STABILITY STUDIES ON PENICILLIN DERIVATIVES

By

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SUMMARY

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By John Michael Hempenstall

A thesis presented for the degree of Doctor of Philosophy in the University of Aston in Birmingham, 1982.

Current analytical assay methods for ampicillin sodium and cloxacillin sodium are discussed and compared, High Performance Liquid Chromatography (H.P.L.C.) being chosen as the most accurate, specific and precise.
New H.P.L.C. methods for the analysis of benzathine cloxacillin; benzathine penicillin V; procaine penicillin injection B.P.; benethamine penicillin injection; fortified B.P.C.; benzathine penicillin injection; benzathine penicillin injection, fortified B.P.C.; benzathine penicillin suspension; ampicillin syrups and penicillin syrups are described.
Mechanical or chemical damage to column packings is often associated with H.P.L.C. analysis. One type, that of channel formation, is investigated. The high linear velocity of solvent and solvent pulsing during the pumping cycle were found to be the cause of this damage.
The applicability of nonisothermal kinetic experiments to penicillin V preparations, including formulated paediatric syrups, is evaluated. A new type of nonisothermal analysis, based on slope estimation and using a 64K Random Access Memory (R.A.M.) microcomputer is described. The name of the program written for this analysis is NONISO.
The distribution of active penicillin in granules for reconstitution into ampicillin and penicillin V syrups, and its effect on the stability of the reconstituted products, are investigated. Changing the diluent used to reconstitue the syrups was found to affect the stability of the product.
Dissolution and stability of benzathine cloxacillin at pH2, pH6 and pH9 is described, with proposed dissolution mechanisms and kinetic analysis to support these mechanisms. Benzathine and cloxacillin were found to react in solution at pH9, producing an insoluble amide.

KEY WORDS

Penicillin, Stability, Nonisothermal, Assay, Dissolution.

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In addition, I would like to thank Lindsay and my family for all the encouragement and patience they have shown during the preparation of this thesis.

To my parents and Lindsay

A little learning is a dang'rous thing;
Drink deep, or taste not the pierian spring:
There shallow draughts intoxicate the brain,
And drinking largely sobers us again.

Alexander Pope (1688-1744)

If a man will begin with certainties,
He shall end in doubts;
But if he will be content to begin with doubts,
He shall end in certainties.

Francis Bacon (1561-1626)

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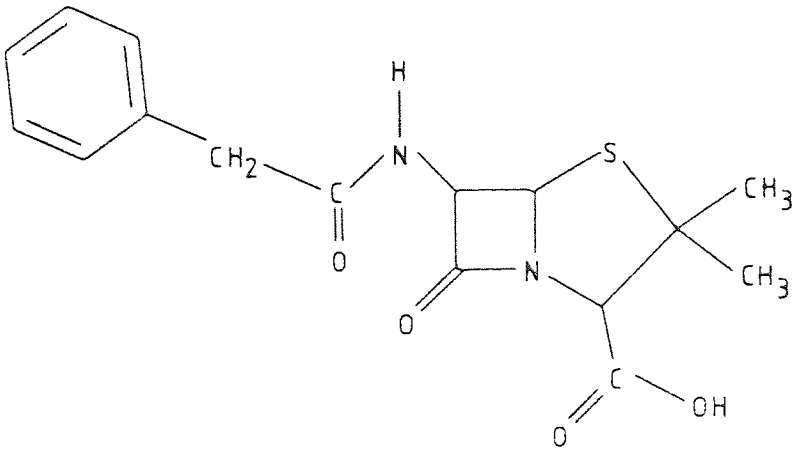


FIGURE 1.1 BENZYLPENICILLIN (PENICILLIN G)

The discovery of penicillin has been attributed to Fleming, although the antibacterial properties of the Penicillium genus had previously been observed (1-8). Fleming was the first researcher to report the low toxicity of Penicillium broth filtrates (9). His rediscovery led to attempts, by Clutterbuck (10) and Reid (11), to concentrate penicillin and encouraged slight clinical use of the material (12). However, it was not until Chain, Florey and Heatley (13,14) became involved that research on penicillin gained real impetus. They improved the yield of penicillin sufficiently to facilitate independent clinical trials and later advised American pharmaceutical companies in developing the fermentation technology required to produce penicillin in large quantities. During the 1940's, much effort was expended on the structure elucidation of penicillin. Eventually, penicillin was found to be a family of closely related chemical entities and not a single substance (15). The addition of phenylacetic acid to the fermentation medium led to an increased yield of one of these penicillins, penicillin G or benzylpenicillin (16). The structure of this compound can be seen in figure 1.1.

Benzylpenicillin was known to be unstable in solution, especially in gastric juice, and to be hydrolysed by penicillinase (beta-lactamase) produced by Escherishia coli (17). It possessed biological activity against Gram positive bacteria but little activity against Gram negative types (18).

In order to overcome these limitations, a search for new penicillins with improved properties took place. Desirable properties were:

(i) improved acid stability

(ii) broadened microbiological spectrum

(iii) improved activity against resistant organisms

(iv) improved metabolic or pharmacokinetic efficiency, such as slow excretion, better tissue diffusion or better oral absorption.

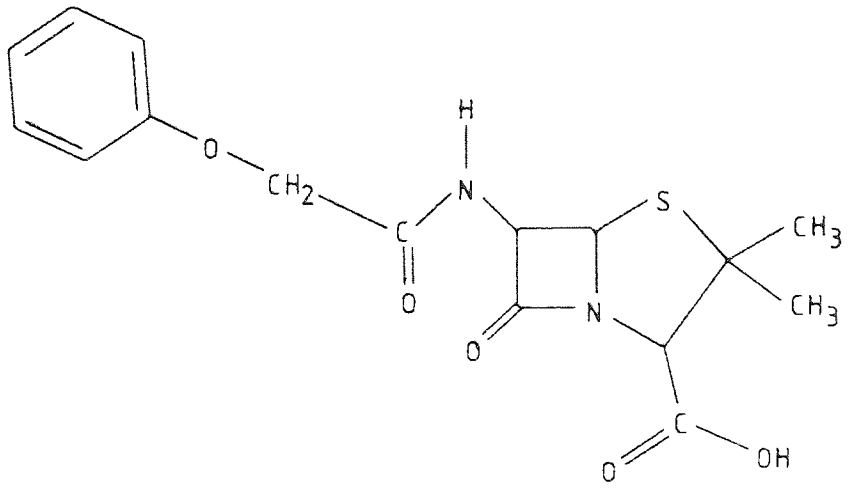


FIGURE 1.2 PHENOXYMETHYLPENICILLIN (PENICILLIN V)

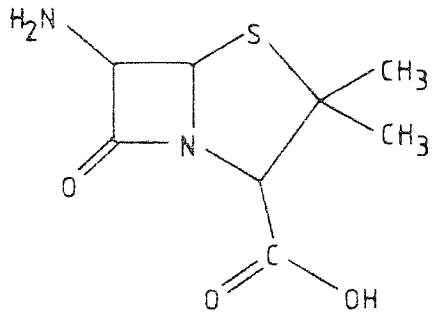


FIGURE 1.3 6 - AMINOPENICILLANIC ACID (6 - APA)

(v) decreased allergenicity

The first useful biochemical modification of the penicillin molecule came with the production of phenoxymethylpenicillin (penicillin V) in 1948 (19). The structure of this compound can be seen in figure 1.2. This penicillin was readily produced during fermentation by adding phenoxyacetic acid to the medium. Phenoxymethylpenicillin resisted hydrolysis by gastric juice and was thus suitable for oral administration.

The most critical discovery in the search for new penicillins came when Batchelor noticed that relatively large amounts of a virtually inactive form of penicillin were formed during fermentation, provided no side-chain precursors were present (20). The inactive molecule proved to be 6-aminopenicillanic acid or 6-APA (figure 1.3).

Simple acylation of 6-APA made possible the synthesis of a vast number of penicillin antibiotics. Methicillin, which is highly resistant to staphylococcal penicillinases (21) and cloxacillin, which possesses similar resistance to staphylococcal penicillinases, but can also be given orally (22), soon appeared. Ampicillin, first produced in 1961, though vulnerable to penicillinases, gave a broad spectrum of action, being effective against a wide range of Gram negative bacilli (23). Six years later, carbenicillin was prepared, extending the effectiveness of the penicillin range to *Pseudomonas aeruginosa* and a number of ampicillin-resistant Gram negative bacilli (24). Since this time, many useful penicillins have been prepared such as pivampicillin (25), talampicillin (26) and carfecillin (27), penicillin esters with improved bioavailability.

Recently, two groups of powerful penicillinase-inhibitors have been derived from *Streptomyces* species. One group show weak intrinsic antibacterial activity *in vitro* and *in vivo*. An example of this group is clavulanic acid (28). Its structure can be seen in figure 1.4.

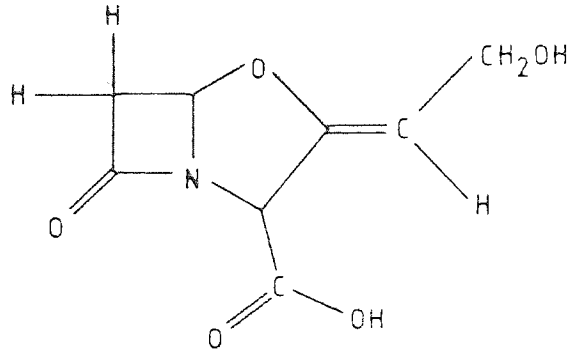


FIGURE 1.4 CLAVULANIC ACID

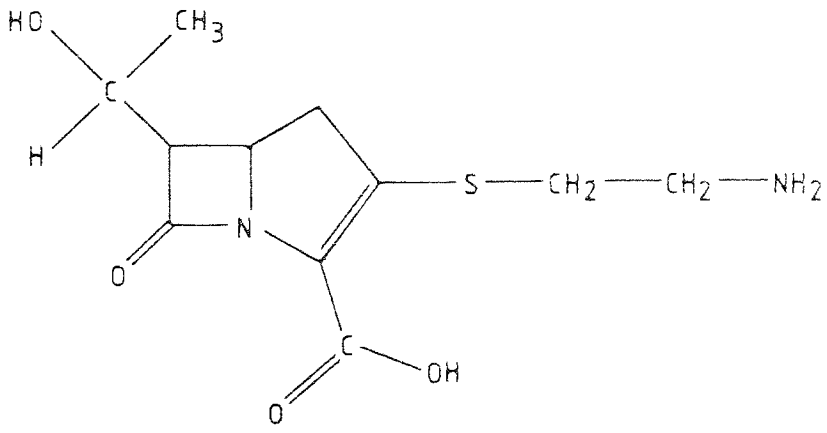


FIGURE 1.5 THIENAMYCIN

It exerts a broad spectrum, irreversible, inhibitory action against penicillinases from staphylococci and most Gram negative bacteria. It is given in combination with other penicillins that are not so penicillinase resistant, most notably amoxycillin. The other group, for example thienamycin (29), irreversibly inhibit many penicillinases and possess useful antibacterial activity against Gram positive and Gram negative organisms. The structure of thienamycin can be seen in figure 1.5. Unfortunately, compounds from this group tend to be unstable in aqueous solution.

The antibacterial action of the various penicillins primarily depends on the fused beta-lactam-thiazolidine ring (the penicillin nucleus), since a break at any point leads to complete loss of activity, irrespective of the side chain (30,31). Penicillin potency, however, is controlled to a great extent by the nature of the side chain (30,31).

The beta-lactam ring of penicillins is labile, there being evidence for a correlation between ring lability and antibacterial activity (32). It is often this ring that breaks open during degradation of penicillins, rendering them inactive. Common degradation pathways are shown in figures 1.6 and 1.7 (33).

The many and varied degradation products produced from penicillins necessitate the use of specific assay methods to selectively analyse the active penicillin (34). For this reason, the initial section of this thesis concentrates on assay methods for ampicillin sodium and cloxacillin sodium, two representative penicillins. This is followed by a section on the develop ment of new analytical assay methods for penicillins, and another on one particular problem associated with High Performance Liquid Chromatography (H.P.L.C.), a modern technique for the analysis of drugs.

Penicillins are chemically reactive compounds, often degrading rapidly in solution (35). It is therefore necessary to study the kinetics of

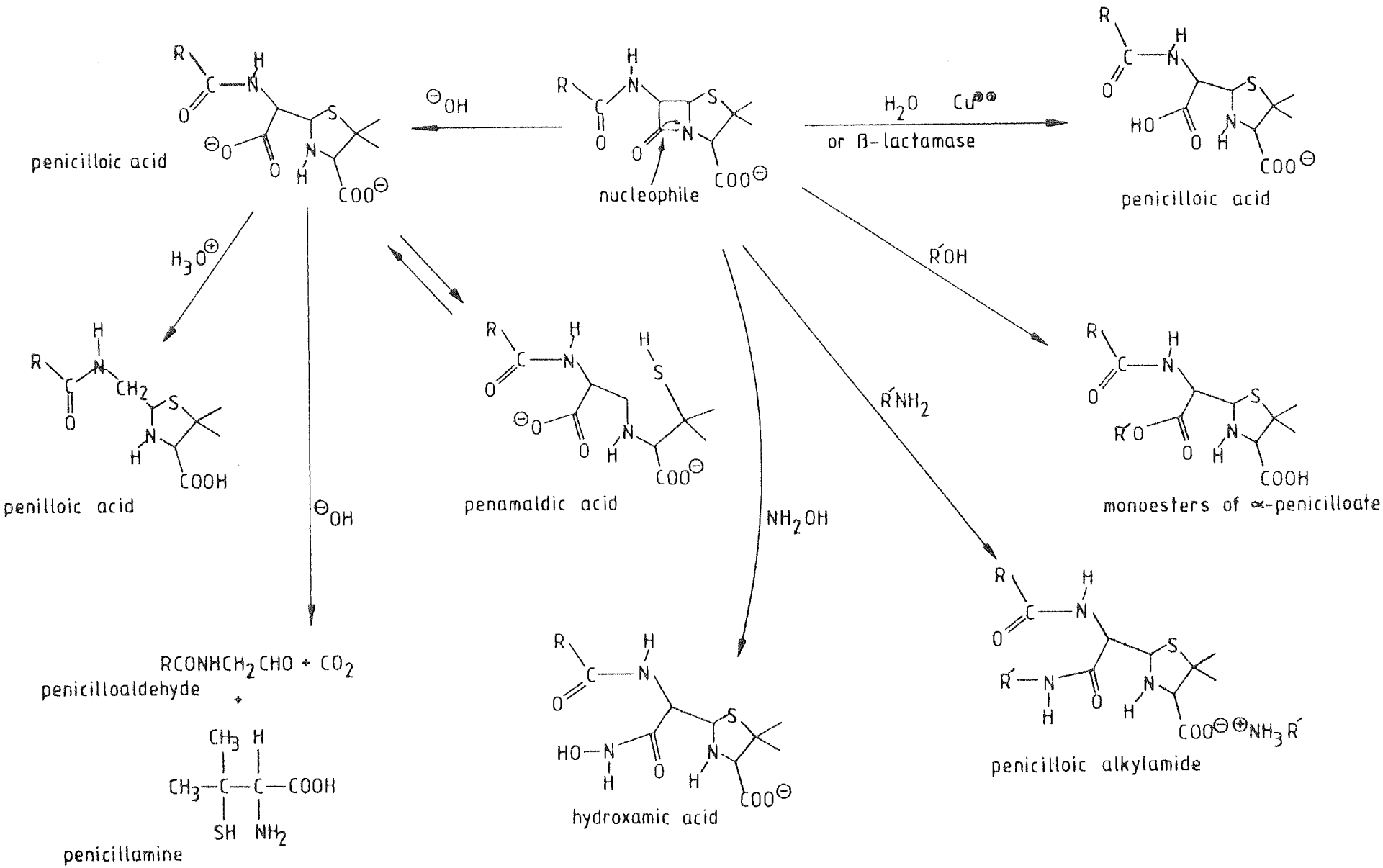


FIGURE 1.6 NUCLEOPHILIC ATTACK REACTIONS

their degradation so that shelf-life estimates can be made for different preparations. Kinetic analysis of penicillins is examined in this thesis with a special emphasis on nonisothermal studies.

Penicillins have been formulated into syrups for oral administration (36). Due to poor stability of the constituent penicillins, these syrups have been manufactured as dry powders, ready for reconstitution with water when required. Segregation of penicillins in the dry powder can occur. This is examined, as is the stability of the reconstituted syrups.

Rapid, high blood levels of relatively short duration usually result after the administration of a penicillin to a patient (37). In order to produce more consistent blood levels for a longer period of time, depot-release penicillins have been developed (38,39). With these compounds, it is essential that release characteristics are understood to ensure the correct blood level and duration of action are achieved. One such depot release penicillin is benzathine cloxacillin.

Its release rate, measured by dissolution in aqueous solution, is examined, as are some chemical reactions involving cloxacillin and benzathine in these systems.

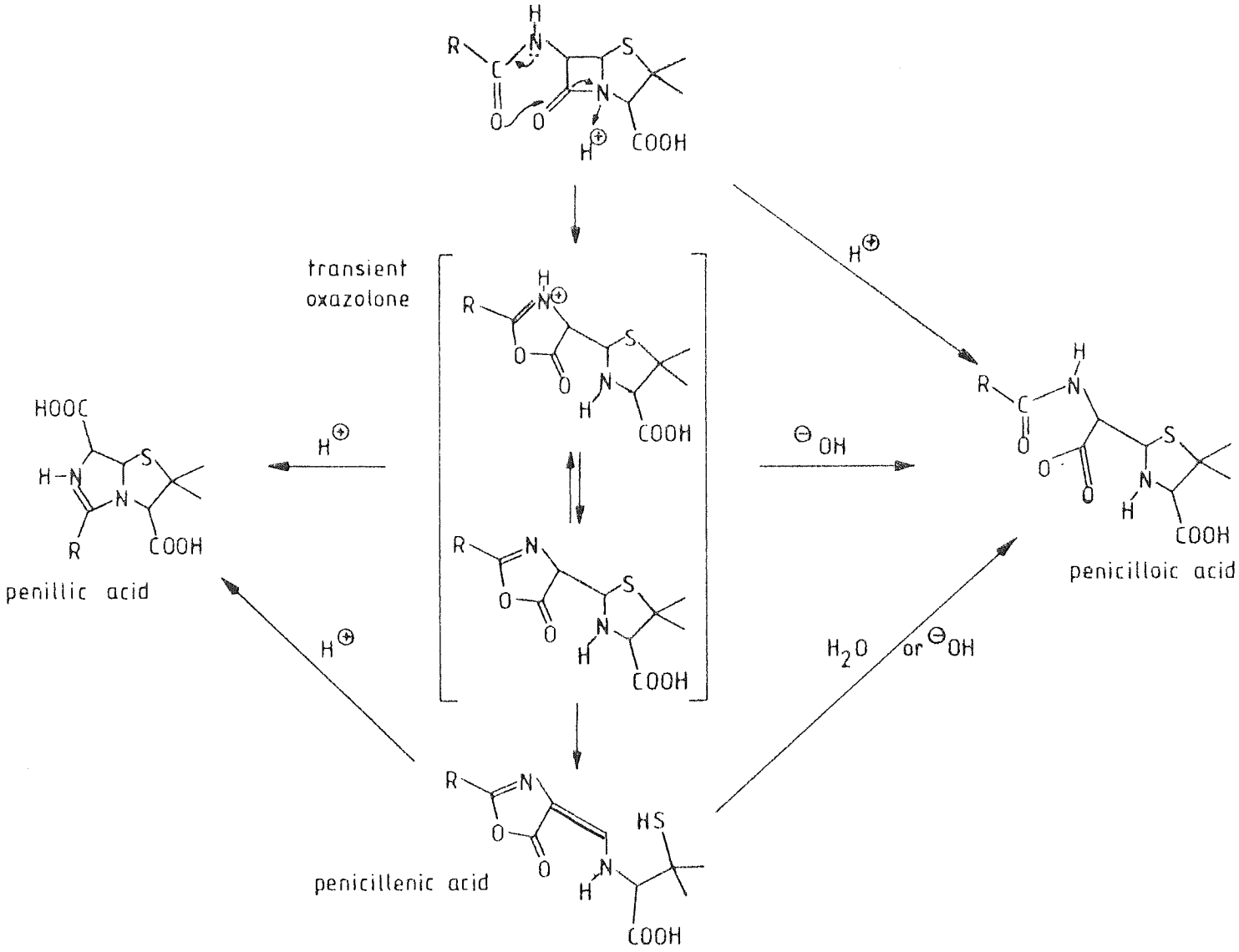


FIGURE 1.7 ELECTROPHILIC ATTACK REACTIONS

CHAPTER TWO : THE COMPARISON OF ASSAY METHODS FOR AMPICILLIN SODIUM AND CLOXACILLIN SODIUM

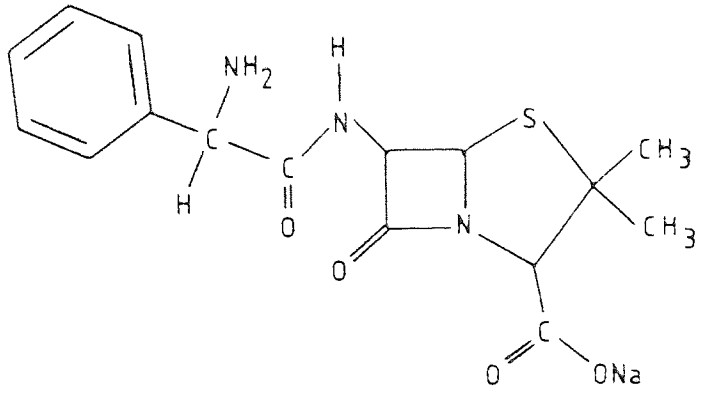


FIGURE 2.1 AMPICILLIN SODIUM

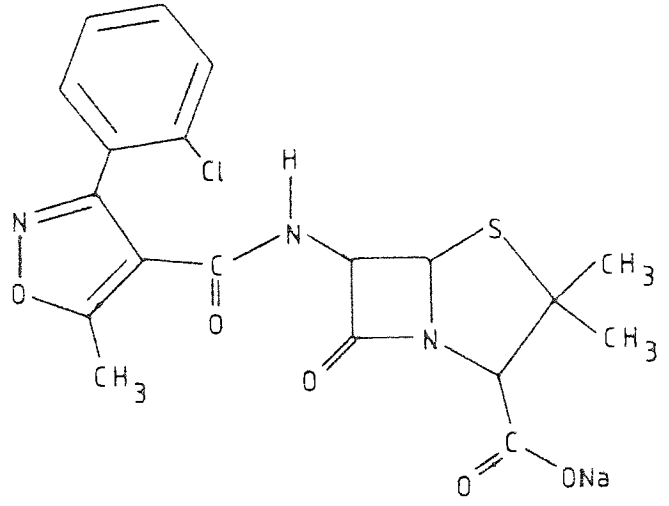


FIGURE 2.2 CLOXACILLIN SODIUM

2.1 INTRODUCTION

The British Pharmacopoeia (40) and Pharmaceutical Codex (41) contain standard monographs for penicillins used in medicines in Britain. These monographs contain details of quantitative analysis, which are usually updated whenever more accurate, precise and specific methods are developed.

To evaluate pharmacopoeial assay methods, an experimental comparison between the British Pharmacopoeia 1973 method and alternative techniques available, was carried out, using ampicillin sodium and cloxacillin sodium as representative penicillins (figures 2.1 and 2.2). The British Pharmacopoeia 1973 method for ampicillin sodium involves heating a solution of the antibiotic in buffered copper sulphate and measuring the ultra violet absorbance of the resulting stabilised penicillenic acid (42).

The British Pharmacopoeia 1973 method for cloxacillin sodium requires the addition of sodium hydroxide, the application of heat to complete reaction and titration of the excess alkali with standard acid solution (43).

A review of the techniques available for the determination of ampicillin sodium and cloxacillin sodium revealed a large number of different methods. These can be divided into eight main categories :

(i) Microbiological

(ii) Titrimetric

(iii) Spectrophotometric

(iv) Fluorometric

(v) Chromatographic

(vi) Infra-Red Spectroscopic (I.R.)

(vii) Nuclear Magnetic Resonance Spectroscopic (N.M.R.)

(viii) Polarimetric

Microbiological assay was the first to be used in the determination of penicillins (14). This technique provided a direct measure of the antibacterial potency, but lacked specificity and precision, and was time consuming. Chemical and physical methods [(ii) to (viii)] were developed to overcome these failings.

Titrimetry includes iodometric determination, an assay based on an observation by Alicino (44), where benzylpenicillin molecules did not react with iodine, but iodine was absorbed by products of alkali inactivation. This method has been adopted as the official method of assay for some penicillins by official compendia (45). A review of titrimetric assays for penicillins has been made (35). More recently, de Leo et. al. (46) used the resistance of cloxacillin to penicillinase deactivation in order to determine both ampicillin and cloxacillin in combined preparations, iodimetry providing the means of analysis. Karlberg and Forsman (47) based their method of assay of penicillins on a method by Grafnetterova (48), where the thiazolidine ring is opened to produce a thiol group which is then titrated with mercury(II) solution. The titration is potentiometric. Two titrations, both at pH 4 to 5 are required, one before hydrolysis (a) and one after (b). (a) determines the degradation products and (b) the intact penicillin. This method was later reported by Paal and Molnar (49). Forsman (50) extended his earlier work by determining penicillamine as well as penicillins and penicilloates, using coulometrically generated mercury instead of mercury(II) salts.

Tawakkol et. al. (51) used the property that cupric ions form 1:1 chelates with ampicillin as the basis of a volumetric determination of the antibiotic. It was claimed that degradation products and other antibiotics (streptomycin and kanamycin) did not interfere.

Non-aqueous titrations were used by Casalini et.al. (52) for assaying basic and acidic penicillins. Basic penicillins were titrated with 0.1N

perchloric acid in acetic acid or 0.1N p-toluenesulphonic acid in ethanol/benzene. 0.1N tetrabutyl-ammonium hydox in benzene/methanol was used for acidic penicillins.

Titrimetric assay methods lack specificity, being suitable for more than one penicillin. Thus some method of differential deactivation (46) or separation (53) is required when assaying mixtures of penicillins by these methods. Iodimetry is not suitable for penicillins with side chains capable of reacting with iodine (54), but these penicillins are not of great commercial importance.

Spectrophotometric methods are the most numerous, involving direct ultra-violet (U.V.) absorption (55) or colour reaction (56). Two main techniques have been developed, one involving the use of hydroxylamine; and the other, the production of penicillenic acids.

Staab et. al. (57) first reacted hydroxylamine with benzylpenicillin to produce a hydroxamic acid from the beta-lactam ring. Complexation of this acid with ferric ions gave a purple product which absorbed spectrophotometrically. Details of the method were reported by Ford (56) and modified by Boxer and Everett (58). Recently, Koprivc et. al. (59) used the method of Boxer and Everett to determine the rate of hydrolysis of cloxacillin and ampicillin in combined preparations. By selecting the optimum conditions, when the reaction rates differed by the greatest amount, they were able to apply a logarithmic extrapolation method to determine both components. The hydroxylamine method distinguishes intact penicillins from precursors and degradation products in which there are no beta-lactam functions. However,compounds which react with hydroxylamine (esters,amides,anhydrides,ketones) may interfere. This necessitates the use of a blanking procedure that ruptures the beta-lactam ring either chemically or enzymatically. Although less sensitive than iodimetry, the method has been adopted as the official method of assay for some penicillins (60).

Penicillins degrade in alkaline solution to produce penicillenic acids that absorb in the U.V. spectrum. These molecules tend to degrade rapidly, but can be stabilised by metal ions. Copper(II) ions were used in the British Pharmacopoeia 1973 assay method for ampicillin sodium (42) and mercury(II) ions have also been used (61). Bundgaard (62) discovered that imidazole catalysed the conversion of penicillins to penicillenic acids and used this property to determine cloxacillin (63) and ampicillin in samples containing ampicillin polymers (64). The assay of penicillins by conversion to their corresponding penicillenic acid is specific to compounds containing intact beta-lactam rings. Major degradation products, therefore, do not interfere.

Mixtures of penicillins, however, cannot be determined using these methods without prior separation or differential deactivation.

Recently, spectrophotometric assay methods for ampicillin, utilising the primary amino group in its side chain, have been reported by Celletti (65), Choudhury (66) and Rao et.al. (67). Other methods for penicillin assay not involving reaction of the beta-lactam ring were developed by Alicino (68), Thomas (69), Ibrahim (70), Patel (71) and Lee (72). Because they do not involve the beta-lactam ring, these methods lack specificity and cannot be used for degraded samples because degradation products are likely to interfere.

Fluorimetry has been used to assay penicillins because this technique shows high sensitivity, comparable with microbiological methods. Jusko (73) utilised the primary amino group of ampicillin to form a fluorescent product, specific to ampicillin among the penicillin group. However, degradation products interfered. More recently, specific methods that differentiate between ampicillin and its degradation products were developed by Miyazaki (74,75) and Dürr and Schatzmann (76). Kŭsnír and Barna (77) reacted fluorescamine with ampicillin, detecting levels down to 14 ng per ml. Degradation products and other

primary amines interfered. Barbhaiya and Turner (78,79) used alkaline hydrolysis of ampicillin at 100°C to produce a fluorescent product, but this was neither specific to ampicillin among penicillins nor free from interference of degradation products.

Chromatographic assay methods include paper chromatography and thin layer chromatography (T.L.C.), but these are mainly used for qualitative analysis. Gas chromatography (G.C. or G.L.C.) of penicillins has proved problematical because of their thermal lability and derivatisation of the penicillin is usually required (80,81). Recently Otani (82) separated ten penicillins by G.C., after ethyl acetate or chloroform extraction and Roy and Szinai (83) used pyrolysis G.C. to quantify fourteen antibiotics.

High-performance liquid chromatography (H.P.L.C.) is a recent technique that provides greater versatility than G.C. and allows separation of components of mixtures of penicillins and their degradation products at ambient temperature. It is particularly suitable for thermo-labile molecules such as penicillins.

Details of I.R., N.M.R. and polarimetric assay methods can be found in a recent review (35).

The methods of analysis chosen for experimental comparison with the British Pharmacopoeia 1973 methods for ampicillin sodium and cloxacillin sodium were :

(i) microbiological methods because these were the original ones used and they directly measure the antibacterial activity of penicillins ;

(ii) imidazole catalysed penicillenic acid methods as representative of spectrophotometric techniques, because ampicillin polymers can be differentiated from ampicillin using this method ;

(iii) a direct U.V. method for determining ampicillin and cloxacillin in combined preparations, because of its simplicity ;

(iv) H.P.L.C. methods as representative of chromatographic techniques.

The aims of this work were to compare the accuracy, specificity and precision of the assay methods chosen, for mixtures of ampicillin sodium and cloxacillin sodium and degraded samples of these antibiotics.

2.2 EXPERIMENTAL

2.2.1 MATERIALS

Ampicillin sodium (858 micrograms per mg. free acid) and cloxacillin sodium (900 micrograms per mg. free acid) were supplied by Beecham Pharmaceutical Research Division, Worthing. Imidazole was obtained from Aldrich Chemical Co. Ltd., Gillingham and was double recrystallised from toluene and ether washed to remove ultraviolet absorbing impurities. Water was double distilled from glass vessels. All other materials were standard laboratory reagents.

2.2.2 METHODS

2.2.2.1 Preparation of Ampicillin and Cloxacillin Samples

Degraded Ampicillin Sodium

25g of ampicillin sodium were dissolved in 100 mls of water. This solution was left for 25 hours, at room temperature, in the dark, before being freeze dried.

Degraded Cloxacillin Sodium

25g of cloxacillin sodium were dissolved in 100 mls of water. After 48 hours at room temperature, shielded from light, the solution was freeze dried.

Degraded 1:1 Mixture of Cloxacillin Sodium and Ampicillin sodium

12.5g ampicillin sodium and 12.5g of cloxacillin sodium were dissolved in 100 mls water. This solution was left for 25 hours, at room temperature, in the dark, before being freeze dried.

1:1 Mixture of Cloxacillin Sodium and Ampicillin Sodium

10g of ampicillin sodium and 10g of cloxacillin sodium were mixed by shaking inside a closed glass vessel.

2.2.2.2 Qualitative Analysis of Samples Prior to Assay

Starch Gel Electrophoresis

Based on the method by Thomas and Broadbridge (84). A higher voltage and current were used (250 volts and 45 mA). Samples, in pH7 buffer, had the concentrations:

Ampicillin sodium - 8 mg / 5 ml

Cloxacillin sodium - 20 mg / 5 ml

Degraded ampicillin sodium - 10 mg / 5 ml

Degraded cloxacillin sodium - 20 mg / 5 ml

1:1 mixture of ampicillin and cloxacillin - 20 mg / 5 ml

Degraded 1:1 mixture of ampicillin and cloxacillin - 20mg / 5ml

Agar Gel Electrophoresis

Based on the method by Lightbown and de Rossi (53). Oxoid medium was used for the analysis of cloxacillin sodium and Noble (Difco) for the analysis of ampicillin sodium. The pH of the media was 6.5. Sample concentration was 20 mg per 5 mls for degraded products and 10 mg per 5 mls for undegraded products. 20 microlitres of each solution was applied to the agar. The samples were separated for 2 hours on pre-seeded (Bacillus subtilis) medium before incubation at 37 degrees centigrade for 18 hours.

Gradient Elution H.P.L.C.

Adapted from the method by Larsen and Bundgaard (85).

Equipment :

Pumps : 2 Waters M6000A pumps.

U.V. Detector : Pye Unicam LC 3 variable wavelength.

Sample Injector : Rheodyne 7120 loop injector with 10 microlitre sample loop

Column : 16 cm x 4 mm Shandon stainless steel,packed with Waters 10 micrometre C-18 reversed phase packing

Solvent Programmer : Waters 660, set at program 7

Conditions of Separation :

Mobile phase : solvent A 10% acetonitrile in pH 7 phosphate buffer (0.1M)

solvent B 50% acetonitrile in pH 7 phosphate buffer (0.1M)

initial concentration - 15% B; 85% A

final concentration - 100% B

Time of program : 10 minutes.

Wavelength of detection : 254 nm.

Flow rate : 2.0 mls per minute

Attenuation : 0.2 a.u.f.s. (absorbance units full scale)

Temperature : ambient

2.2.2.3 Design of the Assay

For all assay methods except the B.P. 1973 method for cloxacillin sodium, the two level microbiological method and the electrophoresis microbiological method, a standard calibration curve was constructed from 5 determinations at 5 concentrations. Samples (5 determinations for each sample) were interpolated onto this line and the mean taken as the best estimate of the potency for that sample.

2.2.2.4 Direct U.V. Assay for Ampicillin and Cloxacillin

Based on the method by Davidson and Stenlake (55). Calibration curves

for ampicillin sodium and cloxacillin sodium were constructed using standard concentrations of 0.32, 0.64, 0.96, 1.28 and 1.60 mg per ml. Sample solutions of ampicillin sodium, degraded ampicillin sodium, cloxacillin sodium and degraded cloxacillin sodium had the concentration of 1.28 mg per ml. All solutions were mixed with equal volumes of pH 5 or pH 9 buffer and their U.V. extinctions measured using a Beckman Acta V spectrophotometer.

2.2.2.5 Official B.P. 1973 Assay Method for Ampicillin Sodium

Based on the method given in the B.P. 1973 (42). Ampicillin sodium standard concentrations in buffered copper sulphate solution pH 5.2 were 33.3, 26.7, 20.0, 13.3 and 6.67 micrograms per ml. All sample concentrations were 20 micrograms per ml. Extinction of samples was measured at 320 nm using a Beckman Acta V spectrophotometer.

2.2.2.6 Official B.P. 1973 Assay Method for Cloxacillin Sodium

Based on the method given in the B.P. 1973 (43). The method was followed exactly.

2.2.2.7 H.P.L.C. Assay Methods for Ampicillin Sodium and Cloxacillin sodium

Equipment :

Altex 100A pump

Pye Unicam LC3 variable wavelength U.V. detector

Rheodyne 7120 injector (20 microlitre loop)

Shandon 16 cm x 4 mm stainless steel column packed with Waters 10 micrometer C-18

reversed phase packing.

Conditions of Separation :

for ampicillin sodium :

Mobile phase 19% acetonitrile in pH 6.2 phosphate buffer (7.53g potassium dihydrogen phosphate + 2.02g disodium hydrogen phosphate per litre).

Attenuation 0.16 a.u.f.s.

Flow rate 1.5 mls per minute.

Wavelength of detection 253 nm.

for cloxacillin sodium :

Mobile phase 28% acetonitrile in pH 6.2 phosphate buffer.

Attenuation 0.08 a.u.f.s.

Flow rate 2.0 mls per minute

Wavelength of detection 270 nm

Preparation of Standards and Samples

Ampicillin Sodium :

Standard concentrations were 0.4, 0.7, 1.0, 1.3 and 1.6 mg per ml. Sample concentrations were 1.0 mg per ml for ampicillin sodium and 2.0 mg per ml for degraded ampicillin sodium, ampicillin sodium/ cloxacillin sodium 1:1 mixture and degraded ampicillin sodium/ cloxacillin sodium 1:1 mixture. All standards and samples contained 0.1 mg per ml of caffeine citrate as an internal standard and were dissolved in water.

Cloxacillin Sodium :

Standard solutions were 0.36, 0.62, 0.88, 1.14 and 1.4 mg per ml. Sample concentrations were 0.88 mg per ml for cloxacillin sodium,

1.0 mg per ml for degraded cloxacillin sodium and 1.76 mg per ml for ampicillin sodium/ cloxacillin sodium 1:1 mixture (degraded and undegraded). All standards and samples were made up in water and contained 0.02 mg per ml caffeine citrate as an internal standard.

