THE DEVELOPMENT OF SOME SPECTROPHOTOMETRIC METHODS FOR THE QUANTITATIVE ANALYSIS OF PENICILLINS

BY

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APRIL 1982

DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree in any other institution or university. Information derived from the published or unpublished work of others has been acknowledged in the text.

Ajayi
Candidate (Signature)

24/3/82
Date

DEDICATION

I dedicate this research effort to the memory of my late uncle; Mr. Simeon Sanya and to my Father; Mr. Oluwatoyin Owolabi Ajayi.

ACKNOWLEDGEMENT

I wish to express my sincere gratitude and appreciation to my supervisors; Dr. M.S. Elmarakby and Dr. S.A. Okecha, whose keen interest, encouragement, inspiration, constructive criticism and useful suggestions helped in carrying me through the project.

I am also grateful to our coordinator; Dr. K. Singh and the Head of Department for providing the necessary guidance and facilities for the research. In the same stance, to Messers Akpeji, Atodo, Obaka and Abubakar for their technical assistance.

I am also grateful to my parents, my cousins, Mr. Tunde Fagoyinbo, Mr and Mrs Omooba, Mr. Bisi Afolabi and my fiancee Ladunni Oluyemi for their moral supports.

ABSTRACTS

The aim of this project was to verify how adaptable. were the proposed methods to the spectrophotometric analysis of penicillins.

The proposed methods are the phosphotungstate and N-ethylmaleimide methods. For both methods, Beer's law is obeyed between 0 and 14 ug/ml with the limit of detection of about 1 ug/ml or less.

These methods were also applied to the quantitative analysis of penicillins in urine and pharmaceutical preparations. For the purpose of comparison, established methods like Pan's method and B.P, 1973

The pharmaceutical preparations employed in the analysis include capsules, injections, tablets and syrups of various trade names and manufacturers.

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CHAPTER ONE

1.1 PENICILLINS

Penicillins belong to a group of therapeutically - important antibiotics of wide usage. They are normally produced wholly or partially as microbial metabolites with ability of inhibiting the growth of, or destroying other microorganisms.

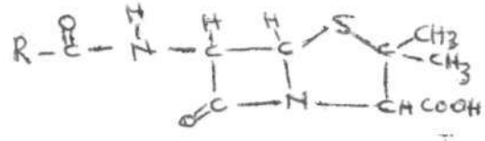
Penicillins form a family of structurally-related antibiotics with more or less the same antimicrobial functions. Penicillin G was the first antibiotic to come into general systemic use, and it still ranks among the best in clinical usage. Indeed some of its properties are unique, and it is nothing short of miracle that so astonishing a substance should have been the first to be discovered. The discovery of penicillin and other antibiotics has helped a great deal in effective control of most bacterial-bourne infectuous diseases that ravaged the world for more than forty-five centuries. With the introduction of penicillins and other antibioties four decades ago, a host of dreaded diseases - from scarlet fever to siphilis-lost their power to disfigure, maim and kill. For this particular reason, antibiotics are known as "miracle drug."

Penicilling G was first discovered in 1929 by Fleming who extracted the substance as a fungal

metabolite of Penicillium notatum from which it derives its name. It was however not immediately possible to obtain the drug in pure forms, until, an exhaustive systemic and chemical studies were carried out by Florey, Chain et al.[2] The first pure form was obtained during the World War II years as benzylpenicillin sodium salt.

Penicillins, chemically are characterised by a bicyclic nucleus comprising of a B-lactam ring fused to a thiazolidine ring with different acylamino side-chains at C-6 position

The activity of this group of antibiotics is therapeutically-dependent upon the intactness of the bicyclic nucleus, in particular, the B-lactam ring. Penicillins and other sister antibioties; cephalosporins, are known as B-lactam antibiotics.



Penicillin

I

The knowledge of the chemistry of penicillins has contributed immensely not only to the striking success in pharmaceutical research, but also to the production of many other equally important derivatives.

Most penicillins have Gram-positive antimicrobial activities, while a few such as ampicillin combine both Gram-positive and negative activities.

Prominent among the clinical uses of penicillins are the treatments of diseases like siphilis, scarlet fever, influenza, gonorrheoea and entritis.

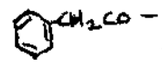
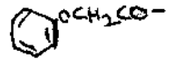
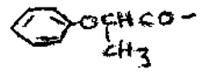
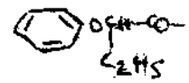
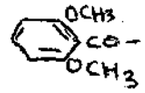
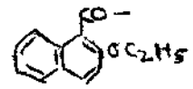
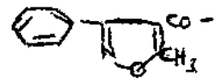
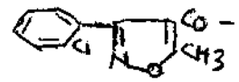
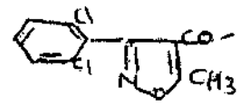
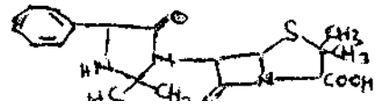
Today, many hundreds of penicillins are available, but only a few are clinically useful. The most useful ones include penicillin G, penicillin ^{V}, ampicillin, cloxacillin and amoxycillin.

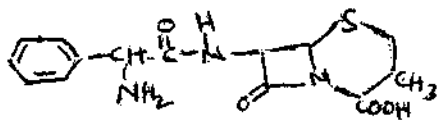
There are a group of other structurally-related antibioties called cephalosporins. Cephalosporins, like penicillins have a bicyclic nucleus with intact B-lactam ring. Unlike penicillins, they have a six-membered dihydrothiazolidine ring instead of five-membered thiazolidine ring.

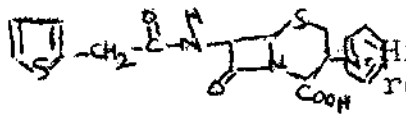
The antibacterial activities are sometimes inhibited by the cleavage of B-lactam ring by secretion of penicillinase enzymes. This constraint, to a great extent, limits the spectrum of activities of a number of penicillins. The structures of cephalosporins are probably responsible for their penicillinase-resistance. The structural modification has also been exploited in production of other penicillinase resistant penicillins like cloxacillin.

Table 1.1 shows a list of some B-lactam antibiotics of general use[8,9,12,20,23].

TABLE 1.1 SOME PENICILLINS

Generic name	Structure (R)	Penicillinase activity	Antibacterial spectrum
Penicillin O	$CH_2=CH_2-S-CH_2CO-$	Susceptible	Gram positive
Benzylpenicillin or Penicillin G		Susceptible	Gram positive
Penicillin V		Susceptible	Gram positive
Phenethiallin		Slightly resistant	Gram positive
Ampicillin		Slightly resistant	Broad Spectrum
Epicillin		Slightly resistant	Broad Spectrum
Propicillin		Slightly resistant	Gram positive Cocei
Methicillin		Highly resistant	Gram positive
Nafcillin		Highly resistant	Gram positive
Oxacillin		Highly resistant	Gram positive
Cloxacillin		Highly resistant	Gram positive
Dicloxacillin		Highly resistant	Gram positive
Hetacillin		Highly resistant	Broad Spectrum

Cephalexin  Highly resistant — Broad Spectrum

Cephaloridine  Highly resistant — Broad Spectrum

Cephalexine and cephaloridine have a dial advantage of penicillinase resistance and broad spectrum of activity over penicillins. These properties though unique, and rare, have not made cephalosporins more versatile than penicillins.

It is essential to distinguish between naturally-produced and semi-synthetic penicillins. The former are produced by selective culturing of fungal molds, while the latter are by total synthesis or acylation of 6-aminopenicillanic acid partially produced by molds. The natural penicillins include benzylpenicillin, penicillin V, penicillin F, penicillin O and penicillin K. Examples of semisynthetic penicillins are ampicillin, amoxycillin, cloxacillin and oxacillin. The effect of modification of the structure of the naturally produced penicillins has further enhanced the versatilities of penicillins.

1.2. MEDICINAL USES OF PENICILLINS

The demonstration of the clinical usefulness of penicillins initially stimulated pharmaceutical industries

and research centres to screening thousands of microorganisms for antibiotics production[23].

The clinical applications of penicillins are wide and varied. It is observed that a bulk of deseases plaguing human and animal lives emanates from bacterial and viral infections. These deseases have been brought under control since the introduction of penicillins. However, the resistance of certain microorganisms to these drugs, has led to the production of new and effective alternatives.

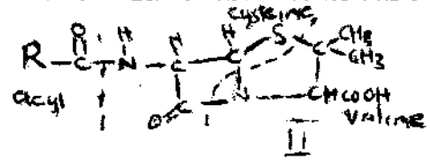
For any drug to be clinically useful, specificity, stability, cost, potency and low incidence of undesirable effect are its most desirable attributes. These attributes are inherent in penicillins, which make them clinically useful. The most important uses of penicillins are in the treatments of ear and eye infections, gonorrheoea, lues, siphilis, scarlet fever, pnuemonia, erysipelas, carbuncles, sinusitis, pyodermia, rheumatic polyarthritis, septicaemia, angina tonisilitis, meningitis, peritonitis, bronchitis and bacterial endocarditis.

1.3. CHEMISTRY OF PENICILLIN

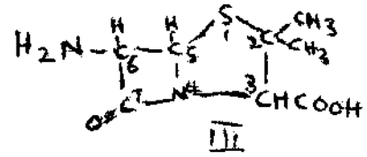
Penicillin is an organic compound with N-substituted and N',N-substituted amides and carboxylic functional groups within its molecular framework. These groups in

one way or the other contribute to the chemical properties of the compound.

(a) Structure:- Penicillins have a bicyclic nucleus of B- lactam ring fused with a thiazolidine ring, which make the molecule unique. Early studies with isotopically-labelled aminoacids established that cysteine and valine form the bicyclic nucleus[1]. This is indicated by the broken line in structure II below.



II



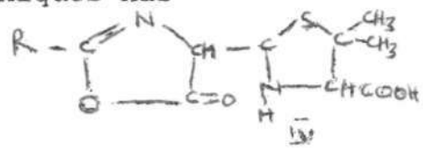
III

L-cysteinyl-L-valine (or an acylderivative of it) appears to undergo an oxidative condensation to yield a (3-lactam ring -B-dehydrogenation of the L-valine residue followed by the addition of sulphur (cysteinyl) to the double bond to form a thiazolidine ring with the D-configuration at C-3 could lead to the formation of 6-aminopenicillenic acid. This acid (6-A.P.A.) has been isolated from the fermentation product of penicillium chrysogenum to which no side-chain precursor was added. (Batchelor, Doyle et al)[1,3]

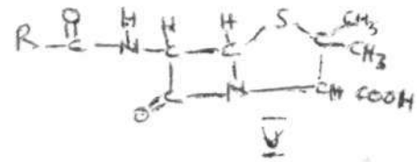
The above information is one of the conclusive information on penicillin structure. The final elucidation of penicillin structures is as a result of bulk of information obtained from chemical degradation, infrared, ultraviolet and nuclear magnetic resonance spectroscopic

data, mass spectrometry, polarimetry and other physico-chemical data. The accounts of their elucidation presented an impressive saga of scientific achievements and afford an insight into the recent development of degradative and diagnostic techniques. The choice of the most probable structure of penicillin has been greatly assisted by the progressive increase in use of physico chemical methods.

Two structural models are proposed as possible structure of penicillins. These are oxazolone (D) and B-lactam structures [18,20]. The use of physico chemical techniques has



IV



V

contributed immensely to reducing the amount of necessary degradative work, and also provided the much needed answers to these otherwise insoluble structural problems. Both structures are probable but structure V is more likely, judging from the following evidences;

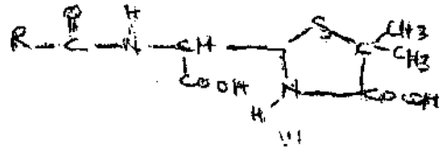
(i) Infra-red spectral studies of models of both structures confirm structure V (B-lactam); due to the characteristic absorption of B-lactam at 1760cm^{-1}. In addition, infra-red screening also confirms the structure[20,29]

(ii) Thermodynamically, B-lactam ring of V is stronger than the lactone ring of structure IV. This is another point in favour of structure V.

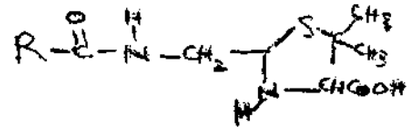
(iii) The x-ray analysis at sodium, potassium, and rubidium salts of benzylpenicillin clearly showed the presence of a B-lactam ring.

(iv) Proton 'H and carbon ^{13}C n.m.r. spectra also confirm B-lactam structure of penicillins.

(v) Another conclusive evidence is obtained from the degradative studies of penicillins. The fact that penicillins decompose to penicillamine and penilloaldehyde on treatment with mercuric chloride indicates the rupturing of the bicyclic nucleus. This reaction is characteristic of thiazolidine ring. In another perspective, the studies of reactions leading to the formation of penicilloic acid (VI) in basic medium strongly indicates the presence of B-lactam ring. This product is due to the cleavage of the B-lactam ring of structure V



penicilloic acid



penilloic acid

The simple fact that penicilloic acid is obtained from penicillin invalidates the stability and possibility of oxazolone (IV) structure and hence supports B-lactam structure. Another supporting evidence is obtained from the fact that methanol-treated penicillins yield methyl-penicilloic acid, which also indicate the cleavage of B-lactam ring by methanol. This derivative also undergoes similar reaction with aqeous mercuric chloride.

(vi) Other less important information are obtained from mass spectra, differential thermal analysis and ultraviolet spectral studies.

(b) Physical and Chemical properties of penicillins

The physico-chemical properties of penicillins are exhaustively discussed by Florey[21], Finar[20], Clark[12], Hilton[26] and British Pharmacopoeae[8,9].

Penicillins in their pure states are usually white, crystalline, odourless powders with bitter tastes. The presence of assymetric carbon atom in their molecules, confer optical activities on them. The solubility is a function of the chemical state; i.e. polar or nonpolar nature of penicillins. The highly polar alkali metal salts are soluble in water, primary alcohols, mineral acids, bases, formamide and dimethysulphoxide. On the other hand, the amine salt and free acid are insoluble in polar solvents but soluble in non-polar solvents like

carbon disulphide, chloroform, pyridine, ethers, esters, acetone and carbon tetrachloride.

Penicillins are characterised by high melting points in the range of 160° to 250°C,

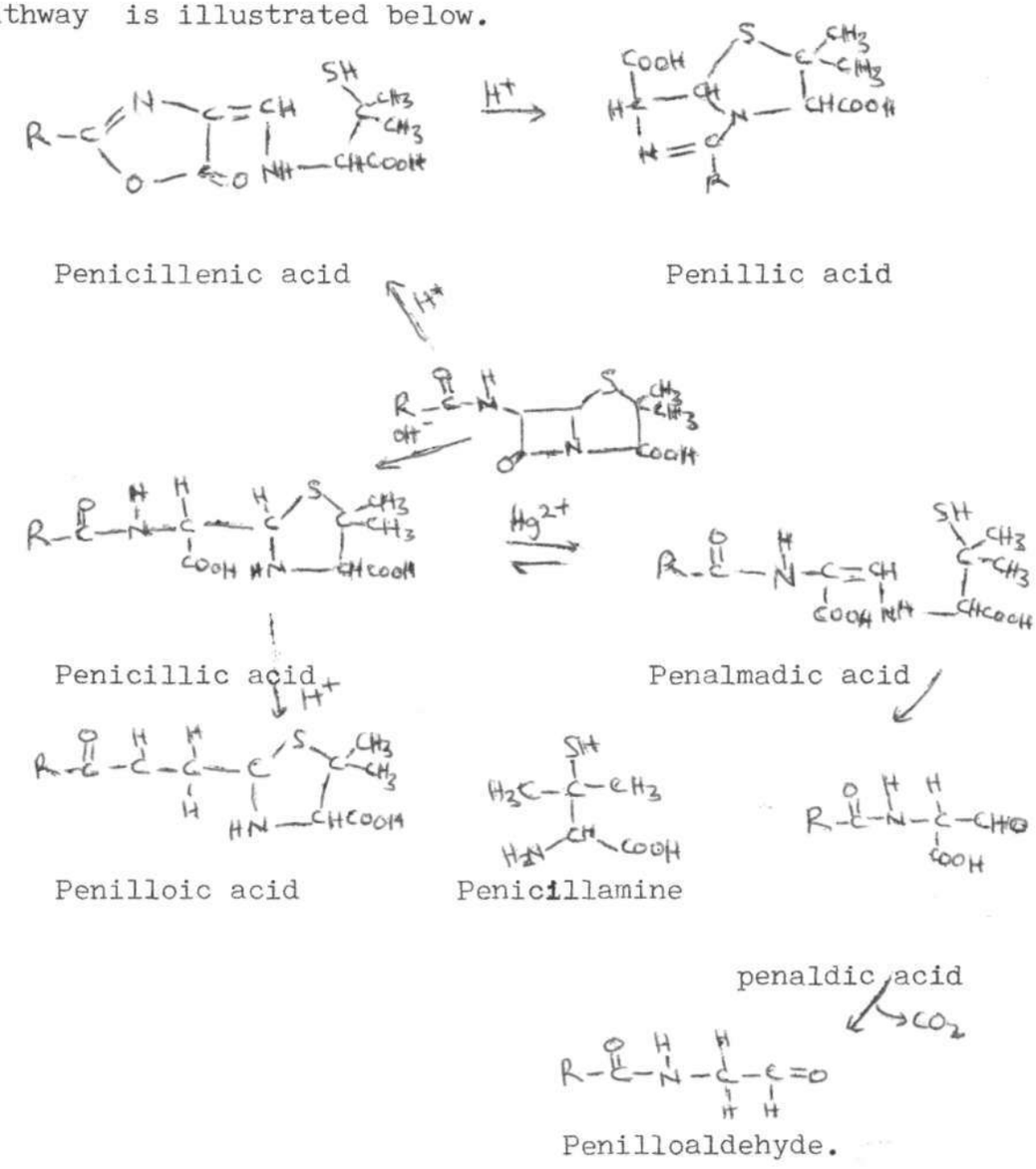
Chemically, penicillins are monobasic acids with pka values between 2.3 and 4.73. Like acids, they are capable of neutralising bases to form salts. The lability of their structures in solution, however, militates against their acid properties. They are susceptible to both nucleophilic and hydrophilic attacks on their B-lactam ring. They are generally stable within the pH range of 5 to 8, beyond which, they rapidly degrade through a serves of reactions. They are also susceptible to degradation by aqeous mercuric salts, a reaction due to the thiazolidine ring. They form esters with diazomethane, the derivatives which undergo similar reaction with aqeous mercuric ions.

