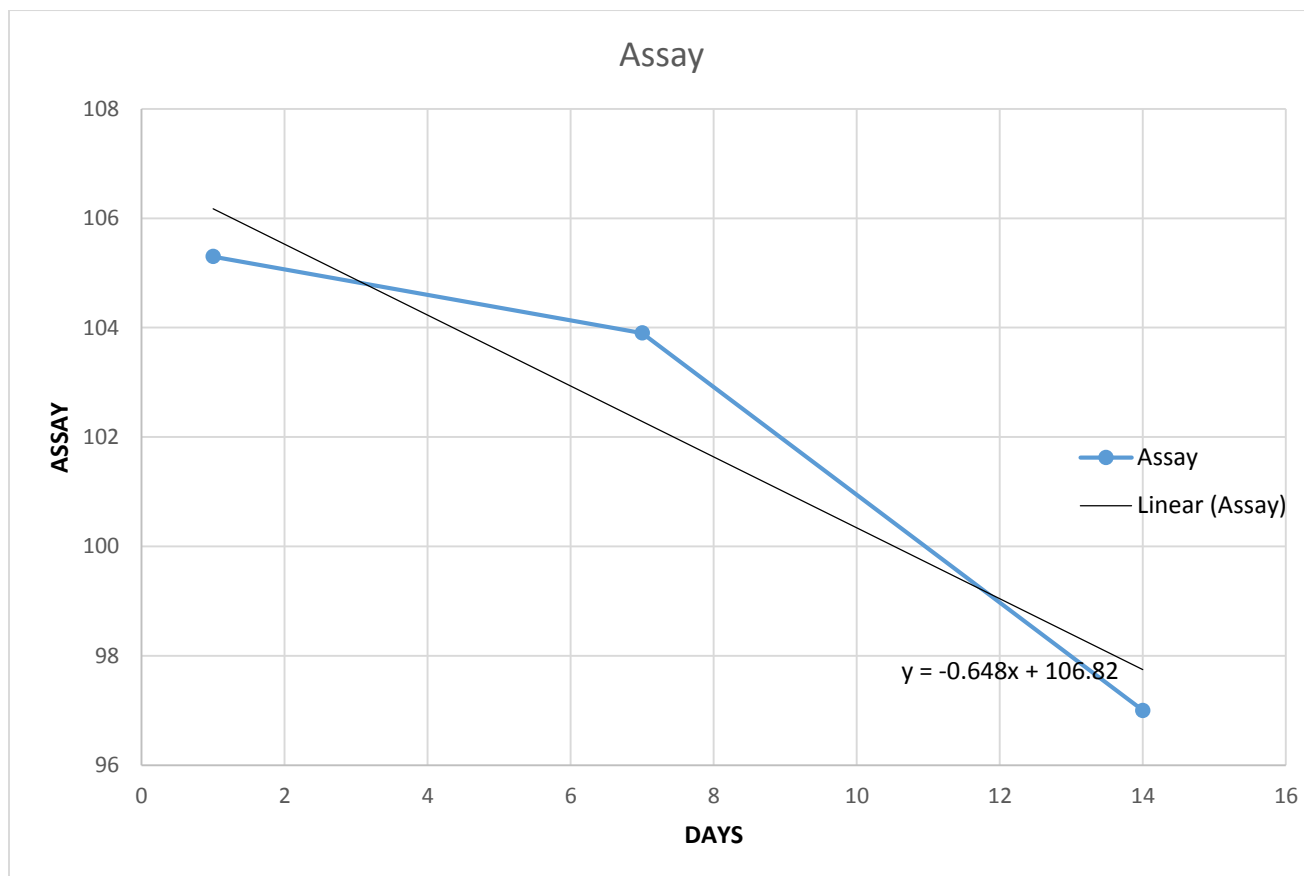
Days	Assay
1	103.4
7	101.8
14	100.4



**Figure31:** Degradation of Reconstituted amoxicillin Trihydrate Suspension  
Sample (B3) versus time under 8 °C

### 9- Sample C3 at 8 °C

Days	Assay
1	105.3
7	103.9
14	97.0



**Figure32:** Degradation of Reconstituted amoxicillin Trihydrate Suspension  
Sample (C3) versus time under 8 °C

#### **4.6- CONCLUSION**

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 the melting ranges for amoxicillin were 193 - 195°C.

The BP states that amoxicillin oral suspension should contain an equivalent of not less than 80.0 percent and not more than 120.0 percent of the labeled amount of amoxicillin. All but for two samples of one of the three brands of oral suspensions of amoxicillin analyzed passed the assay, The samples of that failed the assay which kept in accelerated stability chamber (40°C) had a mean percentage content of 67.4%, 72.6% for amoxicillin trihydrate.

#### **4.7- RECOMMENDATIONS**

Further stability studies should be carried out on the reconstitution of amoxicillin oral powder with different kinds of water kept under nonstandard storage conditions using unofficial methods.

Also in order to prevent antibiotic resistance and achieve maximum therapeutic effect of amoxicillin oral suspension, the first line of storage which is refrigeration of drug at 8°C should be emphasized and encouraged. This will ensure that active ingredient (Amoxicillin trihydrate) is intact for maximum therapeutic benefit.

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