2.2.2.8 Imidazole Spectrophotometric Method

Based on the methods described by Bundgaard and Ilver (63) and Bundgaard (64).

Cloxacillin Sodium :

The method described by Bundgaard and Ilver (63) was followed. Standard concentrations were 0.02, 0.04, 0.06, 0.08 and 0.10 mg per ml. No combined preparations were assayed.

Ampicillin Sodium :

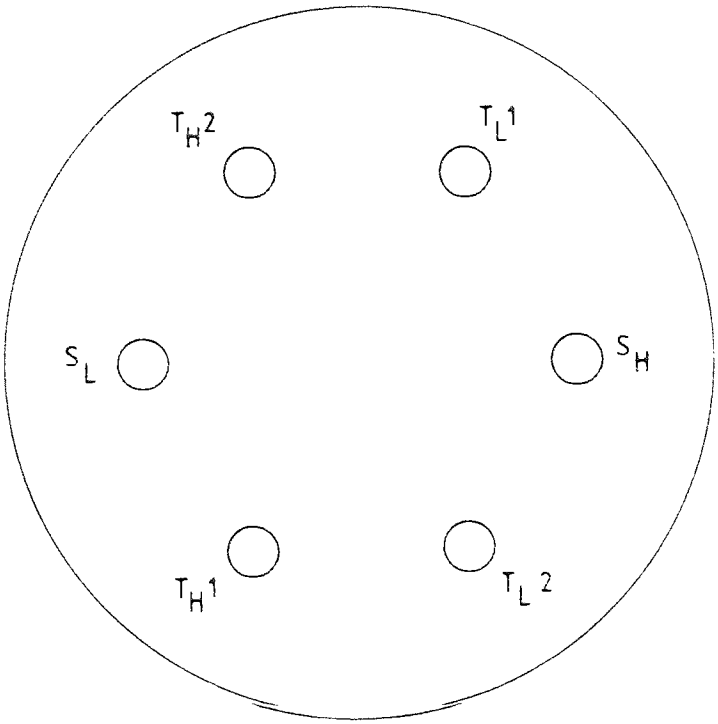
The method described by Bundgaard (64) was followed. Standard concentrations for procedure 1 were 0.02, 0.04, 0.06, 0.08 and 0.10 mg per ml; and 0.4, 0.8, 1.2, 1.6 and 2.0 mg per ml for procedure 2. Sample concentrations were 0.06 mg per ml (procedure 1) and 1.2 mg per ml (procedure 2). The spectrophotometric extinction of all solutions was measured using a Beckman Acta V spectrophotometer.

2.2.2.9 Microbiological Assay Methods

Agar Diffusion Methods -Two Dose and 5x5 Methods

9 cm Petri dishes were used for this method. Each dish was poured with 16 mls of pre-seeded medium. After setting, six identical 6 mm holes were automatically cut in the agar using a cutting tube attached to a vacuum line. These cups were equispaced.

For the assay of all preparations that did not contain a combination of cloxacillin sodium and ampicillin sodium, and the assay of cloxacillin



T_L1 TEST LOW (SAMPLE 1)

T_H1 TEST HIGH (SAMPLE 1)

T_L2 TEST LOW (SAMPLE 2)

T_H2 TEST HIGH (SAMPLE 2)

S_L STANDARD LOW

S_H STANDARD HIGH

FIGURE 2.3 THE ARRANGEMENT OF STANDARD AND TEST SOLUTIONS FOR THE TWO DOSE MICROBIOLOGICAL ASSAY

sodium in the combined preparations, Bacillus subtilis was used as the test organism. The medium used with this organism was Mast antibiotic. The assay of ampicillin sodium in the combined preparations required the use of Sarcina lutea as the test organism, with Sarcina medium. Areas of growth inhibition were measured using an automatic photoelectric method (Quantimet).

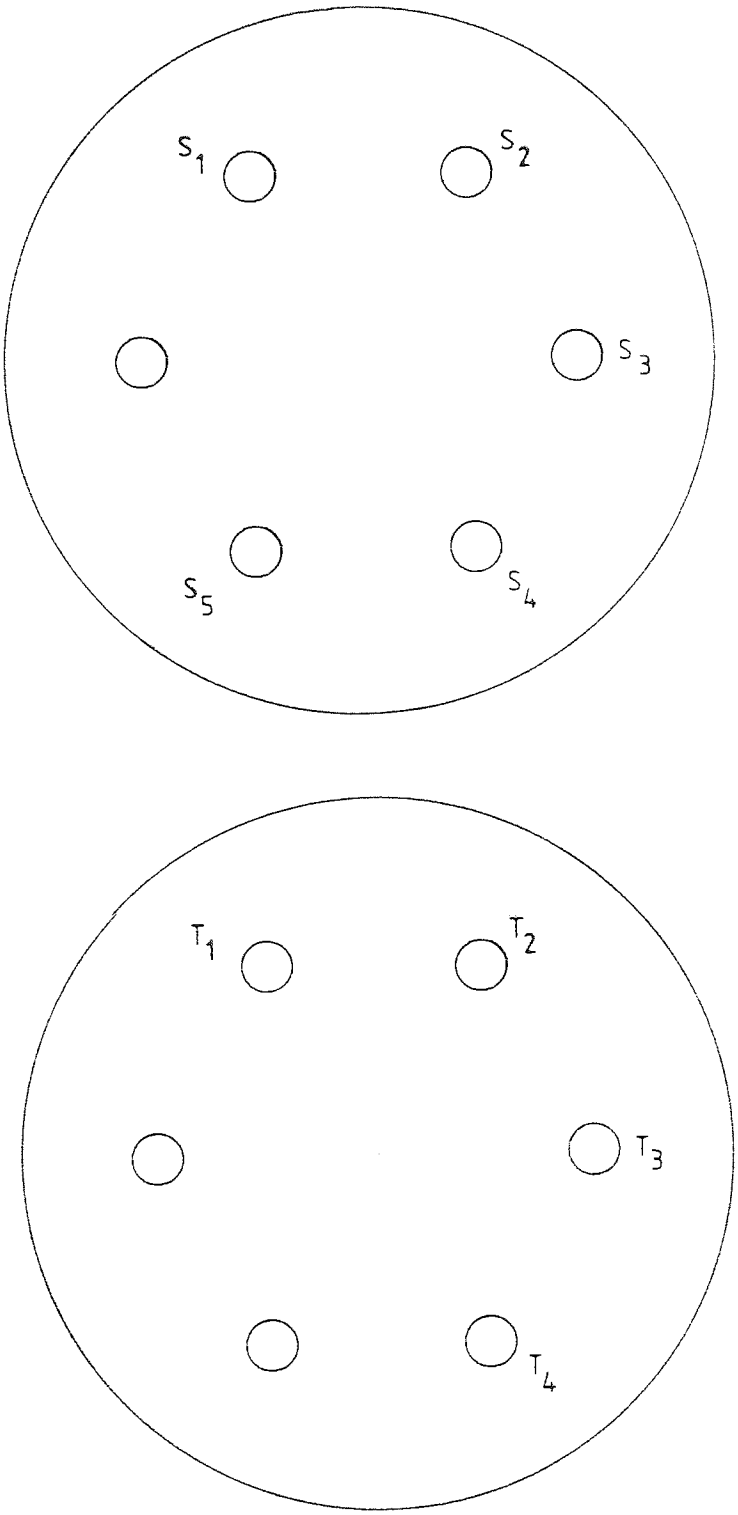
Two Dose Assay

Cloxacillin Sodium

A 20 microgram per ml solution of cloxacillin sodium standard was prepared in pH 7 sodium phosphate buffer (standard high). This solution was diluted 1 to 3 with pH 7 phosphate buffer (standard low). Test samples, not containing ampicillin sodium, were prepared in the same buffer to give similar potencies as the standard solutions (test high and test low). Two test samples (high and low concentrations) and one standard sample (high and low concentrations) were automatically pipetted into the six cups in the agar medium (see figure 2.3).

Ten dishes were prepared for each set of two test samples and one standard. The last dish was filled less than two minutes after the first to minimise variation in the diffusion time of the antibiotic. After diffusion at ambient temperature, the dishes were incubated at 37^{o}C overnight.

Cloxacillin sodium test samples containing ampicillin were prepared at concentrations equivalent to cloxacillin sodium concentrations of 20 micrograms per ml and 6.667 micrograms per ml. They were treated with penicillinase solution (30 minutes at 37^{o}C, then 10 minutes at 80^{o}C) to deactivate ampicillin, then the solutions were pipetted into cups in the medium ,allowed to diffuse and incubated at 37^{o}C overnight.



S_1 S_2 S_3 S_4 S_5 STANDARD SOLUTIONS

T_1 T_2 T_3 T_4 TEST SOLUTIONS

FIGURE 2.4 THE ARRANGEMENT OF STANDARD AND TEST SOLUTIONS FOR THE 5×5 MICROBIOLOGICAL ASSAY

Ampicillin Sodium

A 5 microgram per ml solution of ampicillin sodium standard was prepared in pH 6 sodium phosphate buffer (standard high). This solution was diluted 1 to 4 with the same buffer (standard low). Test samples not containing cloxacillin sodium were prepared at a similar ampicillin sodium concentration (test high and test low). The solutions were pipetted into the cups in the medium, as in figure 2.3, allowed to diffuse and incubated.

Ampicillin test samples containing cloxacillin sodium were diluted to 0.08 micrograms per ml (test high) and 0.0267 micrograms per ml (test low) with pH 6 sodium phosphate buffer and pipetted into cups in dishes poured with Sarcina lutea seeded medium (figure 2.3). After diffusion of the antibiotic, the dishes were inverted and incubated at 37°C overnight.

5 Concentration, 5 Replicate Assay (5x5 Assay)

Cloxacillin sodium standard solutions of concentrations 6.7, 10.4, 13.0, 17.1, 19.2 micrograms per ml were prepared in pH 7 sodium phosphate buffer. Cloxacillin sodium test solutions containing ampicillin sodium were treated with penicillinase. Standard and test solutions were pipetted into cups in the medium as shown in figure 2.4. After diffusion of the antibiotic into the medium, the dishes were inverted and incubated at 37°C overnight.

Ampicillin sodium solutions of 0.98, 1.51, 2.91, 4.10 and 5.13 micrograms per ml were prepared in pH 6 sodium phosphate buffer. Ampicillin sodium test samples, not containing cloxacillin, were prepared at a concentration equivalent to ~3.0 micrograms per ml ampicillin sodium. Standard and test solutions were pipetted into the cups in Bacillus subtilis pre-seeded medium as in figure 2.4. After diffusion, the dishes were inverted and incubated at 37°C overnight.

ANODE CATHODE

S_L	S_H	T_L	T_H	S_L	S_H	T_L	T_H
S_H	T_L	T_H	S_L	S_H	T_H	S_L	T_L
T_L	T_H	S_L	S_H	T_L	S_L	T_H	S_H
T_H	S_L	S_H	T_L	T_H	T_L	S_H	S_L

S_L STANDARD LOW CONCENTRATION

S_H STANDARD HIGH CONCENTRATION

T_L TEST LOW CONCENTRATION

T_H TEST HIGH CONCENTRATION

FIGURE 2.5 THE PLAN OF APPLICATION OF STANDARD AND TEST SOLUTIONS IN THE AGAR GEL ELECTROPHORESIS ASSAY

Ampicillin sodium test samples containing cloxacillin were assayed using Sarcina lutea. Standard concentrations were 0.0224, 0.0368, 0.0512, 0.0656 and 0.08 micrograms per ml. Test samples were prepared at a concentration equivalent to 0.0512 micrograms per ml ampicillin sodium. Standard and sample solutions were pipetted into cups in the medium as in figure 2.4. After diffusion, the dishes were inverted and incubated at 37°C overnight.

Ten standard and test dishes were prepared for each assay. After incubation, five dishes that had no irregularly shaped inhibition zones were measured using the Quantimet.

Agar Gel Electrophoresis Assay

Based on the method by Lightbown and de Rossi (53). Bacillus subtilis was used as the test organism in pre-seeded Oxoid medium. Top layering was not used. 20 microlitres of standard and test samples were applied to the solidified medium using capillary tubes. The plan of application for the solutions can be seen in figure 2.5.

Electrophoretic separation continued for 45 minutes before the plate was incubated at 37°C overnight. The diameter of the inhibition zone was measured using calipers, at 90° to the direction of movement of the antibiotic.

Standard and sample solutions were prepared in pH 6.5 sodium phosphate buffer (1.76g disodium hydrogen phosphate + 2.43g sodium dihydrogen phosphate per litre of water). Standard and test high concentrations (S_H, T_H) for ampicillin sodium and cloxacillin sodium were 0.15 mg per ml. Low concentrations (S_L and T_L) were prepared by diluting high concentrations 1 to 4 with buffer.

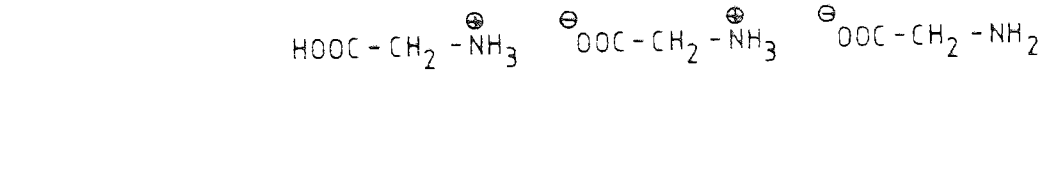


FIGURE 2.6 THE EFFECT OF pH ON THE SIGN AND NET CHARGE OF THE ZWITTERION GLYCINE

2.3 RESULTS AND DISCUSSION

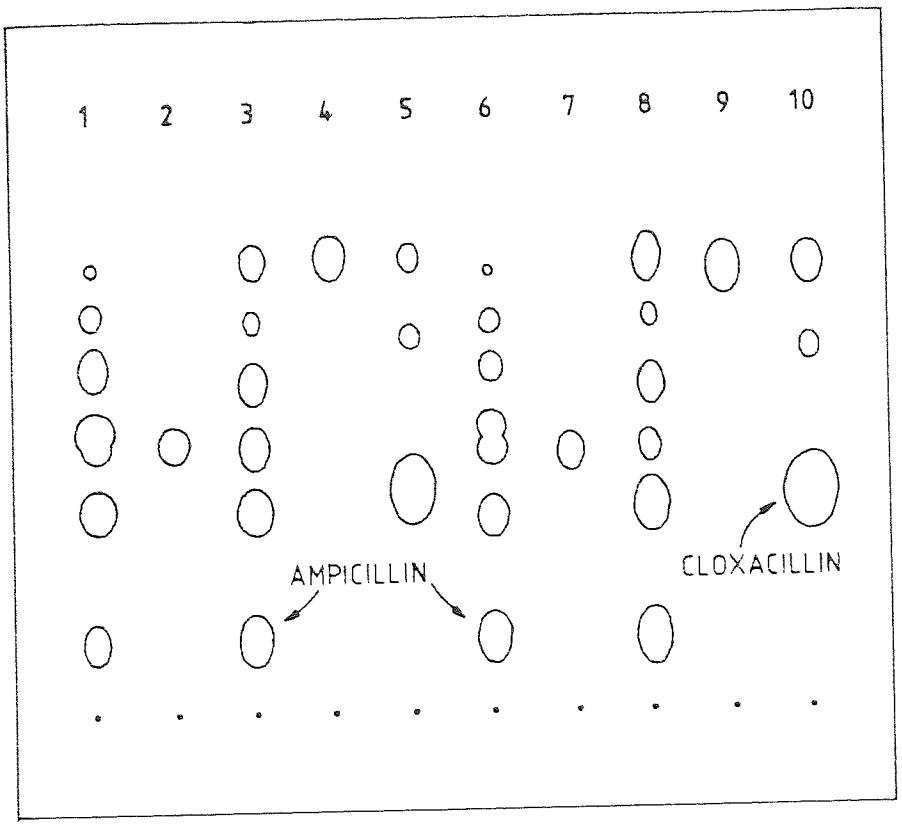
2.3.1 Qualitative Analysis of Degraded Samples

Electrophoresis was the term first used by Michaelis (86) to describe the migration of colloids under the influence of an electric field. Since then the term has been applied to the movement of ions in solution. The basic principle of electrophoresis states that if two electrodes are placed in an aqueous solution containing an electrolyte, the negatively charged ions will travel towards the anode and the positively charged ions towards the cathode. The rate of movement of these ions is determined by the motive force to which they are subjected. The motive force is represented by the multiple of Q, the field strength and N, the net charge on the ion. This motive force is resisted by the frictional force encountered by the ions as they move through the solution and this in turn depends on the size and shape of the ion and the viscosity of the solution.

The value of N for a species containing one ionisable group depends on the amount of ionisation of that group, itself dependent on the pH of the medium. A zwitterion containing two ionisable groups of different charge can have a net positive charge or a net negative charge, depending on the pH of the medium. This can be illustrated using glycine as an example (figure 2.6).

Starch gel and agar gel electrophoresis are examples of zone electrophoresis. Here, an "inert" medium replaces the solution described above. This minimises diffusion of the ions. Cellulose acetate, paper and polyacrylamide are other media used for this type of separation.

Starch gel electrophoresis, as described by Thomas and Broadbridge (84), separates penicilloic acid degradation products from their parent



1, 6) DEGRADED AMPICILLIN SODIUM

2, 7) AMPICILLIN PENICILLOIC ACID

3, 8) DEGRADED COMBINED SAMPLE

4, 9) CLOXACILLIN PENICILLOIC ACID

5, 10) DEGRADED CLOXACILLIN SODIUM

FIGURE 2.7 STARCH GEL ELECTROPHORESIS (AMPICILLIN / CLOXACILLIN)

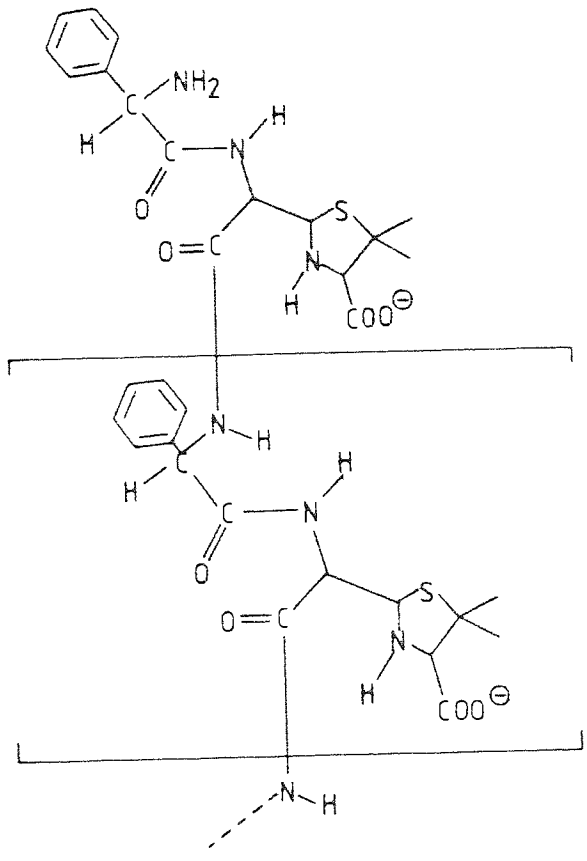


FIGURE 2.8 THE STRUCTURE OF AMPICILLIN POLYMERS

penicillins. Iodine vapour is used to visualise components of the separated samples. It reacts with starch to produce an intense blue colour. Wherever iodine is concentrated, the colour is most intense. Penicilloic acids are known to complex with nine equivalents of iodine (87) and, although Thomas and Broadbridge expected this property to prevent reaction between the complexed iodine and starch, it actually caused intensification of the colour instead. Intact penicillins do not complex iodine, thus require chemical degradation, after electrophoretic separation and prior to iodine exposure, for visualisation. Acid degradation is used because alkali interferes with the iodine/starch reaction.

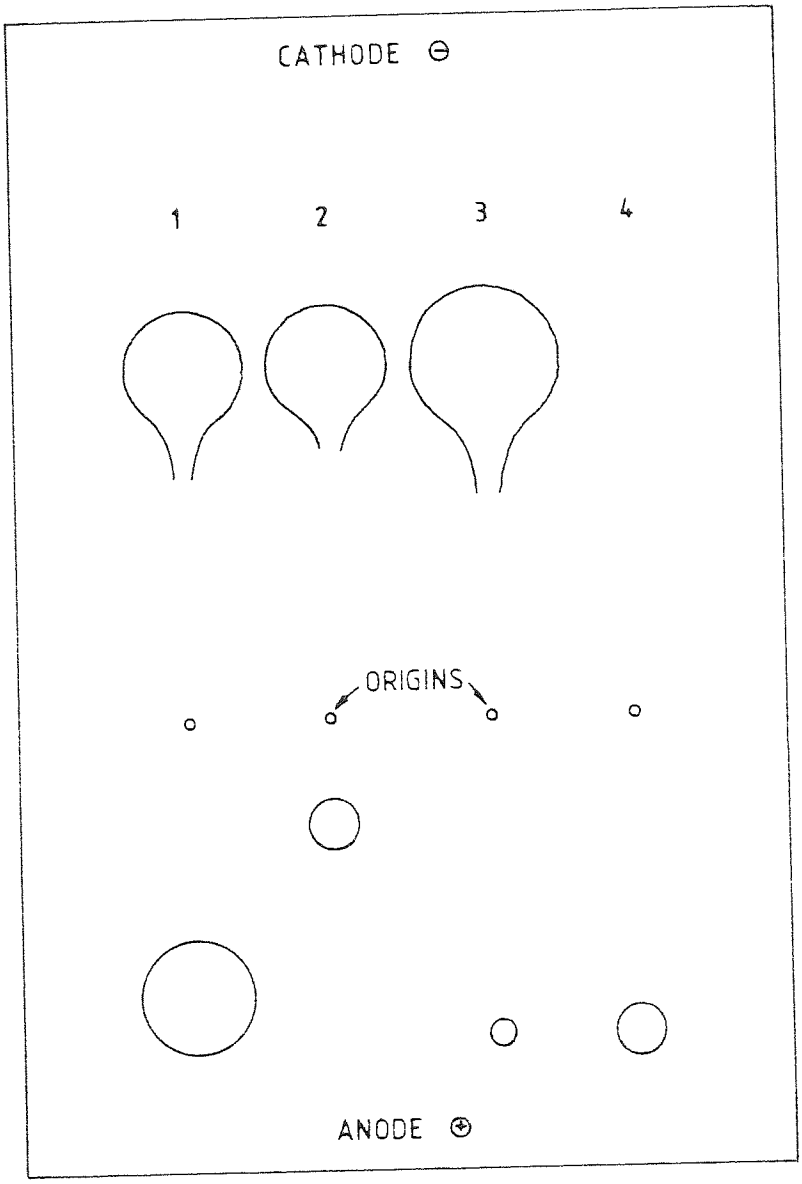
Degraded cloxacillin sodium separated into three components on starch gel electrophoresis plates (figure 2.7). The most mobile of the three was identified as cloxacillin penicilloic acid and the least mobile as cloxacillin, by comparison with reference standards of these compounds. The third component remained unidentified.

Degraded ampicillin sodium separated into many components (figure 2.7). Two of these corresponded to ampicillin penicilloic acid and ampicillin. The remainder were probably polymers of ampicillin, having the structure depicted in figure 2.8. Their "penicilloic acid like" nature (open beta-lactam rings) causes them to concentrate iodine without hydrolysis.

In their paper, Thomas and Broadbridge stated,

"At pH 7.0 the penicilloic acids have twice the mobility of their parent penicillins, thus they were readily separated from their respective penicillins."

This is correct for cloxacillin where the parent penicillin has one fully ionised carboxylic acid group and the penicilloic acid two fully ionised carboxylic acid groups. Ampicillin, however, one of the penicillins studied in their paper, is a zwitterion, possessing one



1 DEGRADED COMBINED SAMPLE

2 DEGRADED AMPICILLIN SODIUM

3 AMPICILLIN SODIUM STANDARD

4 BENZYLPENICILLIN STANDARD

FIGURE 2.9 AGAR GEL ELECTROPHORESIS OF AMPICILLIN AND CLOXACILLIN SAMPLES

ionisable carboxylic acid group of pKa 2.5 (89) and one ionisable primary amine group of pKa 7.25 (89). At pH 7.0, the pH of separation, the carboxylic acid group is fully ionised and the primary amine group 56% ionised. This results in a net charge for the molecule of -0.44. Ampicillin penicilloic acid contains two fully ionised carboxylic acid groups at this pH and one 56% ionised primary amine group. Thus its N value is -1.44. Assuming the size and shape of ampicillin and ampicillin penicilloic acid to be similar and all other factors equal, the mobility of the molecules is proportional to N. Ampicillin penicilloic acid should therefore have a mobility of 3.27 times the mobility of ampicillin, not twice the mobility as stated by Thomas and Broadbridge. Experimental measurements (figure 2.7) gave a mobility for ampicillin penicilloic acid of approximately 3.5 times the mobility of ampicillin. This supports the theoretical treatment above.

Agar gel electrophoresis of degraded cloxacillin samples did not provide any evidence of degradation products. The results from ampicillin samples can be seen in figure 2.9. Ampicillin sodium standard contained a small amount of material corresponding to either benzylpenicillin or cloxacillin. Benzylpenicillin is the more likely contaminant because of its closer chemical structure. Degraded ampicillin contained an unidentified component that exhibited antibacterial properties. This could be the dimer shown in figure 2.10. Evidence in favour of this comes from the direction of movement of the unknown component; and the results of Larsen and Bundgaard (85). In starch gel electrophoresis there are no charged particles in the gel, thus molecules travel towards the cathode if they possess a net positive charge and the anode if they possess a net negative charge. Agar, as used in gel electrophoresis, contains sulphonic acid groups (90). These are attracted towards the anode but cannot move because the medium is stationary. To counter this force, positively protonated

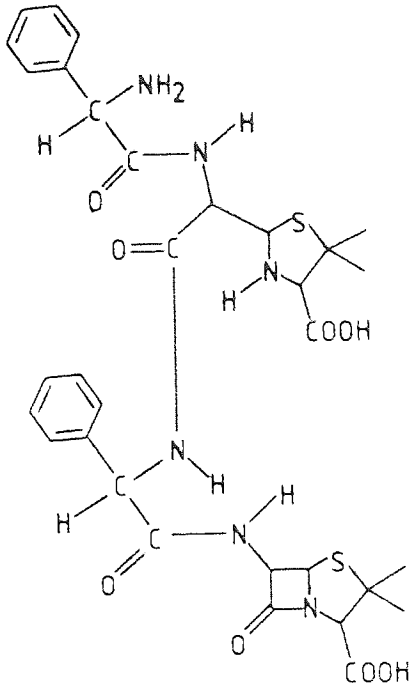


FIGURE 2.10 AMPICILLIN DIMER WITH AN INTACT β-LACTAM RING

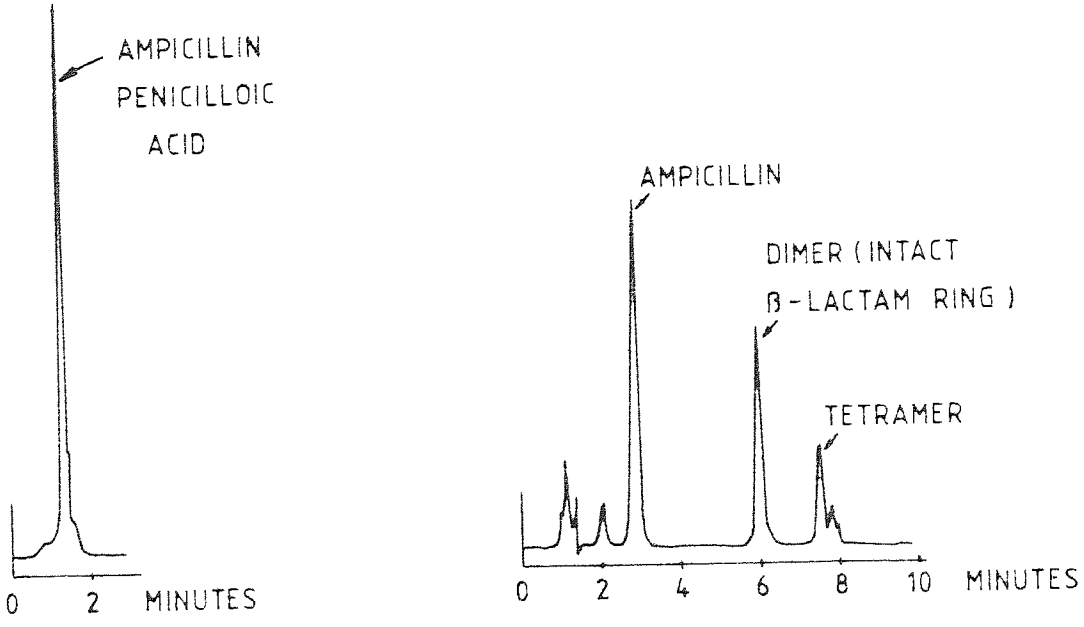


FIGURE 2.11 GRADIENT ELUTION H.P.L.C. OF AMPICILLIN PENICILLOIC ACID AND DEGRADED AMPICILLIN SODIUM

water molecules travel towards the cathode. Thus an osmotic movement of solvent towards the cathode occurs, taking solute molecules with it. This effect is called electroendosmosis. If the net charge (N) on the molecule is negative and large enough, electroendosmosis is overcome and the molecule travels towards the anode. This occurs with cloxacillin where N is -1 per molecule. However, ampicillin at pH 6.5 has an N value of only -0.32 per molecule. This is insufficient to overcome electroendosmosis and it travels toward the cathode. Ampicillin dimer has an N value of -1.32 per molecule, sufficient to overcome electroendosmosis and travel towards the anode. Its large molecular weight retards its mobility, resulting in only slight movement. This is in agreement with experimental observations (figure 2.9). Bundgaard and Larsen kept a 20% w/v solution of ampicillin sodium at room temperature for three days. The major degradation product was the dimer in figure 2.10.

Gradient elution H.P.L.C. (figures 2.11, 2.12, 2.13) supported the results of electrophoretic analysis. Comparison with the chromatogram obtained by Bundgaard and Larsen (85), provided tentative identification of some of the separated peaks. Ampicillin and cloxacillin peaks were identified by the chromatography of standard preparations of these compounds under the same conditions.

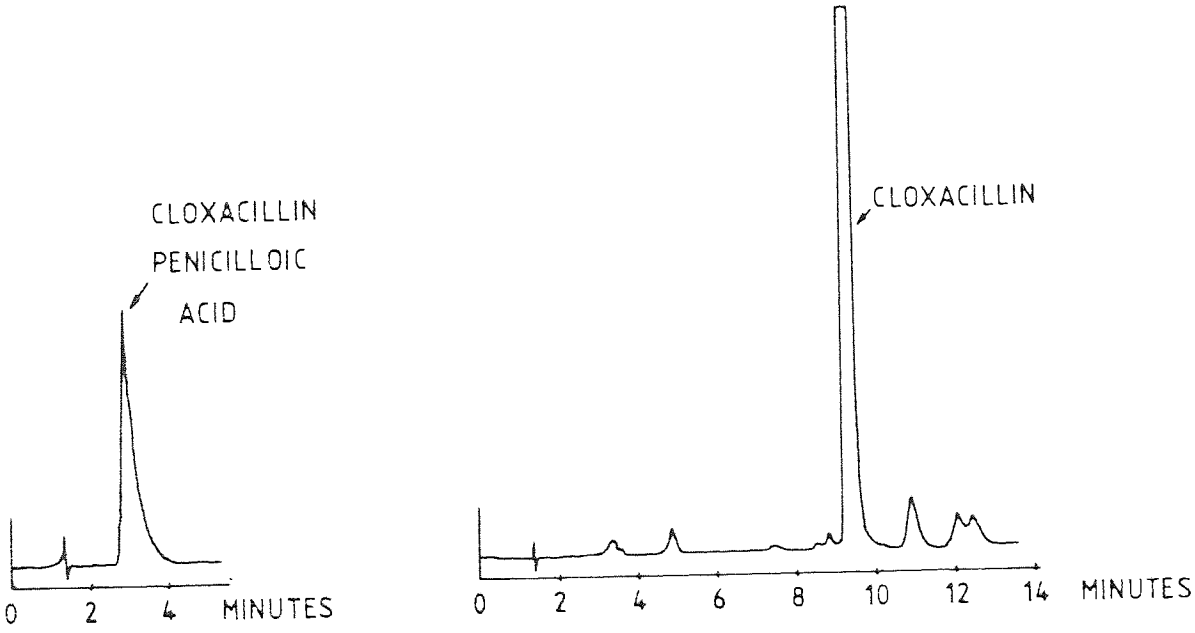


FIGURE 2.12 GRADIENT ELUTION H.P.L.C. OF CLOXACILLIN PENICILLOIC ACID AND DEGRADED CLOXACILLIN SODIUM

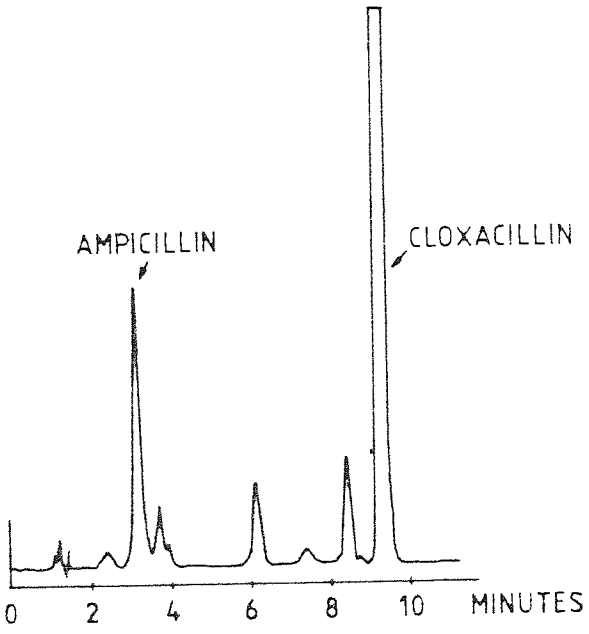


FIGURE 2.13 GRADIENT ELUTION H.P.L.C. OF DEGRADED MIXTURE OF AMPICILLIN SODIUM AND CLOXACILLIN SODIUM

2.3.2 Quantitative Analysis of Undegraded and Degraded Samples

2.3.2.1 Calculation of Assay Results

The objective of this chapter was to assess the accuracy and precision of different assay methods for ampicillin sodium and cloxacillin sodium. To make comparision of assay results simpler, ampicillin sodium standard (858 micrograms per mg free acid) and cloxacillin sodium standard (900 micrograms per mg) were assigned a nominal potency of 100.0% (sodium salts). This necessitated multiplying the B.P. 1973 assay results for cloxacillin sodium (the only absolute assay method) by a factor of 1.0177, the reciprocal of the assay value for cloxacillin sodium supplied with the standard (98.26%).

2.3.2.2 B.P. 1973 Cloxacillin Sodium Method

The B.P. 1973 assay method for cloxacillin sodium (43) refers to the B.P. 1973 assay method for carbenicillin sodium, where intact penicillin is determined by titration and benzylpenicillin impurity by gel electrophoresis. Carbenicillin sodium can be separated, by gel electrophoresis, from benzylpenicillin but cloxacillin cannot (53). Thus the B.P. 1973 assay for cloxacillin sodium is ambiguous and should only involve titration.

The basis for the titration method came from an observation by Abraham *et. al.* (91) and subsequent development by Patterson and Emery (92). The sample solution is neutralised with 0.01M sodium hydroxide. This reacts with any acid degradation products present. Excess 0.1M sodium hydroxide, accurately measured, is then added and the flask heated to facilitate reaction between the beta-lactam moiety of cloxacillin sodium and the alkali. Remaining sodium hydroxide is back titrated with 0.1M hydrochloric acid, the amount of alkali involved in the reaction

being equivalent to the amount of penicillin in the sample. It is an absolute assay, requiring only standard acid and base solutions and not a reference standard of cloxacillin sodium. Total penicillin content is determined, thus this method lacks specificity.

The B.P. recommends precaution against the absorption of carbon dioxide during heating with alkali. These precautions involved purging air from the reaction flask using nitrogen, sealing the flask during heating and titrating the solutions whilst blowing a stream of nitrogen into the titration vessel. Results from this assay method are given in table 2.1.

SAMPLE	ASSAY (%)	MEAN (%)	95% LIMITS	COEFF. OF VARIANCE
Cloxacillin sodium standard	100.2 97.6 99.4 98.8 99.8	99.2	98.0 to 100.4	0.99
Degraded cloxacillin sodium	92.7 89.7 92.0 91.1 91.2	91.4	90.0 to 92.7	1.38

Table 2.1 Results for the B.P.1973 Cloxacillin Sodium Assay Method

It was not possible to determine the cloxacillin sodium content of samples containing both cloxacillin sodium and ampicillin sodium using this method because ampicillin sodium interfered, producing an ill-defined end point to the titration.