Penicillins give characteristic odour reactions with chromotropic acid at 150°C.[8,18,21,25,29].

c. Stability and hydrolytic degradation.

The penicillin structure is generally labile, this a property has been exploited in various chemical conversions. The end-product of these conversions is a function of various factors such as temperature, pH, nature of solvent media and the influence of cations. The common degradative

pathway is illustrated below.

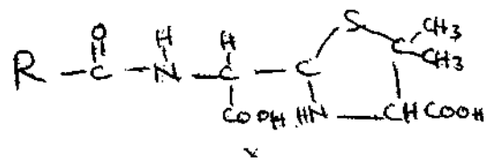


The B-lactam ring is susceptible to nucleophilic attack in aqueous and alkaline media, with resultant formation of corresponding penicilloic acid. In alkaline solution, this is obtained as a stable salt, but in acidification, the free acid rapidly decarboxylates to the corresponding

penilloic acid. In mild acidic solutions, penicillin undergoes an intramolecular rearrangement into penicillenic acid which is partially stable. This penicillenic acid is rapidly converted to penillic acid on further acidification. Penicillin reacts with aqeous mercuric chloride leading to extensive degradations whose final products are penaldic acid and penicillamine. These series of reactions have been exploited in many sensitive analytical techniques for estimation of penicillins. Cressman[14] reported the formation of penicilloic acid in presence of cupric ions side-chains in semi-synthetic penicillins appear to influence the rate of decomposition in acidic solutions rather than the mechanism of reactions[21,25]

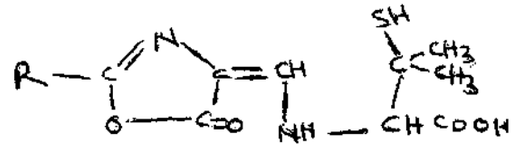
Ampicillin and other penicillins with free amino groups are capable of undergoing intramolecular condensation in acidic and basic media. The formation of these derivatives are due to the presence of free - amino groups. These products are fluorescent in nature. It is also important to briefly discuss some of the degradation products as some of them are of analytical importance.

Penicilloic acid: This is a dibasic, dicarboxylic acid with intact thiazolidine ring.



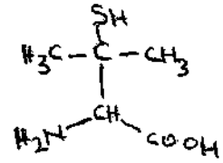
It is formed as a result of the cleavage of the B-lactam ring of penicillins. It is important in both titrimetric and spectrophotometric estimations of penicillins.[29,35] It is unstable as a free acid, since it rapidly decarboxylates to penilloic acid.

Penicillenic acid: This is an intramolecularly-rearranged penicillin with a free thiol group and a five-membered lactone ring. The free SH is formed from therupturing of the thiazolidine ring by treatment with cupric ions in an appropriate pH medium.



This compound is characterized by its ability to absorb in the uv-region, a property exploited in the Herriot, B.P. 1973 methods of spectrophotometric analysis of penicillins[7,8,21,25,29,42]

Penicillamine: This is a universal product obtained from the complete degradation of penicillins. It is an amino acid with a free thiol group also known as B,B'dimethyl-cysteine



XI

As an amino acid, it exhibits zwitterion and isoelectric effects, neutralizes bases, form organic halides and gives colour reaction with ninhydrin.

As a mercaptan, it gives colour reaction with phospho-18-tungstic acid,[8,49] sodium nitroprusside[21].

Elman's reagent (S,S'dithiobis(-nitrobensoic acid)[50,51] and N-ethylmaleimide.[2] The ultimate degradation of penicillins to penicillamine has been exploited in many spectrophotometric techniques of penicillin analysis[21,25, 26,27,36,50,51,48]

(d) Preparation of penicillins

It is important to discuss the various ways penicillins are produced. Penicillins are either prepared from the fungal extract of P. notatum or Pchrysogermn, by chemical synthesis or by the acylation of 6-aminopenicillenic acid.

(i) Preparation from the fermentation media involves the extraction of penicillins as biosynthetic metabolites of the cultured molds. It is noted that the type of penicillin derived from a given medium is related to the type of side-chain precursor present in such medium. There is preferential formation of a penicillin that has its side-chain precursor within the medium. It was discovered that many substituted phenylacetic acids could act as a precursor, and a considerable number of new penicillins have been prepared in this way. There are many limitations to this mode of preparation such as toxicity of the substituent and the non-incorporation of the desired substituent into the penicillin skeleton.

(ii) Total synthesis of penicillins is as a result of the conclusive elucidationof penicillin structure. The first successful synthesis of penicillin was carried out by Sheehan,[1,18,20] who synthesized phenoxymethylpenicillin

starting with t-butylphthalidimidomalonaldehyde.

The penicillin V so synthesized was equally effective as the natural product. The initial problem confronting synthesis was the cyclisation of B-lactam ring which has since been effectively controlled. The ability to synthesize 6-aminopenicillanic acid has also enhanced the synthesis of many penicillins by direct acylation of this acid.

(iii) Semi-synthesis of penicillin resulted from the acylation of 6-aminopenicillanic acid. This acid was successfully extracted from cultured medium devoid of any side-chain precursor. The isolation of this acid also enhanced the study of the probable biosynthetic reactions leading to the formation of penicillins. The acylation of this acid has made it possible not only to produce natural penicillins but also some new semi-synthetic penicillins such as cloxacillin and ampicillin. This acid can also be prepared by enzymatic or chemical hydrolysis of penicillin G.

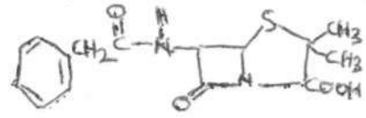
1.4 SOME SELECTED PENICILLINS

For this project, some specially selected penicillins were used to illustrate the findings. The four types of penicillins used were penicillin G, penicillin V, ampicillin and cloxacillin. These were chosen on the basis of their relative abundance, cost and clinical importance.

1.4.1 BENZYL PENICILLIN (PENICILLIN G)

This is the first pure form of penicillins be prepared in large scale and applied for general systemic use. It is one of the most common and important penicillins. Usually marketed as sodium, potassium and calcium salts of injection powder. It is available and prescribed in injection form due to its acid lability. Other forms of the drug are organic amide types like procaine, hydrabamine, dibenzylamine, benzathine and N-ethylpiperidine penicillin: G. These are all non-polar forms prepared to delay the absorption in the body system, and so prolong its effect on the target organ. Physically, the salts are usually white, crystalline, odourless and bitter-tasting powders. The salts are very soluble in water, alcohols and alkali.

Like all penicillins, penicillin G is a monobasic acid with pka value of 2.76 for both its sodium and potassium salt form. By virtue of the benzene ring, it absorbs maximally at 2.57 nm in water, and 263 nm in ethanol. Optical rotation is $[\alpha]_D^{25}$ +301° for monosodium salt and $[\alpha]_D^{25}$ +285° for potassium salt.



XII

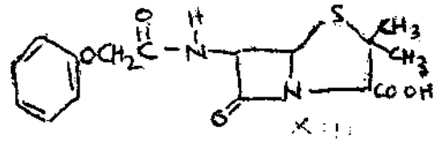
In the chemical abstracts, it is found as 4-thia-1-azabicyclo (3.2.0) heptane-2-carboxylic acid, 3,3dimethyl-7-oxo-6 (phenylacetyl)amino)- Simply as benzylpenicillin.

Chemically, it undergoes all the reactions characteristic of penicillins. It gives a pale yellow colour reaction with chromotropic acid. It has a Gram positive spectrum of action.

1.4.2 PHENOXYMETHYLPENICILLINS (PENICILLIN V)

This is another clinically important penicillin used as alternative for penicillin G in prescriptions requiring oral dosage. Like penicillin G, it has a Gram-postive spectrum of activity.

In the chemical abstract, it is found as 4-Thia-1-azabicyclo(3.2.0)heptane -2=carboxylic acid, 3,3dimethyl-7-oxo-6 (phenoxyacelyl)amino)- It is available potassium, calcium and aluminium salt forms and in some



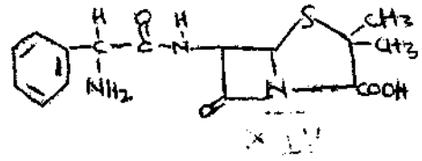
cases as hydrabamine derivative. The phenoxyacetyl side-chain precursor is the probable source of the acid-resistance property.

The compound is white, crystalline, odourless powder with bitter taste. The salt forms are soluble in water, ethanol and dilute alkali solutions. The free acid form is slightly soluble in water and carbon disulphide, while it dissolves in methanol, isopropanol, ethanol and acetone. It is optically active with optical rotation of $[\alpha]_D^{25}$ +265°

in water; and has a pka value of 2.72. It absorbs maximally at 268 nm and 274 nm in water, 263 nm in chloroform and 263 and 268 nm in methanol. It undergoes the characteristic reactions of penicillin. It gives a dark-blue colour reaction with chromotropic acid.

1.4.3 AMPICILLIN

This is a very important, broad spectrum, semi-synthetic penicillin. It is available as trihydrate in capsules, syrup and tablet dosage forms. This drug is very unique in that it is both acid-resistant and broad spectrum in action. It is susceptible to penicillinase - deactivation as a result of which it is sometimes administered as a mixture with cloxacillin.



In the chemical abstract, it is known as 4-Thia-1-azabicyclo (3.2.0)heptane-2=carboxylic acid, 6- (aminophenylacetyl) amino) 3,3dimethyl-7-oxo- Simply known as D-α-(-)amino benzylpenicillin.

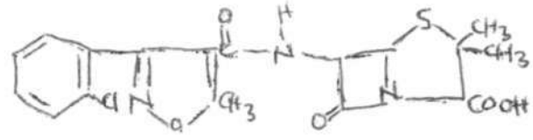
Chemically, it gives colour reactions with ninhydrin, fehling's reagent (potassium cupric tartarate), dimethoxy-quinone, dimethylglyoxal and p-rosaniline. These reactions are characteristic of the compound by the virtue of the presence of amino group. Another unique property is that ampicillin yields fluorescent derivatives with acid, dilute alkali solutions, and uranylacetate[29]. These reactions

form the basis of some sensitive and specific analytical techniques of ampicillin estimation[21,25,26,29]. It gives a violet colour response to chromotropic acid treatment at 150°C.

Physically, ampicillin is white, crystalline, odourless powder with a sharp bitter taste. The presence of an assymetric carbon atom alpha to the benzene ring confers an optical activity of $[\alpha]_D^{25}$ + 281° on the molecule. It melts with decomposition at 202°C. The Pka values are 2.54 and 7.24 due to the zwitterion and isoelectric effects. The compound is slightly soluble in water but more soluble in dilute acid and alkaline solutions, alcohols and pyridine.

1.4.4 CLOXACILLIN

This is another important semisynthetic penicillin of wide usage. It belongs to a family of isoxazolylpenicillins with isoxazole ring adjacent to the benzene ring in the structural skeleton. This molecule is probably responsible for the penicillinase - resistant attribute of these drugs. This may be due to the steric effect exerted by the isoxazole on the B-lactam ring - adjacent to it. Other members of this family are oxacillin, flucloxacillin and dicloxacillin.



Cloxacillin.

It is marketed in sodium salt form as injections, capsule, tablet and syrub dosage forms. Sometimes it is marketed as a mixture with ampicillin known as "ampiclox".

Cloxacillin sodium has a molecular weight of 457.89 and it is known in the chemical abstracts as 4-Thia-1-azabicyclo(3.2.0) heptane-2=carboxylic acid, 6-(3(2-chlorophenyl)-5-methyl-4-isoxazolyl carbonyl)amino), 3,3dimethyl-7-oxo monosodium salt. It is more commonly known as 3-O-chlorophenyl-s-methyl-4-isoxazolypenicillin.

The sodium salt is white, crystalline powder with a bitter taste. The melting point is relatively low (170°C) compared with other penicillins. The pka values are temperature dependent; 2.68 at 35°C and 2.73 at 25°C. The optical activity is $[\alpha]_D^{15}$ + 163° in water. The sodium salt is readily soluble in most polar solvents such as water, acid, bases, alcohols and sulphoxides. It absorbs maximally at 274 nm and 264 nm. The infra-red absorption at 1669 cm^{-1} and 1770cm^{-1} are characteristic of B-lactam penicillins. The n.m.r. spectrum indicates a chemical shift at 2.63 due to the isoxazolyl methyl protons[21].

Cloxacillin is prepared by direct acylation of 6-aminopenicillanic acid. It also exhibits the characteristic reactions of penicillins. It gives a purple colour reaction to chromotropic acid at 150°C.

1.5 SURVEY OF METHODS OF ANALYSIS OF PENICILLINS

The diversity of conditions under which penicillins are assayed makes the choice of method to be a critical decision. The following factors come into play when considering the method of choice; (a) penicillin concentration, (b) sample media (c) desired accuracy and (d) desired selectivity.

The penicillin concentration for assay vary from high purity bulk sample to dilute (mcg/ml or mg/ml) sample of biological origin. For assay of very small concentration (0.02 to 10 ug/ml), a microbiological assay technique may be most suitable. This technique is about the most sensitive analytical tool used in the antibiotic's assay. Other equally sensitive methods are uv-spectrophotometric, and colorimetric methods with sensitivity ranging from 1 to 100 mcg/ml or ug/ml.

The medium of a sample also affects the type of method employed, as no method is conclusively applicable to all media encountered. If a high degree of accuracy is desired, chemical methods are preferable to microbiological techniques.

The achievement of assay specificity is dependent upon many factors. The analyst must be aware of all the assay variables and all potential interferences for each sample. The applicability of assay method to a particular sample must be rigorously investigated. Neither

microbiological nor chemical assay technique have absolute specificity. The various methods of penicillin analysis are reviewed by Hughes and Wilson[29], Hilton and Snell[26] and Higuchi[26].

(a) Microbiological assay: These techniques are many and varied. Microbiological assay techniques are by far the most sensitive and conclusive methods employed.in penicillin estimation. The methods have to their credits; sensitivity, adaptation to biological sample and conclusive consideration by official regulations. The different microbiological techiques employed include cup-agar, paper disc, plate agar diffusion and microbiological turbidimetry. The standard deviation vary between 4% and 8%.

TITRIMETRY:- This is another important analytical tool widely used in estimation penicillins both on conventional and official scale[8,9]. Acidimetric iodometric, and non-aqeous titrimetiic techniques have been employed for this analysis[8,9,21,25,26,29,34].

Iodometric titrimetry is important both as conventional and officially recommended method for penicillin estimation. The most current official British Pharmacopoeae method for penicillin G and penicillin V is based on this principle[9].

Acid-base titrimetry is equally important both as conventional and official method. Infact it is the method recommended by both 1973 and 1980 editions of British

Pharmacopoeae for assay of ampicillin and cloxacillin.[8,9]

Other titrimetric methods, though less frequently used are non aqeous titration, thiomercuric and mercurimetric titration methods. The application of these methods were reported by Mohoric[24], Wronski[54] and Kalberg Bo[31] respectively. The iodometric method has been adapted to automation report given by Ferrari[19] testifies to this development. The methods are less sensitive but precise. The detection limit is about 20ug/ml.