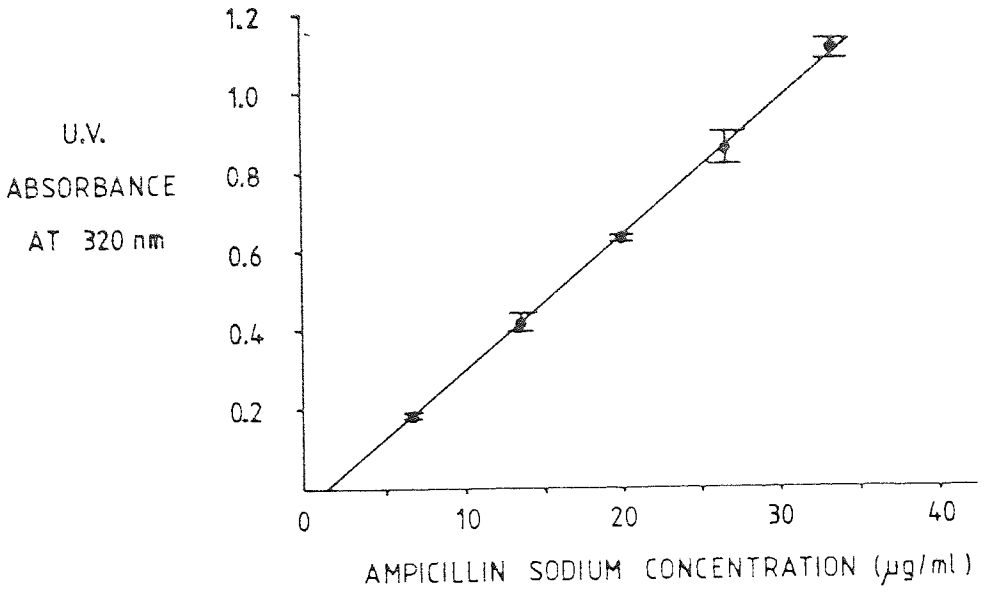


FIGURE 2.14 CALIBRATION CURVE FOR THE B.P. 1973 AMPICILLIN SODIUM ASSAY METHOD WITH 95% LIMIT ERROR BARS

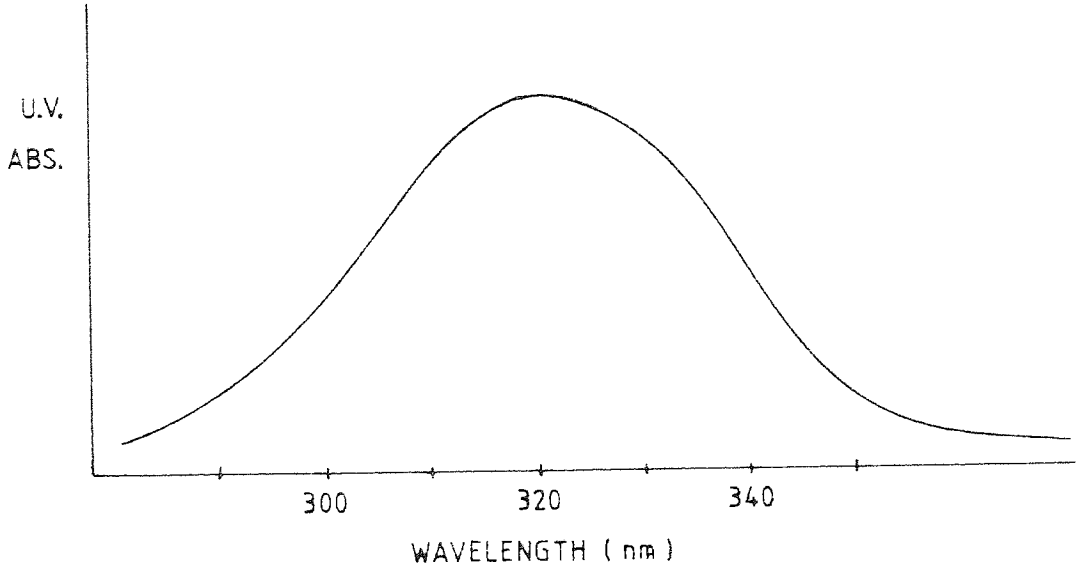


FIGURE 2.15 A TYPICAL U.V. SPECTRUM FROM THE B.P. 1973 AMPICILLIN SODIUM ASSAY METHOD

2.3.2.3 B.P. 1973 Ampicillin Sodium Method

Under controlled conditions of pH, time and temperature, Herriott (93) found that benzylpenicillin could be converted, by acid degradation, into a stable intermediate with an absorption maximum at 322nm. Stock (94) found that copper (II) ions were required to stabilise the intermediate and used this method for the assay of benzylpenicillin. Later, Smith et. al. (95) showed the reaction to be highly specific for intact penicillins and carried out a detailed study concerning ampicillin. He found the optimum pH and copper (II) ion concentration for the method and it was later adopted by the B.P. as a standard assay method, in a slightly altered form.

The stable intermediate was identified by Stock as the penicillenic acid. Smith et. al. agreed with him, although the mechanism of penicillin degradation in the presence of metals appears to be complex and greatly influenced by reaction conditions. In the B.P. 1973 method for ampicillin sodium, the stable intermediate absorbs at 320nm. Degradation products, except polymers, do not interfere; neither does cloxacillin sodium, which undergoes a similar reaction but under different conditions (96).

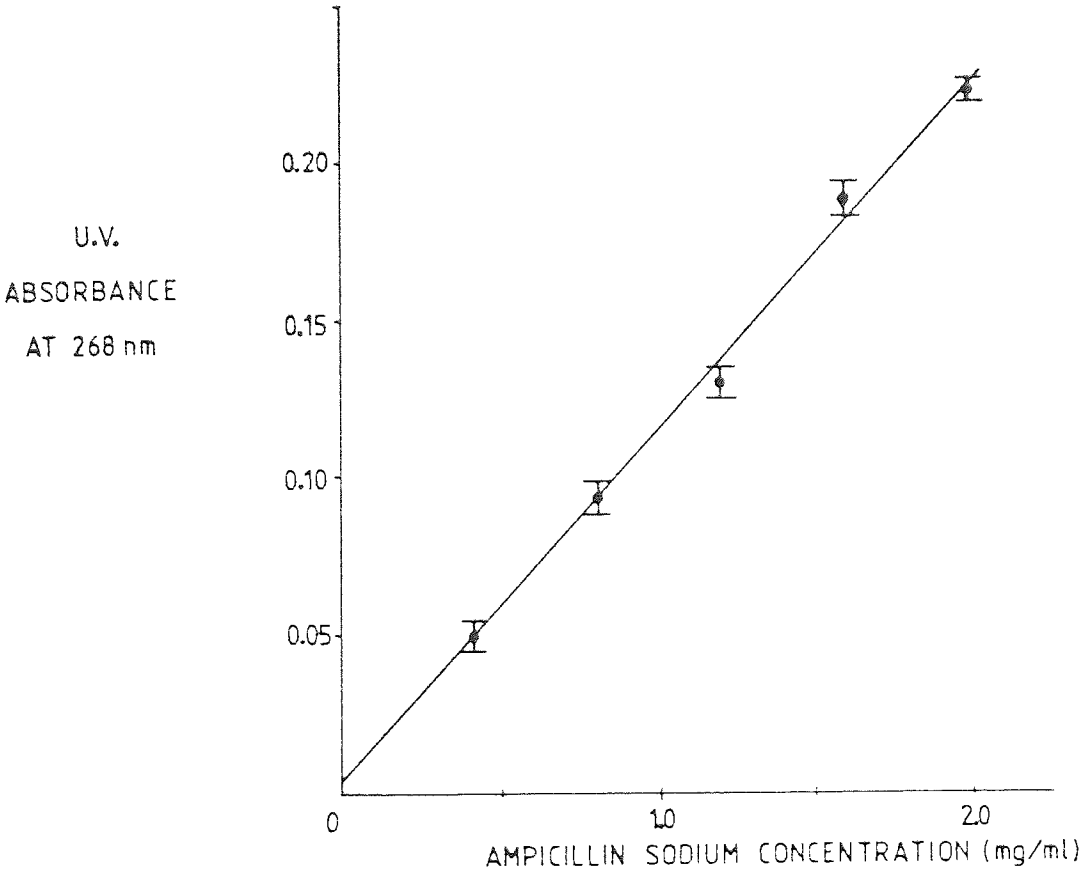
Assay data from this method are included in Appendix 1. Analysis of these values can be seen in table 2.2 and a standard calibration curve in figure 2.14. A U.V. spectrum of an ampicillin sample can be seen in figure 2.15.

AMPICILLIN SODIUM STANDARD CURVE - LINEAR REGRESSION ANALYSIS				
Corr. Coeff.	Slope of Reg. Line	95% C.I. on the Slope	Intercept of Reg. Line	95% C.I. on the Inter.
0.9983	34.26	33.41 to 35.12	-0.0473	-0.0384 to -0.0562

Sample	Interpolated Value (%)	95% C.L. of Interpolated Value	Coefficient of Variation
Ampicillin sodium	100.0	97.7 to 102.3	1.86
Degraded ampicillin sodium	51.7	50.7 to 52.7	1.59
1:1 Ampicillin sodium/ cloxacillin sod. mixt.	47.9	46.0 to 49.8	3.18
Degraded 1:1 Amp. sod./ clox. sod. mixture	37.7	37.1 to 38.4	1.35

C.I. - confidence interval ; C.L. - confidence limits ; Corr. coeff. - Correlation coefficient.

Table 2.2 Assay Results - B.P. 1973 Assay Method for Ampicillin Sodium



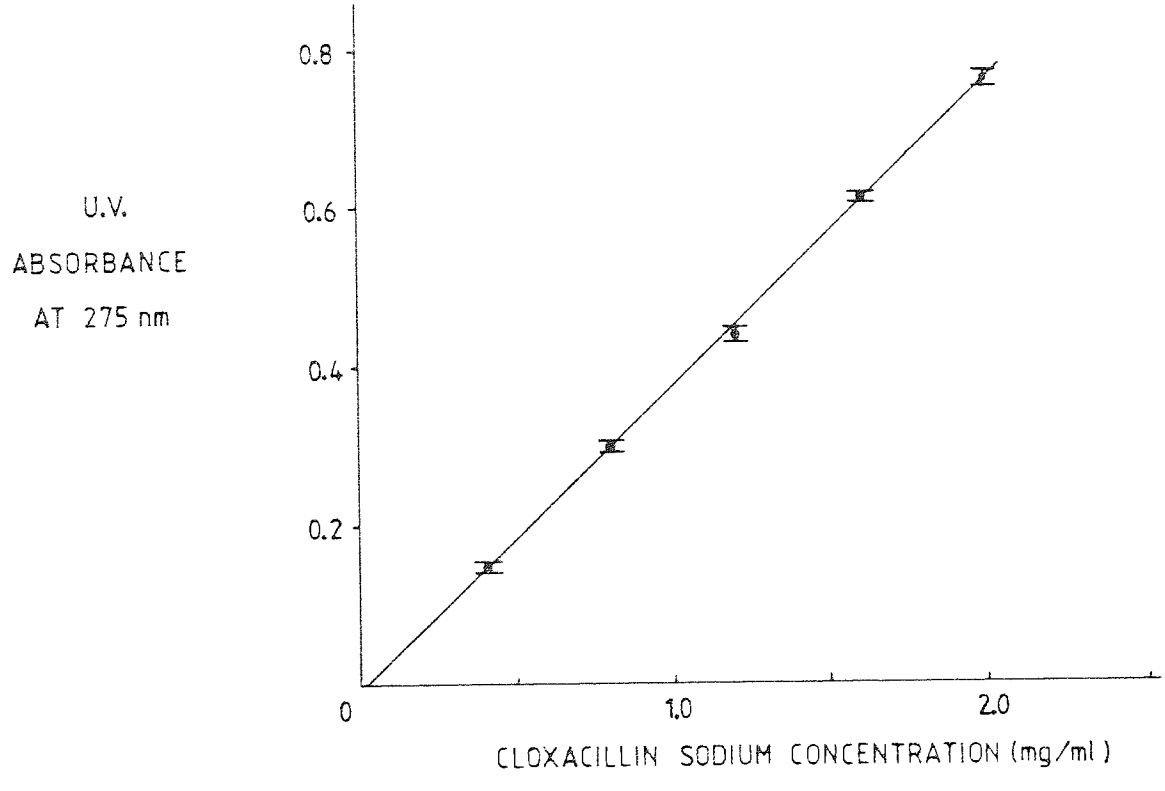
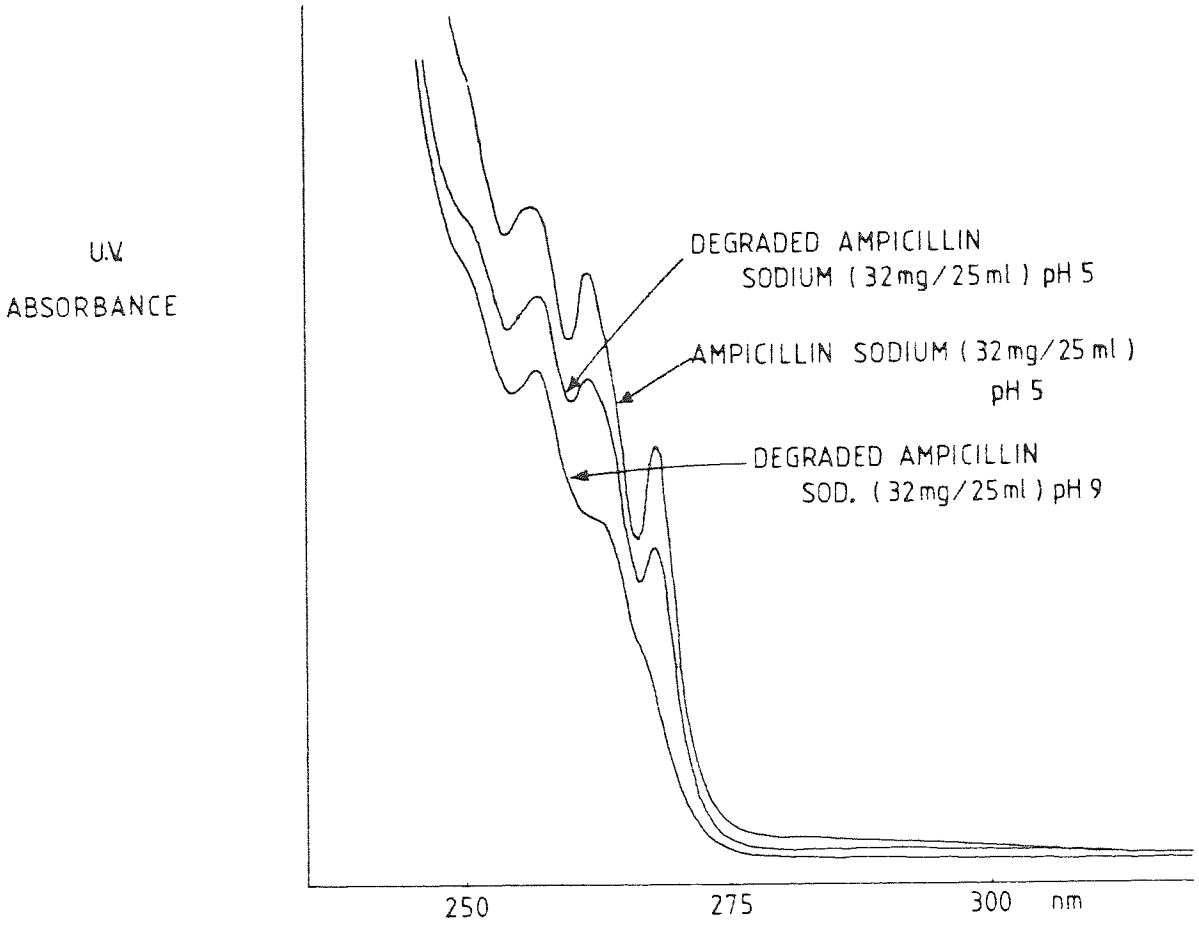


FIGURE 2.16 CALIBRATION CURVES FOR THE DIRECT U.V. ASSAY OF AMPICILLIN SODIUM AND CLOXACILLIN SODIUM WITH 95% LIMIT ERROR BARS

2.3.2.4 Direct U.V. Measurement

Few chemical methods of assay allow simultaneous determination of individual penicillins in a mixture of penicillins, without prior separation of the components. However, Davidson and Stenlake (55) developed a direct U.V. spectrophotometric method to determine both ampicillin and cloxacillin in combined preparations, from a single sample weighing. Solutions of the mixture were prepared in pH 5 and pH 9 buffers and their U.V. extinction measured at 268nm and 275nm. Ampicillin sodium exhibits decreased absorbance at 268nm in pH 9 buffer compared with pH 5 buffer. Cloxacillin has a consistent absorbance in pH 5 and pH 9 buffers. Only cloxacillin has a significant absorbance at 275nm. The method was simple and did not require extraction, separation or colour reaction. For these reasons it was included in the comparison of assay methods.

Assay data are included in Appendix 1. Analysis of these values can be seen in tables 2.3 and 2.4 and standard calibration curves in figure 2.16. Example U.V. spectra can be seen in figure 2.17.



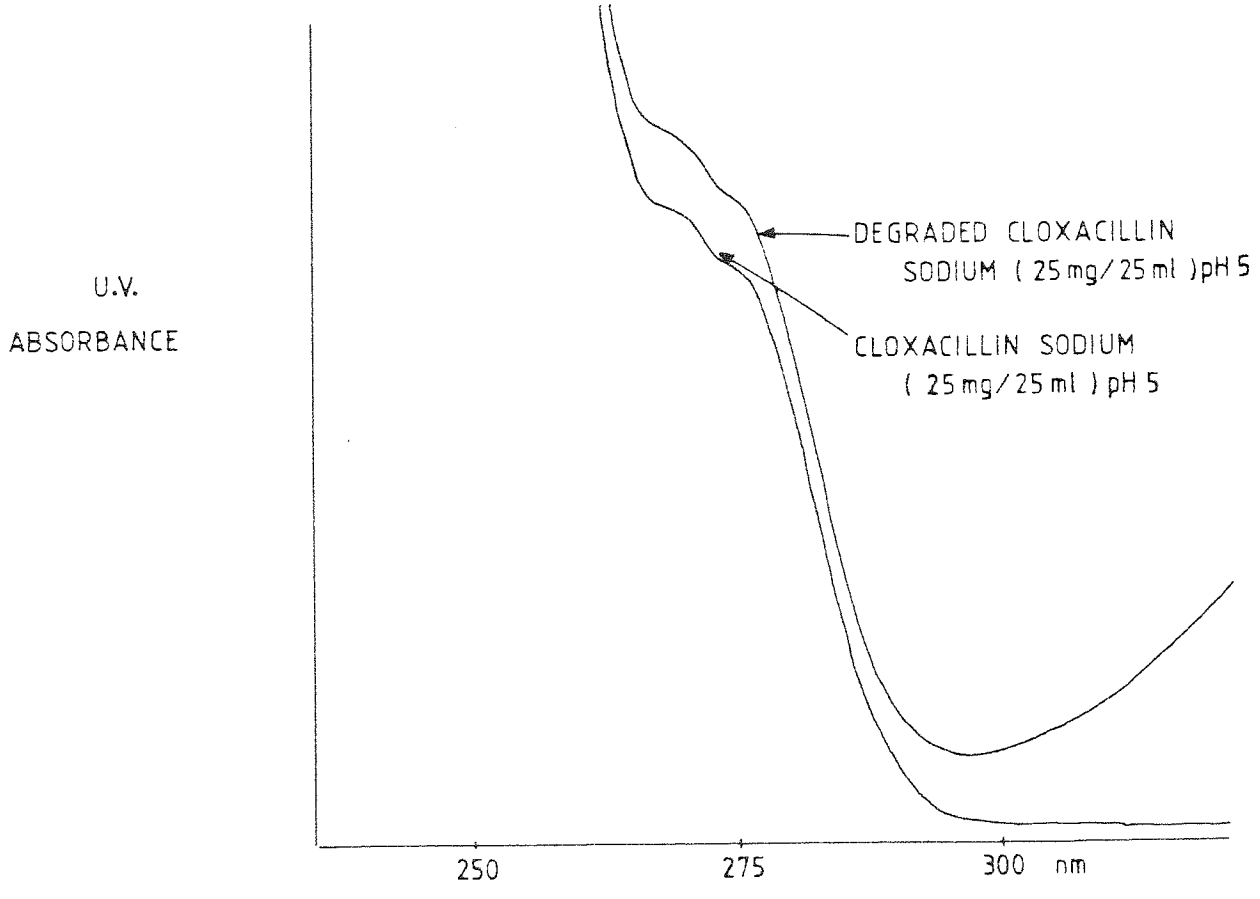


FIGURE 2.17 TYPICAL U.V. SPECTRA FROM THE DIRECT U.V. ASSAY OF AMPICILLIN SOD. AND CLOXACILLIN SODIUM

AMPICILLIN SODIUM STANDARD CURVE - LINEAR REGRESSION ANALYSIS				
Corr. coeff.	Slope of Reg. Line	95% C.I. on the Slope	Intercept of Reg. Line	95% C.L. on the Inter.
0.9957	0.1371	0.1316 to 0.1427	0.0047	-0.0036 to 0.0131

Sample	Interpolated Value (%)	95% C.L. of Interpolated Value	Coefficient of Variation
Ampicillin sodium	102.5	99.0 to 106.0	2.77
Degraded ampicillin sodium	63.1	59.6 to 66.5	4.36
1:1 Ampicillin sod. / clox. sod. mixture	48.1	46.0 to 50.1	3.45
Degraded 1:1 Amp. sod./ clox. sod. mixture	33.9	31.6 to 36.1	5.37

Table 2.3 Assay Results - Direct U.V. Measurement : Ampicillin Sodium

CLOXACILLIN SODIUM STANDARD CURVE - LINEAR REGRESSION ANALYSIS				
Corr. coeff.	Slope of Reg. Line	95% C.I. on the Slope	Intercept of Reg. Line	95% C.I. on the Inter.
0.9992	0.4797	0.4715 to 0.4878	-0.0085	-0.0208 to 0.0039

Sample	Interpolated Value (%)	95% C.L. of Interpolated Value	Coefficient of Variation
Cloxacillin sodium	99.3	97.9 to 100.7	1.14
Degraded cloxacillin sodium	112.1	111.4 to 112.8	0.49
1:1 Ampicillin sod. / clox. sod. mixture	49.2	48.4 to 50.0	1.36
Degraded 1:1 Amp. sod./ clox. sod. mixture	47.6	46.1 to 49.1	2.57

Table 2.4 Assay Results - Direct U.V. Measurement : Cloxacillin Sodium

2.3.2.5 Microbiological Assay Methods

The "Oxford Cup" method for antibiotics was first described by Abraham et.al. (14). The method involved placing an antibiotic solution in a cup, in a layer of agar seeded with a test bacterium, allowing it to diffuse into the agar and measuring the zone of growth inhibition created. The potency of the antibiotic solution was shown by comparing the area of growth inhibition with a similar area of inhibition from a standard solution of antibiotic. This method was further developed by Heatley (97). It was modified by Schmidt and Moyer (98), McKee et.al. (99) and Foster and Woodruff (100, 101). Woodruff also gave a carefully reasoned critique of various microbiological methods (102) and finally recommended the "Oxford Cup" method. In addition, Heatley (103), Lees and Toothill (104, 105) and Kavenagh (106) reviewed this topic. The theory of diffusion through agar was derived by Cooper et. al. (107, 108, 109). Bennett et. al. (110) discussed the type of organism and physical parameters associated with large plate assays, whilst Ciuro (111) evaluated Bacillus pumilis N.C.T.C. 8241 as a test organism in diffusion assays. Ericsson and Malmborg (112) determined antibiotic concentration in only 10 microlitres of plasma by the same method as Druzhinina et. al. (113); and Tebyakina et. al. (114) determined ampicillin and oxacillin in combined preparations by penicillinase deactivation and the use of a specific organism to differentiate between antibiotics. Lightbown and de Rossi (53) identified and assayed mixtures of antibiotics by electrophoresis in agar gel.

Automation in microbiological assays was investigated by Wallhausser (115), along with a review of existing methods for assaying antibiotics. Whyatt et. al. (116) described a precise turbidometric assay for low levels of ampicillin in serum and Smith et. al. (117) developed an assay for ampicillin based on the titration, with 0.05 M

sodium hydroxide, of lactate produced by Escherishia coli on glucose substrate.

The method of analysis chosen for comparison was based on the "Oxford Cup" method. The methods and equipment used in this assay were developed by workers in Beecham Pharmaceutical Research Division.

In order to provide a direct comparison with the format of the other types of assay used in this survey, five concentrations of standard were used to construct a calibration curve for each antibiotic. The Cooper and Woodman equation for the diffusion of antibiotics into gels (109) states :

$$m_1 = m_0 \exp(-x^2/4.D.t) \qquad \text{equation 2.1}$$

where m_0 is the concentration of antibiotic solution in the cup

m_1 is the concentration of antibiotic at a distance x from the cup edge after time t (critical concentration)

D is the diffusion coefficient

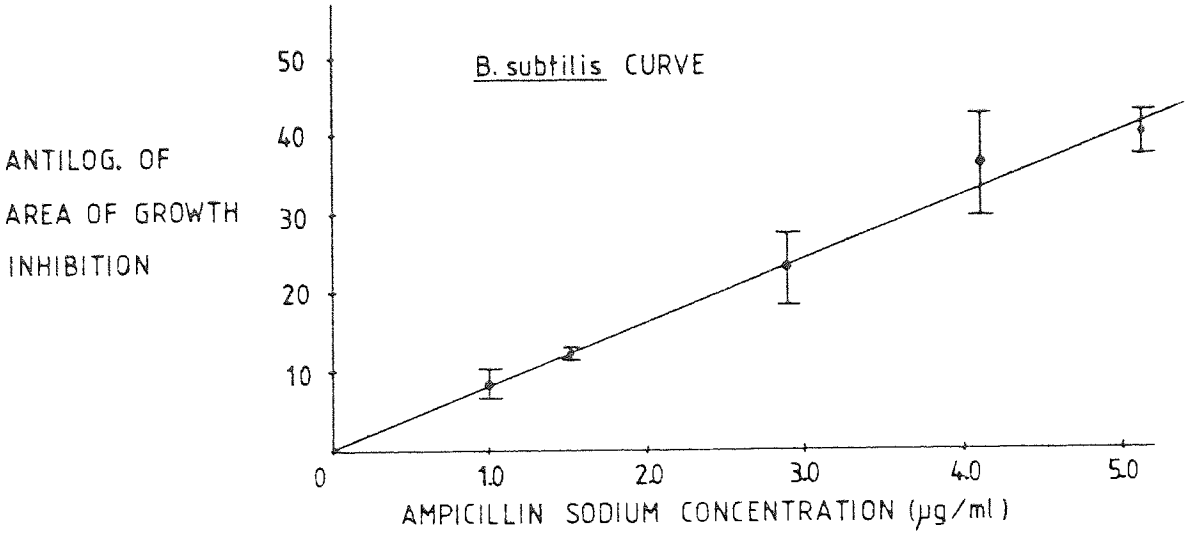
rearranging equation 2.1:

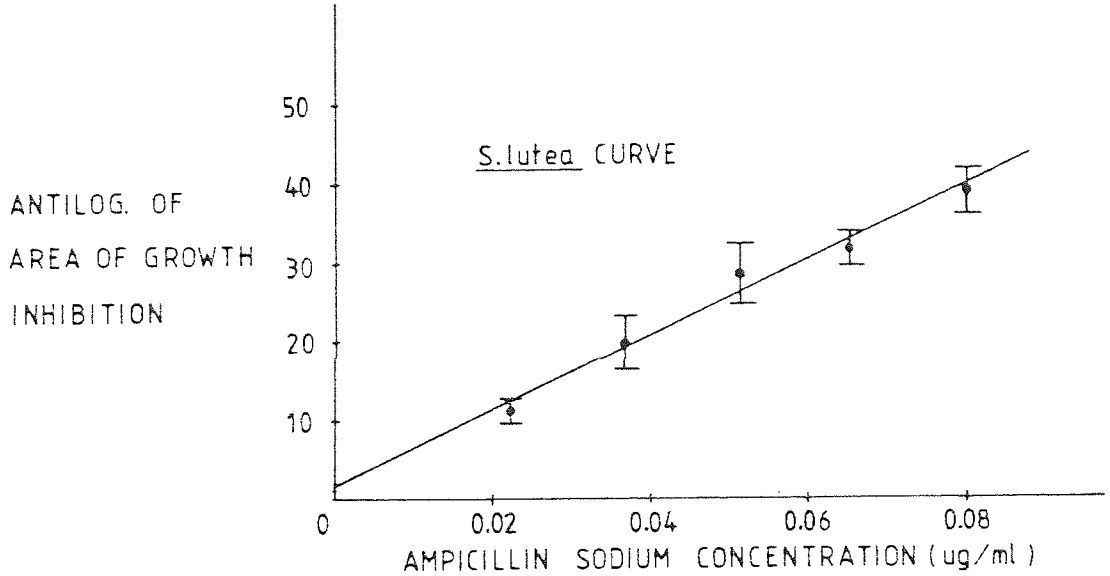
$$x^2 = 4.D.t.\ln (m_0/m_1)$$

Thus the area of the zone of inhibition is proportional to the natural logarithm of the antibiotic concentration in the cup. To obtain a linear calibration curve for the standard, a plot of antilogarithm (area of inhibition) versus antibiotic concentration was made.

Ampicillin sodium in mixtures containing cloxacillin sodium was determined by using Sarcina lutea as the test organism. This organism responds to ampicillin at very low concentrations, concentrations at which cloxacillin sodium is ineffective.

Cloxacillin sodium in mixtures containing ampicillin sodium was





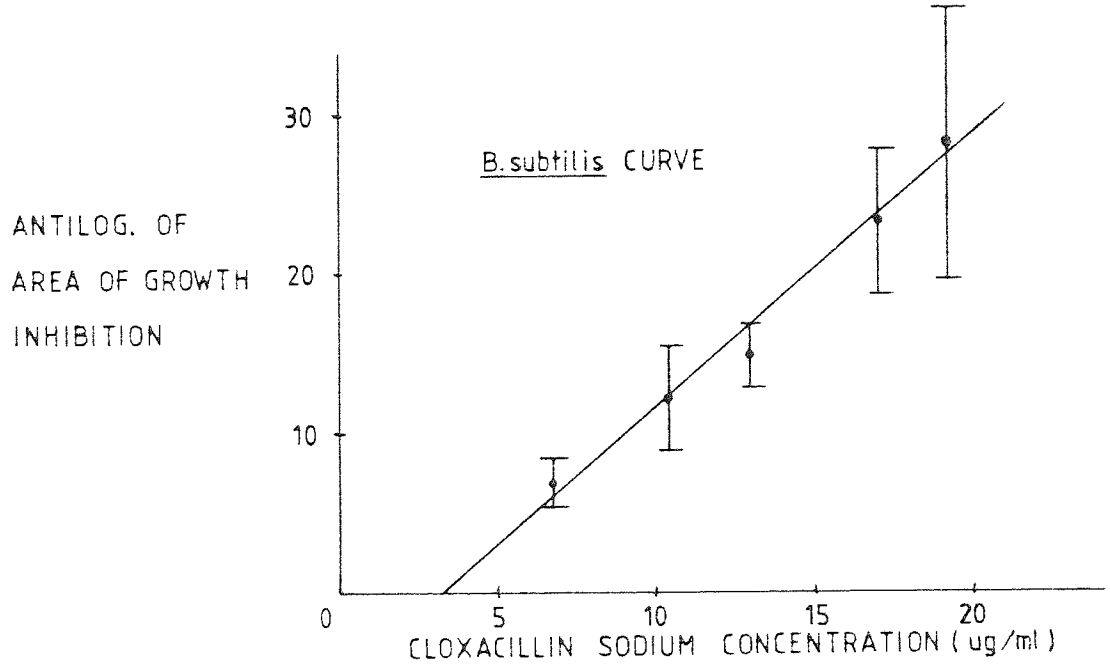


FIGURE 2.18 CALIBRATION CURVES FOR THE 5 REPLICATE / 5 CONCENTRATION MICROBIOLOGICAL ASSAY METHOD WITH 95% LIMIT ERROR BARS

determined by deactivating the ampicillin sodium with penicillinase prior to assay. Penicillinase also deactivates cloxacillin but much more slowly.

Three calibration curves were prepared; Bacillus subtilis curve for ampicillin sodium in preparations not containing cloxacillin sodium ; Sarcina lutea curve for ampicillin sodium in preparations containing cloxacillin sodium; Bacillus subtilis curve for cloxacillin sodium in all samples. Antilogarithm (areas of inhibition) values for samples were interpolated onto the standard curves and the corresponding concentration of antibiotic calculated.

Assay data for this method are included in Appendix 1. Analysis of these values can be found in tables 2.5, 2.6 and 2.7; and the standard calibration curves in figure 2.18.

AMPICILLIN SOD. (B. subtilis) STD. CURVE - LINEAR REGRESSION ANALYSIS				
Corr. Coeff.	Slope of Reg. Line	95% C.I. on the Slope	Intercept of Reg. Line	95% C.I. on the Inter.
0.9688	7.984	7.103 to 8.865	0.640	-6.668 to 7.948

Sample	Interpolated Value (%)	95% C.L. of Interpolated Value	Coefficient of Variation
Ampicillin sodium	89.7	79.1 to 100.3	9.503
Degraded ampicillin sodium	31.4	28.1 to 34.7	8.460

Table 2.5 Assay Results - Microbiological 5 Replicate/5 Concentration Method : Ampicillin Sodium (B. subtilis)

AMPICILLIN SOD. (S.lutea) STD. CURVE - LINEAR REGRESSION ANALYSIS				
Corr. Coeff.	Slope of Reg. Line	95% C.I. on the Slope	Intercept of Reg. Line	95% C.I. on the Inter.
0.9658	468.1	413.9 to 522.2	2.064	0.650 to 3.478

Sample	Interpolated Value (%)	95% C.L. of Interpolated Value	Coefficient of Variation
1:1 Ampicillin sod./ clox. sod. mixture	53.6	49.9 to 57.4	5.63
Degraded 1:1 amp. sod./ clox. sod. mixture	41.3	37.7 to 44.8	7.02

Table 2.6 Assay Results - Microbiological 5 Replicate/ 5 Concentration Method : Ampicillin Sodium (S.lutea)

CLOXACILLIN SODIUM STANDARD CURVE - LINEAR REGRESSION ANALYSIS				
Corr. Coeff.	Slope of Reg. Line	95% C.I. on the Slope	Intercept of Reg. Line	95% C.I. on the Inter.
0.9062	1.687	1.347 to 2.027	-5.370	-26.821 to 16.080

Sample	Interpolated Value (%)	95% C.L. of Interpolated Value	Coefficient of Variation
Cloxacillin sodium	99.0	88.8 to 109.2	8.28
Degraded clox. sod.	91.1	77.2 to 105.1	12.29
1:1 Ampicillin sod./ clox. sod. mixture	38.8	32.7 to 45.0	12.79
Degraded 1:1 amp. sod./ clox. sod. mixture	32.1	26.8 to 37.5	13.39

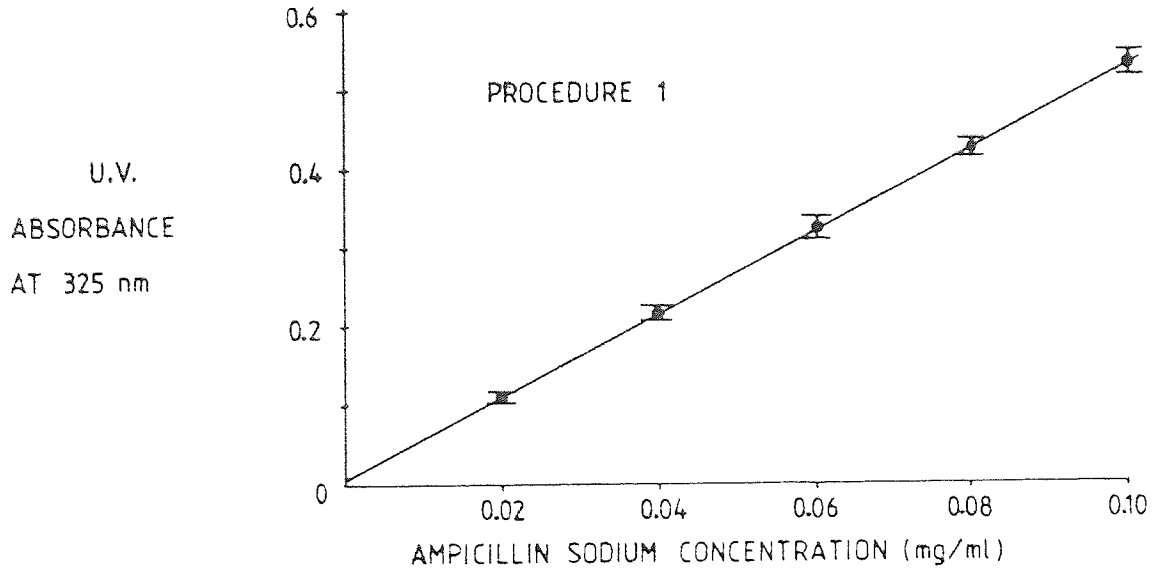
Table 2.7 Assay Results - Microbiological 5 Replicate / 5 Concentration Method : Cloxacillin Sodium

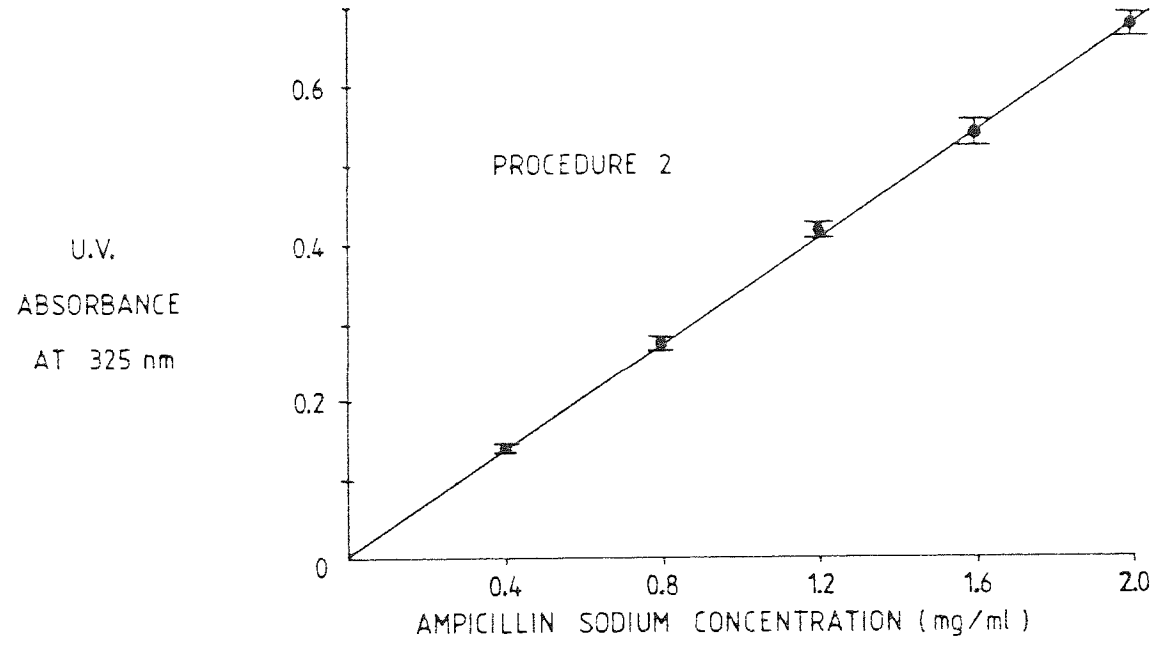
Analysis of the results for this microbiological design indicated a need for repeat assays of the samples, using more standard designs. Thus the samples were assayed by a two dose and an agar gel electrophoretic microbiological method. These two alternative methods were only included to provide accurate assay values for comparison with results from other methods. For this reason, only degraded samples were assayed by these methods. The two dose method was similar to the 5x5 method except that no calibration curve was constucted. Agar gel electrophoresis separated the two penicillins thus obviated the need to deactivate ampicillin. For both types of assay, standard and sample solutions were prepared at two similar concentrations and their inhibition zones compared.