(c) Fluorimetric method: This is another highly sensitive technique, whose sensitivity ranks next to microbiological technique. The apparent constraint in this field is the difficulty of forming fluorimetric derivatives. This has limited its application to very few penicillins, and so make it relatively unexploited to a great extent. An early report by Scudi and Jellinek[42] described an analytical procedure based on the formation of fluorimetric derivative due to a reaction between penicillin and aminoacridine. More recently, Jusco[30] reported a fluorimetric determination of ampicillin based on the formation of the yellow fluorescent 3,3'disubstituted diketopiperazine derivative of ampicillin heated in acid solution. Ampicillin is the anly penicillin so determined due to the presence of free amino group which is capable of forming fluorescent

condensation product in an appropriate medium. Recently, Swatzman and Dior[29] prepared a stable product with blue fluorescence from the reaction between ampicillin and urauylacetate in aqeous medium. Barbhaiya and Turner[3,4] also reported the estimation of ampicillin fluorimetrically in biological sample by heating with 0.1N NaOH solution.

(d) Infra-red spectroscopy:- This is another technique particularly useful in structure elucidation of penicillins. It is less frequently applied to the quantitative estimation of penicillins. The characteristic absorption of penicillins at 1660cm^{-1} provides a conclusive answer for the resolution of the conflicting structure of the drug[19,21,25,26,29]. William, Linsen et al[53] applied this technique to the quantitative estimation of penicillins using selected absorption bands for individual compounds.

(e) Nuclear magnetic resonance spectroscopy: This technique is most applicable to qualitative problems rather than to quantitative estimations. The application in quantitative aspect has been for rough, proximate analysis of crude extract whose results are not accurate and precise. Both proton 'H and carbon ^{13}C n.m.r. have been suitably applied. Hughes, Wilson et al [29] explored the usefulness of 'H n.m.r. for the identification of penicillins and cephalosporins, and consequently developed specific method for the determination of the diastereoisomer ration of phenethicillins.

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(f) Chromatography:- Chromatographic techniques are useful separation methods for penicillins. The applications are largely qualitative. The high speed liquid chromatography is fastly developing as a versatile quantitative technique, recently being used.

Gas chromatography is a suitable qualitative device, though less applied to penicillins. Vagina, Korchagin et al[49] reported the separation and semi-quantitative assay of penicillins on this column. Separation of penicillin on 3.5% S.E-30 column has also been reported[21]

Thin-layer and paper chromatographic procedures have been extensively applied in separation and semi-quantitative determination.[21,2,5,26] The early success of this work was due to Naussbaumer[36], Batchelor[5] and Fischer[25] who not only separated these drugs, but, also developed suitable solvent systems for this purpose.

High speed liquid chromatography as earlier stated is another versatile technique. Wilson[29] reported the h.s.l.c. parameters obtained using reverse phase systems.

(g) Polarography:- This is another versatile technique, though not yet adequately exploited. It is still in its infancy. Its application has been reported for the analysis of penicillin in blood sera.[29] Recently, differential pulse polarography was applied to nitrosated penicillins.[21,29] Cephalosporins appear more amenable to application of this method than penicillins.

(h) The ultraviolet and visible spectrophotometry.

This is about the most important technique widely used in penicillin and pharmaceutical analysis in general. The importance emanated from the striking sensitivity and high precision associated with the technique. The method ranks next to microbiological and fluorimetric techniques in term of sensitivity.

The method has been applied in the following instances:

(i) Direct measurement of the ultraviolet absorption characteristic of individual penicillins. Most penicillins, by virtue of their side chain have peculiar absorptions in the ultraviolet region. For instance Gabor[24] estimated penicillin G spectrophotometrically at 263 and 257 nm. Penicillin V absorbs maximally at 268 nm and 274 nm, while cloxacillin absorbs at 275 nm. These properties are usually recommended for simple assay of these compounds.[8,9]

(ii) Penicillins in appropriate buffer media form penicillenic acid in presence of copper and mercuric ions. The ultraviolet absorption properties of these derivatives have been exploited in estimating these compounds spectrophotometrically. This reaction forms the basis of B.P. 1973 recommended method of analysis of ampicillin and cloxacillin[8]. This

property has also been exploited by Harriot[21], Brandriss[7], Saccani[40] and Smith[43]. The wavelengths usually employed are 320 nm, 339 and 340 nm. The technique is equally sensitive.

(iii) Another instance is the reaction of the degradation products with certain reagents to yield suitable chromophores. In this respect, the reaction between penicilloic and hydroxylamine in presence ferric alum has been extensively exploited in the colorimetric estimation of penicillins.[21,25,26,29,35]. This method was for a long time the official method. Saccani[40] adapted this method to automation. A recent modification of this method using nickel ion instead of ferric alum was reported by Munson et al[35].

The other degradation product equally useful is penicillamine. Its reactions with Elman reagent[50,51], molybdoarsenic acid[27,37] and p-aminodimethyl-N'N'aniline[42] form the basis of some colorimetric assay techniques. Penicillamine is obtained by mercuric ion - influenced degradation of penicillins. The application of Elman's reagent was reported by Vermeij[50,51], while that of molybdoarsenate was by Pans[37] and Holme.[27] Scudi[42], in 1946 determined penicillin colorimetrically using p-aminodimethy-N',N'-aniline.

(iv) Colorimetric estimation of penicillins were carried out by direct reaction of penicillin with

certain reagents. Raychouidry[39] determined penicillin colorimetrically using chloroplatinic acid, while Turner[46] determined penicillin V by nitration in presence of ammonia. A more drastic approach to this was through the oxidation of penicillin by perchloric acid. This was reported by Kondo.[33]

(v) The other premise is the analysis of ampicillin specifically by some colorimetric techniques. Ampicillin by the virtue of its free amino group is able to react with some reagents to give some colour derivatives. These chromophores have been used in the estimation of ampicillin colorimetrically. Chowdri[11] and Smith[43] reported the successful application of 0.1% ninhydrin reagent to colorimetric assay of ampicillin. This was further carried to the estimation of ampicillin in presence of cloxacillin by Smith[43]. Csiba[15] reported the spectrophotometric assay of ampicillin using glyoxal. Thomas[45] also determined ampicillin colorimetrically by reaction with 10% malonic acid solution in acetic anhydride. Elgendi et al[17] described the procedure of colorimetric assay of ampicillin using dimethoxyquinone.

(i) Other methods: Many other methods such as polarimetry[21], circular dichroism[29], electrophoresis[29], and thermal differential analysis have been used in assays of penicillins. Recently, Kidani[32] reported the indirect determination of penicillin by atomic absorption spectrophotometry.

1.6 MOTIVATION AND SCOPE OF THE PROJECT

Penicillins are important antibiotics widely used in health establishments for the treatments of both human and livestocks ailments. It is highly essential to have well-established, precised and specific analytical methods for the estimations of these compounds.

Much of the quantitative analysis of penicillins is carried out by a microbiological method; a type of biological assay technique which is not adequately equiped for rapid, highly precised and accurate analysis. With the importation, production and sales of large quantities of penicillin products in Nigeria, a more suitable technique of assay is necessary to cope with the ever rising issue of quality control of these products.

Since the microbiological procedure is lengthy, needs special equipments and boring statistics as well as it cannot cope with all situations, there is need for the development of alternative techniques. In this direction, the development of new spectrophotometric methods will contribute to providing the much-needed answers to these problems. More credits could be added to the new method if it could do well with dilute concentration of penicillins as well as its pharmaceutical preparations.

Spectrophotometry is a versatile analytical techniques with wide spectrum of applications. This technique is unique in terms of accuracy, sensitivity, precision and

reproducibility. Moreover, its application in various forms is not limited to pharmaceutical analysis, but to microanalysis of biological, geological, clinical and environmental samples.

The aim of this research effort therefore, is to develop new spectrophotometric methods suitable for the analysis of penicillins. In addition, to try and compare such methods with some established methods. In this respect, to investigate and develop some essential attributes of well-defined spectrophotometric techniques in these methods. These attributes include chromophore stability, wavelength of maximum absorption, economy of time, sensitivity, precision and reproducibility.

Moreover, to compare these methods objectively, their applications to assay of penicillins in biological samples and pharmaceutical preparations are essential.

Finally, to make slight modifications (where necessary) to the methods in order to achieve meaningful result.

CHAPTER TWO

EXPERIMENTAL

2.1 THE DEVELOPMENT OF NEW SPECTROPHOTOMETRIC METHODS FOR ANALYSIS OF PENICILLIN

As mentioned before, the aim of this research was to explore the possibility of developing and verifying some proposed methods.

Having surveyed the literatures available on analysis of penicillins, the chemistry and the methods applied to date, two new spectrophotometric methods were proposed.

The principle behind the two proposed methods is besically the same. It depends on the reaction of the totally-degraded products of penicillins with some reagents. The proposal was a trial to extend some colorimetric methods of assay of thiols to penicillins.

The two tried methods are:

(i) Spectrophotometric estimation of penicillins using buffered N-ethylmaleimide-$HgCl_2$ reagent; otherwise known as N.E.M. or N-ethylmaleimide method.

(ii) Colorimetric estimation of penicillins using phospho-18-tungstic acid-$HgCl_2$ reagent. This method is known as phosphotungstate method.

In both instances, the degraded products of pencillins under appropriate conditions were tried to form chromophores with the above reagents.

In the course of the degradation of penicillins, penicillamine and penilloaldehyde are formed. These products were made to reduce N-ethylmaleimide and phosphotungstic acid in each instance. The chromophores resulting from these reactions had their own characteristic absorptions. These reactions are the basic reactions of mercaptans. Penicillamine being a mercaptan liberated from penicillin in presence of mercuric chloride was made to reduce each of the reagents.

(a) N -ethylmaleimide method

The fact that the reaction of N-ethylmaleimide with sulphydryl compounds is a well-documented method of trace analysis of mercaptans, was the motive behind this trial. A mercaptan in form of penicillamine obtained from the degradation of penicillin made to react with this reagent with a view of extending its application to penicillins.

In this perspective, measurement of the absorbance was made at 274 nm, which was found to be the wavelength of maximum absorbance of the chromophore, and not the 300 nm. recommended for the assay of mercaptans.

Alexander and Rouser[2] did apply this method to the assay of cysteine and gluthathione in biological protein samples, by measuring the decrease in absorbance of the reagent at 300 nm. It is important to note that this method, up to the time of this research had not been applied to penicillins mixture with 2% mercuric chloride solution. The mercuric

chloride catalyses the breakdown of the penicillin to penicillamine which mostly simaultaneously react with the reagent as mercaptan.



N-ethymaleimide

The reaction led to the formation of a yellow or cream coloured derivative with characteristic absorption at 274 nm. It was noted that the reaction took place at room temperature, an increase of which resulted in the precipitation of the chromophore. With effective control of temperature, Beer's law was obeyed between 1 and 14ug/ml. of penicillin. The detection limit was found to be lower than 1ug/ml.

However, the chemistry of this reaction and the principle as applied to mercaptans is not yet established. The probable reaction taking place is the hydrolysis of the reagent at imino bonds with the simaultaneous reduction by mercaptan or penicillamine as the case may be.

The concentration of the reagent employed was 0.001M which was prepared in 0.1M phosphate buffer solution pH 6.8.

(b) Phosphotungstate method

Phospho-18-tungstic acid otherwise known as folin-Denis reagent has been in use for the trace analysis of mercaptans

since 1929.[52] What was done in this case was to extend the application to penicillins via the liberation of penicillamine which is also a marcaptan. Its application to trace analysis of mercaptans in air, water, food and petroleum products is well-documented.[6] The method was originally proposed by Folin, later modified by Shinohara and Wild.[52] However, its application up to date, has not been extended to penicillins. The only use of the reagent in penicillin chemistry, is in the identification test of penicillamine (B.P. 1980)[9].

In the present investigation, the scope of this technique was extended to the determination of penicillins via a colour reaction with penicillamine, set free by the hydrolysis of penicillins.

In a typical set-up, a mixture of this reagent with 2% mercuric chloride solution was reacted with penicillins to produce a greenish-blue chromophore with a characteristic absorption at 764 nm. The absorbance at this wavelength was found to be directly proportional to the concentration of penicillin used. This was in accordance to Beer's law, which was obeyed between 1 and 14ug/ml of penicillin. The detection limit was about 1 ug/ml.

The probable reaction taking place is illustrated by the equation below[52];

$$(H_2O)_3P_2O_5(WO_3)_{18} + 2R-SH \longrightarrow RS-SR + (H_2O)_3P_2O_5(WO_3)_{17}WO_2 + H_2O$$

penicillamine

This reaction was found to be instant but it usually took a long time for the maximisation and the stabilization of the intensity.

However, the maximization of the intensity can be enhanced by warming on steam-bath for about 10 minutes.

2.2 PRELIMINARY APPLICATION OF THE METHODS

Before these methods were applied to samples, their validities were tested as regards the verification of Beer's law. Beer's law is very important in visible and ultra-violet spectrophotometry. Other factors or features considered here were the stability of the chromophores, wavelength of maximum absorption, effects of temperature and pH.

(a) N-ethylmaleimide method

As earlier stated, this method has been extensively used in the analysis of mercaptans and sulphydryl compounds. The reagent was prepared in phosphate buffer solution pH 6.8. The aim of this exercise was to establish the wavelength of maximum absorbance of the chromophore, stability of the chromophore and the validity of Beer's law.

Reagent and equipment

(1) Phosphate buffer solution pH 6.8 was prepared by mixing 245 ml of 0.1M Na_2HPO_4 solution and 255 ml of 0.1M NaH_2PO_4 solution. Both salts were of analytical (A.R) grades.

(ii) 0.001M solution of N-ethylmaleimide was prepared by dissolving 62-5 mg of N-ethylmaleimide A.R. in 500 ml of the phosphate buffer solution (prepared in i)

(iii) 2 % Mercuric chloride solution was prepared by dissolving 5g of $HgCl_2$ A.R. and 5.0g KCl A.R. in sufficient volume of water to prepare 250 ml solution.

(iv) Stock solutions of cloxacillin sodium, ampicillin trihydrate, benzylpenicillin sodium and penicillin V potassium, 0.5 mg/ml each were prepered by dissolving 50mg each of the powders or penicillins in sufficient volumes of 0.1N NaOH solution to prepare 100 ml.

(v) Unicam SP 1703 spectrophotometer.

Procedure

There were experiments carried out to determine the wavelength of maximum absorbance, suitable time to take absorbance i.e. stability of the chromophore and the verification of Beer's law.

Firstly, a 1:1 mixture of N-ethylmaleimide and 2% $HgCl_2$ solutions was prepared. From this mixture, a 5 ml aliquot

was pipetted into 25 ml flask, followed by 0.5ml of penicillin solution. The mixture was thoroughly shaken and diluted to 25 ml mark with water. The wavelength of maximum absorption was determined by scanning a portion of chromophore against a water blank on Unicam SP 1703 spectrophotometer.

Having determined the wavelength of maximum absorption, the chromophore stability was then determined by repeatedly measuring the absorbance over a period of time. From this, the suitable time for taking absorbance was determined as time it took the chromophore to reach its maximum instensity.

Into various 25ml-flasks, were pipetted aliquots of pemicillin equivalent to 50, 100, 150, 200, 250, 300 and 350ug. These aliquots were equivalent to volumes of stock solution ranging from 0.1 ml to 0.7 ml. The contents of these flasks were expected on the final dilution to be equivalent to a concentration ranging from 2 to 14ug/ml.

After tne transference of penicillin into the flask, 5ml of the reagent mixture (N.E.M) was pipetted into each flask followed by thorough shaking. The blank containing water instead of drug was similarly prepared. The content of each flask having been diluted to mark with water, was allowed to stand for 20 minutes before reading the absorbance. The absorbance was read at 274 nm against the blank.

The result of these experiments are illustrated graphically in figs. 4 - 6.

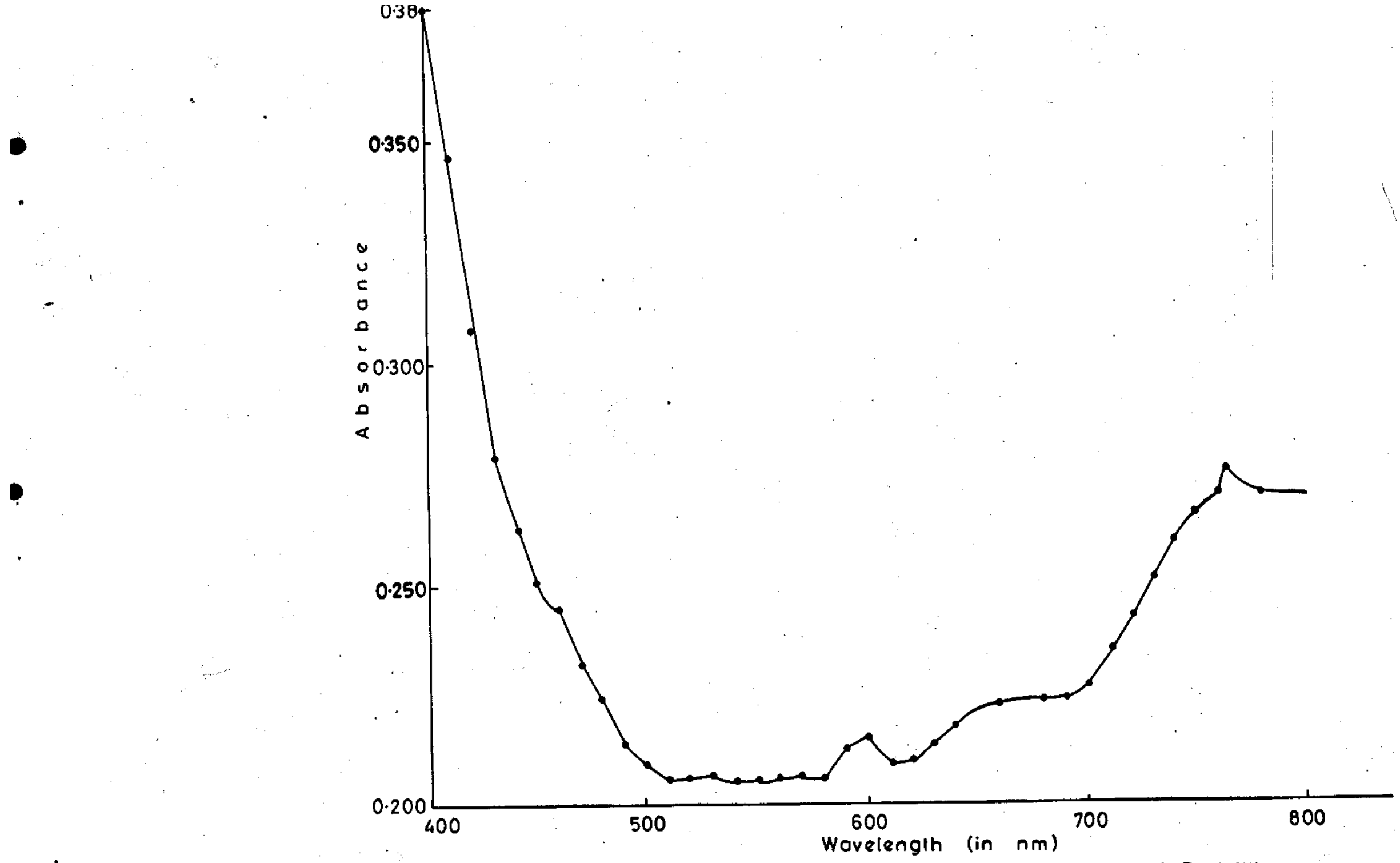


Fig. 1 **Absorption spectrum of** Phosphotungstate Chromophore of Penicillin

(b) Phospho-18-tungstate method

Phospho-18-tungstic acid otherwise known as Folin-Denis reagent, formed a greenish-blue chromophore in presence of mercuric ions. The resulting chromophore was found to absorb maximally at 764 nm, the intensity of which was proportional to the concentration of penicillin.

Materials

(i) Stock solutions of penicillins containig 0.5mg/ml drug were prepared by dissolving equivalent of 50 mg in 100 ml of 0.1N NaOH solution. The stock solutions of this strength of penicillin V potassium, cloxacillin sodium, benzylpenicillin and ampicillin trihydrate were prepared.

(ii) Phospho-18-tungstic acid was prepared by refluxing a mixture containing 25 ml of 85% H_3PO_4, 50 ml of conc. HCl, 50gm of sodium tungstate, 25g of sodium molybdate and 350ml of water for 10 hours. On cooling; 75 gm of Lithium sulphate and a few drops of bromine were added. The mixture was then heated until the greenish colour disappeared.

(iii) 2% Mercuric chloride solution was similarly prepared as in section 2.2a.

(iv) Acetate buffer solution pH 4.7 was prepared by mixing equal volumes of 0.4M sodium acetate and 0.4M acetic acid solutions. The pH was adjusted to 4.7.

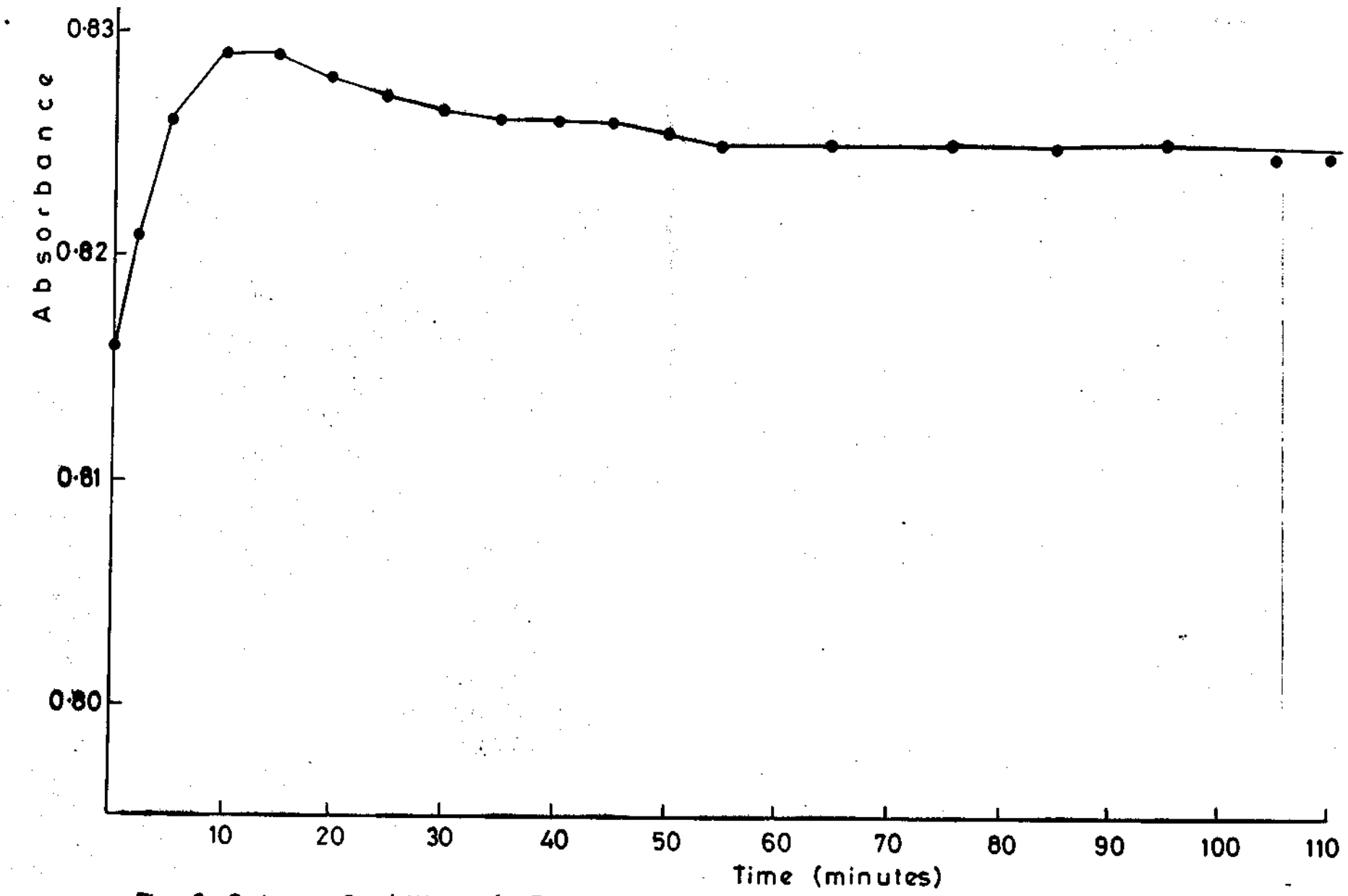


Fig. 2 Colour Stability of Phosphotungstate Chromophore of Ampicillin

Equipment

(i) Unicam SP 1703 Spectrophotometer

(ii) Gallenkamp water-bath.

Procedure

Firstly, a 1:1 (by volume) mixture of 2% $HgCl_2$ solution and the reagent was prepared. Three experiments were performed; aimed at establishing the wavelength of maximum absorption, suitable time to read the absorption and the verification of Beer's law.

Into a 25 ml flask, were added 5 ml of acetate buffer, 0.5ml of penicillin solution, and 2ml of the reagent mixture. The content of the flask was heated for 10 minutes on water-bath to enhance the maximisation of the colour intensity. On cooling, the content of the flask was diluted to mark with water. Using water as blank, the wavelength of maximum absorption was determined by scanning the chromophore between 400 nm and 800 nm on the spectrophotometer. The spectrum of this chromophore is illustrated graphically in fig 1.

Using the same preparation, the colour stability was then determined by repeatedly measuring the absorbance over a period of time.

For the third experiment, chromophores were similarly prepared using varied quantities of penicillins. Into various 25 ml flasks, were transfered 5 ml aliquots of

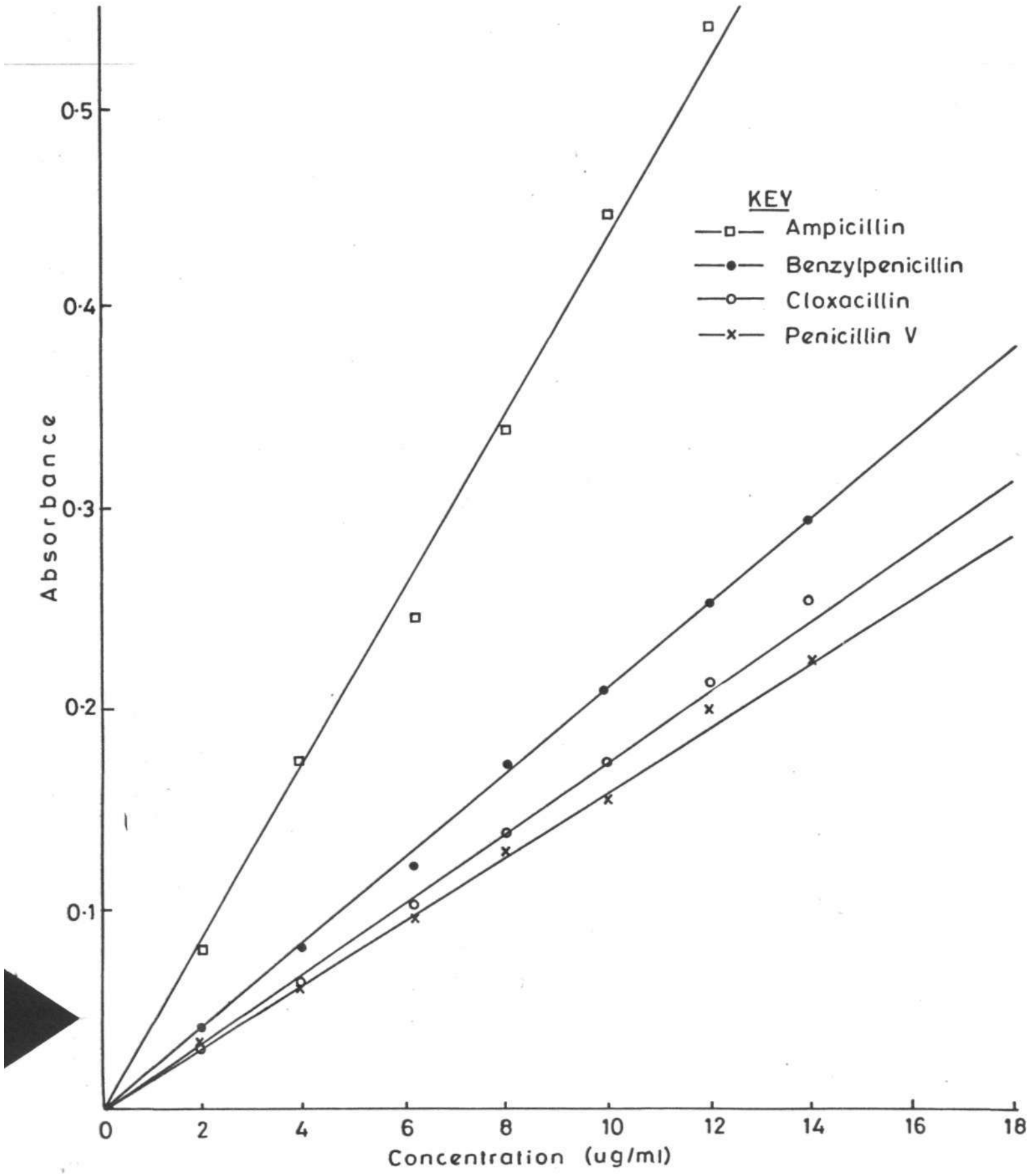


Fig. 3 Calibration Curve of Reagentgrade Penicillins by Phosphotungstate Method (max = 764 nm)

acetate buffer solution, followed by 0.1ml to 0.7ml aliquots of penicillin solutions. These aliquots of penicillin were equivalent to between 50ug and 350ug of drug, which on final dilution amounted to between 2ug/ml and 14ug/ml. Then, 2ml aliquot of the reagent mixture was pipetted into each preparation. Blank solution was similarly prepared to contain water, buffer and reagent only. The content of each flask was warmed for 10 minutes, and cooled before reading the absorbance at 764 nm against the blank preparation. These readings (absorbances) are displayed on table 3.4 and graphically in fig. 3.

2.3. APPLICATION OF THE PROPOSED METHODS AND COMPARISONS WITH THE STANDARD METHODS

For the proper appraisal of these methods, it is essential to apply them along with other standard methods in the analysis of some samples with a view of comparing them objectively. This process will among other things help in deciding whether or not the proposed methods could be equally effective as the established ones. Moreover, this will also help in detecting any apparent flaw or deficiency in either of the proposed or established method.

In this perspective, the methods concerned (proposed and established) were applied to the estimation of penicillins in urine and pharmaceutical preparations.

The established methods used were Pan's method based on the colour reaction of molybdoarsenic acid with penicillins [27,37] and B.P. (1973) standard method based on the uv-absorptions of penicillenic acid derivatives of ampicillin and cloxacillin.

The principle behind the Pan's method is similar to that of the proposed method i.e. reaction of penicillamine liberated by mercuric chloride with molydoarsenic acid. This mercaptan (penicillamine) being a reducing agent, characteristically reduces the reagent to molybdenum-blue complex. The absorbance was measured at 674 nm as a function of penicillin concentration.

As earlier discussed in the chemistry of penicillin, penicillins rearrange into penicillenic acid in appropriate media and in presence of a trace amount of copper or mercuric ions. This derivative absorbs in the uv-region of the electromagnetic spectrum. This method until recently, used t the B.P. (1973) official method of estimation of ampicillin and cloxacillin.[8,9]

2.3.1. QUANTITATIVE ESTIMATIONS OF PENICILLINS IN URINE

In a number of literatures, estimation of penicillins in biological fluids were mostly carried out by micriobiological assay techniques. The aim of this research was to propose alternative analytical techniques that would be

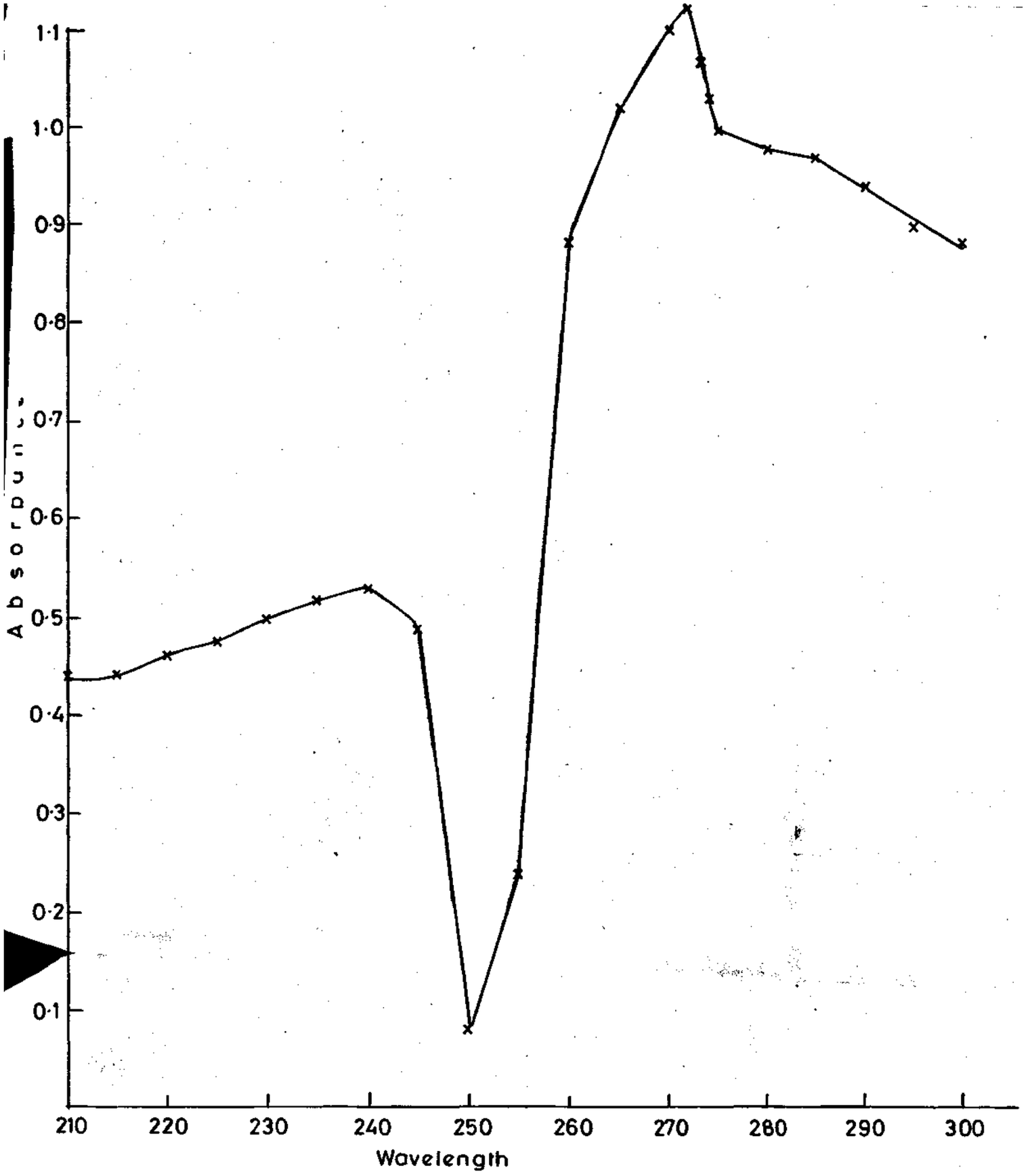


Fig. 4 UV – Spectrum of N–Ethylmaleimide Chromophore of Cloxacillin

equally effective as this technique. Microbiological assay technique, with its apparent dificiencies cannot be exclusively relied upon as analytical tool for this purpose. In order to achieve this objective, the two proposed methods were employed along with two standard methods mentioned in sention 2.3.