Results from the two dose and agar gel electrophoresis microbiological methods are in table 2.8.

Sample	Two Dose Microb.		Gel Electro. Micro.	
	Mean	95% C.L.	Mean	95% C.L.
AMPICILLIN SODIUM :				
Degraded amp. sod.	34.6	31.9 to 37.4	36.6	35.3 to 37.9
Degraded 1:1 amp.sod./ clox. sod. mixture	35.8	34.0 to 37.6	39.6	36.1 to 43.0
CLOXACILLIN SODIUM :				
Degraded clox. sod.	92.7	87.7 to 97.9	95.0	88.4 to 101.8
Degraded 1:1 amp.sod./ clox. sod. mixture	33.2	30.5 to 36.0	40.1	38.7 to 41.6

Table 2.8 Assay Results - Microbiological Two Dose and Gel Electrophoresis Methods





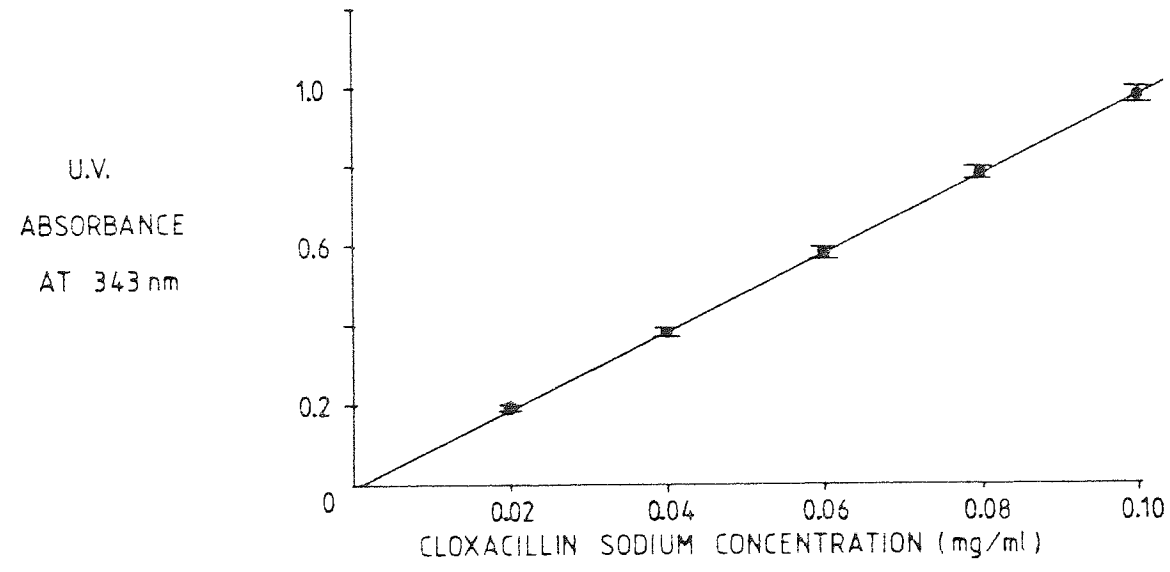


FIGURE 2.19 CALIBRATION CURVES FOR THE IMIDAZOLE SPECTROPHOTOMETRIC ASSAY METHOD WITH 95% LIMIT ERROR BARS

2.3.2.6 Imidazole Spectrophotometric Method

Only one spectrophotometric assay method for ampicillin sodium, the imidazole spectrophotometric method, differentiates between polymers of ampicillin and ampicillin sodium (64). The degraded products used as samples in the comparison of assay methods were prepared from 25% w/v aqueous solutions, conditions under which polymers of ampicillin are known to form (88). Thus the imidazole spectrophotometric method was chosen for experimental evaluation. The method involved imidazole catalysed re-arrangement of the penicillin to a penicillenic acid intermediate stabilised by mercuric ions, followed by subsequent measurement of the U.V. extinction of the species. Ampicillin sodium required prior treatment with acetic anhydride to acetylate the primary amine group which interfered with the assay. Polymers of ampicillin sodium were differentiated from ampicillin sodium by differential hydrolysis prior to assay.

Mixtures of ampicillin sodium and cloxacillin sodium could not be assayed using this method without previous separation and, therefore, were not assayed. Ampicillin sodium was assayed using procedures 1 and 2 in the method for ampicillin sodium (64). Cloxacillin sodium was assayed using the general method for penicillins (63). Assay data are included in Appendix 1. Analysis of this data can be found in tables 2.9, 2.10, and 2.11. Standard calibration curves are drawn in figure 2.19.

AMPICILLIN SOD. STD. CURVE (Proc. 1) - LINEAR REGRESSION ANALYSIS				
Corr. Coeff.	Slope of Reg. Line	95% C.I. on the Slope	Intercept of Reg. Line	95% C.I. on the Inter.
0.9988	5.259	5.150 to 5.368	0.0071	0.0031 to 0.0110

Sample	Interpolated Value (%)	95% C.L. of Interpolated Value	Coefficient of Variation
Ampicillin sodium	99.6	97.5 to 101.7	1.72
Degraded amp. sod.	49.4	48.0 to 50.9	2.36

Table 2.9 Assay Results - Imidazole Spectrophotometric Method : Ampicillin Sodium Procedure 1

AMPICILLIN SOD. STD. CURVE (Proc. 2) - LINEAR REGRESSION ANALYSIS				
Corr. Coeff.	Slope of Reg. Line	95% C.I. on the Slope	Intercept of Reg. Line	95% C.I.on the Inter.
0.9989	0.3358	0.3290 to 0.3426	0.0066	-0.0075 to 0.0208

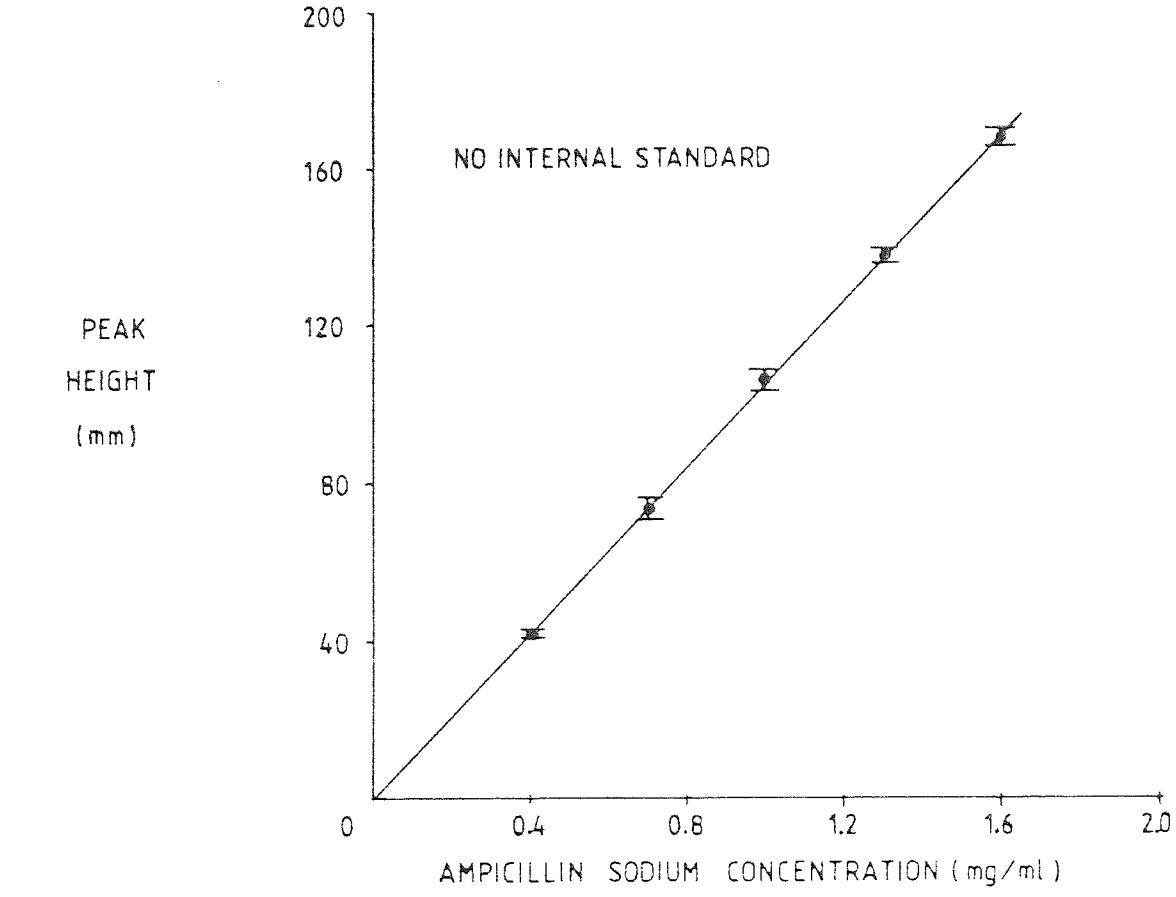
Sample	Interpolated Value (%)	95% C.L. of Interpolated Value	Coefficient of Variation
Ampicillin Sodium	101.4	97.4 to 105.4	3.16
Degraded amp. sod.	37.7	36.4 to 39.0	2.78

Table 2.10 Assay Results - Imidazole Spectrophotometric Method : Ampicillin Sodium Procedure 2

CLOXACILLIN SODIUM STANDARD CURVE - LINEAR REGRESSION ANALYSIS				
Corr. Coeff.	Slope of Reg. Line	95% C.I.on the Slope	Intercept of Reg. Line	95% C.I. on the Inter.
0.9996	9.797	9.677 to 9.917	-0.0064	-0.0107 to -0.0020

Sample	Interpolated Value (%)	95% C.L. of Interpolated Value	Coefficient of Variation
Cloxacillin sodium	100.1	98.8 to 101.3	1.01
Degraded clox. sod.	90.1	88.4 to 91.8	1.53

Table 2.11 Assay Results - Imidazole Spectrophotometric Method : Cloxacillin Sodium



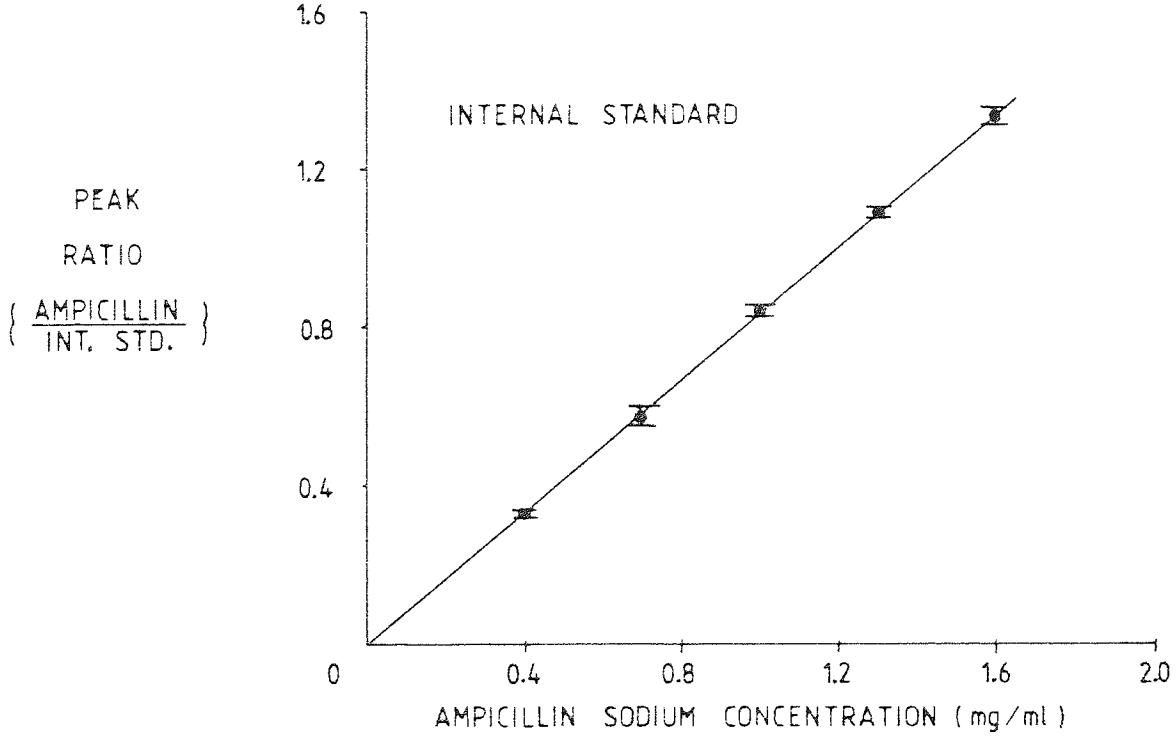
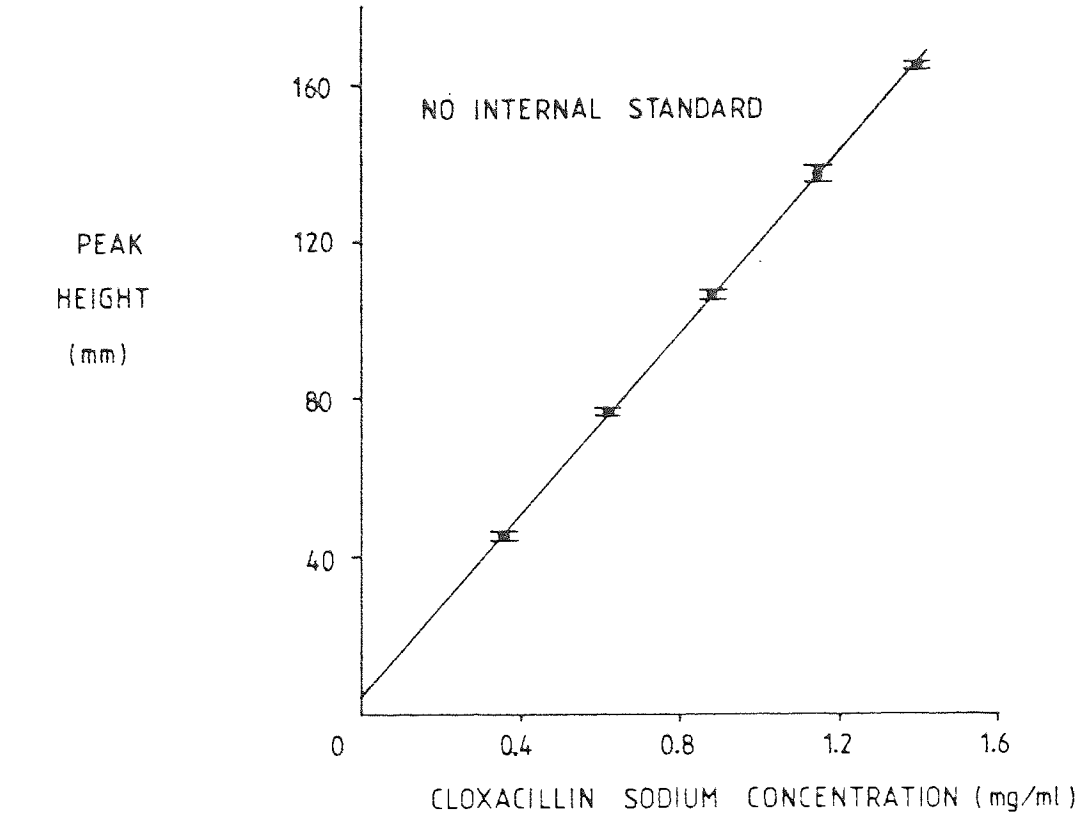


FIGURE 2.20 CALIBRATION CURVES FOR THE HPLC ASSAY METHOD WITH 95% LIMIT ERROR BARS - AMPICILLIN SODIUM

2.3.2.7 High Performance Liquid Chromatographic Method

Tsugi (118) used a 1m anion exchange column to separate ampicillin from its degradation products. All three peaks were quantifiable in less than 12 minutes. Peak profiles were poor, however. Hartmann et. al. (119) used reversed phase columns, preferring partition to ion exchange chromatography. A variety of column materials and solvents were investigated. Acetonitrile was found to be the most suitable organic component in the aqueous mobile phase. White et. al., having previously used medium efficiency, pellicular columns (120), found an improvement in performance with octadecyl microparticulate column supports (121). Larsen and Bundgaard (85) studied the polymerisation of ampicillin by using a similar column support to that used by Hartmann (Lichrosorb RP8) and a similar mobile phase. Tsugi later improved on his previous method, using a C-18 microBondapak (30 cm x 3.9 mm internal diameter) column to analyse ampicillin (122). The mobile phase consisted of acetonitrile : water : 0.2M ammonium acetate buffer (15:75:10) at pH 6.0.

For the comparison of assay methods, H.P.L.C. systems similar to that used by Tsugi (122) and White (121) were developed. It was not possible to develop a single system for quantification of both penicillins because of the difference in their retention volumes. Ampicillin sodium and cloxacillin sodium were assayed with and without an internal standard to assess the need for an internal standard in the samples. Data for this method can be found in Appendix 1. Analysis of the data can be found in tables 2.12, 2.13, 2.14 and 2.15. Standard calibration curves are in figures 2.20 and 2.21; and H.P.L.C. traces of the samples in figures 2.22 and 2.23.



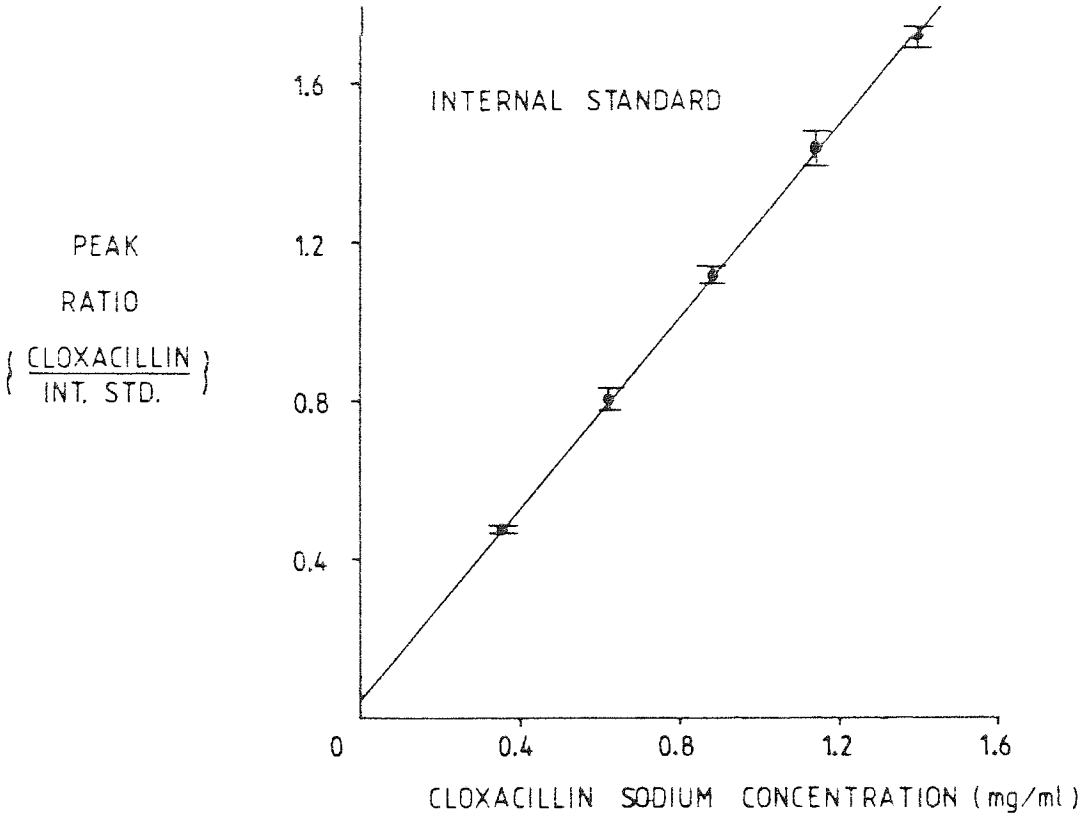


FIGURE 2.21 CALIBRATION CURVES FOR THE HPLC ASSAY METHOD WITH 95% LIMIT ERROR BARS - CLOXACILLIN SODIUM

AMPICILLIN SODIUM STANDARD CURVE - LINEAR REGRESSION ANALYSIS				
Corr. Coeff.	Slope of Reg. Line	95% C.I. on the Slope	Intercept of Reg. Line	95% C.I. on the Inter.
0.9993	105.3	103.5 to 107.0	0.099	-2.551 to 2.749

Sample	Interpolated Value (%)	95% C.L. of Interpolated Value	Coefficient of Variation
Ampicillin sodium	100.9	98.7 to 103.2	1.783
Degraded amp. sod.	36.1	35.2 to 37.0	2.016
1:1 Ampicillin sod./ clox. sod. mixture	47.6	46.4 to 48.8	1.979
Degraded 1:1 amp.sod./ clox. sod. mixture	36.2	35.3 to 37.0	1.993

Table 2.12 Assay Results - H.P.L.C. Method : Ampicillin Sodium (No Internal Standard)

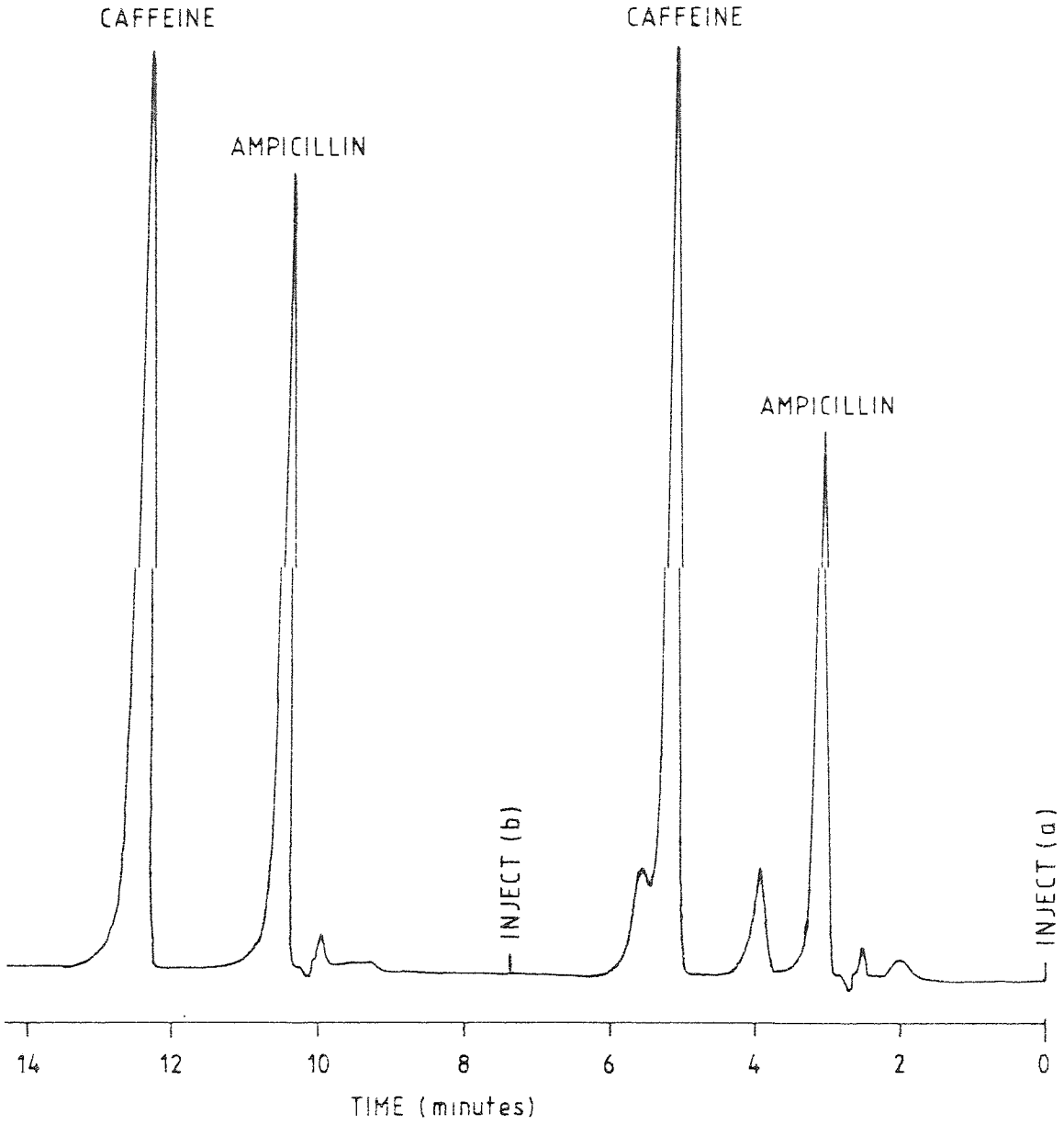


FIGURE 2.22 AMPICILLIN SYSTEM H.P.L.C. CHROMATOGRAMS OF (a) DEGRADED 1:1 MIXTURE OF AMPICILLIN SODIUM AND CLOXACILLIN SODIUM (b) AMPICILLIN SODIUM

AMPICILLIN SODIUM STANDARD CURVE - LINEAR REGRESSION ANALYSIS				
Corr. Coeff.	Slope of Reg. Line	95% C.I. on the Slope	Intercept of Reg. Line	95% C.I. on the Inter.
0.9995	0.830	0.818 to 0.842	0.0009	-0.0169 to 0.0186

Sample	Interpolated Value (%)	95% C.L. of Interpolated Value	Coefficient of Variation
Ampicillin sodium	100.9	99.3 to 102.4	1.234
Degraded amp. sod.	36.0	35.6 to 36.4	0.936
1:1 Ampicillin sod./ clox. sod. mixture	47.2	46.2 to 48.1	1.590
Degraded 1:1 amp.sod./ clox. sod. mixture	35.9	35.2 to 36.6	1.514

Table 2.13 Assay Results - H.P.L.C. Method : Ampicillin Sodium (Internal Standard)

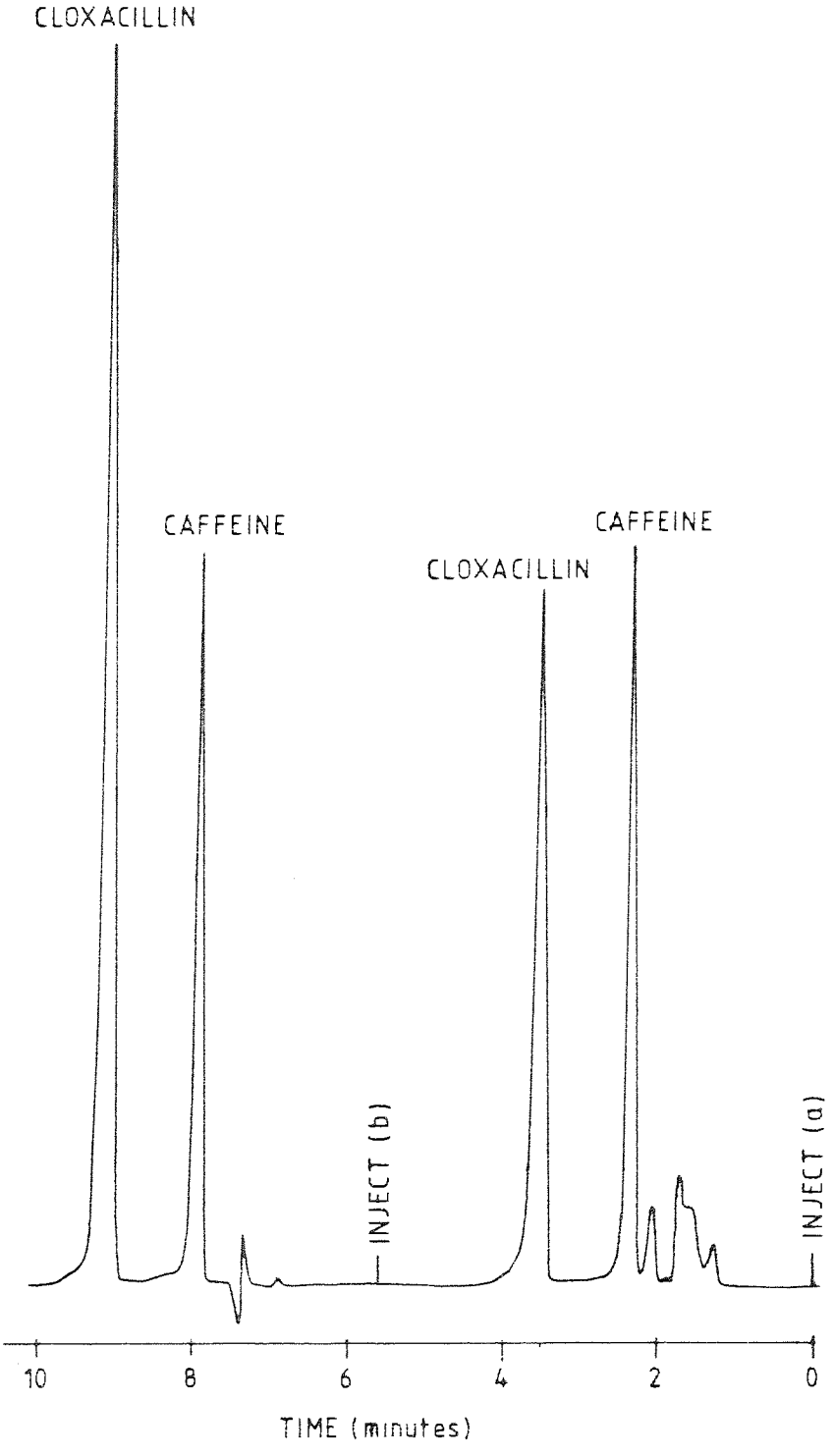


FIGURE 2.23 CLOXACILLIN SYSTEM H.P.L.C. CHROMATOGRAMS OF (a) DEGRADED 1:1 MIXTURE OF AMPICILLIN SODIUM AND CLOXACILLIN SODIUM (b) CLOXACILLIN SODIUM

CLOXACILLIN SODIUM STANDARD CURVE - LINEAR REGRESSION ANALYSIS				
Corr. Coeff.	Slope of Reg. Line	95% C.I. on the Slope	Intercept of Reg. Line	95% C.I. on the Inter.
0.9994	114.9	113.1 to 116.6	4.73	2.55 to 6.91

Sample	Interpolated Value(%)	95% C.L. of Interpolated Value	Coefficient of Variation
Cloxacillin sodium	100.2	99.1 to 101.2	0.814
Degraded clox. sod.	88.9	88.5 to 89.2	0.307
1:1 Ampicillin sod./ clox. sod. mixture	50.2	49.7 to 50.8	0.804
Degraded 1:1 amp.sod./ clox. sod. mixture	41.6	41.3 to 41.9	0.597

Table 2.14 Assay Results - H.P.L.C. Method : Cloxacillin Sodium (No Internal Standard)

CLOXACILLIN SODIUM STANDARD CURVE - LINEAR REGRESSION ANALYSIS				
Corr. Coeff.	Slope of Reg. Line	95% C.I. on the Slope	Intercept of Reg. Line	95% C.I. on the Inter.
0.9986	1.197	1.169 to 1.224	0.0557	0.0211 to 0.0903

Sample	Interpolated Value(%)	95% C.L. of Interpolated Value	Coefficient of Variation
Cloxacillin sodium	100.4	98.3 to 102.5	1.702
Degraded clox. sod.	87.8	86.6 to 89.1	1.125
1:1 Ampicillin sod./ clox. sod. mixture	50.5	49.1 to 51.8	2.181
Degraded 1:1 amp.sod./ clox. sod. mixture	42.1	41.4 to 42.7	1.143

Table 2.15 Assay Results - H.P.L.C. Method : Cloxacillin Sodium (Internal Standard)

2.3.2.8 Comparison of Assay Results from the Different Methods

Accuracy and precision are the most important parameters in quantitative assays. Accuracy is high if the mean assay value approximates to the true value. Specificity of an assay is part of the accuracy. A non-specific assay is not accurate for a specific component if interfering compounds are present. Assay precision is the measure of spread of results, usually represented by parameters such as 95% confidence limits. It is desirable to have high precision (low spread) in order to get confidence in the accuracy of an assay. High precision does not guarantee accuracy, however, because the assay may suffer from interference (be non-specific).

Tables 2.16 and 2.17 compare the assay results for ampicillin sodium and cloxacillin sodium obtained from the different assays.

Sample	Assay Method	Assay(mean)	95% C.L.
Ampicillin Sodium	B.P. 1973 Ampicillin	100.0	97.7 to 102.3
	Direct U.V.	102.5	99.0 to 106.0
	5 rep./5 conc. Microb.	89.7	79.1 to 100.3
	Imidazole (proc. 1)	99.6	97.5 to 101.7
(Section 1)	Imidazole (proc. 2)	101.4	97.4 to 105.4
	H.P.L.C. (no int.std.)	100.9	98.7 to 103.2
	H.P.L.C. (int. std.)	100.9	99.3 to 102.4
Amp. sod./ Clox. sod. 1:1 Mixture	B.P. 1973 Ampicillin	47.9	46.0 to 47.9
	Direct U.V.	48.1	46.0 to 50.1
	5 rep./5 conc. Microb.	53.6	49.9 to 57.4
	H.P.L.C. (no int.std.)	47.6	46.4 to 48.8
(Section 2)	H.P.L.C. (int. std.)	47.2	46.2 to 48.1
Degraded Ampicillin Sodium	B.P. 1973 Ampicillin	51.7	50.7 to 52.7
	Direct U.V.	63.1	59.6 to 66.5
	5 rep./5 conc. Microb.	31.4	28.1 to 34.7
	Two Dose Microb.	34.6	31.9 to 37.4
	Electrophor. Microb.	36.6	35.3 to 37.9
	Imidazole (proc. 1)	49.4	48.0 to 50.9
(Section 3)	Imidazole (proc. 2)	37.7	36.4 to 39.0
	H.P.L.C. (no int.std.)	36.1	35.2 to 37.0
	H.P.L.C. (int. std.)	36.0	35.6 to 36.4
Degraded Amp. sod./ Clox. sod. 1:1 Mixture	B.P. 1973 Ampicillin	37.7	37.1 to 38.4
	Direct U.V.	33.9	31.6 to 36.1
	5 rep./5 conc. Microb.	41.3	37.7 to 44.8
	Two Dose Microb.	35.8	34.0 to 37.6
	Electrophor. Microb.	40.1	37.4 to 42.9
(Section 4)	H.P.L.C. (no int.std.)	36.2	35.3 to 37.0
	H.P.L.C. (int. std.)	35.9	35.2 to 36.6

Table 2.16 Comparison of Assay Results for Ampicillin Content

Sample	Assay Method	Assay(mean)	95% C.L.
Cloxacillin Sodium	B.P. 1973 Cloxacillin	99.2	98.0 to 100.4
	Direct U.V.	99.3	97.9 to 100.7
	5 rep./5 conc. Microb.	99.0	88.8 to 109.2
	Imidazole	100.1	98.8 to 101.3
(Section 5)	H.P.L.C. (no int.std.)	100.2	99.1 to 101.2
	H.P.L.C. (int. std.)	100.4	98.3 to 102.5
Amp. sod./ Clox. sod. 1:1 Mixture (Section 6)	Direct U.V.	49.2	48.4 to 50.0
	5 rep./5 conc. Microb.	38.8	32.7 to 45.0
	H.P.L.C. (no int.std.)	50.2	49.7 to 50.8
	H.P.L.C. (int. std.)	50.5	49.1 to 51.8
Degraded Cloxacillin Sodium	B.P. 1973 Cloxacillin	91.4	90.0 to 92.7
	Direct U.V.	112.1	111.4 to 112.8
	5 rep./5 conc. Microb.	91.1	77.2 to 105.1
	Two Dose Microb.	92.7	87.7 to 97.9
	Electrophor. Microb.	95.0	88.4 to 101.8
	Imidazole	90.1	88.4 to 91.8
(Section 7)	H.P.L.C. (no int.std.)	88.9	88.5 to 89.2
	H.P.L.C. (int. std.)	87.8	86.6 to 89.1
Degraded Amp. sod./ Clox. sod. 1:1 Mixture	Direct U.V.	47.6	46.1 to 49.1
	5 rep./5 conc. Microb.	32.1	26.8 to 37.5
	Two Dose Microb.	33.2	30.5 to 36.0
	Electrophor. Microb.	40.1	38.7 to 41.6
	H.P.L.C. (no int.std.)	41.6	41.3 to 41.9
(Section 8)	H.P.L.C. (int. std.)	42.1	41.4 to 42.7

Table 2.17 Comparison of Assay Results for Cloxacillin Content

Assay Precision

Microbiological assay, especially the 5x5 design, gave low precision. Biological variation could be a factor in this, as described by Wallhausser (123), who stated,

"Every estimate of potency determined in a biological assay is subject to a random error due to the variability characteristic of all biological results."