The process involved the extraction of penicillin from urine samples containing these drugs. This was followed by the purification of the extracts before assaying them for penicillins.

The aim of this exercise was to try the applicability of the techniques, establish the effiency of the extraction, and also that of each method used. This was aimed at putting the new methods on sound footing.

Materials

(i) Urine samples collected from students who had not taken any drug four days previous to the test.

(ii) Penicillin stock solutions containing 0.5mg/ml each of penicillin V potassium, cloxacillin sodium, ampicillin trihydrate and penicillin G sodium. Their preparations are described in section 2.2 (a)

(iii) 0.001M N-ethylmaleimide solution; prepared as described in section 2.2(a) .

(iv) Phosphotungstic acid solution; prepared as described section 2.2(b).

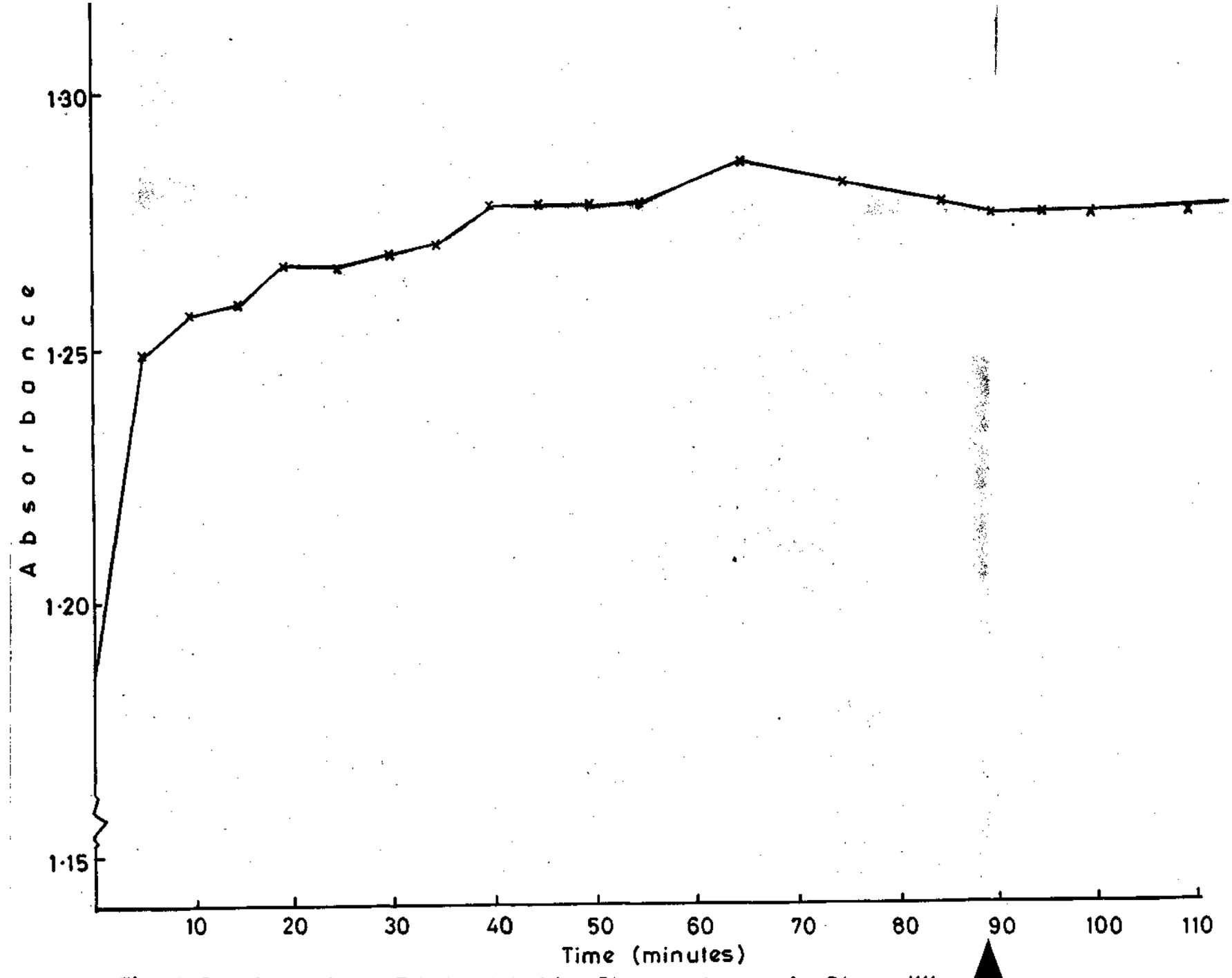


Fig. 5 Stability of N-Ethylmaleimide Chromophore of Cloxacillin

(v) 2% $HgCl_2$ solution; prepared as described in section 2.2(a).

(vi) 0.5% $HgCl_2$ solution specially prepared for Pan's method. This solution contained 0.5gm of $HgCl_2$ A.R. and some crystals of KCl in 100 ml of solution.

(vii) Molybdoarsenic acid was prepared by dissolving 25 gm of ammonium molybdate in 21 ml of conc. H_2SO_4 before transfering into 350 ml of deionized water. Other constituents of this solution included sodium arsenate solution prepared by dissolving 3g of arsenic trioxide A.R. in 25 ml of 0.5N NaOH solution. The resulting mixture was made up to 500 ml mark with water before being kept for 24 hours in water-bath regulated at 37°C.

(viii) Copper sulphate solution containing 1mg/ml of copper ion. This was prepared by dissolving 0.345g of $CuSO_4$ A.R, ir sufficient quantity of water to make 100 ml solution.

(ix) Buffered copper solution pH 5.2 was prepared by mixing 15 ml of copper solution (viii) with 528 ml of 0.2M Na_2HPO_4 solution and 457 ml of 0.1M citric acid solution. The pH was adjusted to 5.2.

(x) Buffered copper solution pH 3.2 (for cloxacillin) was prepared by mixing 22.5ml of copper solution (from Viii) with 366.7ml of 0.1M potassium hydrogen phthalate solution and 110.8ml of 0.1M HCl solution. The pH was adjusted to 3.2.

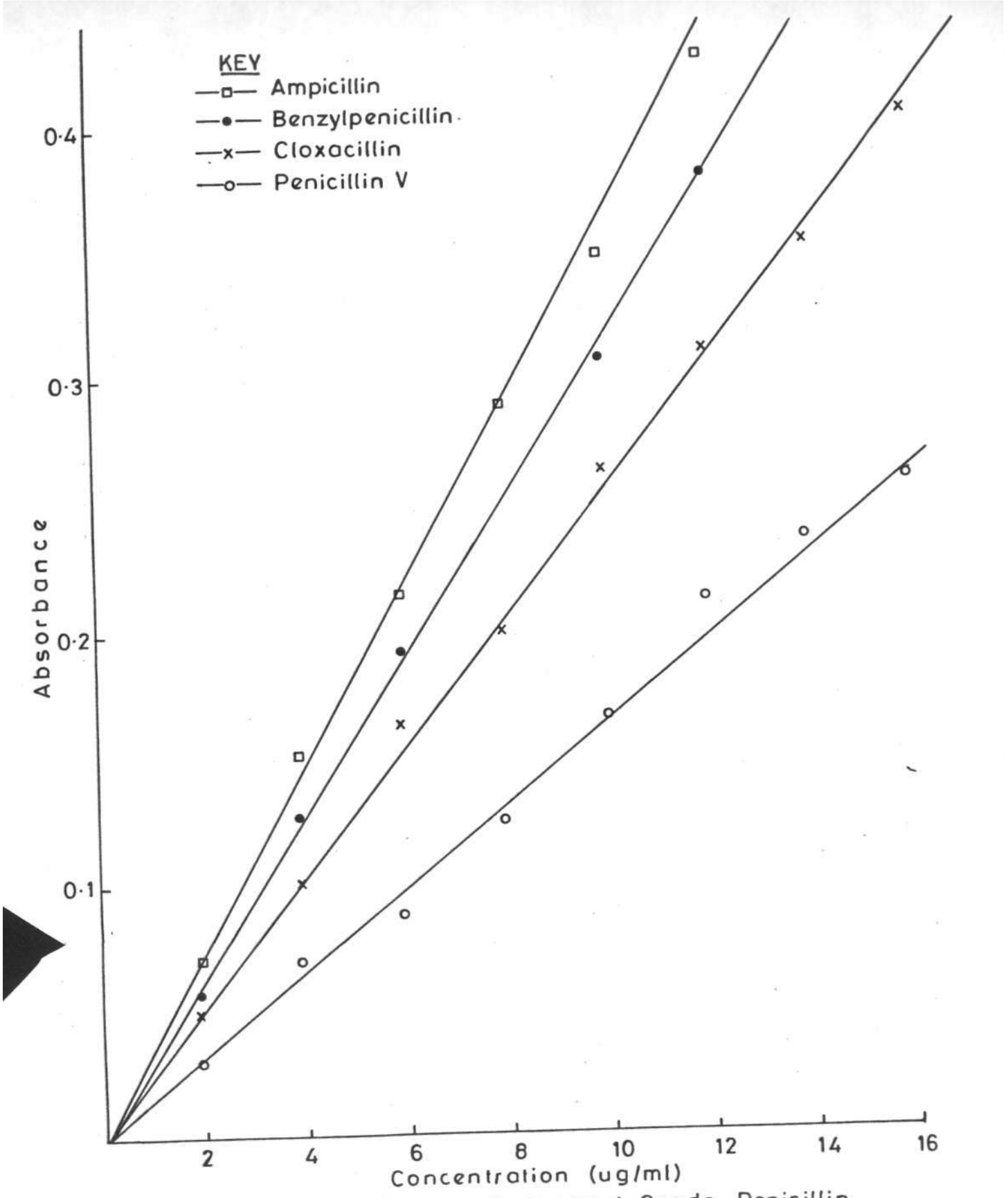


Fig. 6 Calibration Curve of Reagent Grade Penicillin by N-Ethylmaleimide method (λ_{max} 274nm)

(xi) Acetate buffer; prepared as described in section 2.2(b).

(xii) Chloroform.

(xiii) Water-bath

(xiv) Unicam Sp-1703 spectrophotometer.

Procedure

(i) Sample pretreatment and extraction from urine

100ml aliquot each of urine was transfered into four separating funnels. Into each funnel was added 50mg of penicillin; followed by 10ml of 0.1N HCl to acidify the urine. The sample was extracted with 3 aliquots of 100 ml chloroform. The non-aqeous layer was removed and transfered into a distillating flask. Having distilled off the chloroform, the extract was dissolved in 100 ml of 0.1N NaOH solution and filtered into 100 ml flask. These extracts were used in the subsequent analysis.

(ii) Estimation by phosphotungstate method.

Using the stock solution of penicillins, the calibration curve of each drug was prepared as described in section 2.2(b).

For the extract, 0.5 ml aliquots were pipetted into 25ml flask containing 5ml of acetate buffer solution. Then 2ml aliquot each of the reagent mixture was added into the flasks. After 10 minutes warming, the content of each flask was diluted to mark with water and cooled. The absorbance of the resulting chromophore was read at 764 nm against the blank preparation. From the calibrationcurve, the

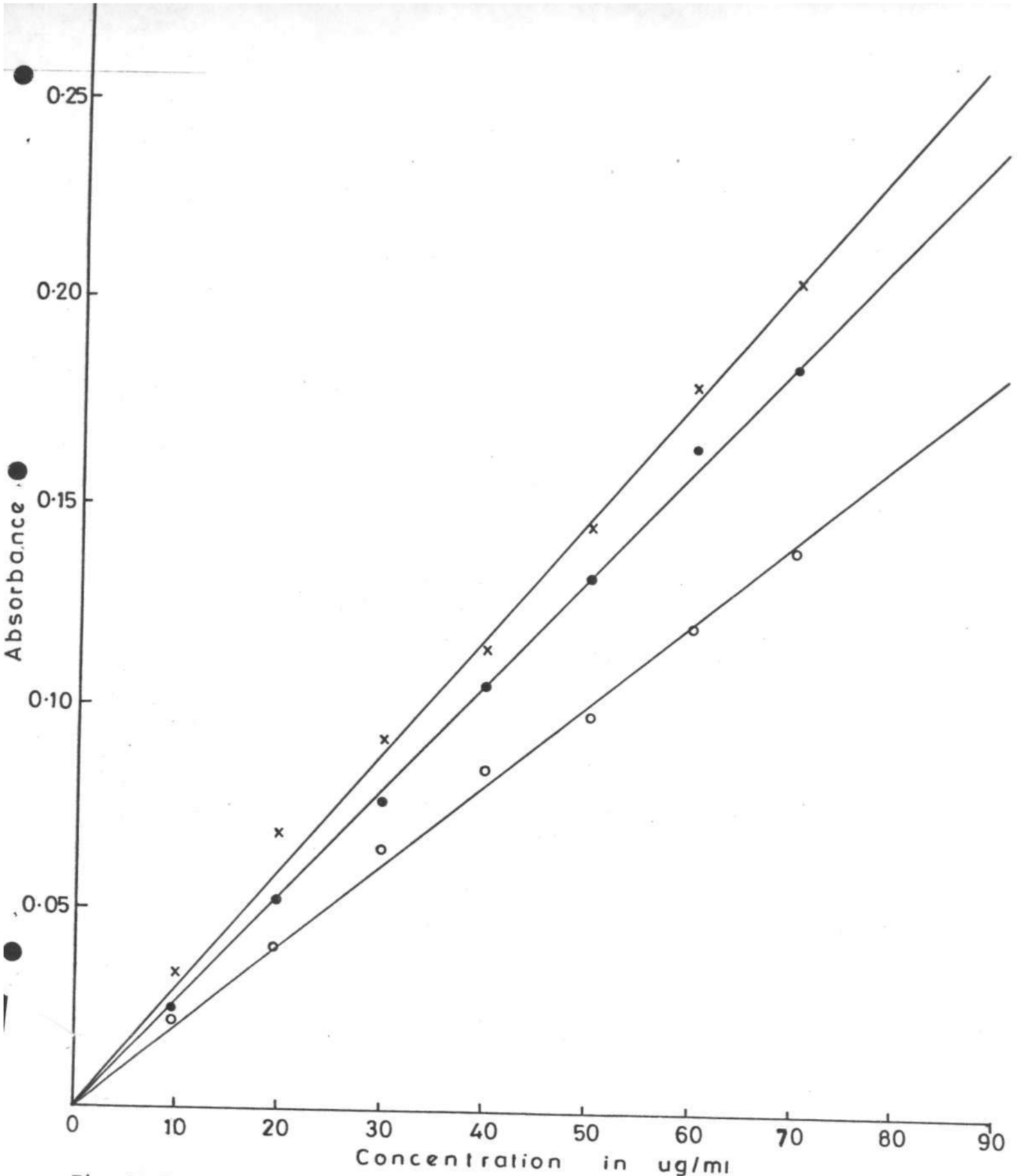


Fig. 7 Calibration Curve of Penicillin by Pan's method (Molyboarsenate Chromophores at 674 nm)

concentration in the final solution was read and the original amount of penicillin was calculated.

(iii) N-ethylmaleimide method

Using the stock solutions of the penicillins, the calibration curve of each was prepared as described in section 2.2(a).

For each extract, 0.5ml aliquots were pipetted into 2 separate 25ml flasks. This was followed by the addition of 5ml aliquot of the reagent mixture in each case. Having thoroughly shaking the content of each flask, this was diluted to mark with water, and kept for 20 minutes before reading the absorbance. From the calibration curve, the concentration in the final solution was read and the original amount of penicillin was calculated.