Operator technique is critical in microbiological assays because of the many physico-chemical parameters involved.

The other assay method that produced poor precision was the direct U.V. method by Davidson and Stenlake. This is explained by the small U.V. absorbance difference between pH 5 and pH 9 solutions of ampicillin in the presence of cloxacillin, an observation made by the developers of the method (55).

Assay Accuracy

Several assay results from tables 2.16 and 2.17 require discussion. The poor precision of the 5x5 microbiological assay does not give confidence in the accuracy of the result. The mean values should have approximated to the mean values from the two dose microbiological assay, since the technique was similar. For this reason the 5x5 assay results will be ignored in this comparison of accuracy.

The results in section 1 of table 2.16 were as expected. Mean assay results in section 2, table 2.16, were consistently low (means ranged from 47.2% to 48.1% and should have been 50.0%). If poor mixing of the penicillin standards had been the cause, assay results in section 6, table 2.17, would have been high (approximately 52%). However, the means from section 6 ranged from 49.2% to 50.5%. Starch gel electrophoretic analysis of the 1:1 mixture suggested that ampicillin sodium had

degraded slightly, although the cause of this remained unexplained.

In section 3, table 2.16, the H.P.L.C., imidazole (procedure 2) and microbiological methods provided the probable accurate result (36.0%, 36.1%, 37.7%, 34.6%, 36.6%). The B.P. 1973, direct U.V. and imidazole (procedure 1) methods gave high results, due to degradation product interference.

In section 4, table 2.16 the H.P.L.C., B.P. 1973, direct U.V. and two dose microbiological assays gave the probable accurate result (35.9%, 36.2%, 37.7%, 33.9%, 35.8%). Ampicillin polymers would be expected to interfere with the B.P. 1973 assay result. However, H.P.L.C. qualitative analysis of the sample showed few polymeric degradation products. Also, less degradation of ampicillin had occurred (14% compared with 64% for ampicillin sodium alone), thus fewer interfering degradation products were present. The direct U.V. assay result of 33.9% suggests that little penicilloic acid was present in the sample or that other effects compensated for this, e.g. the presence of a degradation product that produced a higher U.V. absorbance at pH 9 than at 5. Amoxycillin exhibits this effect (55), as could other phenolic compounds. Agar gel electrophoresis microbiological assay gave a slightly high result (40.1%) but the assay had low precision (95% confidence limits of 37.4% to 42.9%), which provides an explanation.

Results in sections 5 and 6, table 2.17, were as expected. A probable accurate result for section 7,table 2.17, was provided by the H.P.L.C., B.P. 1973, two dose microbiological and imidazole assays (87.8%, 88.9%, 91.4%, 92.7%, 90.1%). Direct U.V. assay gave a high result (112.1%) due to strong U.V. absorbing degradation products of cloxacillin sodium. Agar gel electrophoresis assay gave a slightly high result with low precision.

In section 8, table 2.17, the H.P.L.C. and agar gel electrophoresis assay gave the probable accurate result (42.1%, 41.6%, 40.1%). The

direct U.V. assay suffered from inteference by stong U.V. absorbing degradation products and the two dose microbiological produced a low result. This could be explained by the differential deactivation required for the assay of cloxacillin in combined ampicillin/ cloxacillin preparations (incubation with penicillinase at 37°C for 30 minutes and 80°C for 10 minutes). The high temperatures and penicillinase action, although calculated to have minimal effect, could degrade the cloxacillin in the sample.

Table 2.18 contains a summary of the characteristics of the assay methods investigated.

METHOD	PRECISION	ACCURACY	INTERFERENCE FROM :		ADVANTAGES	DISADVANTAGES
		OTHER PENICILLINS	DEGRADATION PRODUCTS	OTHER COMPOUNDS		
B.P. 1973 CLOXACILLIN SODIUM	HIGH	INTERFERENCE FROM AMPICILLIN AND OTHER PENICILLINS	INTERFERENCE FROM COMPOUNDS REACTING WITH SODIUM HYDROX. ON HEATING . e.g. ESTERS	INTERFERENCE FROM COMPOUNDS REACTING WITH SODIUM HYDROX. ON HEATING . e.g. ESTERS	SIMPLE, RAPID . REQUIRES STANDARD LABORATORY EQUIPMENT AND REAGENTS	-
B.P. 1973 AMPICILLIN SODIUM	HIGH	NO INTERFERENCE FROM CLOXACILLIN. INTERFERENCE FROM OTHER PENICILLINS	INTERFERENCE FROM POLYMERS OF AMPICILLIN AND PENICILLENIC ACID	INTERFERENCE FROM CMPD. THAT DEGRADE UNDER ASSAY CONDITIONS TO GIVE INCREASED ABS. AT 320 nm	SIMPLE, RAPID. REQUIRES STANDARD LABORATORY EQUIPMENT AND REAGENTS	REQUIRES AN ULTRA VIOLET SPECTROPHOTOMETER
DIRECT ULTRA VIOLET SPECTROPHOTOMETRIC	HIGH FOR CLOXACILLIN. MEDIUM FOR AMPICILLIN	CLOXACILLIN : ANY PENICILLIN WITH A U.V. ABS. AT 320nm AMPICILLIN : AMOXYCILLIN	CLOXACILLIN : DEG. PRODUCTS WITH A U.V. ABS. AT 320nm AMPICILLIN : PENICILLOIC ACID	COMPOUNDS WITH U.V. ABS. AT 273 nm. COMPOUNDS THAT HAVE A DIFFERENT U.V. ABS. AT 268nm AT pH5 & pH9	SIMPLE, RAPID. DETERMINES AMPICILLIN AND CLOXACILLIN FROM ONE WEIGHED SAMPLE	REQUIRES AN ULTRA VIOLET SPECTROPHOTOMETER
MICROBIOLOGICAL : (i) 5x5	LOW	INTERFERENCE FROM OTHER PENICILLINS	NO BIOLOGICALLY INACTIVATED DEG. PRODUCTS INTERFERE	ANTIBIOTIC MOLECULES e.g. PRESERVATIVES	SIMPLE. DIRECT MEASURE OF ANTIBIOTIC ACTIVITY	SLOW
(ii) TWO DOSE	MEDIUM	INTERFERENCE FROM OTHER PENICILLINS	NO BIOLOGICALLY INACTIVATED DEG. PRODUCTS INTERFERE	ANTIBIOTIC MOLECULES e.g. PRESERVATIVES	SIMPLE. DIRECT MEASURE OF ANTIBIOTIC ACTIVITY	SLOW
(iii) AGAR GEL ELECTROPHORESIS	MEDIUM	SOME INTERFERENCE e.g. PENICILLIN G AND CLOXACILLIN	NO BIOLOGICALLY INACTIVATED DEG. PRODUCTS INTERFERE	LESS INTERFERENCE	SIMPLE. DIRECT MEASURE OF ANTIBIOTIC ACTIVITY	SLOW. REQUIRES SPECIALISED EQUIPMENT
IMIDAZOLE SPECTROPHOTOMETRIC	HIGH	INTERFERENCE FROM OTHER PENICILLINS	PENICILLENIC ACIDS COULD INTERFERE	COMPOUNDS THAT DEGRADE UNDER ASSAY CONDITIONS TO GIVE INC. ABS. AT ASSAY WAVELENGTH	SIMPLE, RAPID.	REQUIRES AN ULTRA VIOLET SPECTROPHOTOMETER
H.P.L.C.	HIGH	NO INTERFERENCE	NO INTERFERENCE	NO INTERFERENCE	SIMPLE, RAPID	REQUIRES SPECIALISED AND EXPENSIVE EQUIP

TABLE 2.18 SUMMARY OF THE CHARACTERISTICS OF THE ASSAY METHODS INVESTIGATED

2.4 CONCLUSION

The one technique producing accuracy, specificity and precision for all the samples was H.P.L.C. However, this technique requires expensive equipment and suffers from the possibility of co-elution for parent penicillins and one, or more, of their degradation products. Co-elution of peaks cannot easily be diagnosed unless the U.V. spectrum of the two species is significantly different. Despite these disadvantages, it is the technique most suitable for the analysis of penicillins, quantifying degradation products (124, 125, 118), parent penicillins (118,119,125,121) and polymeric derivatives (85).

Of the other techniques, electrophoresis microbiological assay was the most versatile, separating components in the mixture and differentiating degradation products, even polymeric derivatives, from their parent penicillins. However, the time of assay, the need for special equipment and the lack of precision were disadvantages. The other microbiological methods lacked precision, the 5x5 assay proving to be of poor design. Some measure of specificity was possible by using selective organisms, e.g. Sarcina lutea, but mixtures of penicillins required differential deactivation, by penicillinase, to assay cloxacillin. Microbiological assays, apart from the 5x5 design, required a good estimate of the concentration in the samples. This is a large disadvantage. However, these methods provide a measure of antibacterial potency, albeit against non-pathogenic organisms.

Direct U.V. spectrophotometric assay of mixtures of penicillins was found to be rapid, but lacked precision for the estimate of ampicillin in the presence of large amounts of cloxacillin. It was neither specific, nor accurate, U.V. absorbing compounds, including degradation products, causing interference.

The imidazole catalysed penicillenic acid method could not be used for

mixtures of penicillins but was specific and precise for penicillins in the presence of their degradation products, even polymeric derivatives. The official B.P. 1973 cloxacillin sodium method, a method involving direct acid/ base titration, lacked specificity and interference could occur from compounds degraded by hot sodium hydroxide.

The official B.P. 1973 ampicillin sodium method differentiated between cloxacillin and ampicillin, but suffered from interference from polymeric derivatives of ampicillin.

Both of these B.P. methods were rejected by the B.P. Commission in 1980 in favour of the imidazole spectrophotometric methods of Bundgaard and Ilver (63) and Bundgaard (64). Whilst this is an improvement for the determination of cloxacillin sodium, there is no mention in the B.P. 1980 of polymeric derivatives of ampicillin sodium, or any reference to the differential hydrolysis technique developed by Bundgaard for the determination of ampicillin in the presence of its polymers.

CHAPTER THREE : THE DEVELOPMENT OF ANALYTICAL ASSAY METHODS

3.1 THE H.P.L.C. ANALYSIS OF PREPARATIONS CONTAINING BENZATHINE CLOXACILLIN, BENZATHINE PENICILLIN V, BENZATHINE PENICILLIN G, BENETHAMINE PENICILLIN G AND PROCAINE PENICILLIN G

The structures of these compounds can be found in figures 7.1 and 7.2

3.1.1 PREPARATIONS ANALYSED :

i) Benzathine cloxacillin B.P.C. (unformulated)

ii) Benzathine penicillin V (unformulated)

iii) Procaine penicillin injection B.P. (containing procaine penicillin B.P.)

iv) Benethamine penicillin injection, fortified B.P.C. (containing benethamine penicillin B.P.C., procaine penicillin B.P. and benzylpenicillin sodium B.P.)

v) Benzathine penicillin injection (containing benzathine penicillin B.P.)

vi) Benzathine penicillin injection, fortified B.P.C. (containing benzathine penicillin B.P., procaine penicillin B.P. and benzylpenicillin potassium B.P.)

vii) Benzathine penicillin suspension (containing benzathine penicillin B.P.)

3.1.2. OFFICIAL METHODS OF ASSAY

The standard monograph for benzathine cloxacillin in the B.P.(Vet.) 1977 (126) contains the methods of analysis for benzathine and cloxacillin. They are essentially the same as for benzathine and penicillin G in the standard monograph for benzathine penicillin B.P. (127). Penicillin G is determined by reaction with iodine and back-

titration with sodium thiosulphate. Benzathine is determined by non-aqueous titration with perchloric acid after a complex extraction procedure.

No official standard exists in the B.P. 1980 or the B.P.C. 1979 for benzathine penicillin V. However, a standard monograph was found in the U.S.P. 20th edition (128). It referenced, but did not give, details of analysis.

Official methods of analysis for benzathine penicillin injection, fortified B.P.C. are given in the B.P.C. 1973 (129). The standard monograph requires determination of :

i) the content of benzylpenicillin potassium

ii) the content of procaine penicillin

iii) the content of benzathine penicillin

iv) the content of total penicillins

i), ii) and iv) are determined in a similar way to the determination of these compounds in benethamine penicillin injection, fortified B.P.C.

iii) is determined by non-aqueous titration with perchloric acid, after a complicated preparation. An accurate value for procaine penicillin content is required for its calculation.

The official standard for benethamine penicillin injection, fortified B.P.C. (130) requires estimation of i), ii) and iv) above, as well as the content of benethamine penicillin.

i) is determined by reaction with iodine and back-titration with sodium thiosulphate, after separation of benzylpenicillin sodium (benzylpenicillin potassium for benzathine penicillin injection, fortified B.P.C.) by differential solubility and centrifugation.

ii) is determined by direct U.V. absorbance measurements, after dilution and filtration.

iv) is determined by reaction of the whole sample with iodine and back-titration with sodium thiosulphate.

Benethamine penicillin content is determined by a complicated extraction procedure, reaction with 0.01 N sulphuric acid and back-titration of excess acid with 0.01 N borax. A value for the content of procaine penicillin is required for its calculation.

The standard monograph for procaine penicillin injection B.P. (131) requires an assay for total penicillins and for procaine. Total penicillins are determined by reaction with iodine and back-titration with sodium thiosulphate; and procaine is determined by extraction, reaction with sulphuric acid and titration of excess acid with sodium hydroxide.

Whilst these methods require only readily available laboratory equipment and reagents, they are time consuming, non-specific and often complicated.

3.1.3 ALTERNATIVE METHODS OF ASSAY

Total penicillin concentration in all of the above preparations was determined by iodimetry. Other methods available for the assay of penicillins could be adapted to determine penicillin content. These include U.V. and visible spectrophotometry [hydroxylamine (58), copper (II) catalysed breakdown to penicillenic acid (132), imidazole catalysed breakdown to penicillenic acid (63), complex formation (133)], titrimetry [reaction with iodine monochloride (134)], fluorimetry [reaction with aminoacridine (135)] and microbiology [cylinder plate method (97)]. However, none of these methods offer a significant improvement on the existing official methods.

Chromatographic separation, however, does have significant advantages. It is more specific, simple and can quantify more than one component at a time. The main disadvantage is the need for expensive equipment (G.C. and H.P.L.C.). Several methods for the assay of penicillins by

chromatographic separation have been developed. T.L.C. and paper chromatography lack precision, so tend to be used for qualitative work (136), although quantitative methods have been reported (137). G.C. analysis of penicillins most often requires derivatisation (80, 81) and this decreases precision. H.P.L.C., carried out at ambient or near-ambient temperatures, provides the precision and specificity of G.C., without the need for derivatisation.

Literature methods for the H.P.L.C. analysis of penicillins are numerous. Relevant ones are:

i) the separation of penicillin G from penicillin V by reversed-phase H.P.L.C. (138, 121)

ii) reversed-phase H.P.L.C. of penicillin G and its degradation products (124, 125)

iii) reversed-phase H.P.L.C. of penicillin V and its degradation products (139)

iv) reversed-phase, ion-pair chromatography of cloxacillin (140)

Procaine has been assayed by H.P.L.C. (141) and by other methods [amperometric titration (142), colorimetry (143, 144)].

Few methods of assay relate specifically to slow release preparations of penicillins like those analysed here. Holbrook (132), developed a spectrophotometric assay for the assay of penicillin G in procaine penicillin and benzathine penicillin. Hishta *et. al.* (80) analysed procaine penicillin using G.C. where procaine chromatographed as procaine and penicillin G as T.M.S.-penicillanic acid. Derivitisation was by H.M.D.S. at room temperature. La Belle *et. al.* developed an H.P.L.C. system for benzathine penicillin V oral suspension (145) when they encountered problems with the iodimetric assay method given in the American official standard (146). They only determined penicillin V content, benzathine being invisible at their chosen wavelength of detection. Under the conditions of the assay, benzathine eluted as

a broad peak (detectable at a lower U.V. wavelength) with short retention. Tsugi et. al. developed a system for procaine penicillin G (147), which determined both penicillin G and procaine from a single chromatogram. Retention of procaine was long (22 minutes), making routine analysis slow.

H.P.L.C. was chosen as the method of analysis for the slow release preparations under study, since simultaneous quantification of the penicillin and its amine adjunct were possible using this approach. Systems were developed using information from the literature sources above, with the intention of making them similar to each other. This has the advantage that reagents are common and one bulk mobile phase can be readily converted into another.

3.1.4 EXPERIMENTAL

3.1.4.1 EQUIPMENT

Pump : Altex 100A dual piston liquid pump

U.V. detector : Unicam L.C.3

Recorder : JJ dual pen recorder

Column : Shandon 10 cm x 4.6 mm stainless steel, packed with 5 micrometre ODS-hypersil

Injector : Rheodyne 7120 with 20 microliter loop

3.1.4.2 MATERIALS

1-heptane sulphonic acid was supplied by Fluka A.G.

Cloxacillin sodium, benzathine cloxacillin and benzathine diacetate came from Beecham Pharmaceuticals Research Division Laboratories, Worthing.

Benethamine diacetate came from Glaxo Operations U.K. Ltd., Ulverston, Cumbria.

Procaine penicillin injection B.P., Benethamine penicillin injection, fortified, Benzathine penicillin injection, Benzathine penicillin injection, fortified B.P.C. and Benzathine penicillin suspension were obtained from a retail pharmacy.

Solvents were H.P.L.C. grade. Water was double-distilled from glass vessels.

All other materials were standard laboratory reagents.

3.1.4.3 METHOD

All ion-pair separations were carried out at 34°C, at a flow rate of 1 ml per minute and a detection wavelength of 258 nm.

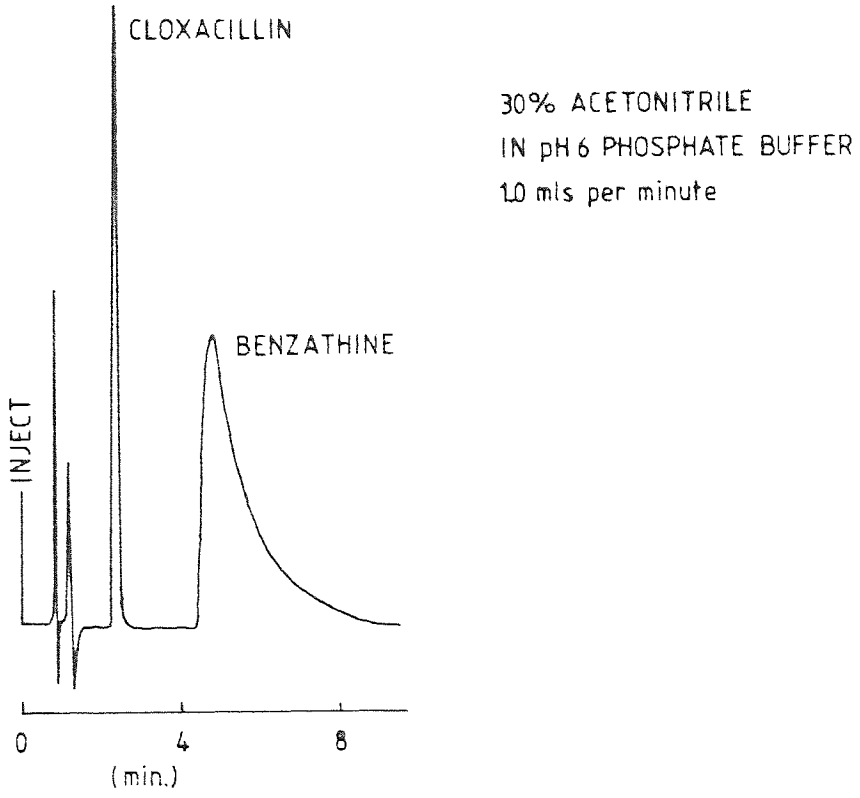


FIGURE 3.1 CHROMATOGRAPHY OF BENZATHINE CLOXACILLIN - A

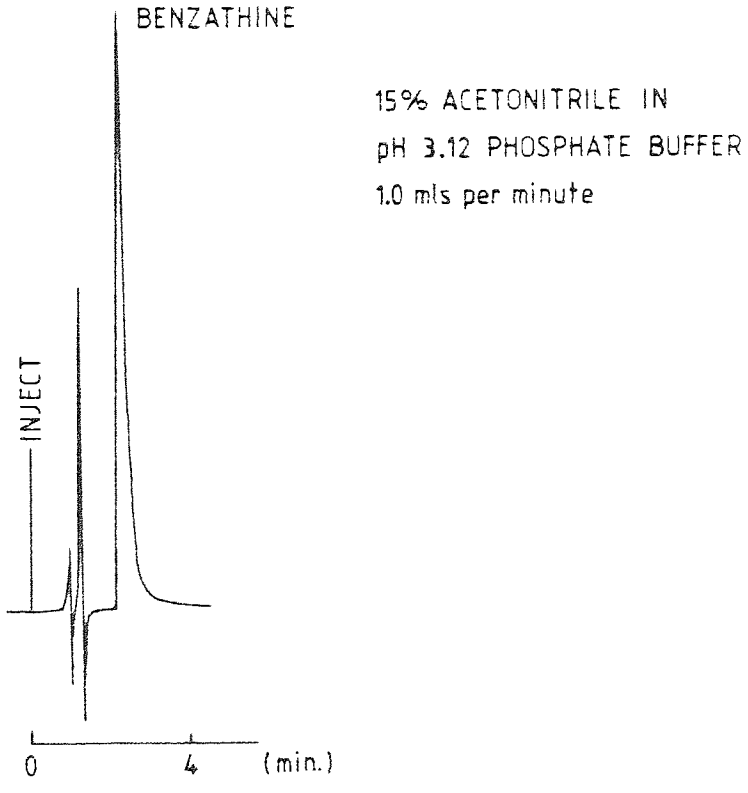


FIGURE 3.2 CHROMATOGRAPHY OF BENZATHINE CLOXACILLIN - B

3.1.5. RESULTS AND DISCUSSION

3.1.5.1 Benzathine Cloxacillin

Development of the System

Reversed-phase chromatography was preferred because the system was to be used for the assay of aqueous samples. Benzathine and cloxacillin were ionisable, thus a buffer was necessary to control the degree of ionisation (148).

Figure 3.1 illustrates the chromatography of both components in a buffered system at pH 6. Cloxacillin produced a sharp peak profile, but the benzathine peak tailed badly. A lower pH improved the peak shape of benzathine, but caused an unacceptable increase in the retention of cloxacillin (figure 3.2).

Ion-pair chromatography was considered at this stage in order to improve the peak shape of benzathine and to increase its retention, facilitating simultaneous determination of cloxacillin and benzathine. Temperature can affect the column performance, an increase in temperature usually creating higher efficiencies (148). For this reason, the temperature was raised to 34^{o}C by placing it in a water bath.

Figures 3.3 and 3.4 illustrate the effects of ion-pair concentration and pH on the separation of benzathine and cloxacillin, whilst table 3.1 shows that buffer salt concentration also affects the system.

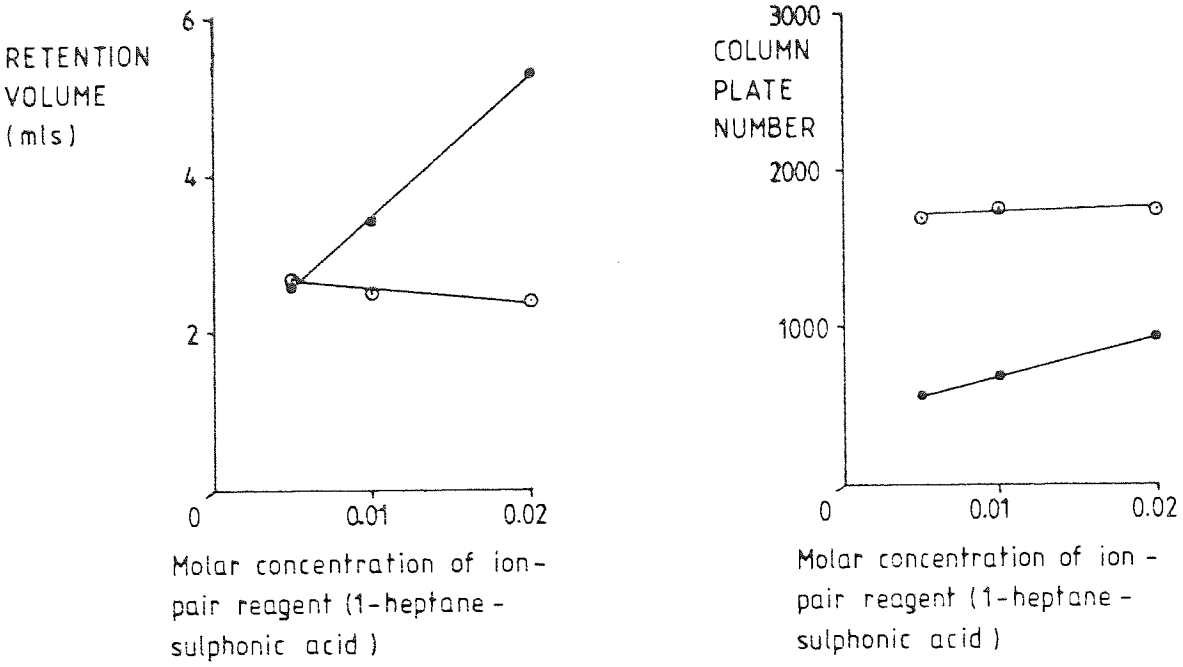


FIGURE 3.3 EFFECT OF ION-PAIR CONCENTRATION ON THE CHROMATOGRAPHY OF BENZATHINE (•) AND CLOXACILLIN (⊙). MOBILE PHASE: 30% Acetonitrile ; 70% Aqueous Component ($9.08 g.l^{-1}$ KH_2PO_4 + 1-heptane-sulphonic acid adjusted to pH 4.5 with H_3PO_4)

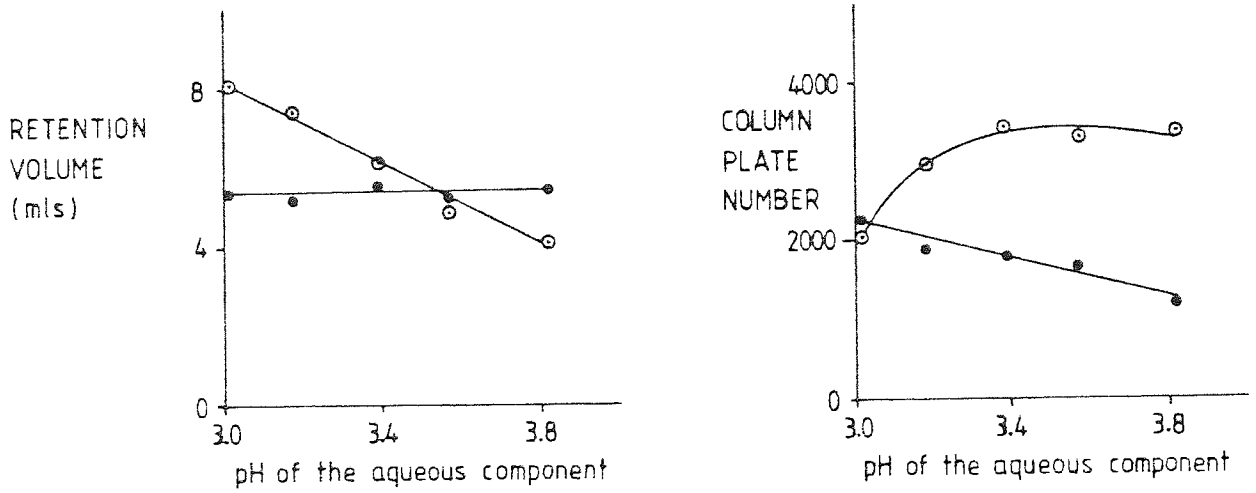


FIGURE 3.4 EFFECT OF pH ON THE CHROMATOGRAPHY OF BENZATHINE (•) AND CLOXACILLIN (⊙). MOBILE PHASE: 30% Acetonitrile ; 70% Aqueous Component ($9.08 g.l^{-1}$ KH_2PO_4 + $6.3 g.l^{-1}$ 1-heptane-sulphonic acid adjusted to required pH with H_3PO_4)

Mobile Phase: 33% Acetonitrile

67% Aqueous phase (6.3 g per litre 1-heptane sulphonic acid + KH_2PO_4*

Apparent pH 3.76

*KH_2PO_4 conc. (g/l)	Benzathine		Cloxacillin	
	Retention Vol.(mls)	Column Plate Number	Retention Vol.(mls)	Column Plate Number
4.54	4.89	828	6.03	2152
9.08	3.06	1072	5.43	2437

Table 3.1 The Influence of Salt Concentration on the Chromatography of Cloxacillin and Benzathine.

Figure 3.3 shows that ion-pair concentration changes the retention time and plate number of benzathine, but has little effect on cloxacillin. Benzathine has two secondary amine groups [pKa values approx. 6.2 and 9.2, (150, 150a) - see appendix 3] of which one or both are available for ion-pairing with a suitable anion (149). As the ion-pair concentration increases, the degree of pairing increases and benzathine is retained longer. At the same time, benzathine assumes a less ionic form and its chromatography improves, seen as an increase in plate

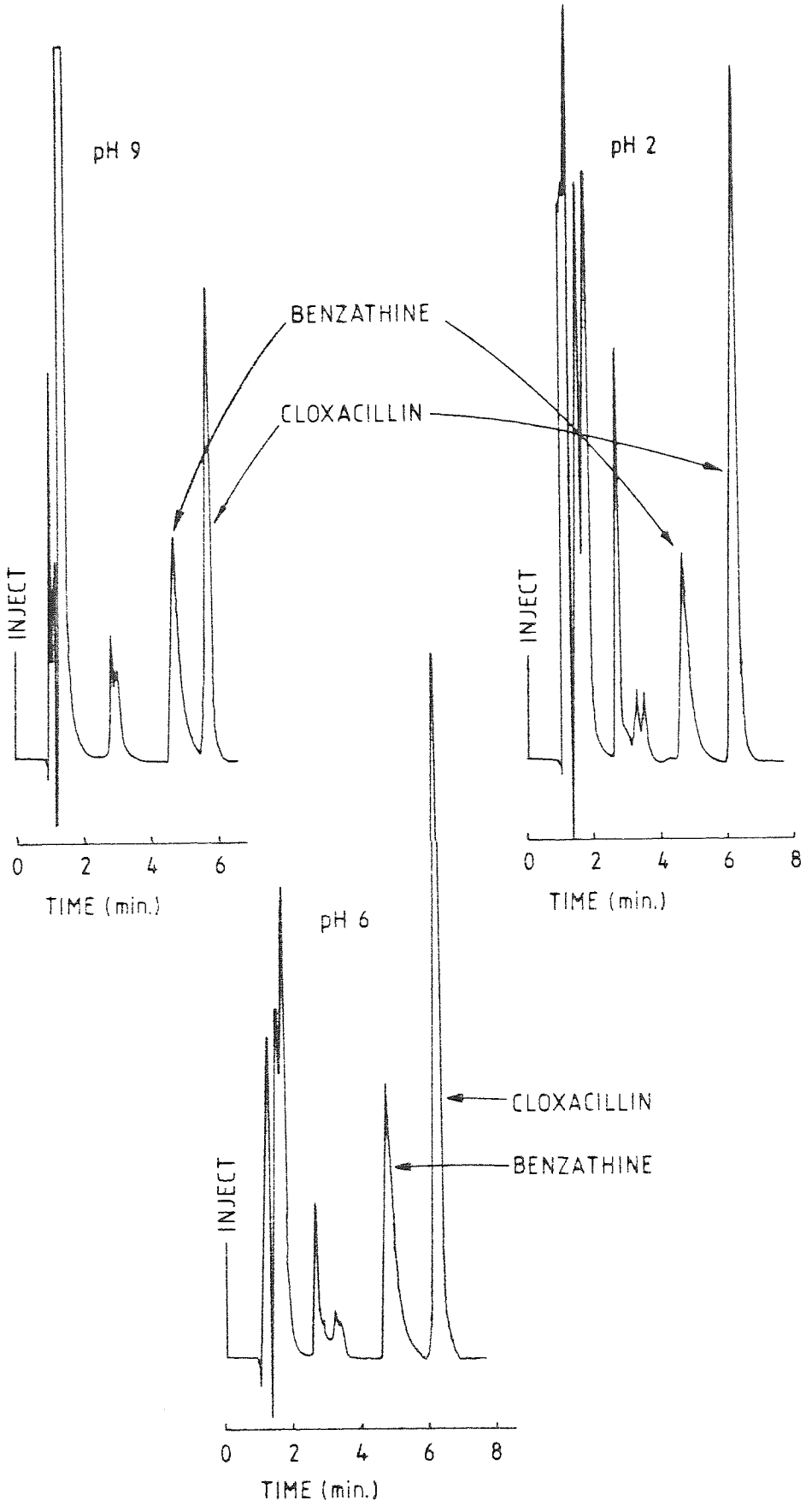


FIGURE 3.5 SEPARATION OF BENZATHINE AND CLOXACILLIN FROM DEGRADATION PRODUCTS OF CLOXACILLIN

number.

pH affects the chromatography of both compounds (figure 3.4). Decreasing pH improves the peak shape of benzathine (increasing plate number), but has little effect on retention. This is unusual since retention usually increases as the pH maximises the concentration of the ionic form of the solute (151), an effect indicated by the improved shape of the peak with decreasing pH.

Cloxacillin exhibits increased retention and decreased plate number with decreasing pH. This is due to cloxacillin [pKa 2.7 (33)] becoming increasingly protonated as the pH approaches its pKa value.

The results in table 3.1 illustrate that an increase in buffer salt concentration causes a decrease in retention volume. This indicates that benzathine and cloxacillin are present in their ionic forms to some degree at an apparent pH of 3.76 (149).