(iv) Pan's method (molybdoarsenate)

As earlier stated, the technique operates basically on the same principle as either of the proposed methods.

Firstly, a 1:1 (by volume) mixture of this reagent with 0.5% $HgCl_2$ solution was made. Using the stock solutions, calibration curve were prepared for 2 to 16ug/ml of ampicillin and 10 to 80ug/ml of other penicillins. Into separate 25ml flasks, 5ml aliquots of the reagent were transfered followed by addition of 0.1 ml to 0.8ml of ampicillin and 0.5ml to 3.5ml in case of other penicillins.

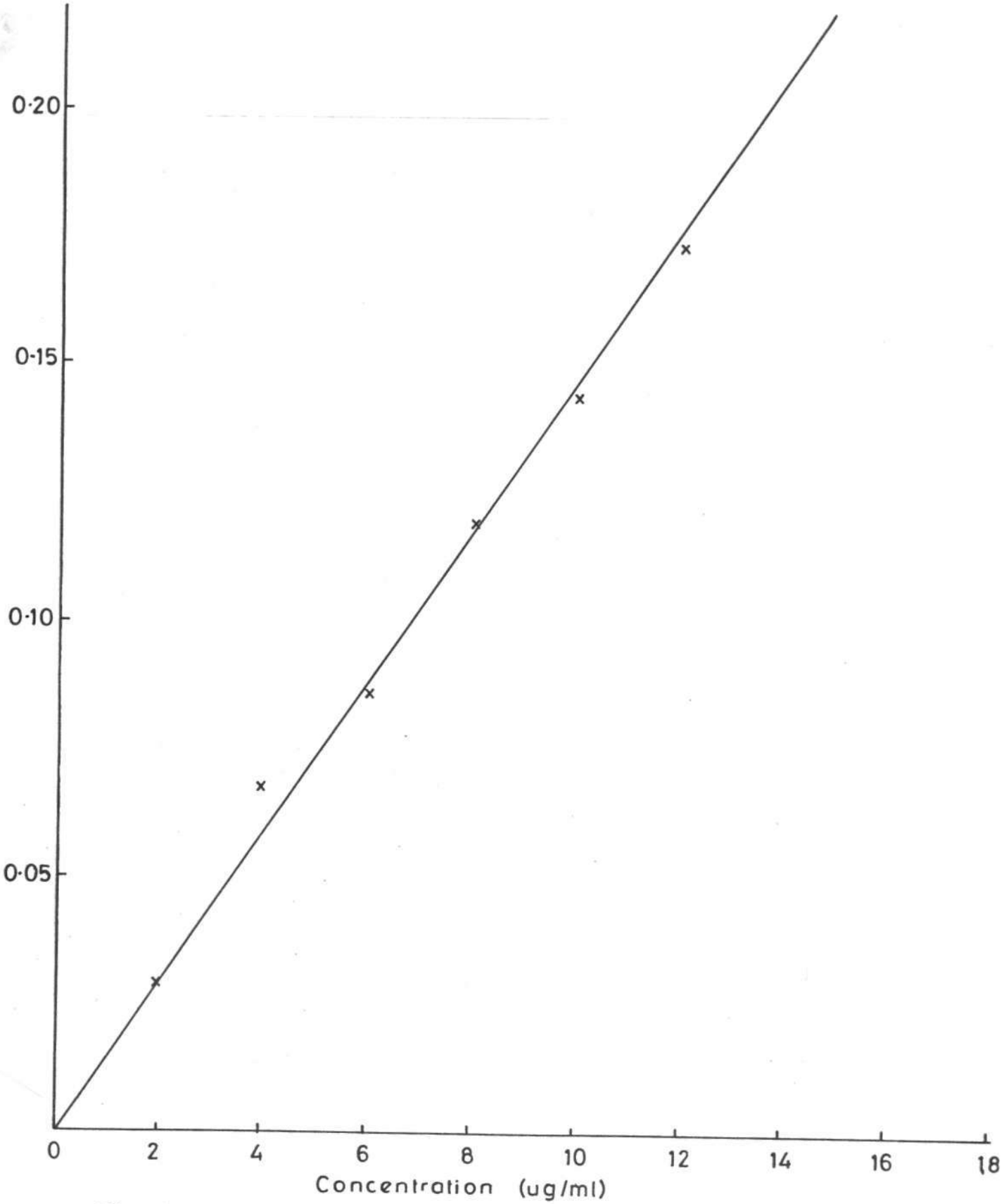


Fig. 8 Calibration Curve of Ampicillin by B.P. (1973) method (max = 339 nm)

The content of each flask was left for 30 minutes before diluting to mark with water. The absorbance of each preparation was measured at 674 nm against a blank preparation.

The treatment of the chloroformic extract involved the transference of 1.0 ml aliquot of each of (penicillin V, cloxacillin and penicillin G) into separate 25 ml flasks. The same procedure enumerated above was repeated for the extracts. In the other extracts; i.e. ampicillin, 0.5 ml aliquot was used. The calibration curves are displayed in figs 7 and 12.

From the calibration curves, the concentration in the final solution was read and the original amount of penicillin was calculated.

(v) Estimation of ampicillin and cloxacillin by B.P. (1973) methods.

Both methods operate on the absorption of uv-energy by the penicillenic acid derivatives of both penicillins. The differences are only in the constituents and the pH of the buffer solutions employed.

Calibration curve of ampicillin was prepared using penicillin concentration between 2 and 16ug/ml. Into various 25ml - flasks, were transfered between 0.1ml and 0.8ml of ampicillin, with the content of each flask diluted to mark with buffer solution pH 3.2. Having warmed the

flask for 30 minutes on water-bath and colled, the absorbance of each was read at 339 nm against blank preparation 0.5 ml aliquots of extract were similarly treated for analysis. From the calibration curve, the concentration of the final solution was read and the original amount of penicillin was calculated.

Cloxacillin, being less sensitive to detection than ampicillin, required higher concentration for calibration curve. Aliquots ranging from 0.5ml to 3.5ml were pipetted into separate 25ml - flasks, and diluted to mark with buffer solution pH 5.2. The contents of each flask was warmed for 50 minutes on water bath and cooled rapidly. The absorbances of the preparations were read at 340 nm against a blank preparation. 1.oml of the chloroform extract was similarly treated and prepared for absorbance reading at 340 nm. The concentration of cloxacillin in the extract was read off from the calibration curve and calculated accordingly.

(vi) Calculation for contents of penicillins in urine extract using the previous four methods.

Assuming that the corresponding concentration read off from calibration is y ug/ml.

This implies that the 25ml-flask bearing the preparation contains 25y ug.

Since 25 y.ug/ml is equivalent to 0.5ml of original extract.

$$\therefore \quad 1\text{ml of the extract contains} = \frac{25y}{0.5}$$

$$= 50y \text{ ug/ml.}$$

Hypothetically, the extract should contain 500 ug/ml of penicillin, provided that the extraction is 100% efficient.

$$\therefore \quad \% \text{ of penicillin found} = \frac{\text{Quantity estimate}}{\text{Hypothetical quantity}}$$

$$= \frac{50y}{500} \times 100$$

$$= 10y\%$$

The percentage of penicillin estimated in the extract is directly proportional to y read from the curve.

2.3.2. QUANTITATIVE ESTIMATION OF PENICILLINS IN PHARMACEUTICAL PREPARATIONS

In order to assess the proposed methods critically, it is essential to apply them along with other methods for comparative purpose.

In this exercise, the proposed and established methods were comparatively applied to the estimation of penicillins in various products. This was aimed at comparing and establishing the efficiency of various methods in quality control processess. As a result of this, it could be possible to objectively appraise, and critise the various methods. Moreover, this was also to pin-point the apparent deficiency of each method, and so provide basis for possible modification of the method concerned.

This in assence could enhance the application of the methods on wider scale.

The four methods used were the phosphotungstate, N-ethylmaleimide, Pan's and B.P. (1973) methods. Various drug samples made by different manufacturers and in different forms were used. These were however, commercially available drugs widely used in the Nigerian Health Institutions.

Materials

(a) For the preparation of standard curves, pure, analytical grades (standard of ampicillin sodium, cloxacillin sodium, penicillin G sodium and penicillin V potassium were used. For each drug stock solution containing 0.2mg/ml of drug was prepared. 250ml of each solution contained equivalent of 50mg penicillin (free acid). This 50mg equivalent corresponds to 54.94mg of cloxacillin sodium, 53.1mg of ampicillin sodium, 54.9mg of penicillin V potassium and 52.0mg of penicillin G sodium.

(b) Other reagents include

(i) 0.5% Mercuric chloride solution; prepared as described in section 2.3.1.

(ii) Molybdoarsenic acid solution; prepared as described in section 2.3.1.

(iii) 0.001M N-ethylmaleimide solution prepared as described in section 2.2(a).

(v) 2% Mercuric chloride solution; prepared as described in section 2.3(a).

(vi) Acetate buffer; prepared as described in section 2.2(b).

(vii) Buffered copper solution pH 5.2; was prepared as described in section 2.3.1.

(viii) Buffered copper solution pH 3.2. (for cloxacillin assay); was prepared as described in section 2.3.1.

(c) The pharmaceutical products employed were mainly capsule, injection, syrups and tablets. Each product was coded with a given number.

A_1 = Roscillin capsule (ampicillin trihydrate) a product of Ranbaxy (Nig) Ltd, Lagos. Batch no: 54 - 81.

A_2 = Penbritin capsule (ampicillin trihydrate a product of Beecham Laboratory, U.K. batch number 3152.

A_3 = Elkacillin (ampicillin trihydrate) capsule; a product of Elko Pharmacy, Milan, Italy. Batch No: 577.

A_4 = Danpicillin (ampicillin trihydrate) syrup; a product of Dangi Pharmacy, Lagos. batch number 81F17

A_5 = Pentrexyl (ampicillin trihydrate) syrup; a product of Bristol Lab, N.Y., U.S.A. batch no: MA004

A_6 = Ampicillin (trihydrate) syrup, (yellow grains) a product of Rajrab (Nig) Ltd., Lagos. Batch no: 7821.

G_1 = Penicillin G injection (Hoechst) (penicillin G sodium); a product of Hoechst, Western Germany,

batch number C806

G_2 = Crystapen injection (penicillin G sodium); a product of Glaxo Laboratory U.K. batch number: OFP650C.

G = Cloxacillin capsule (cloxacillin sodium); a product of Helm Pharmacy, Harmburg. Batch number: 149

C_2 = Orbenin capsule (cloxacillin sodium); a product of Beecham Laboratory, U.K. Batch number 273V

C_3 = Staphydox injection (cloxacillin sodium); a product of Dianic U.S.S.R. Batch number: 82FO

C_4 = Cloxacillin injection (cloxacillin sodium) a product of Rajrab, Lagos. Batch no: 7179.

C_5 = Cloxacillin syrup (pink granules) (cloxacillin sodium), a product of Rajrab (Nig) Ltd. batch number: 8240.

V_1 = Ospen 400 syrup (penicillin V potassium); a product of Sandoz, Austria, Batch no: 55569.

V_2 = Scanicillin V tablet (penicillin V potassium); batch number 5876.

Procedure

(a) Sample pretreatment

It was necessary to prepare each sample specially for analysis; the method of which varied from sample to sample.

Tablets: Ten tablets were weighed and ground to powder. Having mixed the powder, quantity equivalent to one tablet was weighted and dissolved in 250ml solution.

Capsules: The contents of 10 capsules were weighed and mixed. Quantities equivalent to 50mg were accurately weighed and dissolved in 250ml of 0.1N NaOH solution.

Syrups: Volume of water sufficient eough to prepare 60ml of syrup was added into each bottle and shaken. From each solution, about 2ml equivalent to 50mg was withdrawn, and diluted to 250ml with 0.1N NaOH solution.

Injections: Contents of 2 vials of the same injection were weighed and mixed. From the mixture, quantity equivalent of 50mg was withdrawn and dissolved in 250ml of 0.1N NaOH solution

(b) Spectrophotometric assays of the drugs

(i) Phosphotungstate method

The standard curve of each drug was prepared using the stock solution of the standard samples. Into 25ml-flask, aliquots ranging from 0.25ml to 1.5ml were pipetted, followed by 5ml of acetate buffer and 2ml of the reagent mixture. After a brief warming on water-bath, the content of each flask was diluted to mark with water and allowed to cool. The absorbance of each solution was read at 764 nm against a blank preparation. For the calibration curves, see fig. 9.

For samples analyses, 1.0ml aliquot of each was transfered into 25ml-flask with the same procedure followed.

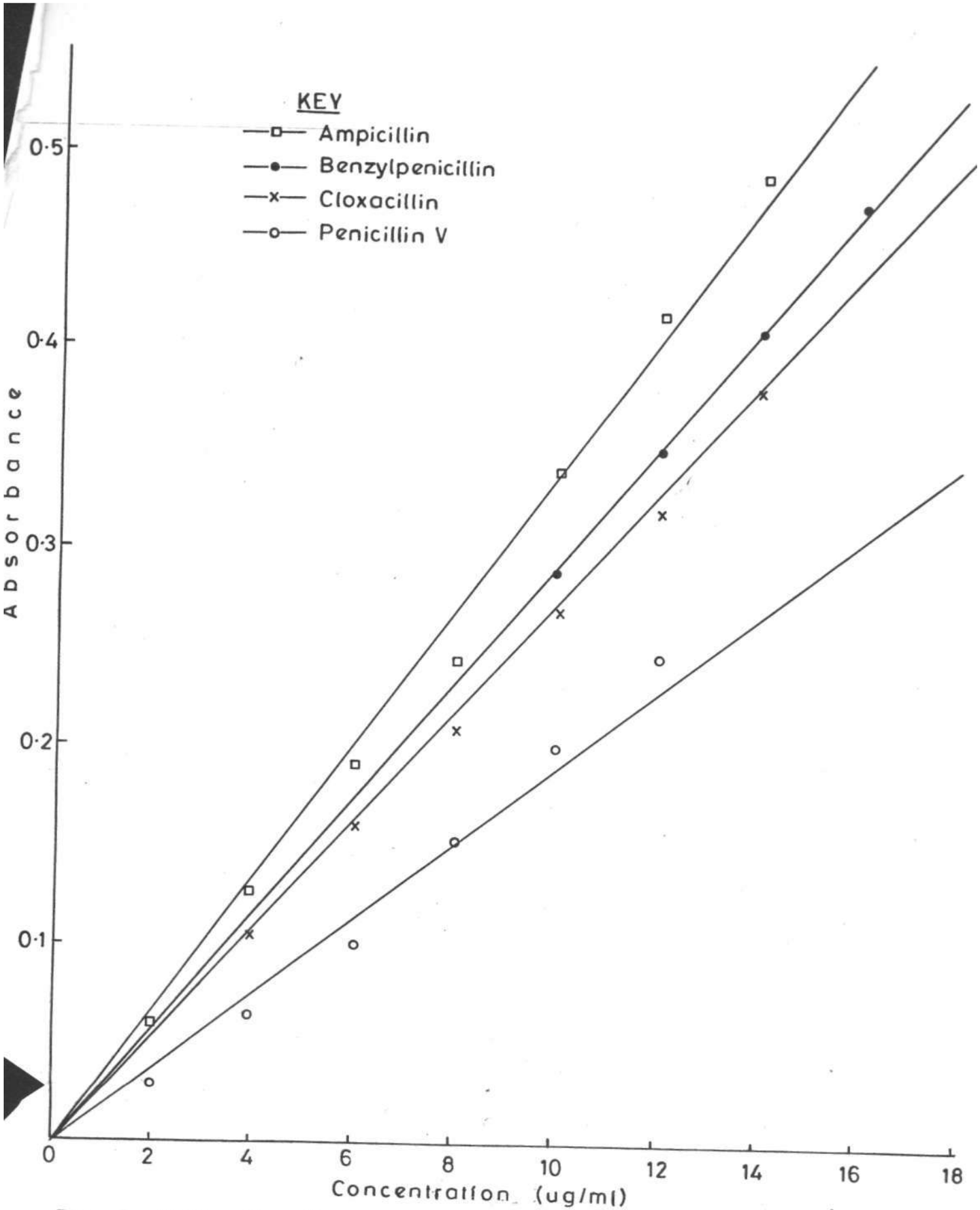


Fig. 9 Calibration Curve of Beecham Standard Penicillin by Phosphotungstate method

From the calibration curve, the concentration in the final solution and the original amount of penicillin was calculated.

(ii) N-ethylmeleimide method

The calibration curve of each drug was prepared from their respective stock solutions. Since Beer's law was obeyed between 2ug/ml and 14ug/ml, aliquots equivalent to 50, 100 --- 350ug were accurately pipetted into 25ml flask. In which case, 0.25ml to 1.75ml of the 0.2mg/ml stock solution were pipetted into the flasks. This was followed by transference of 5ml of N-ethylmaleimide - $HgCl_2$ reagent with thorough shaking. Having diluted the content of each flask to mark with water after 20 minutes, the absorbance was read at 274 nm against a blank preparation.