Control of pH, salt, ion-pair and organic modifier concentration provided great flexibility in the optimisation of a chromatographic system for benzathine and cloxacillin, and enabled these compounds to be separated from the degradation products of cloxacillin produced in pH 9 borate, pH 2 citrate and pH 6 citrate buffers (figure 3.5). The optimum mobile phase had the composition:

33% Acetonitrile

67% Aqueous component (4.54 g/l KH_2PO_4
(6.3 g/l 1-heptane sulphonic acid

Adjusted to an apparent pH of 3.40 with H_3PO_4

Sample solvent effect

Samples to be analysed by H.P.L.C. are usually dissolved in the mobile phase. This minimises interference between the sample solvent and the mobile phase. However,in the work associated with this analytical

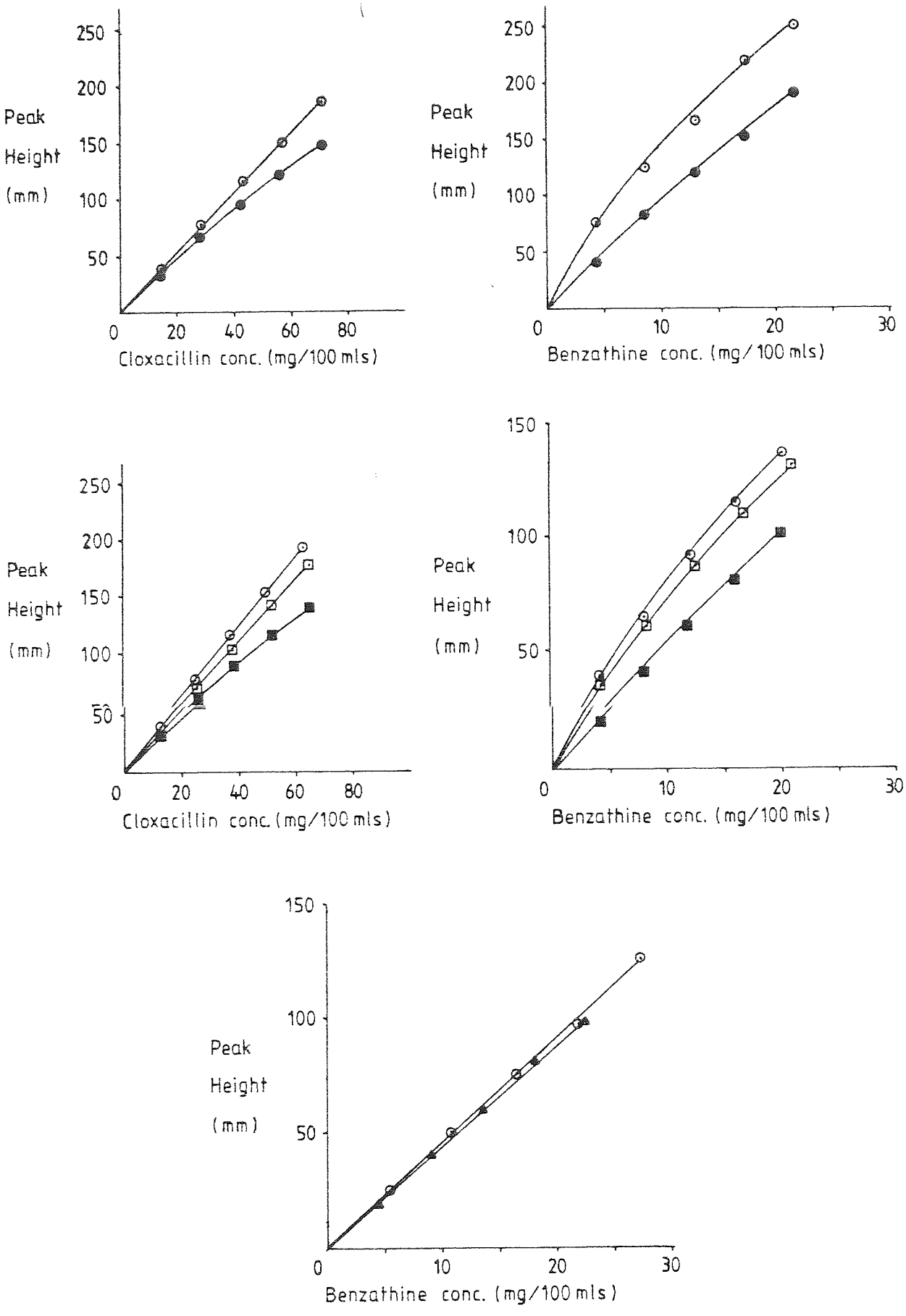


FIGURE 3.6 SAMPLE SOLVENT EFFECTS FOR BENZATHINE AND CLOXACILLIN IN WATER (⊙); pH 6 BUFFER (●); pH 9 BUFFER (■); pH 9 BUFFER DILUTED 1 TO 6 (⊡); pH 2 BUFFER (▲)

method (chapter 7), the sample was already dissolved in pH 2 citrate, pH 6 citrate or pH 9 borate buffer. This prevented use of the mobile phase as the sample solvent. Williams et. al. (152) demonstrated the influence of sample solvent composition on column chromatography. They reported that the greatest effect occurred when the organic modifier concentration in the mobile phase was exceeded in the sample solvent. This condition was not found in the system developed for benzathine cloxacillin, because all of the samples were aqueous, but its complex chromatographic mechanism (ion-pairing for benzathine and ion-suppression for cloxacillin) makes it susceptible to similar effects. The effect of different sample solvents on the chromatography of benzathine and cloxacillin was therefore investigated by dissolving these compounds in water, pH 6 citrate buffer, pH 9 borate buffer , pH 9 borate buffer diluted 1 to 6 with water and pH 2 citrate buffer (the sample solvents found in chapter 7). (Figure 3.6). It was not possible to examine the effects of injecting cloxacillin in pH 2 citrate buffer due to its rapid rate of degradation in this solvent (approximately ten times more rapid than at pH 9).

Figure 3.6 shows that a considerable sample solvent effect was found with this chromatographic system. The precise cause of these effects was not known, but is probably associated with the buffering action of the sample solvent. The sample solvents used in this study were of a different pH to the mobile phase. The greater the buffering action of these sample solvents and the greater the difference in pH between the sample solvent and the mobile phase, the greater the effect they would have on the pH of the mobile phase. pH is known to have a marked effect on the peak shape and separation of benzathine and cloxacillin. Thus change in the pH of the mobile phase (apparent pH 3.4) would affect the chromatography.

The only practical way to overcome sample solvent effect is to prepare

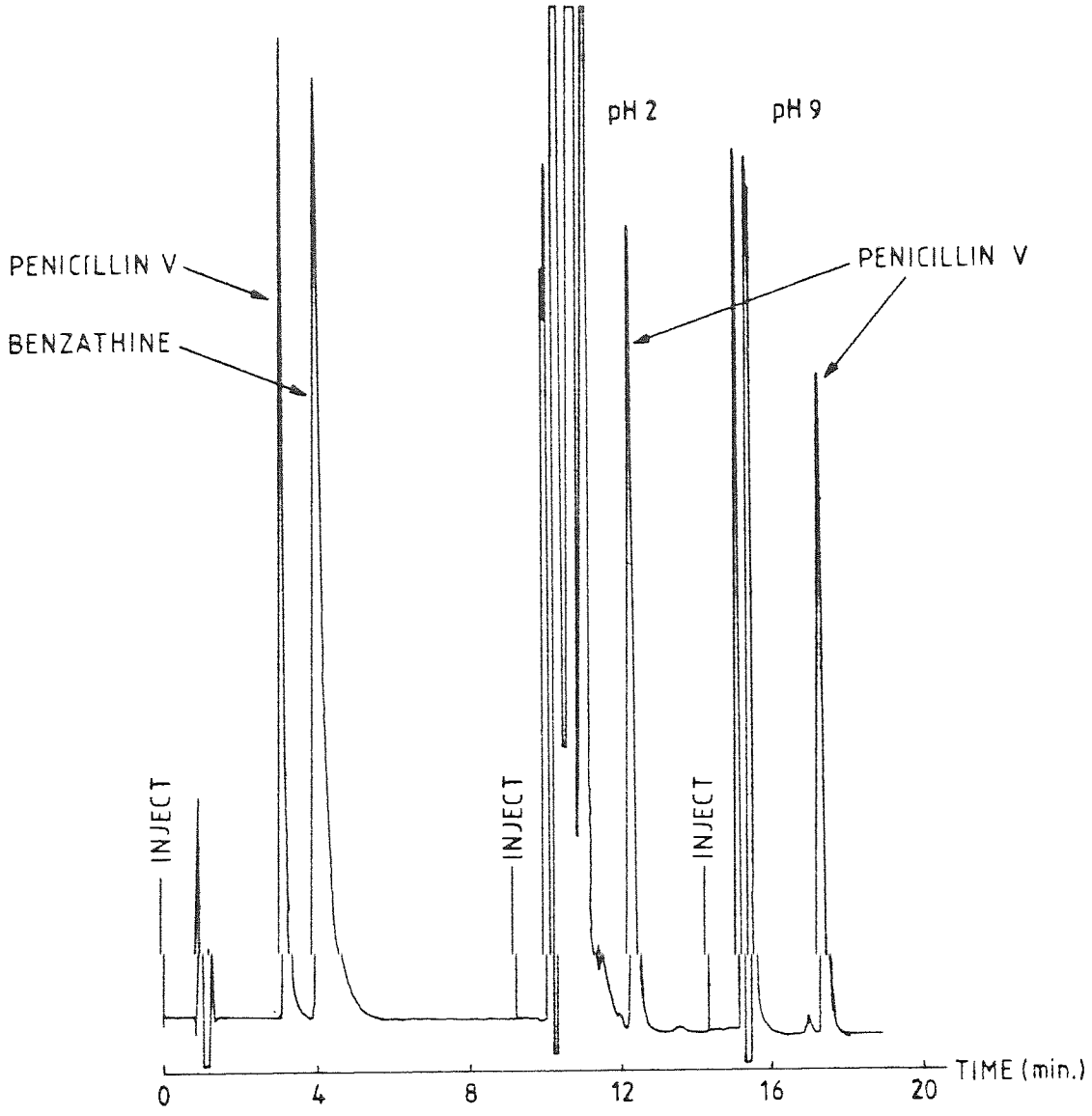


FIGURE 3.7 SEPARATION OF BENZATHINE AND PENICILLIN V FROM DEGRADATION PRODUCTS OF PENICILLIN V

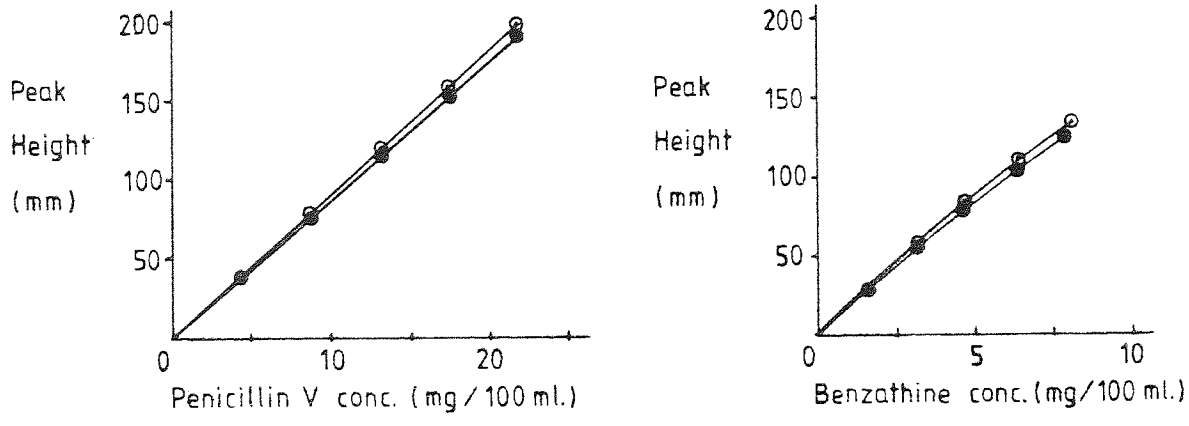


FIGURE 3.8 SAMPLE SOLVENT EFFECTS FOR BENZATHINE AND PENICILLIN V IN WATER (⊙) AND pH 9 BUFFER DILUTED 1 TO 11 WITH WATER (●)

standards in solvents of the same composition as the samples.

Linearity of plots of peak height against concentration

Figure 3.6 shows that the peak height was not always linearly dependent upon concentration. It was unexplained and necessitated interpolation of the sample peak heights onto standard calibration curves.

3.1.5.2 Benzathine Penicillin V

The mobile phase developed for the analysis of benzathine cloxacillin was adjusted to an apparent pH of 3.5 with H_3PO_4. This facilitated separation of benzathine and penicillin V (figure 3.7). Penicillin V was degraded in pH 9 borate and pH 2 citrate buffers, and analysed using this system (figure 3.7). No interference between penicillin V and its degradation products was apparent.

Sample solvent effect and linearity of response

Benzathine penicillin V was only analysed in a sample solvent consisting of pH 9 borate buffer diluted 1 to 11 with water (chapter 7). Therefore, standard solutions were prepared in water and in pH 9 borate buffer diluted 1 to 11, and their chromatography compared (figure 3.8).

Slight sample solvent effect was evident from figure 3.8. Penicillin V exhibited a linear response *vs* concentration relationship, unlike benzathine which produced a curve, necessitating interpolation of values onto a standard calibration curve.

3.1.5.3 Procaine Penicillin Injection B.P.

The mobile phase used for the separation of benzathine and cloxacillin was diluted with water to an acetonitrile concentration of 28.5% and the apparent pH adjusted to 3.17. The mobile phase had the composition:

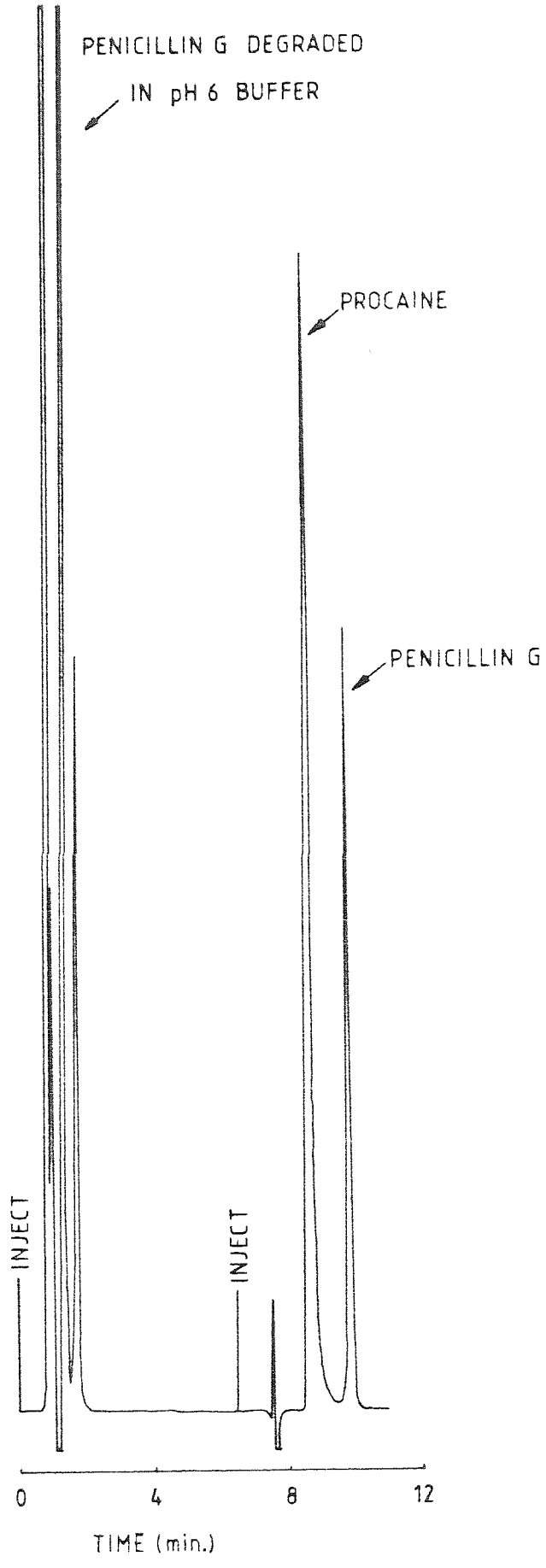


FIGURE 3.9 SEPARATION OF PROCAINE AND PENICILLIN G FROM DEGRADATION PRODUCTS OF PENICILLIN G

28.5% Acetonitrile

71.5% Aqueous component (4.32 g/l KH_2PO_4
(6.00 g/l 1-heptane sulphonic acid

Adjusted to an apparent pH of 3.17 with H_3PO_4

Penicillin G was degraded in pH 6 citrate buffer to produce its major degradation products. Figure 3.9 illustrates the separation of procaine, penicillin G and the major degradation products of penicillin G. The separation of procaine from its degradation products was not investigated.

Sample preparation

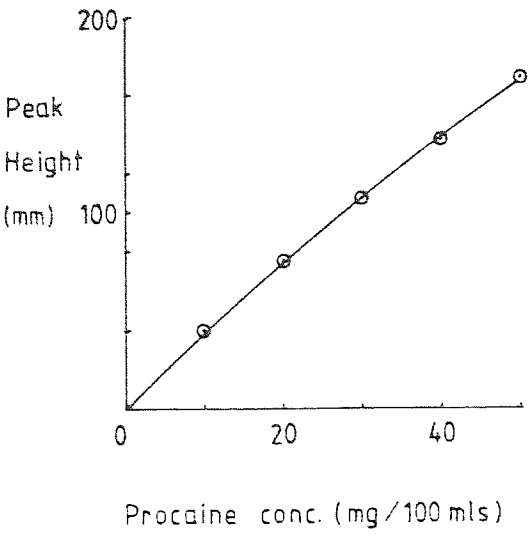
The dry powder for injection was reconstituted by adding 8 mls of water and shaking. One ml of this suspension was diluted to 250 ml with water. All of the suspended material dissolved. Formulation details of the injection were not known, thus it was not possible to prepare standards of procaine and penicillin G in identical solvents to the samples. However, dilution of the injection was high (1 to 250), thus the solvent composition was taken to approximate to water. Standards were therefore prepared in unbuffered water.

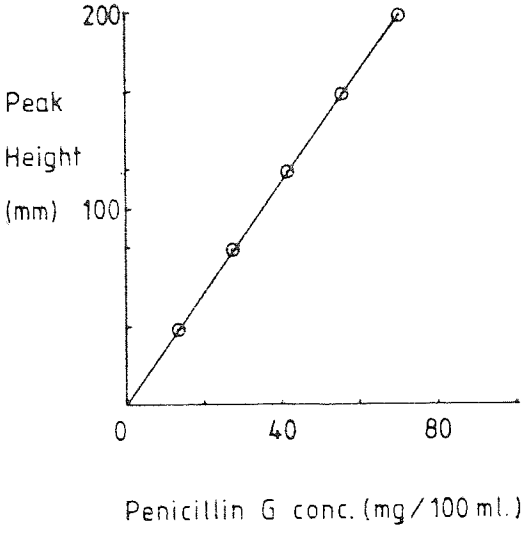
Linearity of response

One sample of procaine penicillin injection B.P., already diluted 1 to 250, was further diluted with water to produce samples of 80%, 60%, 40% and 20%. A standard containing 56.5 mg of procaine hydrochloride and 73.1 mg of penicillin G in 100 mls was similarly diluted. Figure 3.10 illustrates the linearity of response of these preparations.

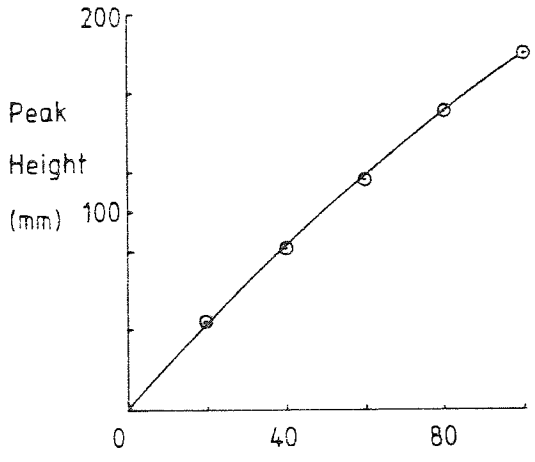
Penicillin G exhibits a linear peak height *vs* concentration relationship. Ion-paired procaine, in a similar way to benzathine, produced a curved line.

STANDARD PREPARATION





SAMPLE PREPARATION



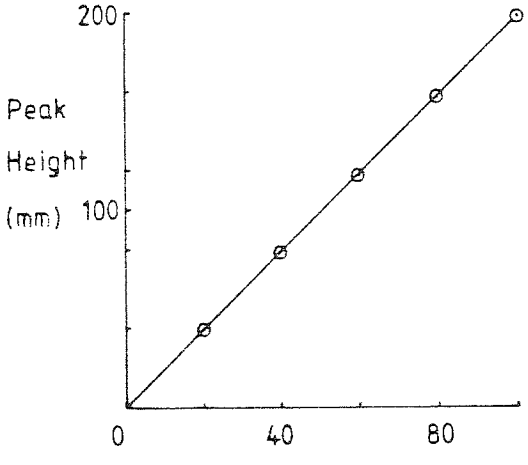


FIGURE 3.10 LINEARITY OF CALIBRATION CURVES FOR PROCAINE AND PENICILLIN G IN PROCAINE PENICILLIN INJECTION B.P.

Procaine penicillin injection B.P. assay results

The results from the assay of three ampoules of injection can be seen in table 3.2

Ampoule		Procaine Concentration (mg/ml)	Penicillin G Concentration (mg/ml)	Labelled Claim
A	1	132.5	174.8	300 mg/ml procaine penicillin (equivalent to 170.4 mg/ml penicillin G ; 120.4 mg/ml procaine)
	2	132.5	175.8	
B	1	136.8	180.0	
	2	135.5	179.3	
C		131.3	174.8	

Two samples were analysed from ampoule A and B.

Table 3.2 Procaine and Penicillin G Content of Procaine Penicillin Injection B.P.

The standard for procaine penicillin injection B.P. (131) requires the total penicillin content to be 90% to 110% of the labelled claim (153.4 mg/ml to 187.4 mg/ml). All assay values were within this range. The procaine content should be 36.0% to 44.0% of the labelled claim (108 mg/ml to 132 mg/ml). Four of the H.P.L.C. assay values slightly exceeded this range.

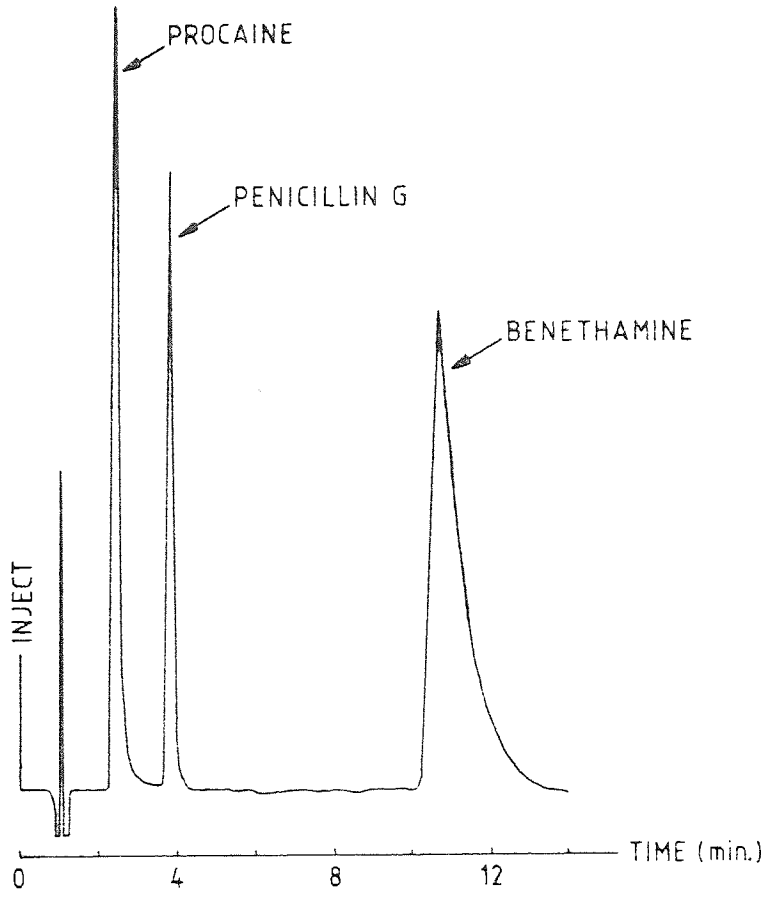


FIGURE 3.11 SEPARATION OF PROCAINE, PENICILLIN G AND BENETHAMINE

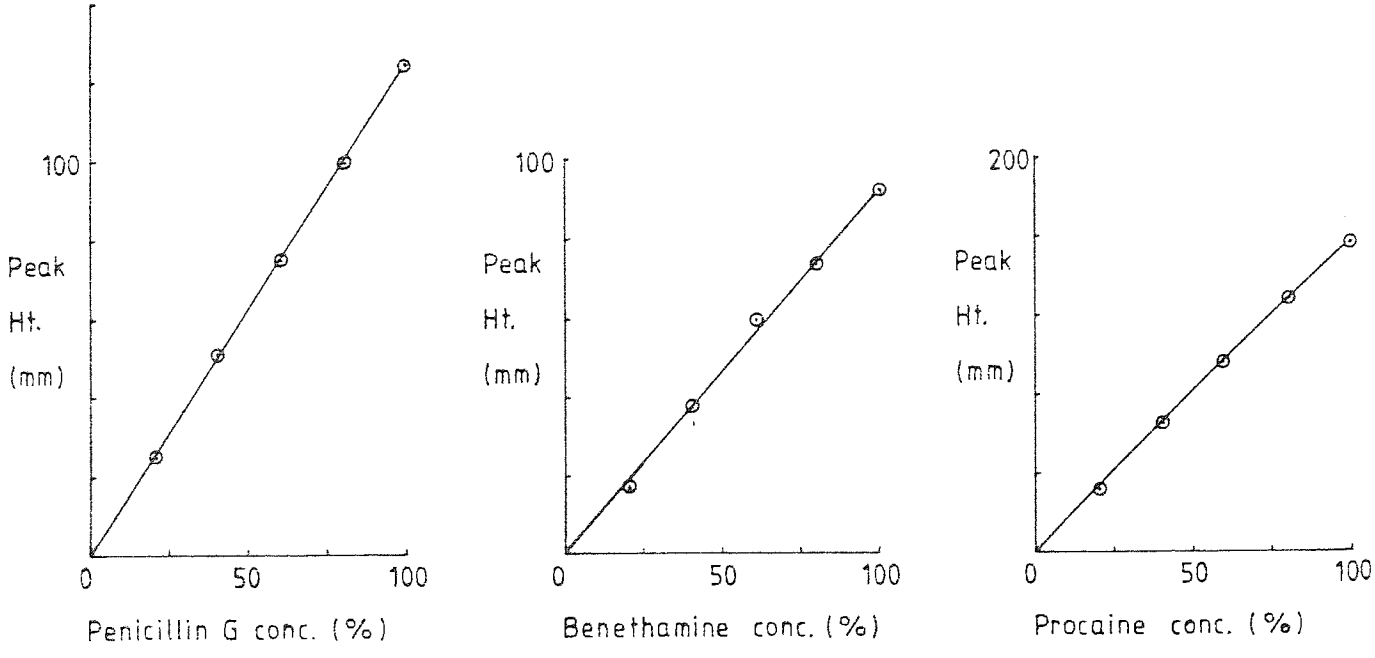


FIGURE 3.12 LINEARITY OF CALIBRATION CURVES FOR PENICILLIN G; BENETHAMINE AND PROCAINE IN BENETHAMINE PENICILLIN INJECTION, FORTIFIED B.P.C.

3.1.5.4 Benethamine Penicillin Injection, Fortified B.P.C.

Slight modification of the mobile phase used for procaine penicillin injection B.P. provided a suitable system for this preparation:

28.5% Acetonitrile

71.5% Aqueous component (4.54 g/l KH_2PO_4
(6.30 g/l 1-heptane sulphonic acid

Adjusted to an apparent pH of 3.17 with H_3PO_4

Figure 3.11 illustrates the separation of benethamine, penicillin G and procaine using this system. The chromatography of the degradation products of benethamine and procaine were not investigated. Benethamine chromatographed as a very broad peak at a retention time of 11-15 minutes. No method of improving the peak shape of benethamine was found.

Sample preparation

The contents of one ampoule were reconstituted with 1.3 mls of water. The resulting suspension was dissolved in 100 mls of methanol and then diluted to 500 mls with water. Standard preparations were prepared by dissolving procaine hydrochloride and penicillin G sodium in 20% methanol in water. The standard did not contain benethamine because no pure benethamine salt was available, making quantification of benethamine difficult. However, identification of benethamine in the chromatogram was carried out using a sample of benethamine diacetate of unknown purity.

Linearity of response

A linear response between concentration and response was found for benethamine and penicillin G. Procaine produced a slightly curved response (figure 3.12).

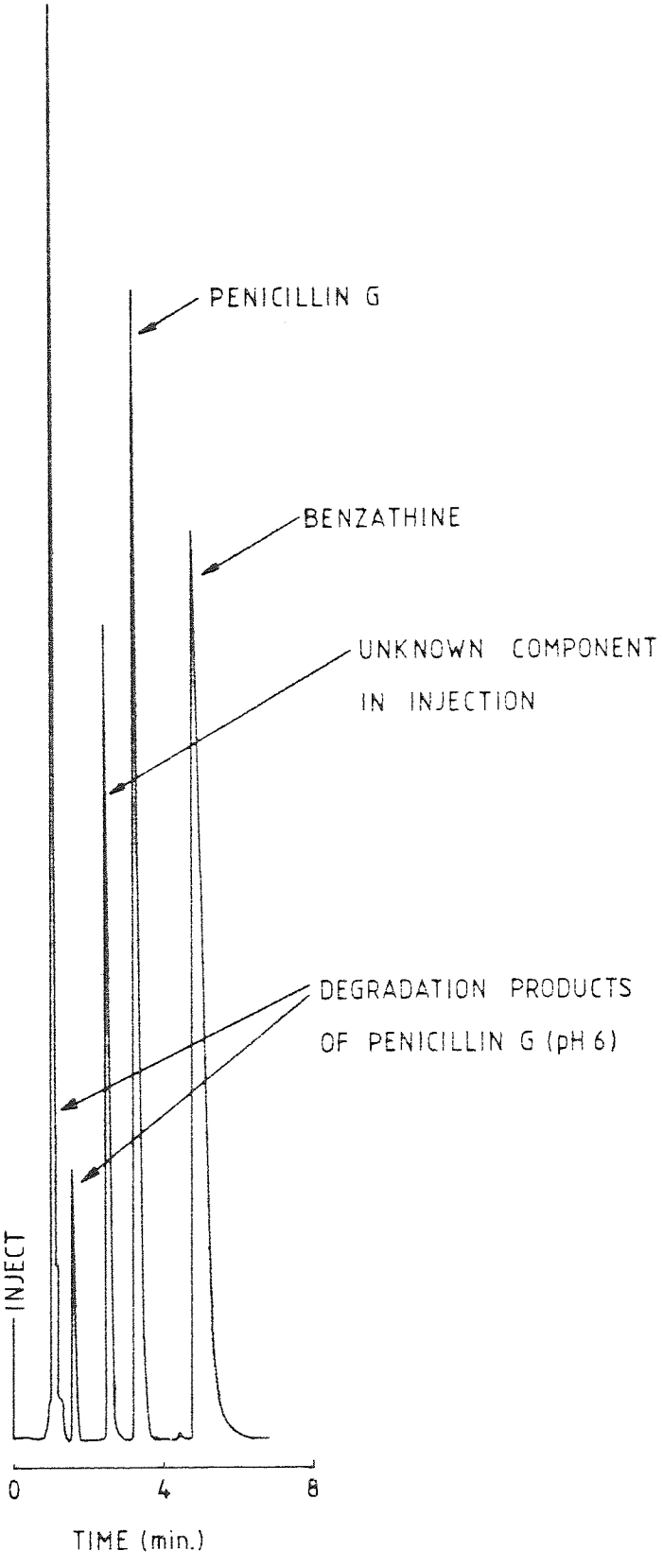


FIGURE 3.13 SEPARATION OF PENICILLIN G AND BENZATHINE FROM DEGRADATION PRODUCTS OF PENICILLIN G

Benethamine penicillin injection, fortified B.P.C. assay results

Ampoule	Procaine Concent. (mg/amp.)	Penicillin G Concent. (mg/amp.)	Benethamine Concent. (mg/amp.)	Labelled Claim (mg/ampoule) *
A	114.0	790.0	Not quantified	475 mg beneth. penicillin BPC 250 mg proc. penicillin BP 300 mg pen. G sodium
B	110.0	780.0		

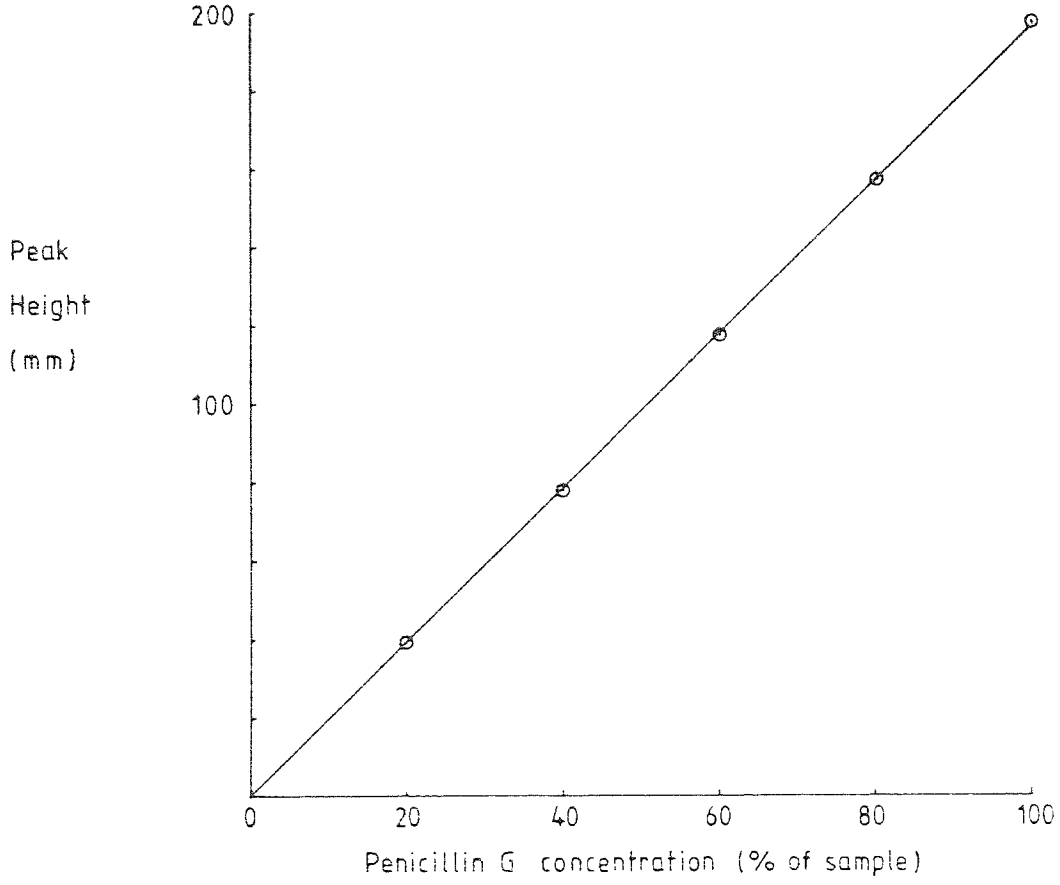
* equivalent to 736 mg penicillin G sodium and 108 mg procaine

Table 3.3 Procaine and Penicillin G Content of Benethamine Penicillin Injection, Fortified B.P.C.

The B.P.C. standard for benethamine penicillin injection, fortified B.P.C. requires the penicillin G content to be 95.0% to 130.0% and the procaine penicillin content 95.0% to 125.0% of labelled claim. This standard is met by the assay results in table 3.3.

3.1.5.5. Benzathine Penicillin Injection

The mobile phase developed for procaine penicillin B.P. was suitable for this preparation. Figure 3.13 illustrates the separation between benzathine, penicillin G and the degradation products of penicillin G



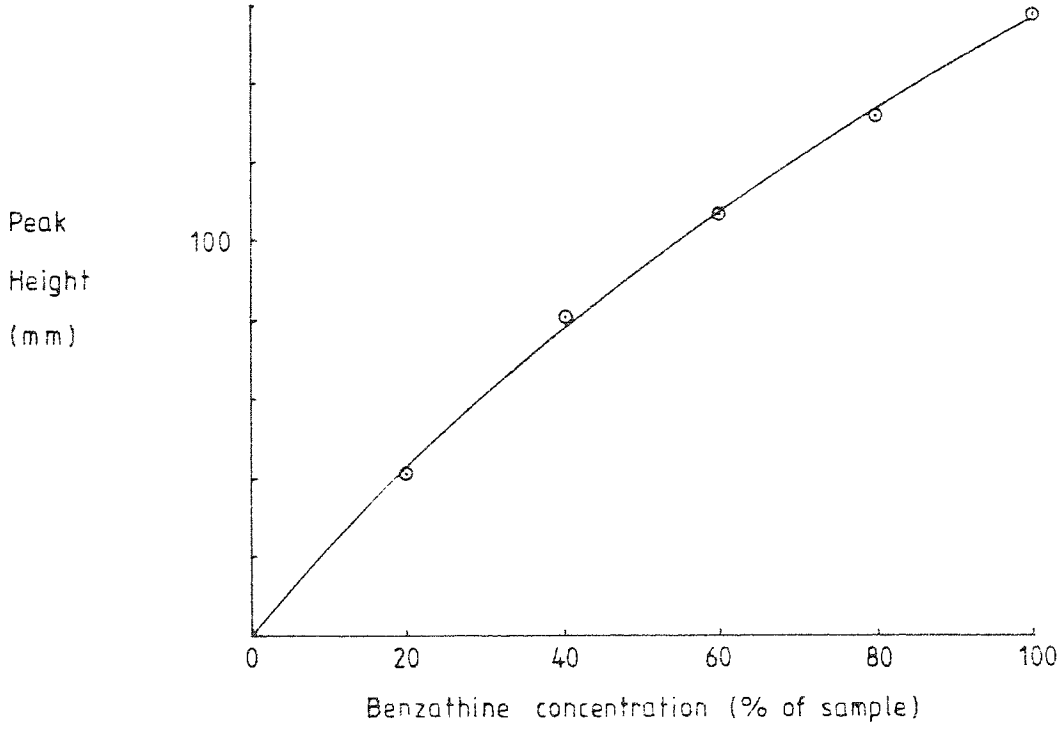


FIGURE 3.14 LINEARITY OF CALIBRATION CURVES FOR PENICILLIN G AND BENZATHINE IN BENZATHINE PENICILLIN INJECTION

(pH 6 buffer).

Sample preparation

Due to its low solubility in common solvents, benzathine penicillin was dissolved in formamide before dilution with water. One ml of injection was dissolved in 50 mls of formamide and diluted to 250 mls with water. A standard was prepared by dissolving benzathine diacetate and penicillin G sodium in 20% formamide in water.

Linearity of response

Penicillin G produced a straight line relationship between concentration and response, but benzathine gave a curved calibration line (figure 3.14).

Benzathine penicillin injection assay results

Ampoule		Benzathine Concentration (mg/ml)	Penicillin G Concentration (mg/ml)	Labelled Claim *
A	1	63.5	173.5	229 mg benzathine penicillin G per ml
	2	63.5	173.8	
B		60.8	168.8	

* equivalent to 173.6 mg/ml penicillin G and 60.5 mg/ml benzathine.

Table 3.4 Benzathine and Penicillin G Content of Benzathine Penicillin Injection

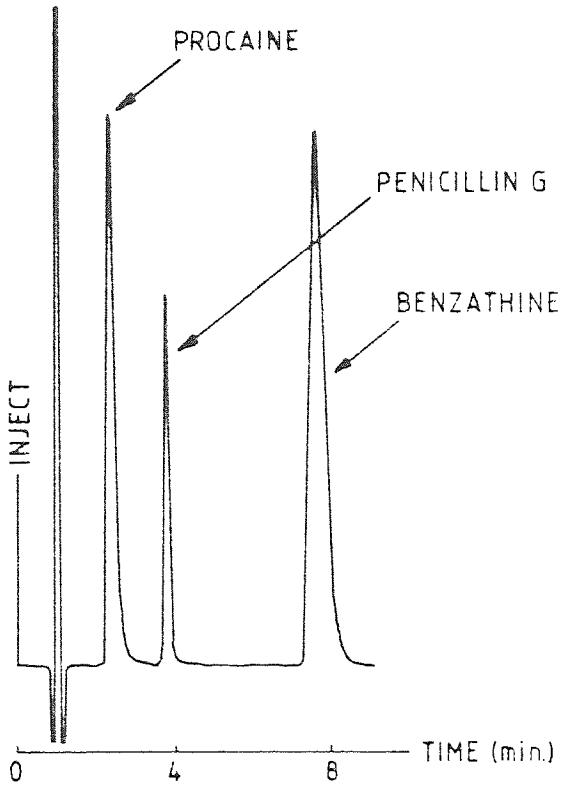


FIGURE 3.15 SEPARATION OF PROCAINE, PENICILLIN G AND BENZATHINE

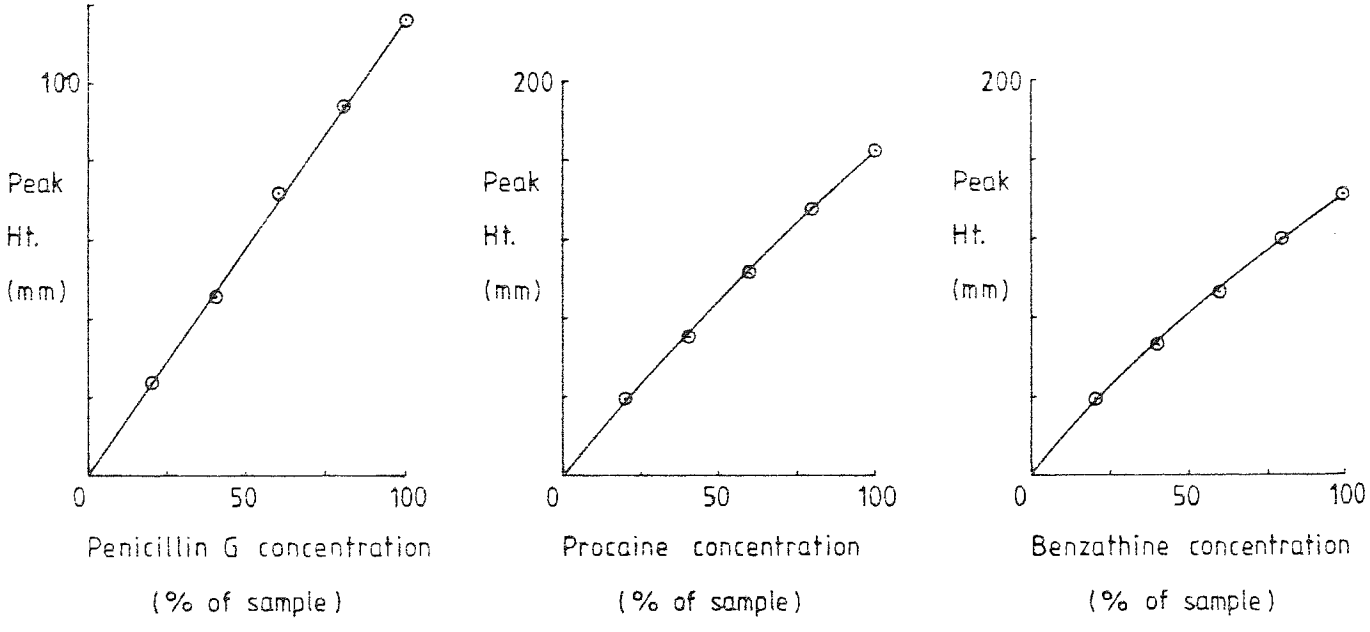


FIGURE 3.16 LINEARITY OF CALIBRATION CURVES FOR PENICILLIN G, PROCAINE AND BENZATHINE IN BENZATHINE PENICILLIN INJECTION, FORTIFIED B.P.C.

There was no standard for this injection in the B.P. or B.P.C. The measured concentration of benzathine and penicillin G is within 10% of the labelled claim.

3.1.5.6. Benzathine Penicillin Injection, Fortified B.P.C.

The mobile phase developed for benethamine penicillin, fortified B.P.C. was suitable for this preparation. Figure 3.15 illustrates the separation of benzathine, procaine and penicillin G.

Sample preparation

The contents of one ampoule were reconstituted with 1.5 mls of water, dissolved in 100 mls of formamide and diluted with water to 500 ml.

Linearity of response

Penicillin G exhibited linearity of response, but benzathine and procaine produced slightly curved calibration lines (figure 3.16).

Benzathine penicillin injection, fortified B.P.C. assay results

Ampoule		Benzathine Concent. (mg/amp.)	Procaine Concent. (mg/amp.)	Penicillin G Concent. (mg/amp.)	Labelled Claim (mg/ampoule) *
A	1	127.5	126.0	745.0	458mg benz.pen G 300 mg proc. pen. 190 mg pen. G pot.
	2	124.8	123.7	751.5	

* equivalent to 677.8 mg penicillin G, 121.0 mg benzathine and 120.4 mg procaine per ampoule

Table 3.5 Benzathine, Procaine and Penicillin G Content of Benzathine Penicillin Injection, Fortified B.P.C.

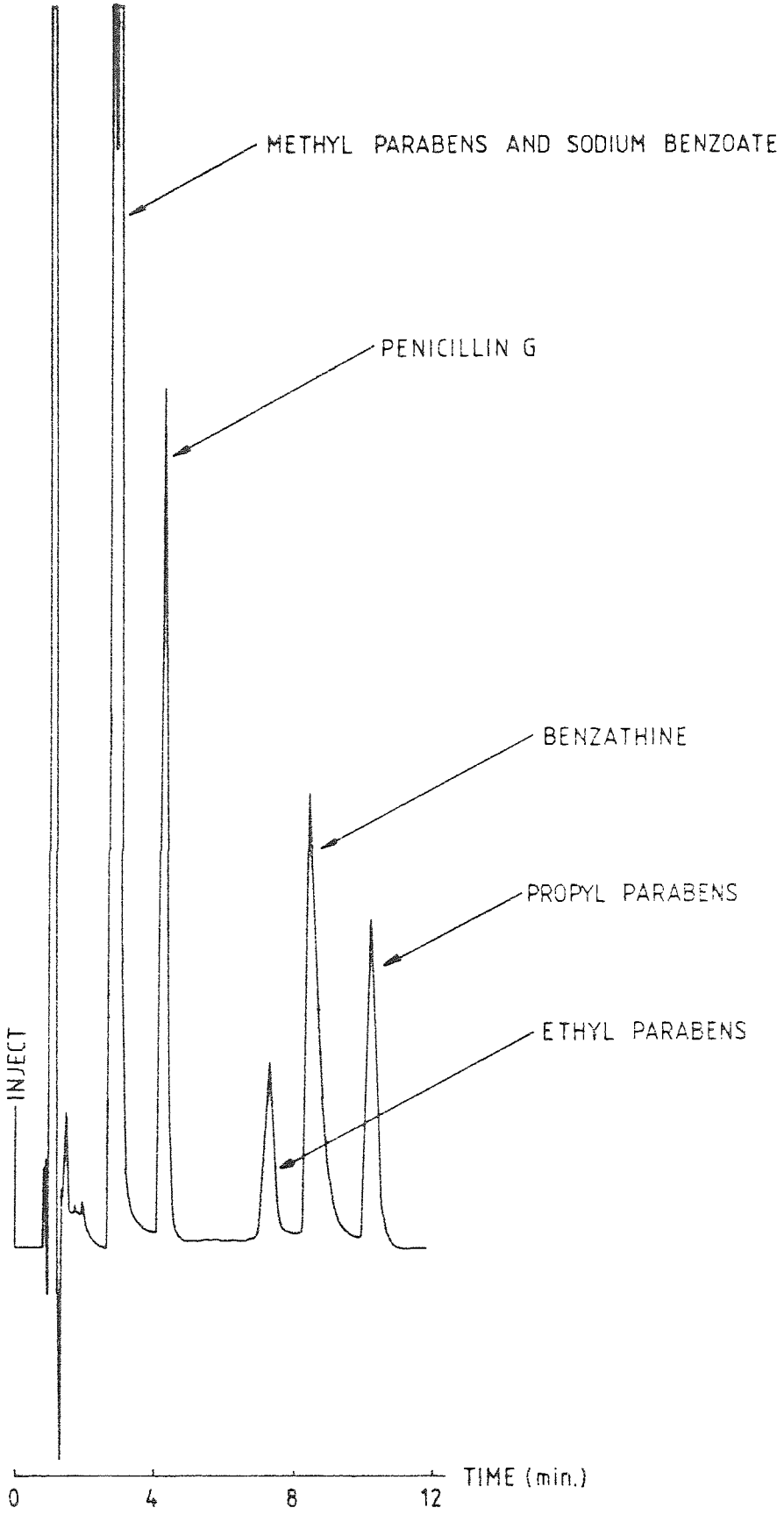


FIGURE 3.17 SEPARATION OF PENICILLIN G AND BENZATHINE FROM EXCIPIENTS IN BENZATHINE PENICILLIN SUSPENSION

The B.P.C. standard for this preparation is :

Content of penicillin G potassium : 95.0 to 130.0% labelled claim

Content of procaine penicillin : 95.0 to 125.0% labelled claim

Content of benzathine penicillin : 95.0 to 125.0% labelled claim

All of these requirements are met by the assay results in table 3.5.

3.1.5.7 Benzathine Penicillin Suspension

The mobile phase producing the optimum separation for this multi-component preparation had the same composition as the mobile phase for benethamine penicillin injection, fortified B.P.C. Separation of benzathine, penicillin G and several excipients can be seen in figure 3.17.

Sample preparation

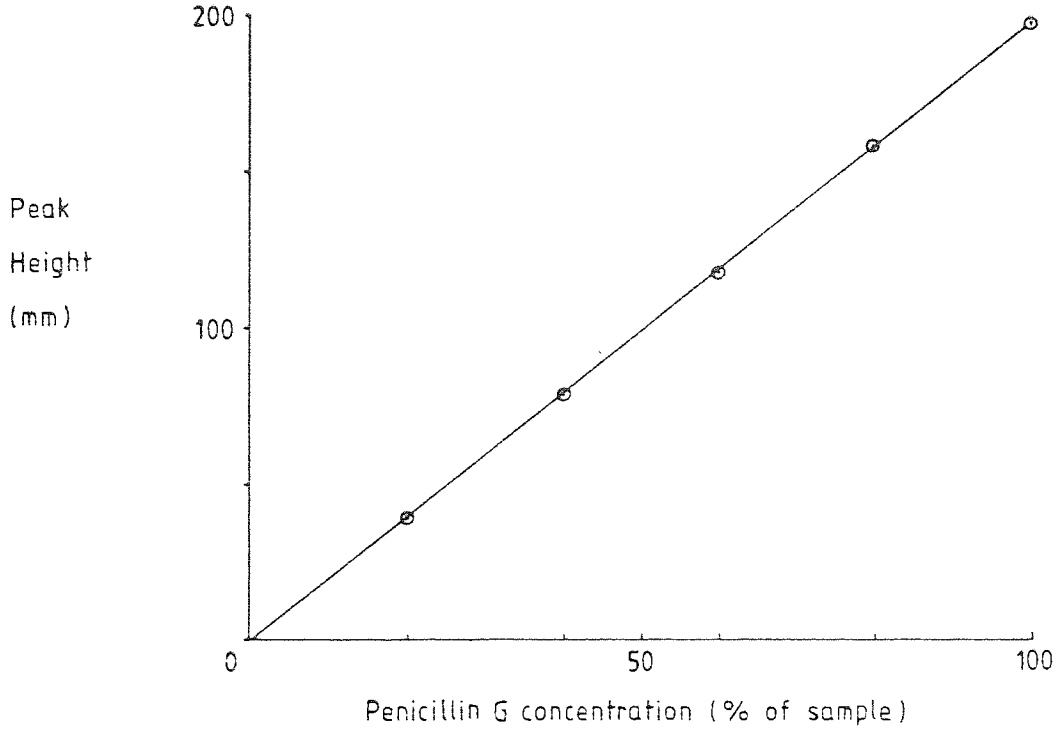
Two mls of suspension were dissolved in 20 mls of formamide and diluted to 100 mls with water. A standard was prepared in the same solvent (20% formamide in water).

Linearity of response

Penicillin G gave a linear response, but benzathine produced a slightly curved relationship (figure 3.18).

Benzathine penicillin suspension assay results

The assay values were within 10% of the labelled claim (table 3.6)



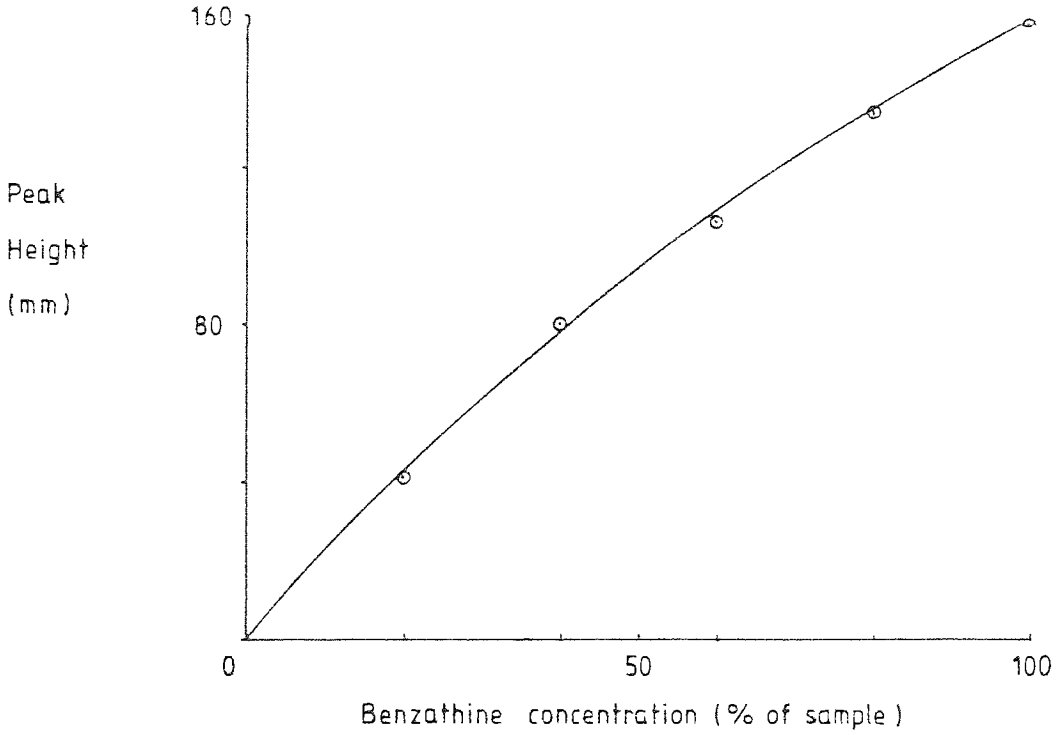


FIGURE 3.18 LINEARITY OF CALIBRATION CURVES FOR PENICILLIN G AND BENZATHINE IN BENZATHINE PENICILLIN SUSPENSION

Sample	Benzathine Concentration (mg/5ml)	Penicillin G Concentration (mg/5ml)	Labelled Claim (mg/5ml)
1	63.0	183.3	229 mg benzathine penicillin B.P. *
2	62.3	177.7	

* equivalent to 60.5 mg benzathine and 168.4 mg penicillin G

Table 3.6 Benzathine and Penicillin G Content of Benzathine Penicillin Suspension

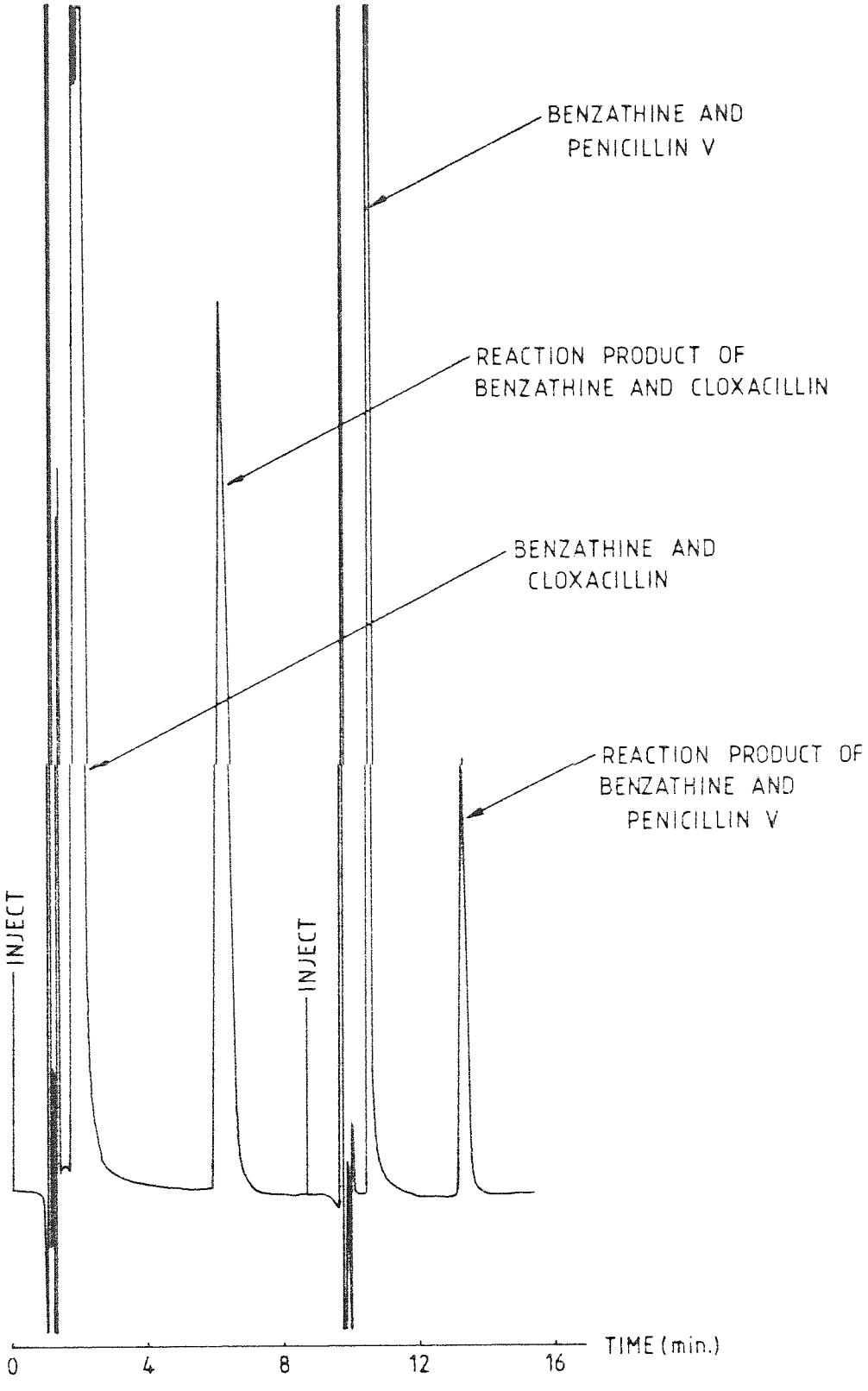


FIGURE 3.19 CHROMATOGRAPHY OF REACTION PRODUCTS OF BENZATHINE AND PENICILLIN V; AND BENZATHINE AND CLOXACILLIN

3.2 THE H.P.L.C. ANALYSIS OF THE REACTION PRODUCTS FROM BENZATHINE AND CLOXACILLIN AT pH 9; AND BENZATHINE AND PENICILLIN V AT pH 9

The experimental details were as in section 3.1

The reaction products were known to have long retention times when chromatographed with mobile phases developed in section 3.1 (see chapter 7). A suitable modification to the system used for the analysis of benzathine cloxacillin in section 3.1 yielded a mobile phase with the composition :

50% Acetonitrile

50% Aqueous component (4.54 g/l KH_2PO_4
(6.30 g/l 1-heptane sulphonic acid

Adjusted to an apparent pH of 3.55 with H_3PO_4

A simpler mobile phase may have been successful in separating the components, but benzathine was present in the injected samples, thus the retention of of an ion-pairing agent (1-heptane sulphonic acid) ensured that benzathine eluted as a sharp peak. Also, the nature of the complexation products was unknown and they may have required ion-pairing themselves. Figure 3.19 illustrates the separation of the various components.

Linearity of response of the primary complexation product between benzathine and penicillin V

All the samples analysed using this system were in pH 9 borate buffer diluted 1 to 11 with water. In order to see if an increase in peak height was associated with a corresponding increase in concentration, a sample from the complexation of benzathine and penicillin V was diluted 1 to 5, 2 to 5, 3 to 5 and 4 to 5 with diluted pH 9 buffer (100

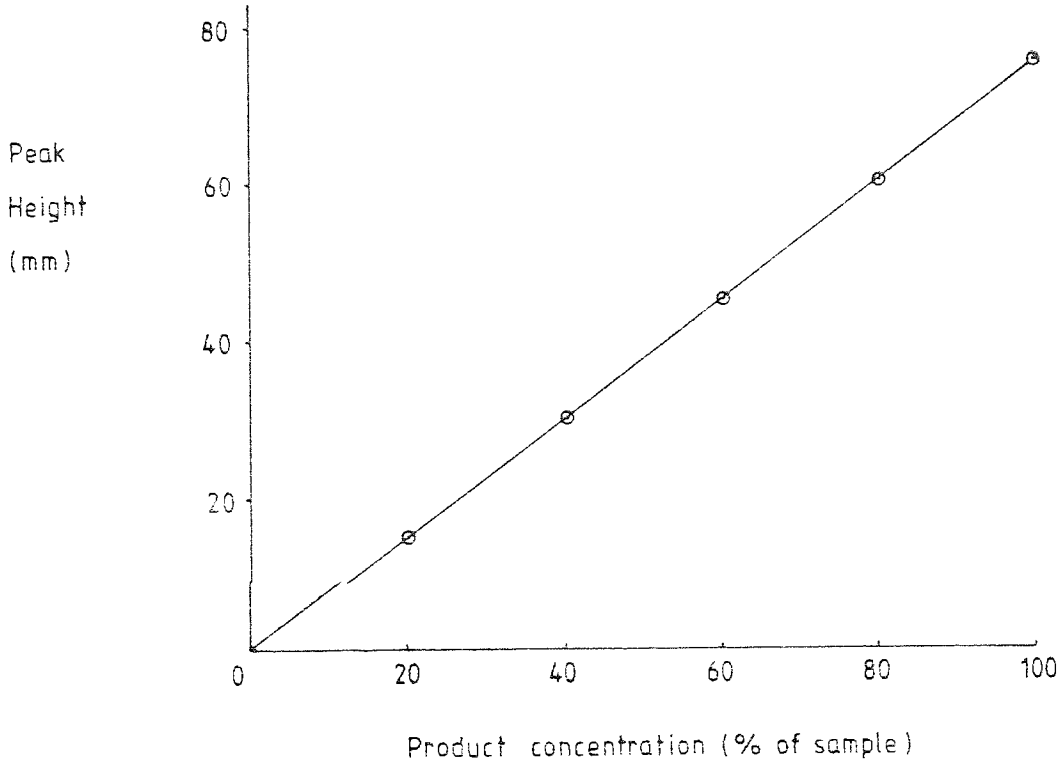


FIGURE 3.20 LINEARITY OF CALIBRATION CURVE FOR THE REACTION PRODUCT OF BENZATHINE AND PENICILLIN V

mls of buffer diluted to 1100 mls with water). Linearity of response was found (figure 3.20).

The linearity of response vs concentration for the primary reaction product between benzathine and cloxacillin was not investigated because this product precipitated from solution.

3.3 THE H.P.L.C. ANALYSIS OF PENICILLIN V AND AMPICILLIN SYRUPS

The official methods of assay for these preparations are given in the B.P.C. 1973 (36). Ampicillin in ampicillin syrups (or mixtures) is determined by the hydroxylamine method and total penicillins in penicillin V syrups by iodimetry (see chapter 2 for details of the methods). Neither assay is specific to the penicillin being assayed. Alternative quantitative analytical methods for ampicillin have been discussed in chapter 2 and alternative methods for penicillin V at the beginning of this chapter.

Specific methods of assay were developed, based on the H.P.L.C. methods for ampicillin mixtures and penicillin syrups reported by Tsugi (122).

3.3.1 EXPERIMENTAL

3.3.1.1 EQUIPMENT

As in section 3.1.4.1

3.3.1.2 MATERIALS

As in sections 2.2.1 and 3.1.4.2.

3.3.1.3 METHODS

H.P.L.C. separations were carried out at ambient temperature. The mobile phase flow rate was 1.0 mls per minute. Detector wavelengths were:

258 nm for ampicillin and 271 nm for penicillin V.

Internal standards for the samples were, unless otherwise stated :

Ampicillin - caffeine citrate (0.1 mg per ml)

Penicillin V - phenol (0.1 mg per ml)

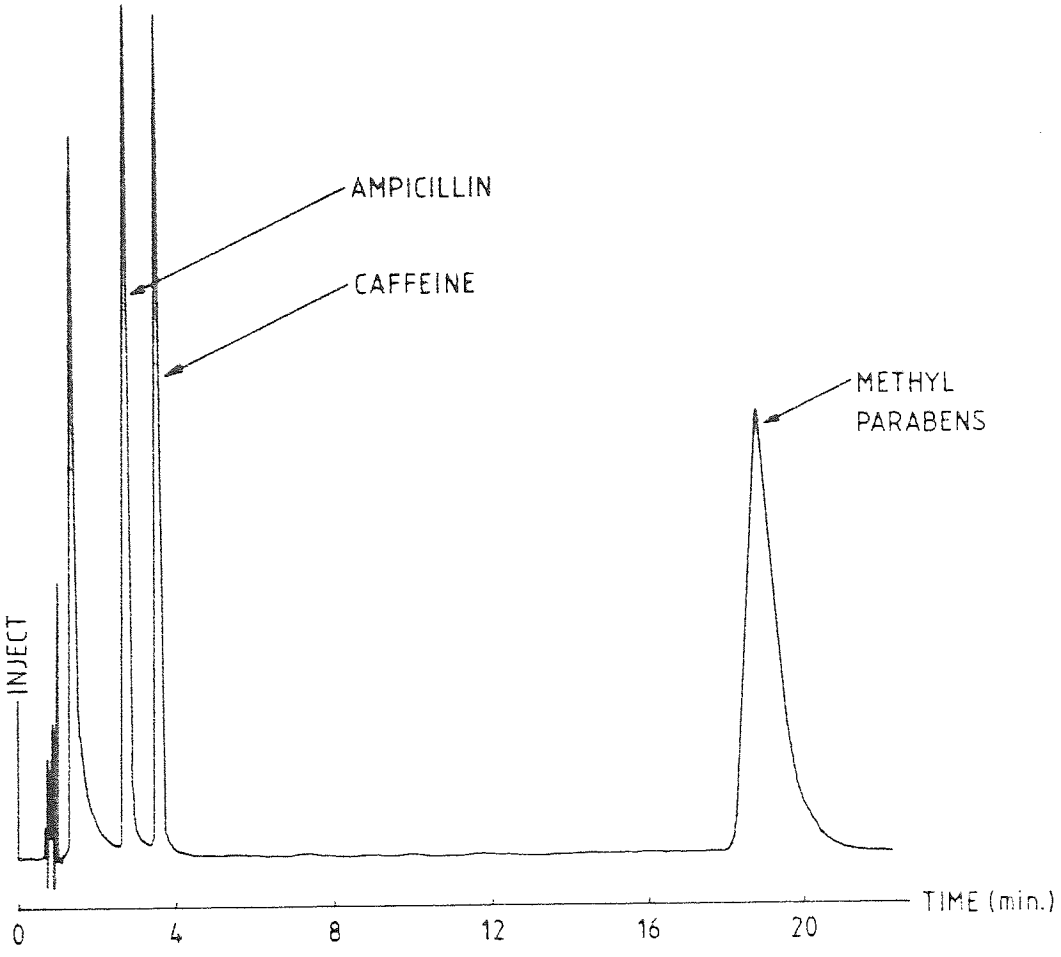


FIGURE 3.21 SEPARATION OF AMPICILLIN AND CAFFEINE FROM EXCIPIENTS IN AMPICILLIN SYRUP

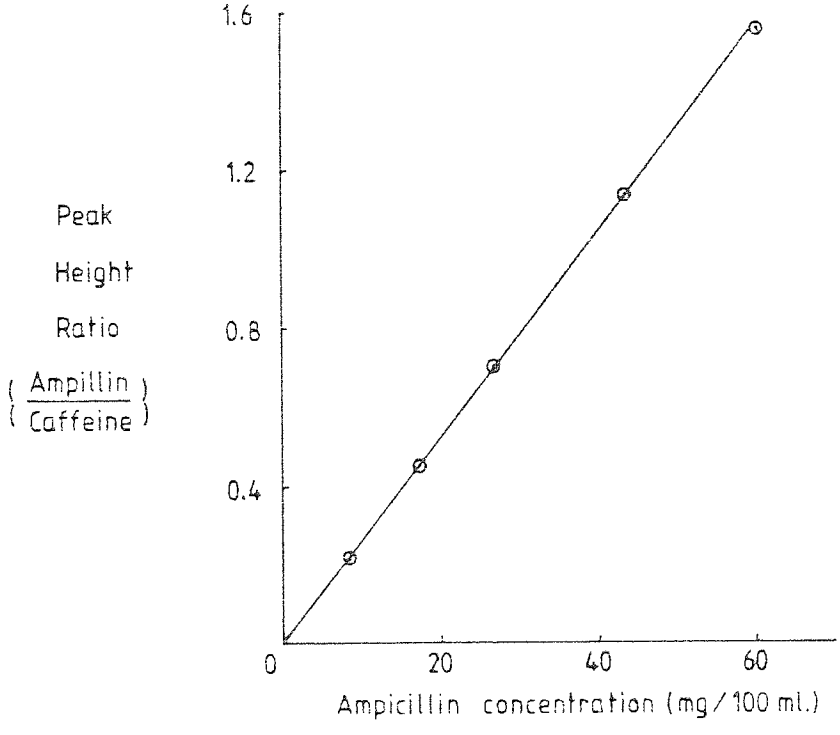


FIGURE 3.22 LINEARITY OF CALIBRATION CURVE FOR AMPICILLIN IN AMPICILLIN SYRUP

3.3.2 Ampicillin in Ampicillin Syrups (Mixtures)

A suitable mobile phase for the separation of the various components in the mixtures had the composition:

10% Acetonitrile in pH 6 buffer (1.32 g $Na_2HPO_4.2H_2O$ + 8.07 g KH_2PO_4
(made up to 1000 mls with water.

A typical H.P.L.C. separation can be seen in figure 3.21

Linearity of response

Ampicillin gave a linear peak height *vs* concentration response over the concentration range 10 to 60 mg per 100 mls ampicillin, free acid (figure 3.22).

Recovery of ampicillin from ampicillin mixtures

Ampicillin recovery from ampicillin mixtures was approximately 100% for the concentration range 24 to 50 mg per 100 mls ampicillin, as free acid (table 3.7).

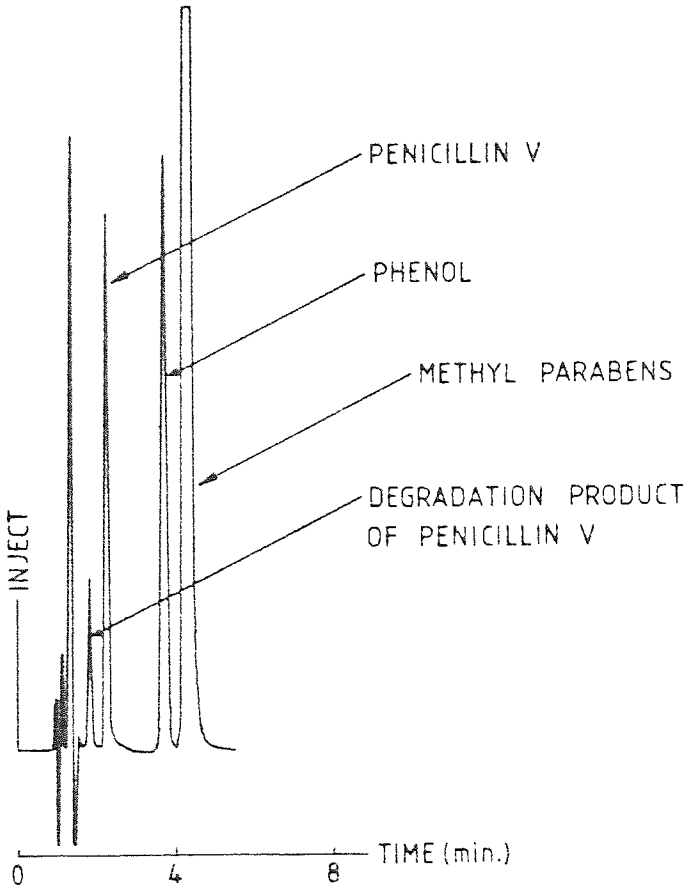


FIGURE 3.23 SEPARATION OF PENICILLIN V AND PHENOL FROM EXCIPIENTS AND DEGRADATION PRODUCTS OF PENICILLIN V SYRUP

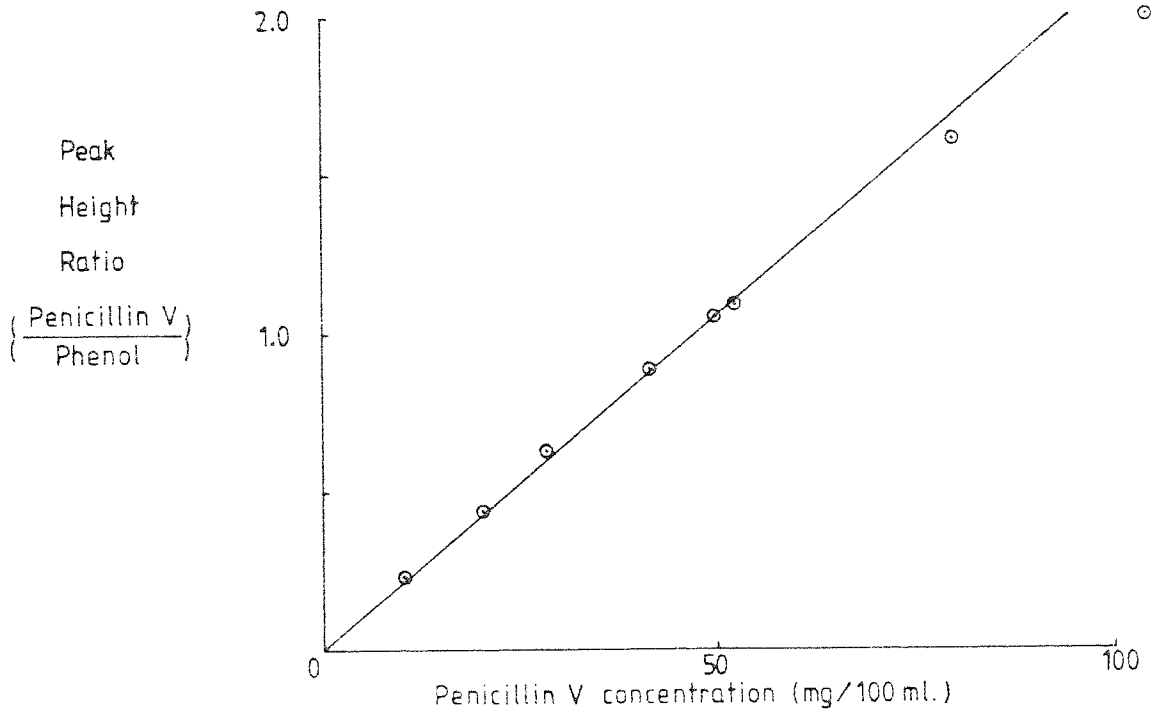


FIGURE 3.24 LINEARITY OF CALIBRATION CURVE FOR PENICILLIN V IN PENICILLIN V SYRUP

Sample	Ampicillin Conc. (mg per 100 mls free acid)	Recovery (%)
Ampicillin mixture	24.48	—
Ampicillin mixture (100 mls) + 8.32 mg Ampicillin (free acid)	32.79	99.9
Ampicillin mixture (100 mls) + 16.63 mg Ampicillin (free acid)	41.48	102.2
Ampicillin mixture (100 mls) + 24.95 mg Ampicillin (free acid)	49.24	99.2

Table 3.7 Recovery of Ampicillin from Ampicillin Syrup

3.3.3 Penicillin V in Penicillin V Syrups

A mobile phase providing suitable separation of the components of the syrups had the composition :

28% Acetonitrile in pH 6 phosphate buffer (buffer - as in 3.3.1.4)

A typical separation can be seen in figure 3.23.