For the analysis of samples, 10ml aliquot of the sample was used and similarly treated. From the calibration curve, the concentration was calculated accordingly.

(iii) Estimation of penicillin G and penicillin V by Pan's method

Pan's method was used for the estimation of penicillin G and penicillin V. The calibration curve was prepared using aliquots containing between 10 and 70 ug/ml of penicillin.

For the sample preparation, 5ml aliquots of the solution were pipetted into separate 25ml-flasks. This was followed by addition of 5ml aliquot each of the reagent

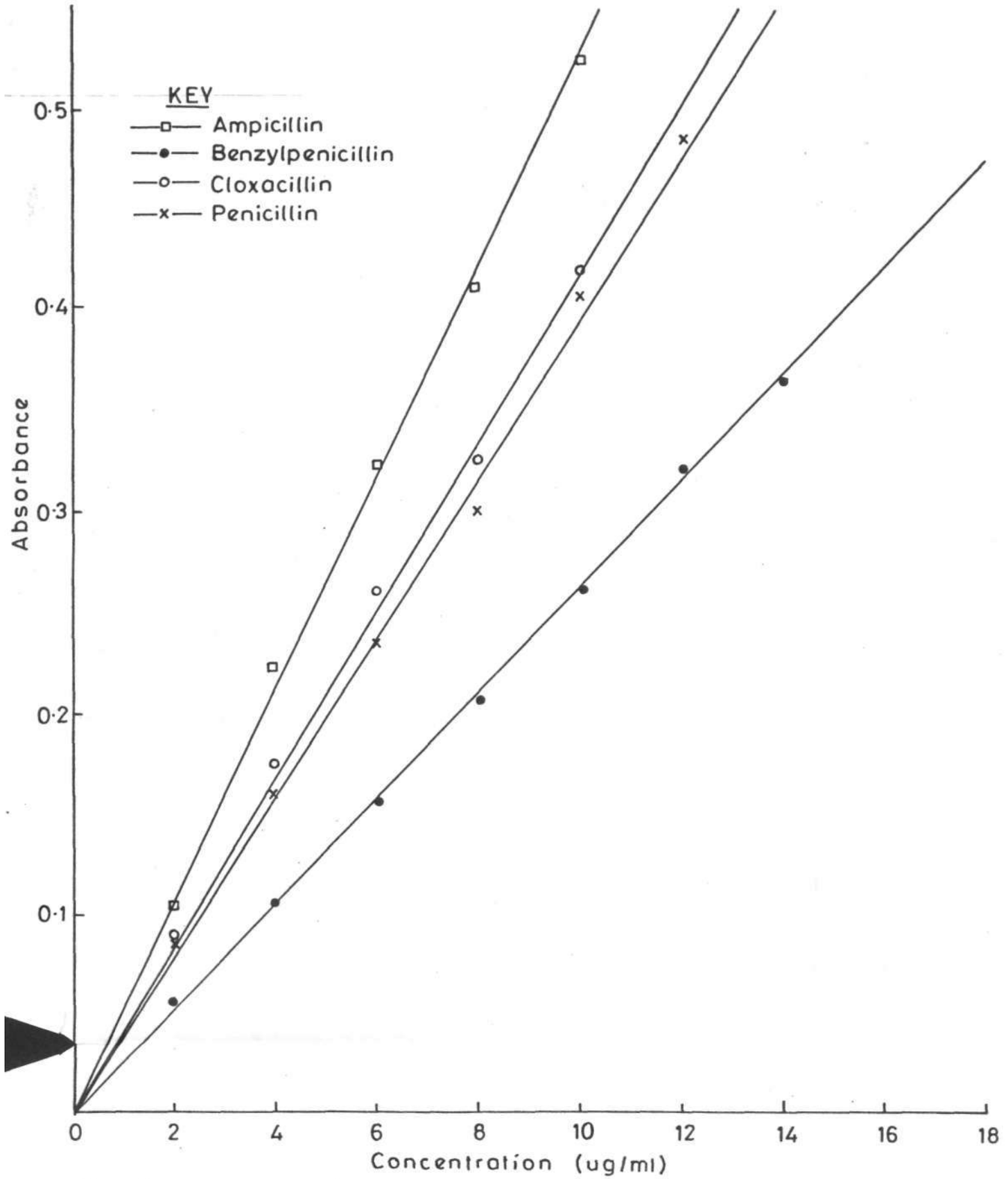


Fig. 10 Calibration Curve of Penicillin (Beechamstandard by N-Ethylmaleimide method)

mixture (molybdoarsenic - $HgCl_2$). After 30 minutes, the content of each flask was diluted to mark with water. The absorbance of each preparation was read at 674 nm against a blank preparation. From the calibration curve, the concentration in the final solution was read and the original amount of penicillin was calculated.

(iv) Estimation of ampicillin and cloxacillin by the B.P. (1973) method

The techniques are basically the same as described 2.3.1.

Calibration curves of ampicillin was prepared using the standard solution. Since Beer's law was obeyed between 2 and 16 ug/ml in this instance, aliquots ranging from 0.25 ml to 1.75 ml were transfered into separate flasks. The content of each flask was diluted to mark with the buffered copper solution pH 5.2 and heated for 30 minutes on water-bath. After cooling, the absorbances of the resulting chromophores were determined at 339 nm against blank preparation. Following the same procedure, 1.0ml aliquots of sample preparation were similarly treated. From the calibration curve, the concentration of each sample was calculated accordingly.

Calibration curves of cloxacillin was prepared using buffered copper solution pH 3.2. The procedure was

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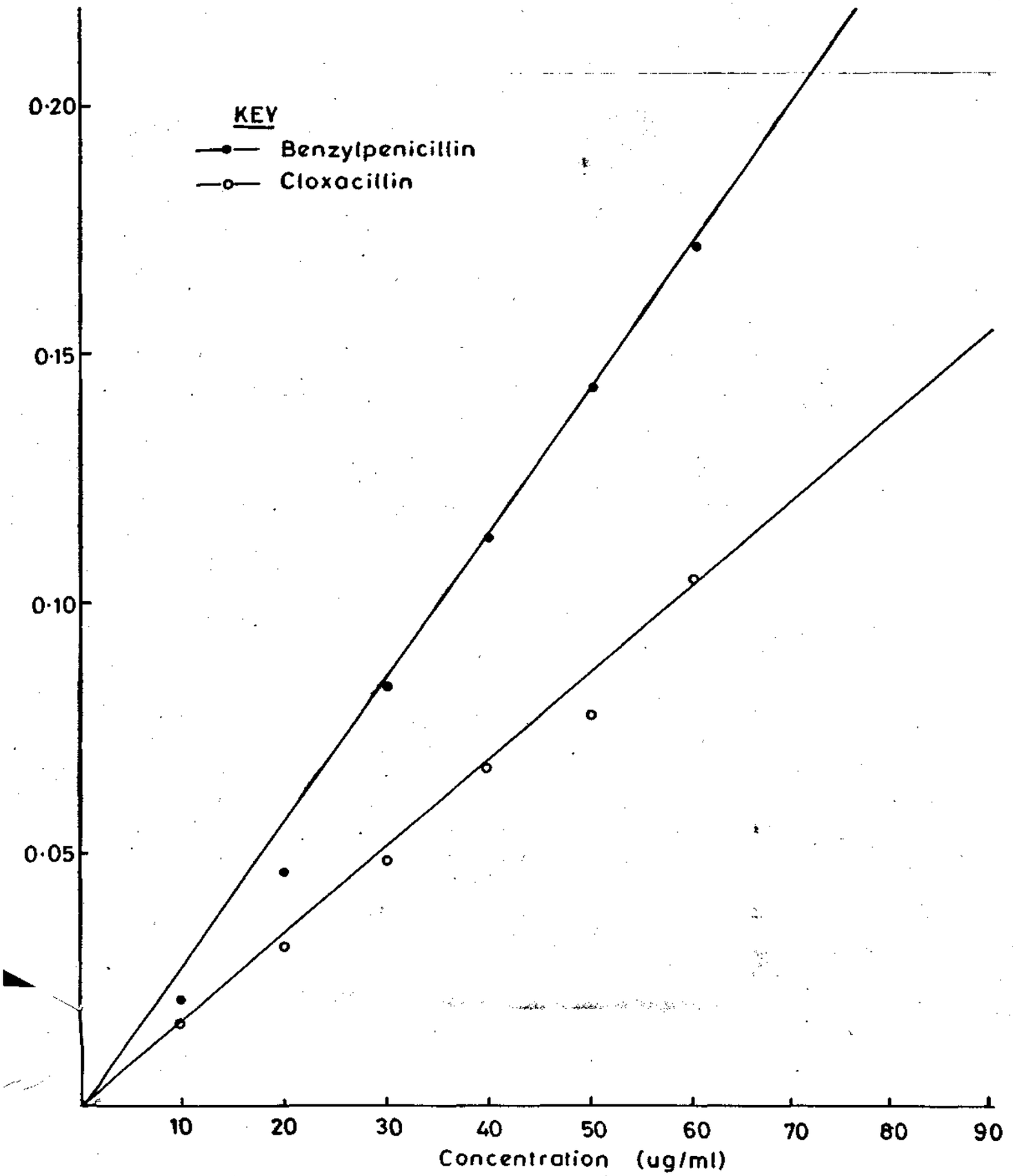


Fig. 11 Calibration Curve of Cloxacillin and Benzylpenicillin by uv – method (B.P. 1973)

similar to that of ampicillin solution of cloxacillin between 2.5ml and 10ml were pipetted into 25ml - flasks and diluted to mark with the buffer solution. After 50 minutes of warming, and rapid cooling, the absorbance was read at 340 nm against a blank preparation. Following the same procedure, 5ml aliquots of samples solution, were similarly treated. From the calibration curve, the concentration of the penicillin in each sample was calculated accordingly.

(v) Calculation:

Since the four methods operate on the same principle, their calculations should follow the same pattern.

Assuming that the concentration of sample corresponds to Z ug/ml on the standard curve.

25ml sample preparation contain 25 zug.

Since 1 ml aliquot of original sample = 25 z ug

$\therefore$ 250ml sample preparation contain

= 250 x 25z ug.

= 6250z ug.

Hypothetically, the sample preparation contains 50mg or 50000ug of penicillin.

$\therefore$ % penicillin found $= \frac{\text{Quantity found}}{\text{Claimed quantity}} \times 100$

$= \frac{625z}{50000} \times 100 = 12.5z\ \%$

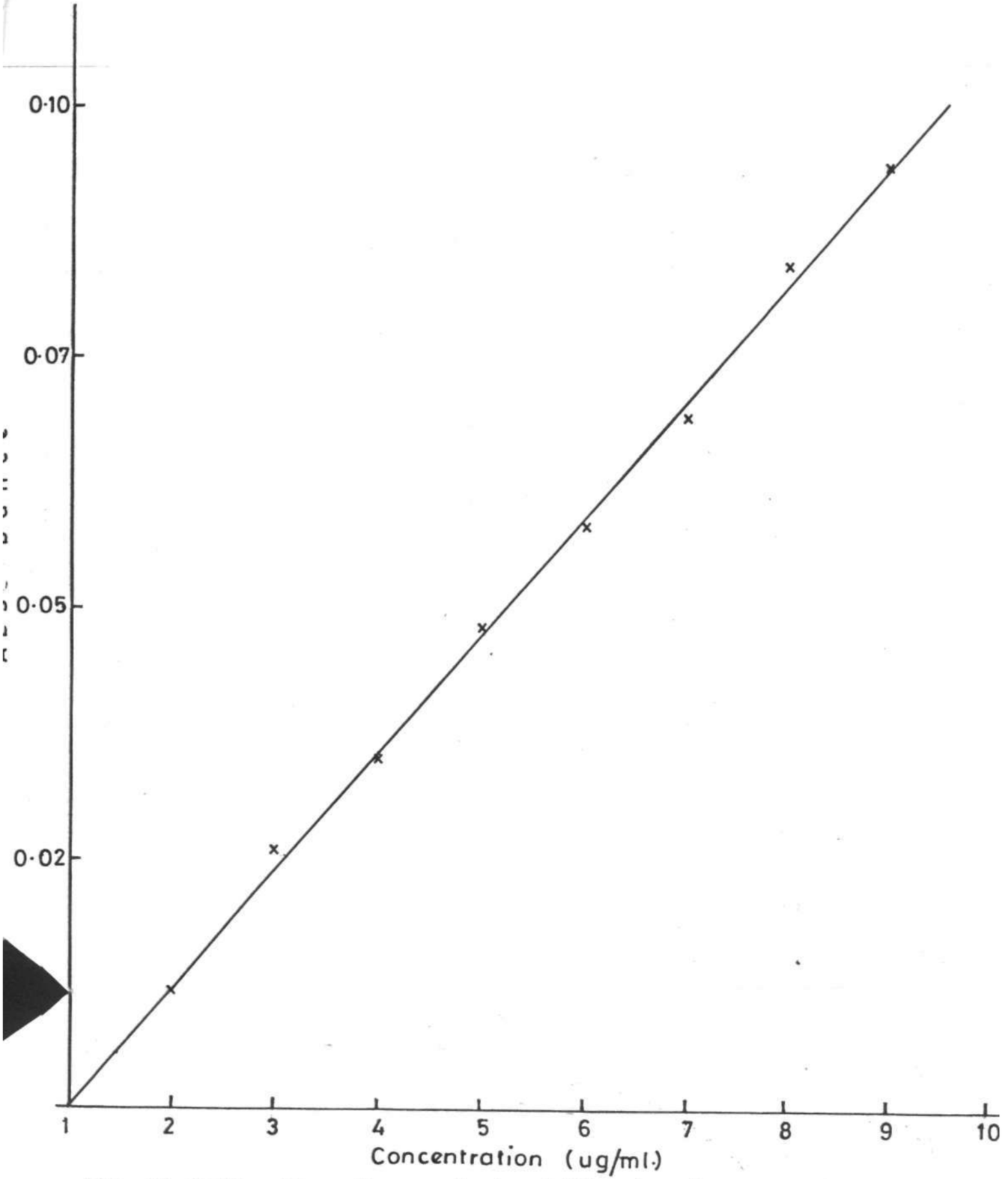


Fig. 12 Calibration Curve of Ampicillin by Pan's method

The percentage of penicillin found is directly proportional to value of zug/ml read off from the calibration curve.

CHAPTER THREE

RESULTS AND DISCUSSION

The results of the various experiments for the verifications, applications and comparisions of the proposed method are discussed as follows.

3.1 N-ETHYLMALEIMIDE METHOD

This reagent in presence of mercuric chloride reacted instantly with penicillins to give rise to yellow or whitish chromophore. The chromophore so produced was found to absorb in the ultra-violet region of the electromagnetic spectrum. Coupled with this, the chromophore is appreciably stable for about 2 hours. The reagent is extremely sensitive to penicillins, provided the temperature and pH are carefully controlled. The main constraint was found to be the tendency to form precipitate at high concentration (i.e. above 26 ug/ml), high temperature and acidic medium.

Wavelength of maximum absorption: From the ultra-violet spectrum of this chromophore (see fig. 4), it was found that for most penicillins, the derivative absorbs maximally at 274 nm. For the various experiments, this wavelength was chosen for the assay of the penicillins. Moreover, the absorbance at this wavelength was found to be devoid of flunctuation as experienced with other

chromophores.

Chromophore stability: The chromophore was found to be appreciably stable reaching its maximum intensity after 60 minutes. Relatively, readings could be taken between 20 and 40 minutes where the absorbance is very close to maximum. As indicated on fig. 5, the absorbance fell after 65 minutes when it became stable. However, there is appreciable stability between 20th and 25th minutes, 35th and 55th, and 65th minutes. As for the acceptable timing for measurement of absorbance, 20 and 40 minutes are recommended. (see fig. 5).

Beer's law:- This law was observed to be obeyed between 0 and 14 ug/ml in most cases; to some extent between 0 and 16 ug/ml. The limit of detection being about 1ug/ml,, can be further lowered by some modifications. However, the fluctuation effect of pH and temperature may affect the linearity of the Beer's law curve particularly when these factors flunctuate. In essence, pH, temperature and interferent must be strictly controlled, in order to get meaningful results. The most desirable pH range is between 6.8 and 8.0

Table 3.1 shows that absorbances of the different penicillins increase with concentration.

On the other hand, fig. 6 illustrates that Beer's law is obeyed for the different penicillins. From the experiment, it is possible to detect and determine penicillins even at micro-level.

TABLE 3.1: ABSORBANCES OF PENICILLINS BY N-ETHYLMALEIMIDE METHOD (at 274 nm)

Concentration (ug/ml)	Absorbance			
	Ampicillin	Penicillin G	Cloxacillin	Penicillin V
2	0.053	0.059	0.060	0.030
4	0.117	0.128	0.151	0.079
6	0.176	0.193	0.214	0.088
8	0.268	0.250	0.318	0.125
10	0.353	0.270	0.425	0.166
12	0.380	0.381	0.489	0.223

Temperature:- This an important factor whose strict control is essential for this reaction. It was observed that this reaction took place at room temperature particularly between 20°C and 28°C. Moreover, any slightest increase in temperature usually results in destabilization of the reaction, coupled with the precipitation of the chromophore.

pH: The chromophore was observed to be stable in neutral or slightly basic medium. Below pH 5.0, the chromophore tends to precipitate. Precisely, it is unstable at the extremes of pH.

This reagent was found to most sensitive to ampicillin, a factor reminiscent of the auxochromic effect of the free amino groupings. The sensitivity to micro-quantity of penicillins makes it suitable for even trace analysis of these drugs; a feature which is uncommon with most techniques.

3.2. PHOSPHOTUNGSTATE METHOD

Phospho-18-tungstic acid, otherwise known as Folin-Denis reagent, in presence of 2% $HgCl_2$ solution was observed to react instantly with penicillins to form a stable, grenish-blue chromophore. The reaction was rapid with the chromophore attaining its maximum intensity after about 2 hours. However, a brief warming on water-bath hastened the attainment of the maximum intensity.

The intensity of the colour was found to be directly proportional to the concentration of penicillin.

Wavelength of maximum absorption:- The chromophore being greenish-blue, was found to absorb maximally at 764 nm in the red region of the visible spectrum. The colour of the chromophore is an asset to its analytical application. Fig. 1 illustrates the absorption spectrum of the chromophore. Another important point is that this chromophore absorbs intensely in the ultraviolet region, with highly flunctuating intensities at any given wavelength. As a result, reading of absorbance in this region is unreliable; and hence restricted to visible region.

Stability of colour:- Provided the chromophore is warmed for 5 minutes, the colour is very stable; in fact for several hours. Fig. 2 illustrates the colour stability of this chromophore. The unheated preparation was found to attain

its maximum intensity after a few hours. It is important to note that the life-span of the chromophore is in order of 18 hours. Another important advantage of this method is that it is devoid of precipitation even at the extremes of concentration, pH and temperature. Moreover, the intensity does not much flunctuate with time. The stability of this chromophore as indicated in the graph (fig 1) and by the life-span is strong indication of the versatility of this method.

Beer's law:- This law was observed to be obeyed between 1 and 14 ug/ml, with a detection limit of about 1 ug/ml or less. Table 3.2 shows that the absorbances of the different penicillins increase with concentration.

On the other hand, fig.3 illustrates that Beer's law is obeyed for the different penicillins.

TABLE 3.2. ABSORBANCES OF PENICILLINS BY THE PHOSPHOTUNSTATE METHOD AT 764 nm

Concentration (ug/ml)	Absorbances			
	Ampicillin	Penicillin G	Cloxacillin	Penicillin V
2	0.082	0.069	0.030	0.033
4	0.178	0.092	0.066	0.061
6	0.248	0.149	0.104	0.096
8	0.338	0.183	0.138	0.128
10	0.445	0.287	0.186	0.152
12	0.534	0.324	0.228	0.200
14	0.613			

pH: This is an important factor in this experiment. The absorbance of a given quantity of penicillin was found to be higher in the basic medium. In absence of acetate buffer, the absorbance was found to be higher. The use of buffer was to diminish the effect of interferents. The intensity of the chromophore (both mercaptans and penicillamine derivatives), tends to decrease with increasing acidity[52]. The most suitable pH for the experiment is 4.7 and to some extent 8.0.

Interferents:- The study of the effect of interferents has not been rigorously pursued; but Wild[52] indicated that reducing entities such as cation, urea, uric acid, and mercaptans could affect the results. On the other hand, acetone, acetaldehyde tend to inhibit the colour formation[52]. It was observed that the chromophore assumed greenish-blue colouration as against blue with penicillamine[9]. This change in the usual colour may be due to the cumulative effect of penilloaldehyde and mercuric chloride.

This method like N.E.M. method is highly sensitive and hence, it can be applied to trace analysis of penicillins.

3.3. ESTIMATION OF PENICILLINS IN URINE

As one of the objectives of this research, application of the methods to assay of penicillin in biological fluid is essential. To this extent, the methods were applied to estimation of penicillins in urine, the results of which

are displayed in table 3.3.

TABLE 3.3. RESULTS OF ESTIMATES OF PENICILLINS IN URINE

Compound	Phosphotungstate method	N.E.M. method	B.P.(1973) method	Pan's method
	Percentage (%) estimated	Percentage (%) estimated	Percentage estimated (%)	Percentage estimated (%)
Ampicillin	73.3 ± 0.97	62.3 ± 0.88	61.0 ± 1.0	62.6 ± 0.44
Penicillin V	132.0 ± 1.8	100.0 ± 1.2	-	95.0 ± 1.0
Cloxacillin	180. ± 1.0	107 ± 1.3	105.0 ± 1.25	105 ± 1.0
Penicillin G	61.6 + 0.88	40.0 ± 2.0	52.5 ± 0.5	60.6 ± 1.2

The results show a great deal of variation in the quantity estimated in each sample from one method to the other. These variations are reminiscent of the relative influence of the interferents within each extract, adaptation of each technique to the problem, and the difference in operating principle of the methods employed. From the results, ~~the values~~, the values of drug estimated by Phosphotungstate method are highest, reason being that the reagent appears to have reacted with urine component which might have been extracted along with the drugs. It must be borne in mind that phosphotungstic acid also react with urea, and uric acid which are also present in urine[52].

From this experiment, it was observed that the proposed methods were more sensitive than the standard methods employed. This is indicated by the relatively high absorption of the chromophores of both methods compared to the low absorbance of the same concentration by the B.P. and Pan's methods. Except for the higher values with the phosphotungstate method, the 3 other methods prove suitable for estimation of penicillins in urine. The relatively low absorption of the Pan's and B.P. methods chromophores makes the methods relatively susceptible to errors.

As a result, none of these method is adequately equiped for the assay of all types of penicillins.

In the course of this experiment, the B.P. (1973) method for assay of ampicillin was modified, a modification which shifted the wavelength of absorption from 320 nm to 339 nm. Using ampicillin dissolved in 0.1N NaOH, the sensitivity was improved with detection limit of about 2ug/ml compared to 12 ug/ml recorded by the literature[26].

3.4. QUANTITATIVE ESTIMATIONS OF PENICILLINS IN PHARMACEUTICAL PREPARATIONS

This exercise was purely a comparative spectrophotometric analysis of penicillins in various pharmaceutical products. This was aimed at appraising, criticising and pin-pointing the apparent deficiencies in the methods employed in each

case. The contents of the various brands of drugs were calculated from the corrected calibration curves using standard samples of penicillins. The results of the quantity estimated in each group of drugs are discussed separately in order to pin-point the possible causes of variation encountered in the analysis. The results are displayed in Table 3.4.

(a) Ampicillin

6 Different samples of various brands and dosage forms of this drug were assayed. The sample were mainly capsules and syrups.

From the result in table 3.4, it was observed that the estimate of any given sample vary from one method to the other. These variations may be due to the relative reaction of the additives of each drug to the reagent used for each method. Other probable reasons may be the differences in the operating principle of the various methods, inhibiting or modifying influence of the additives, efficiency of the methods and the experimental conditions. It was observed that the value estimated by B.P. method in each drug is higher than those of the two proposed methods. This may be due to the low absorbance obtained using B.p. method, and hence the error associated with the low sensitivity. The standard deviation obtained for each drug is a measure of precision and comparison.

TABLE 3.4: RESULTS OF PENICILLIN ESTIMATED IN PHARMACEUTICAL PREPARATIONS

Code number	Drug (Brand name)	Claimed quantity	Active ingredient	Mean weight	Mean percentage estimated per claimed label (%)				
					Phosphotungstate method (%)	N-ethylmaleimide (N.E.M) method (%).	B.P.(1973) method (%)	Pan's method (%)	Standard deviation
A_1	Roscillin capsule (Ranbaxy) capsule	250mg	Ampicillin trihydrate	315.6mg	102.8	104.3	109.4	-	2.82
A_2	Penbritin capsule (Beecham)	"		299.1mg	108.1	105.6	111.8	-	2.55
A_3	Elkocillin capsule(Elko)	"		297.4mg	104.6	103.1	102.8	-	0.787
A_4	Danpicillin syrup (Dangi)	125mg/5ml		-	114.3	115.0	122.8	-	3.85
A_5	Pentrexyl syrup	125mg/5ml		-	108.3	105.6	115.6	-	4.22
A_6	Ampicillin syrup(Rajrab)	125mg/5ml		-	95.0	93.8	93.8	-	0.565
G_1	Penicillin G injection (Hoechst)	600mg	Penicillin G Sodium	617.4mg	99.5	97.1	-	100.9	1.57
G_2	Crystapen injection (Glaxo)			620.1mg	100.5	100.9	-	100.9	0.188
C_1	Cloxacillin capsule (Helm)	250mg caps.	Cloxacillin sodium monohydrate	270.5	106.0	109.3	111.4	-	2.22
C_2	Orbenin capsule (Beecham)			308.1	119.5	123.2	120.1	-	1.622
C_3	Staphyclox injection (Dianic)	250mg injection		265.0	106.0	105.0	105.0	-	0.471
C_4	Cloxacillin injection (Rajrab)	"		267.4	115.6	115.0	113.1	-	1.06
C_5	Cloxacillin syrup	125mg/5ml		-	102.5	103.8	105.8	-	1.36
V_1	Scanicillin V tablet	250mg tab.	Penicillin V potassium	327.1	114.7	111.2	-	114.6	1.62
V_2	Ospen 400 syrup		"	-	116.0	112.0	-	117.5	2.32

Low value of this deviation is an indication that the methods compare favourably with each other. The values are particularly high for pentrexyl syrup and Dangi syrup, which may be due to the effect of additives used to fortify them. The result obtained for Elkocillin and Ampicillin syrup are the best as suggested by their values of standard deviation and the estimates. The problem of variation is less encountered in most capsule than the syrups.

For a new method, mean deviation of about 3.5% is still tolerable, in view of the fact that these methods are still in their infancy and hence the insensitivity of the B.P. method used to compare them.

It would be noted that the values estimated in most cases are higher than 100%, this being so for the fact that a given drug usually contain excess of the claimed content in order to compensate for the possible losses in degradation. The B.P. standard recommends that a given capsule, injection, syrup and tablet must contain between 97.4 to 107.4% of the claimed content.

(b) Penicillin G products

Two products of this drug were employed and they were both injection powders. The result of both products are to a great extent remarkably consistent, with standard deviation of less than 1.6%. This is also true for other injections.

This is as a result of the apparent purity of injections in general, with little or no additive.

Crystapen injection was found to give the most correlated or concordant result in the whole analysis. The result varied between 100.5 and 100.9% with a variation of 0.4% which is the least. Moreover, the values of penicillin estimate by various method are still within the B.P. standard (97.4% to 107.4%.

On the other hand, the results, of sample G_2 Penicillin G (Hoechst) are less consistent. The results vary from 97.1% to 100.9%, with the standard deviation of 1.57% which is still within a tolerable limit.

The performances of the various methods are good, except for the Pan's method which is less sensitive than either of the proposed method. The sensitivity is indicated by the relatively low absorbance of highly concentrated penicillin preparation. This is however an advantage of the proposed method over the Pan's method. It should be noted that there has been no specific spectrophometric method recommended for the assay of this drug. In fact, the official methods recommended for penicillin G is titrimetry[8,9].

(c) Cloxacillin products

5 Various products such as injections, capsules and syrups were assayed. The results also follow the same pattern of other drugs.

From table 3.4, the injections particularly staphyclox and cloxacillin injections gave fairly consistent result with standard deviation in tune of about 1.06% Staphyclox injection was found to contain between 105% and 106% with a variation of about 1% and mean deviation of 0.471% while the other sample is 1.06%. Moreover the estimates were found to be within B.P. limit of purity.

The results of both injections also confirm the impression of the apparent purity of injection powders. From these assays, it is right to deduce that most injections are apparently devoid of additives.

The two capsules, were found to give highly variable results as indicated by their standard deviation. The standard deviation values are 2.22% for cloxacillin capsule and 1.62% for orbenin capsules. The values estimated for orbenin capsule are particularly high (i.e. 119.5% to 123.2%). These high values are characteristic of Beecham products (ref penbritin). It was also observed that in most cases, the estimates obtained by B.P. method are higher than those of the proposed method. The reason for this may be due to the effect of additives and the apparently low absorbances and hence apparent in sensitivity of the method.

Moreover the additives of capsules syrups and tablets cannot but contribute to the highly flunctuating results

obtained for any given sample. The additives usually employed in pharmaceutical preparation include antioxidant, sweetening and coloring agents. Their influence may be either inhibiting or modifying.

The cloxacillin syrup was estimated to contain between 102.5% and 105.8% of the drug. This result is particularly good with standard deviation of about 1.36% compared to 4.32% of pentrexyl syrup.

(d) Penicillin V products

Two samples; syrups and injections were assay. The methods used were the two proposed methods and the Pan's method. From the Table 3.4, Scanivillin V tablet was estimated to contain between 111.2% and 114.7% of drug. These values vary with standard deviation of 1.62% which are still tolerable for the comprison of new methods. Moreover, the Pan's method was incapacitated by its low absorption and hence its apparently low sensitivity. The variation may be due to a high content of additives used to fortify the drug.

The ospen syrup, pink in colour, was estimated to contain between 112.0% and 117.7% of drug with a standard deviation of about 2.32% which is high. This value is however tolerable in view of the fact that the sample is heavily fortified with colouring, sweetening, adhesive and preservative

reagents. These additives cannot but seriously influence the results.

(f) Important note

The high value of percentage estimate of drug per labelled claim is a common phenomenon in pharmaceutical analysis. This being so due to the fact that every drug; capsules, injections and tablets, are packed to contain excess of the content claimed. The excess of ingredient in any particular package is to compensate for possible losses of potency by degradation. It is also to help in maintaining the required potency of the drug. These high values; usually more than 100%, are also manifested in the results of colorimetric assay of ampicillin by Choudry[11], Patel *et al*[38] and Thomas[45].

The variation in the results of any given sample by various methods are direct inference of the selectivity, suitability, sensitivity and precision of each method to a given analysis.

3.5. SUGGESTION FOR FURTHER STUDIES

Relatively, the proposed methods are yet to be extensively applied to the analysis of penicillins. Moreover, a lot more needs to be done to bring them to a sound analytical footing. In view of this, the following suggestions are made:-

(i) Thorough investigation of the potential interferents like other antibiotics, aldehydes, ketons, mercaptans, urea, cations and uric acid.

(ii) Trial of this method to the analysis of sister antibiotic; cephalosporins.

(iii) Detailed studies of the chemistry of the reaction involved.

(iv) Studies of the electrode potential of the reagents and the resulting chromophores with a view of establishing the chemistry of the reaction.

(v) Possible modifications and refinement that can be made to improve these methods.

It is believed that the follow-up of these steps or suggestions can help to some extent in establishing these methods as suitable alternative approaches to penicillin analysis.

3.6 CONCLUSION

From the results obtained from the various experiments, the two proposed methods appear to compare favourably with the established procedures.

In all ramifications, the proposed methods appear to be more suitably equiped for penicillin assays than any of the B.P. and Pan's method. It was noted that both could be applied to the assay of penicillin in biological medium,

provided the drug is efficiently extracted from the medium. The main constraint in the assay of penicillin in biological fluids; particularly urine, is the interaction of urea or uric acid with phosphotungstic acid. However, the N-ethyl-maleimide method is equally as effective as the B.P. method in this regards. Provided the extract is urea-free, phosphotungstate method can be applied.

In analysis of pharmaceutical products, the two methods were very effective, in fact, they gave better results than the established method.

The variation of results obtained for a given sample stems from weak intensity of the coloured species of the B.P. and Pan's method. It can be therefore be deduced that the proposed methods are more sensitive, than either of the established methods. For instance, the absorbances of penicillins by both methods are relatively higher compared to the low absorbances of the same concentration of drug by either of the two methods. Also, the limit of detection by phosphotungstate and N-ethylmaleimide methods is about 1ug/ml for all penicillins compared to 10 - 20ug/ml in Pan's and B.P. method. Coupled with this, the absorbance of 10ug/ml sample of cloxacillin by B.P. method is 0.022 compared to 0.060 of 2ug/ml sample by phosphotungstate and N-ethylmale-imide method - same applies to Pan's method.

Another advantage of these method is the relative stability of their chromophores. The chromophores employed in both methods are quite stable, devoid of precipitate and they absorb distinctly at their characteristic wavelength. The penicillenic acid derivative of B.P. method is very unstable.[8,26,43]

In terms of duration of experiment, the time required for a single analysis by phosphotungstate method is about 30 minutes, N-ethylmaleimide method; 25 minutes, compared to 60 - 75 minutes for B.P. method and 35 minutes for Pan's method.

The proposed methods are quite simple and their requirements are quite easy to obtain.

In essence, the two methods are simple, sensitive, precise and relatively reproducible. The best method among the two is the phosphotungstate method in that it is more reliable though less sensitive than the N-ethylmaleimide method.

With the yearning need for a sensitive, convenient and precise method of analysis of penicillins, it is believed that these methods will help in providing the much-sought solution to this problem.

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