Linearity of response

Penicillin V gave a linear response for the concentration range 10 to 50 mg per 100 mls penicillin V, as free acid (figure 3.24).

Recovery of penicillin V from penicillin V syrup

Penicillin V recovery from penicillin V syrup was approximately 97% for the concentration range 30 to 55 mg per 100 mls penicillin V ,as free acid (table 3.8).

Sample	Penicillin V Conc. (mg per 100 mls free acid)	Recovery (%)
Penicillin V syrup	30.57	—
Penicillin V syrup (100 mls) + 8.65 mg Pen. V (free acid)	39.26	100.5
Penicillin V syrup (100 mls) + 17.30 mg Pen. V (free acid)	47.04	95.2
Penicillin V syrup (100 mls) + 25.95 mg Pen. V (free acid)	55.16	94.8

Table 3.8 Recovery of Penicillin V from Penicillin V Syrup

CHAPTER FOUR : MECHANICAL DAMAGE IN REVERSED-PHASE H.P.L.C. COLUMNS - ITS CAUSE AND REPAIR

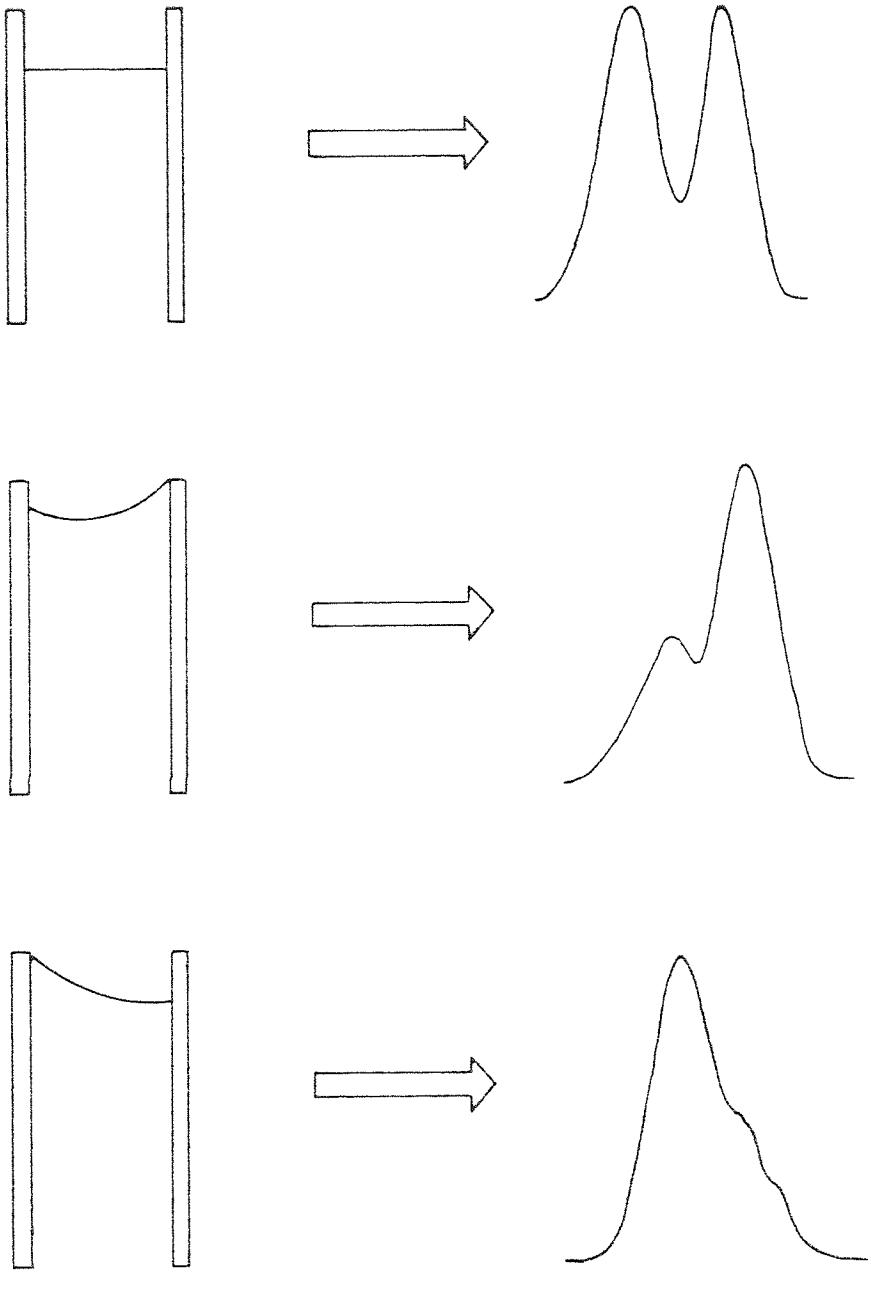


FIGURE 4.1 EFFECT ON CHROMATOGRAPHY OF VOIDS IN THE COLUMN PACKING CAUSED BY SILICA DISSOLUTION

4.1 INTRODUCTION

Increasing peak asymmetry, accompanied by loss of column efficiency, was noticed after several weeks of routine analysis using "Swagelok" design H.P.L.C. columns containing reversed-phase packing material. A change to "Shandon" design columns led to an increase in this problem and a need to understand its cause.

The care of microparticulate H.P.L.C. columns, and the role of pre-columns in this, has recently been discussed by Rabel (153, 154). He pointed out that mechanical damage to H.P.L.C. columns could occur by the use of mobile phases that dissolve the column support. This usually occurred at high pH values (> pH 7.5). Bonded-phase ion-exchange columns showed this effect to a greater extent than bonded reversed-phase columns, the dissolution rate of the silica backbone being dependent on the pH and ionic strength of the mobile phase. Dissolution of the silica caused sinking of the column bed, resulting in poor efficiency, peak shoulders and peak splitting (figure 4.1).

The most suitable way to overcome dissolution of silica was to saturate the mobile phase with silica, prior to analysis, by incorporating a guard column in the apparatus.

Rabel also noted the intense linear velocity of the mobile phase as it entered the column from a small bore capillary inlet tube, suggesting that mechanical damage, as in figure 4.1, could be caused by a jet of mobile phase. Other causes of split peaks were given as partially clogged injectors, inlet tubing and inlet frits.

Williams *et. al.* (152) discussed the importance of sample solvent composition to efficiency and peak symmetry. Tseng and Rogers (155) found that a single component eluted as a double or split peak when methanol was used as the sample solvent with a mobile phase consisting of water. They concluded that the difference in polarity between

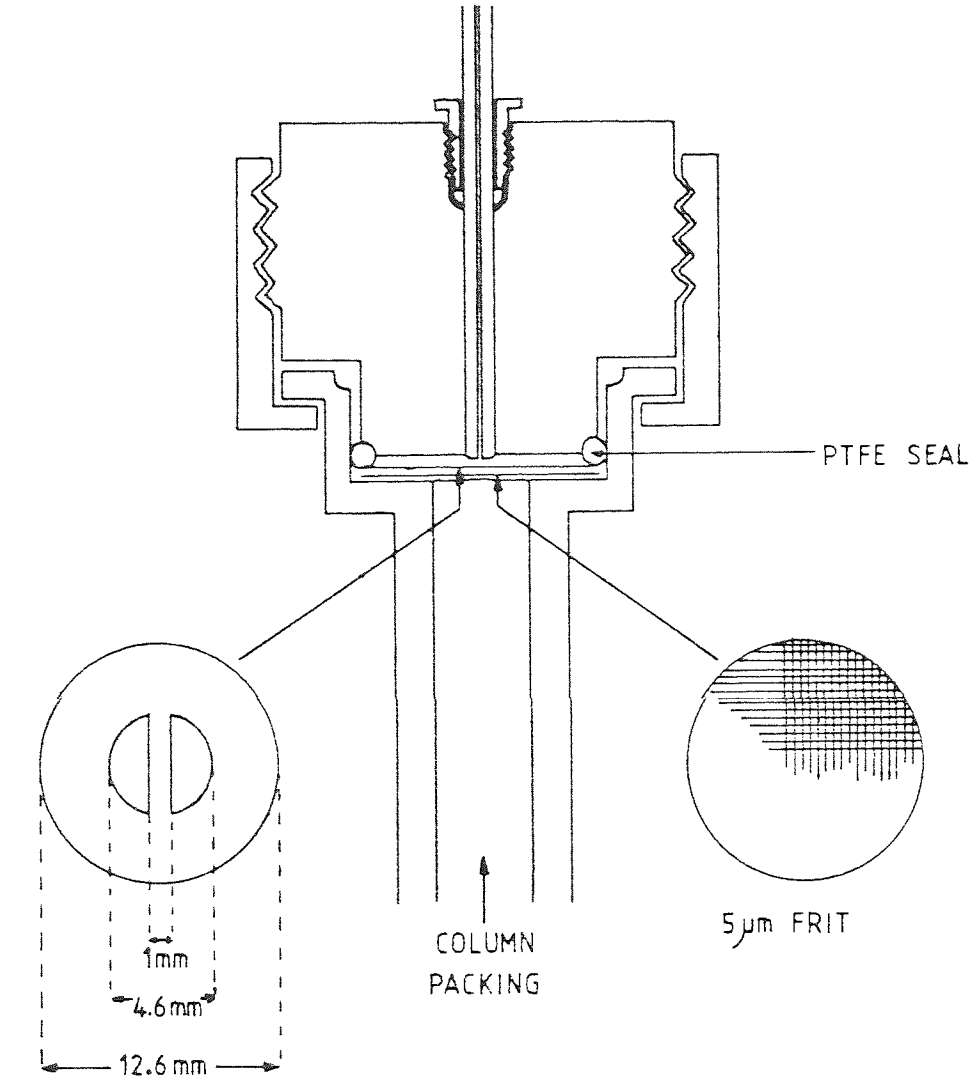


FIGURE 4.2 "SHANDON" COLUMN INLET DESIGN

methanol and water was the cause of this effect.

The cause of the observed loss in efficiency and increase in peak assymmetry, with reversed-phase "Swagelok" and "Shandon" columns, was thus taken to be due to one, or more, of the following effects :

(i) sample solvent effect

(ii) partitioning on the column (the column absorbs material that has a different partitioning characteristic to the stationary phase)

(iii) dissolution of the column packing, causing :

a) voids or channels in the column

b) a depression in the column inlet bed

(iv) mechanical damage by the jet of mobile phase from the inlet tubing, causing :

a) depression of the column inlet bed

b) other damage

(v) partially blocked injector

(vi) partially blocked inlet tubing

(vii) partially blocked inlet frit

(i) was discounted because loss of column efficiency occurred whilst the same samples were repeatedly injected.

(iii) b) and (iv) a) were discounted because no depression was observed in the column inlet bed when the column was carefully dismantled.

The remaining possible causes were investigated.

4.2 EXPERIMENTAL

4.2.1 EQUIPMENT

Pump : Altex 110 single piston pump with no pulse damper

U.V. detector : Unicam L.C.3

Recorder : JJ dual pen recorder

Column : Shandon 10 cm x 4.6 mm stainless steel column, packed with 5 micrometre ODS-hypersil

Injector : Rheodyne 7120 with a 20 microliter loop

Column Packer : Shandon

Peak Integrator : Infotronics CRS 304

4.2.2 MATERIALS

Penicillin V potassium was supplied by Beecham Pharmaceuticals Research Division, Worthing. Acetonitrile was H.P.L.C. grade. SurfaSil was purchased from Pierce Chemical Company, Rockford, Illinois, U.S.A. All other materials were standard laboratory reagents. Water was double distilled from glass.

4.2.3 METHODS

Sample

The sample chosen for this study consisted of penicillin V (0.5 mg per ml, in water) and phenol (0.1 mg per ml, in water). A fresh sample was prepared daily.

H.P.L.C. system

The mobile phase used in this study had the composition :

28% Acetonitrile in pH 6 buffer (1.32 g $Na_2PO_4.2H_2O$ + 8.07 g KH_2PO_4 made up to 1000 mls with water

The flow rate was 1.0 mls per minute and the detection wavelength 271 nm. The sensitivity of the detector was set at 0.32 a.u.f.s.

Method of column packing

1.7 g of ODS hypersil was suspended in 33 mls propan-2-ol, placed in an ultra-sonic bath for 5 minutes, poured into the packing reservoir and packed under a pressure of 7000 pounds per square inch (p.s.i.). The packing solvents used were hexane (100 mls), then propan-2-ol (100 mls). The first 140 mls was collected with the column inverted. After release of the packing pressure, the column was allowed to pressure equilibrate for at least 15 minutes before the equipment was dismantled.

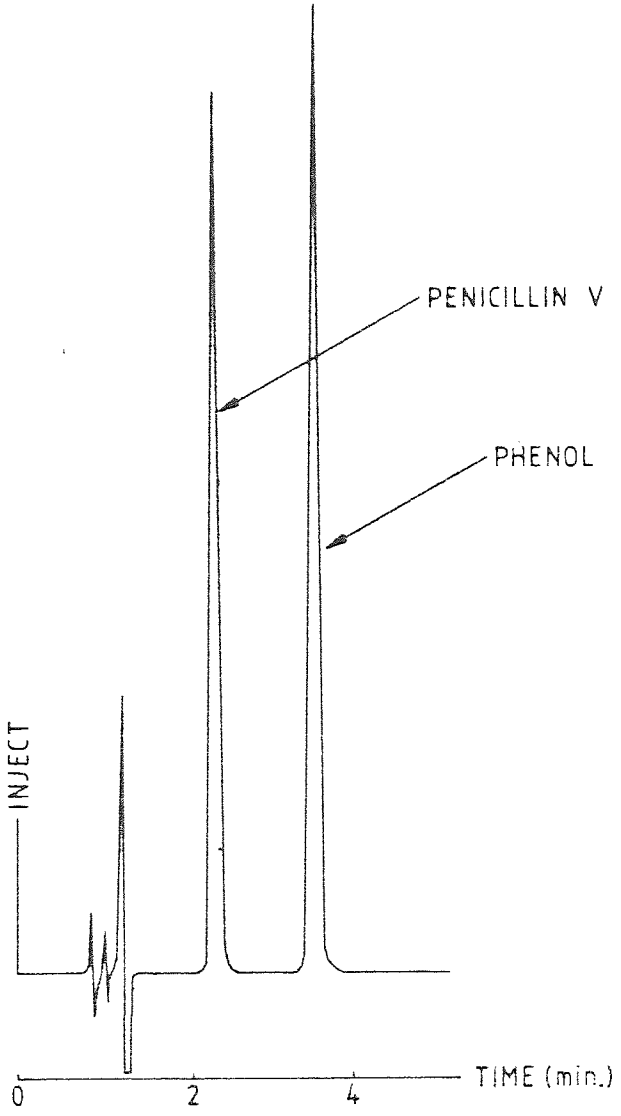


FIGURE 4.3 CHROMATOGRAM OF PENICILLIN V AND PHENOL - NEWLY PACKED COLUMN

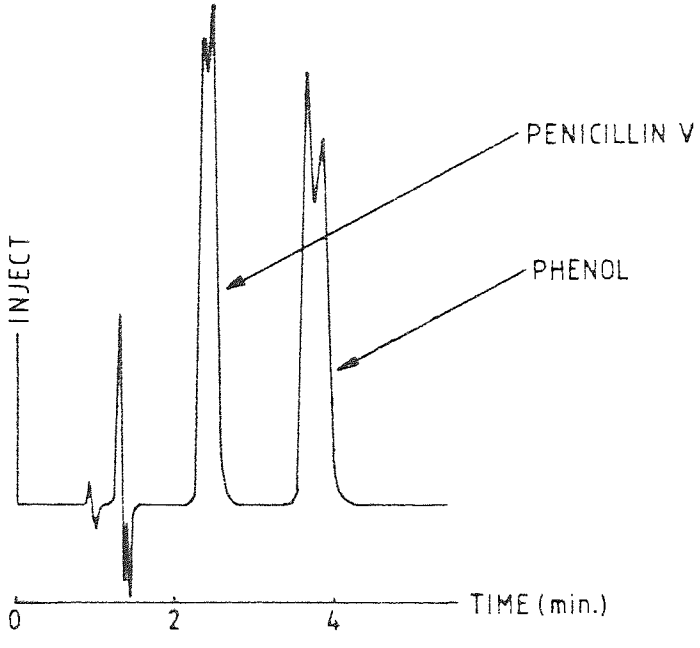


FIGURE 4.4 CHROMATOGRAM OF PENICILLIN V AND PHENOL - AFTER 2 HOURS SOLVENT PUMPING WITH A SINGLE PISTON PUMP

4.3 RESULTS AND DISCUSSION

Analysis of penicillin V and phenol, using a newly packed column, produced the chromatogram seen in figure 4.3. The peak heights of penicillin V and phenol were 114 mm and 124 mm, respectively, and their peak areas 98.4 and 111.4, respectively. After 2 hours of continual pumping, the peaks had reduced in height (62 mm and 55 mm) and split (figure 4.4). The peak areas remained consistent (100.4 and 113.4). The flow of mobile phase was stopped and the column carefully dismantled. (It was necessary to loosen the connection between the column head and the valve injector before dismantling the column. This was to prevent negative pressure being formed at the top of the column when dismantling). The flow distributor was rotated by 90 degrees, the column reassembled, the pump started and a sample injected (figure 4.5). Peak shape and peak height were restored (106 mm and 105 mm). Peak area remained constant (101.3 and 113.3).

This result discounted causes (v) and (vi) because the tubing and the injector had not been changed, yet column efficiency had recovered when the flow distributor was rotated by 90 degrees.

This experiment was repeated, peak splitting becomming apparent after 1 hour. The column head was carefully dismantled, the flow distributor position accurately marked and the inlet frit removed. The column showed no signs of depression and no obvious damage. Using a metal rod with a 3 mm flat surface, the column bed was tamped very gently. No significant amount of packing material was removed. The frit and flow distributor were replaced in their original positions and the column reassembled. Injections of sample indicated a return of column efficiency similar to that at the start of the experiment.

These observations rule out causes (iii) a), (ii) and (vii) because the frit was unchanged and in the same orientation; any voids or channels

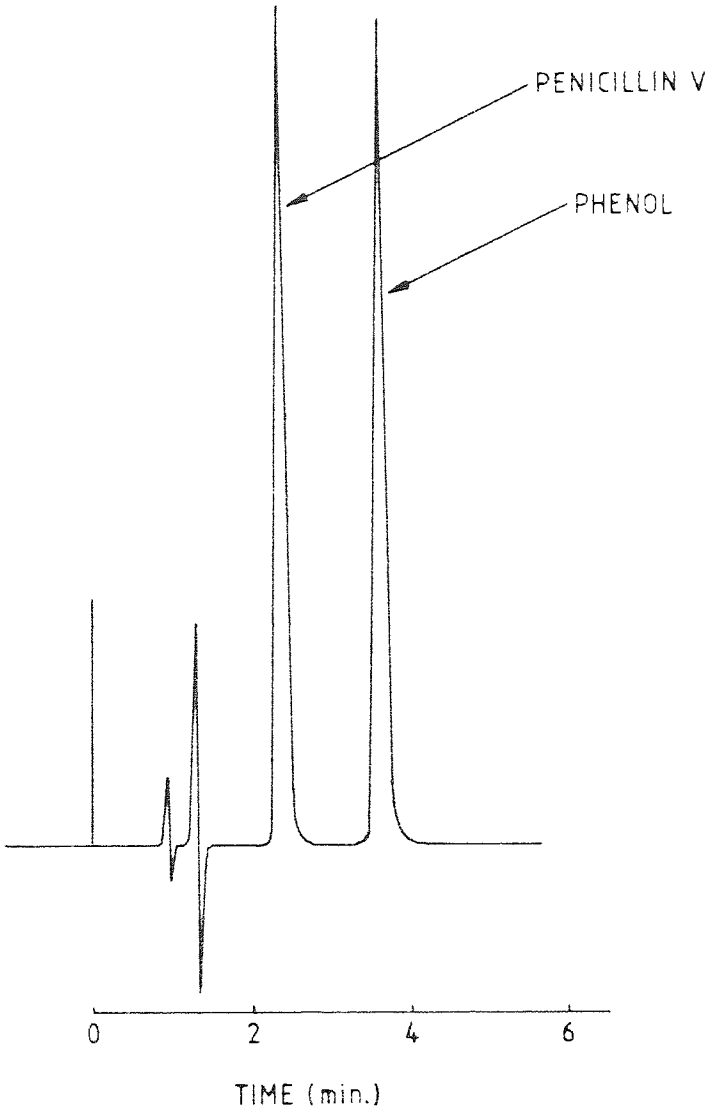


FIGURE 4.5 CHROMATOGRAM OF PENICILLIN V AND PHENOL - FLOW DISTRIBUTOR ROTATED 90° AFTER 2 HOURS SOLVENT PUMPING WITH A SINGLE PISTON PUMP

in the main body of the column would have been unaffected by the tamping and in the same orientation; column packing was not removed and it remained in the same orientation.

This only left cause (iv) b) ; mechanical damage caused by the jet of mobile phase. Initially, no evidence could be found for this, because there was no apparent mechanical damage.

The retention time of the split peak was next investigated to decide whether the material in the split peak was eluting more rapidly or more slowly than the original single peak. The results can be seen in table 4.1.

Injection Number	Retention Time (seconds)		Peak Height (mm)		Peak Area (units)	
	Pen.V	Phenol	Pen.V	Phenol	Pen.V	Phenol
1	150	232	110	111	97.7	112.0
2	150	233	103	97	97.3	112.0
3	151	233	98	87	97.9	112.7
4	151	234	91	78	98.3	112.6
5	151	227 234	84	69	98.3	41.4 71.5
6	151	226 235	77	63	98.2	49.0 64.3
7	152	226 234	72	59	99.1	50.9 61.9
8	147 152	225 234	67	58	33.4 65.5	53.6 58.7
9	147 152	225 234	68	58	32.5 66.4	55.2 59.1
10	147 152	225 235	62	60	42.4 56.5	56.5 59.2
11	146 152	224 235	59	61	42.9 56.2	60.0 53.4

Table 4.1 Retention Characteristics of Split Peaks

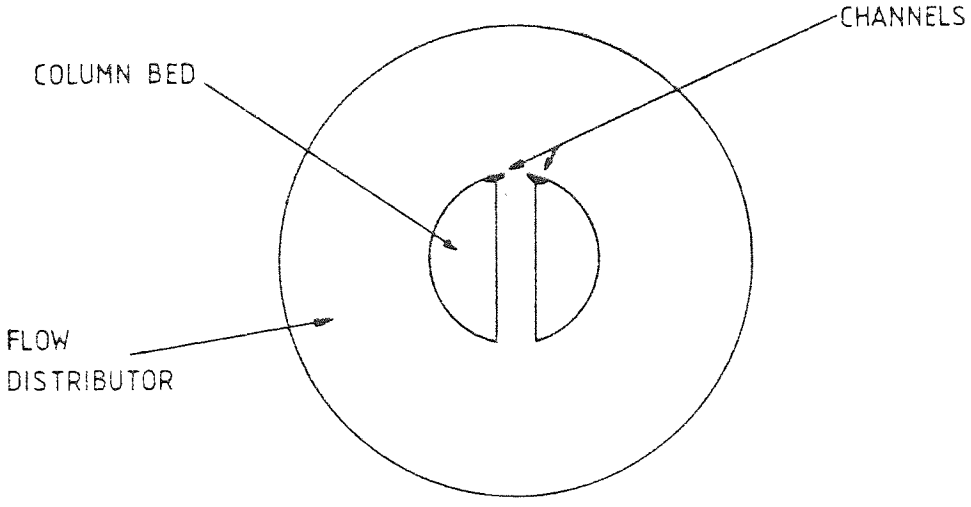


FIGURE 4.6 THE POSITION OF CHANNELS IN THE COLUMN BED

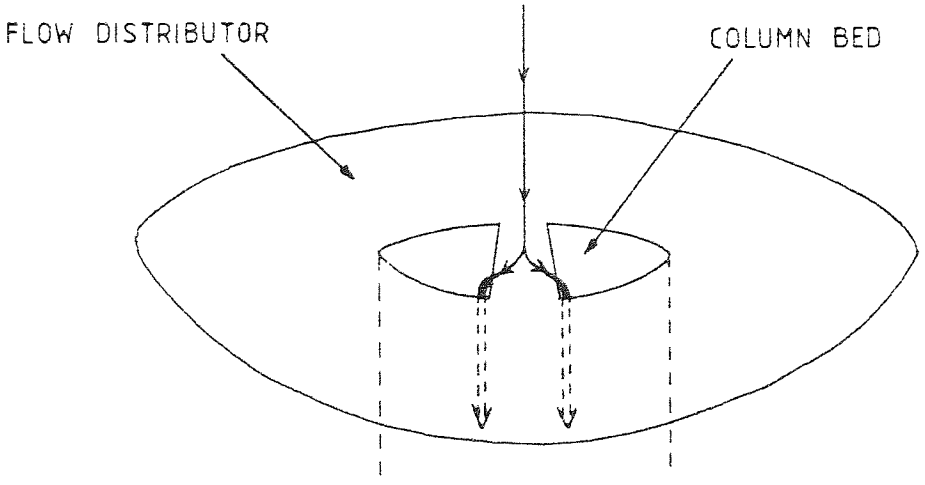


FIGURE 4.7 PROPOSED FLOW OF MOBILE PHASE THROUGH COLUMN

In table 4.1, peak height was taken as the maximum height of either peak in the split peak. Two values in the retention time and peak area columns represent split peaks recognised by the integrator. Results in table 4.1 show that, when splitting occurred, the split peak eluted at a shorter retention time than the original single peak. This indicated that the distance travelled by the solute in the column was reduced when splitting occurred.

At the end of this experiment, the column was dismantled, the position of the flow distributor marked and the column bed critically examined. Two small holes, or channels, were seen. Their position, in relation to the flow distributor, is shown in figure 4.6. No other signs of damage were evident.

Rotation of the flow distributor and reassembley of the column produced peak heights and efficiencies similar to those at the start of the experiment.

This experiment was repeated several times and similar results obtained. Channels were predominantly formed at the positions shown in figure 4.6.

Split peaks showed an apparent solute distribution of about 50 : 50 in each half of each peak. This suggests that 50% of the solute travels down the channels. The surface area of the channels compared with the surface area of the entire column is small, thus this is strong evidence in favour of a narrow point of injection onto the column, contrary to the band expected to be produced by the flow distributor. Figure 4.7 illustrates restricted flow over the flow distributor and into the column bed. If this did occur, the velocity of the mobile phase issuing from the inlet tube would not be greatly reduced by the flow distributor before it hit the column bed. This could explain how the channels were formed.

From table 4.1, the ratio of the retention time of the first half of

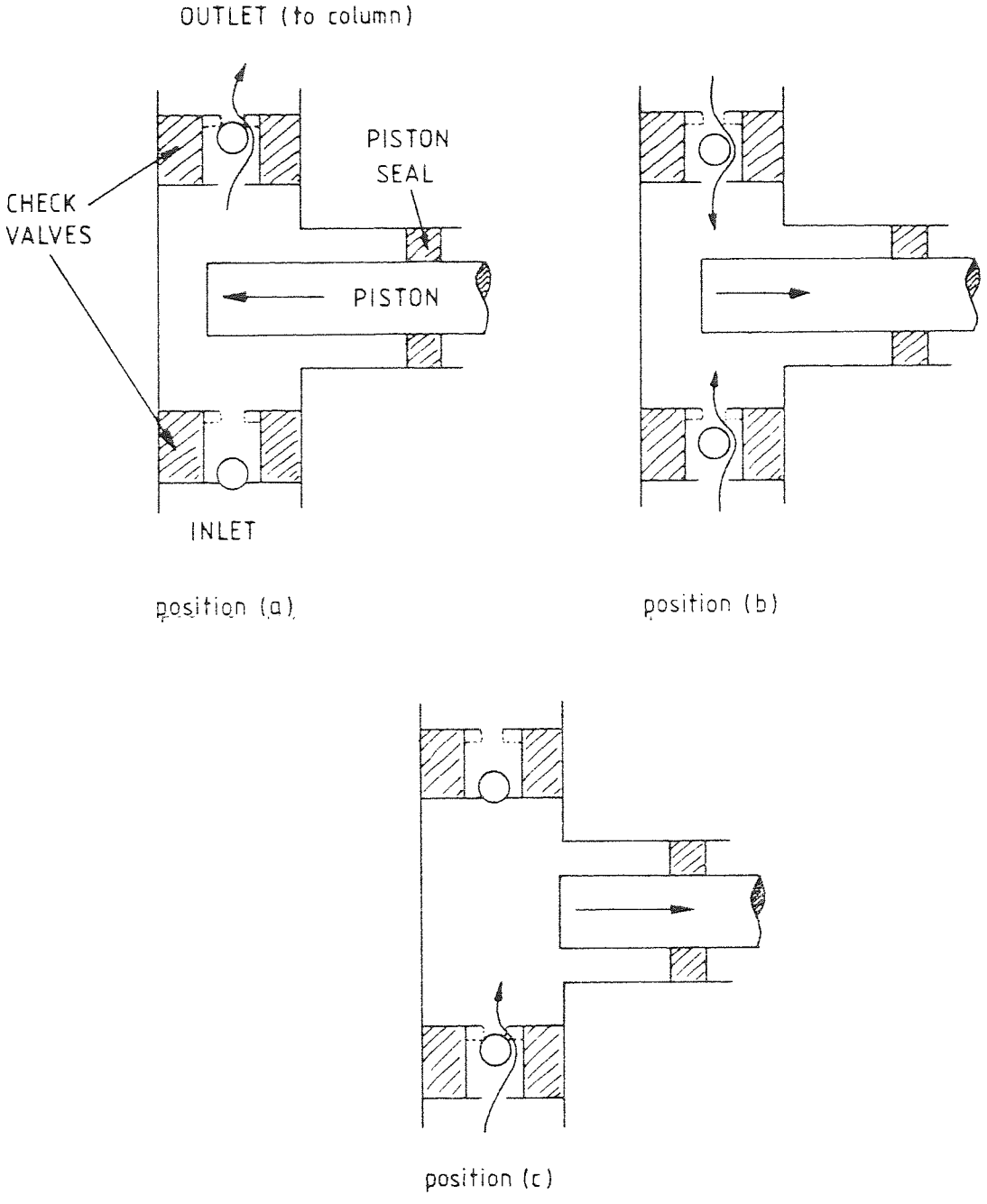


FIGURE 4.8 FLOW OF SOLVENT DURING PUMP CYCLE OF SINGLE PISTON PUMP (a) DELIVERY ; (b) AND (c) DRAWBACK

the split peak compared to the retention time of the second half, was 0.961 (penicillin V) and 0.953 (phenol). Since the column was 100 mm in length, these values suggest channel lengths of 4 to 5 mm, assuming the retention time of the solute was directly proportional to the distance it travelled in the column.

When two channels were formed, as in figure 4.6, it was possible that they were of different lengths. This should have caused a single peak to split into three peaks. This was observed in some experiments.

Split peaks occurred very quickly when using the Altex 110 pump to deliver the solvent onto the column bed. During the pumping cycle, the piston expelled solvent at a slow rate during solvent delivery. At the end of each delivery stroke, the piston rapidly drew back to refill. During the draw-back, flow stopped, then reversed very slightly, until the balls in the check-valves took up their new positions (figure 4.8). At the start of a new forward stroke, the piston caused a pulse of solvent onto the column. This pulse lasted until the working pressure of the system was achieved. Thus the column bed was subjected to a negative flow of solvent and a solvent jet, at a higher linear velocity than necessary to achieve the intended flow rate, every pump cycle. To show that this effect caused the mechanical damage seen as channels in the column, a pulse damper (pressure filter) was attached between the pump head and the injector. This piece of equipment consisted of a coil of metal tubing, with an internal volume of approximately 3 mls. Its geometry and large internal volume (compared with the volume of other H.P.L.C. tubing) utilised the property of solvent compressibility to minimise the effects of negative flow and solvent jets. Loss of column efficiency and eventual splitting were seen, but the time required to achieve these conditions was much longer (several hours for peak height to be affected and 12 hours continual pumping before splitting was seen). The use of a two piston pump (Altex 100A), instead of the Altex

110 pump plus pulse damper, reduced the rate of mechanical damage still further. No significant loss of efficiency was noticed during the first 20 hours of pumping. Two piston pumps were designed to reduce solvent pulsing. After 5 days continual pumping with the 100A pump, however, split peaks occurred. The Altex 100A pump does not exhibit negative flow in its pump cycle (as seen in the Altex 110 pump) because the pistons overlap in their solvent delivery strokes. This isolated the cause of the channels to the high linear velocity of the jet of solvent issuing from the inlet tube, although negative flow probably potentiates this effect.

The cause of the channels having been discovered, pre-coating of the column with an alkyl silylating agent (SurfaSil), the use of different conditioning solvents after packing and the re-design of the flow distributor, were tried in order to overcome their formation. The Altex 110 pump was used without a pulse damper throughout these experiments.

Pre-coating of the column

Channels were always seen at the sides of the column, thus it was reasoned that an improvement in the binding between the column wall and the column packing would reduce the incidence of channel formation. A clean, dry, column was treated with SurfaSil, at room temperature, prior to packing. No significant improvement in the maintenance of column efficiency was seen.

Different conditioning solvents

Column packing was carried out as described in the experimental section, but, after propan-2-ol, an additional solvent (conditioning solvent) was pumped through the column. The effect of this conditioning solvent on the top of the column was difficult to quantify, but methanol appeared to produce a more rapid loss of efficiency than ethanol, propan-1-ol or propan-2-ol.

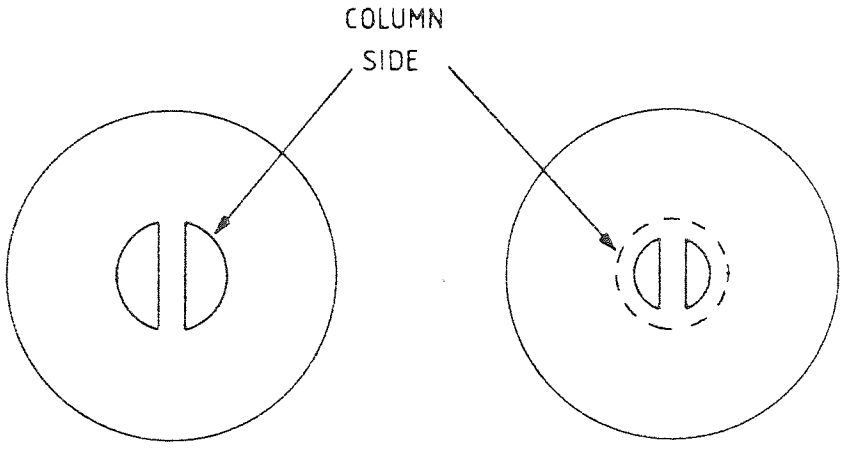


FIGURE 4.9 RE-DESIGN OF FLOW DISTRIBUTOR

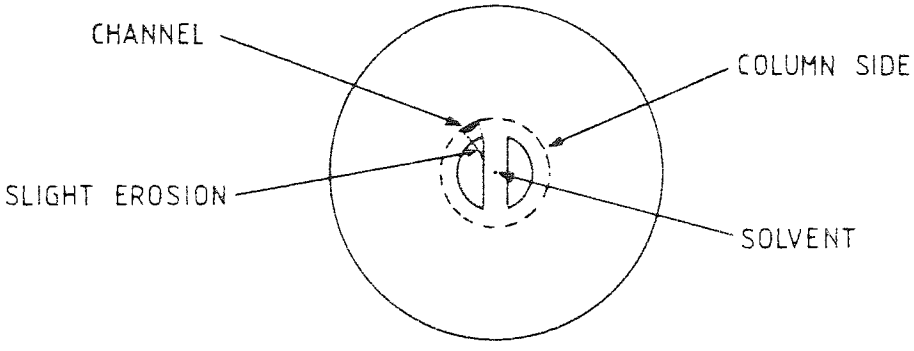


FIGURE 4.10 POSITION OF THE COLUMN CHANNEL WITH THE RE-DESIGNED FLOW DISTRIBUTOR

When levelling the column bed, after packing and prior to use, it was critical to prevent the column bed from drying because this made it prone to mechanical damage. Methanol, being more volatile than the other solvents, evaporated quickly from the column bed, increasing the probability of this occurring.

No solvent was found that significantly decreased channel formation.

Re-design of the flow distributor

A flow distributor with a narrow column inlet hole was designed to keep solvent flow away from the column side (figure 4.9). Loss of efficiency occurred more slowly with this design of flow distributor, peak splitting occurring after 4 hours. Inspection of the column, after careful dismantling, revealed a single channel in the column inlet bed (figure 4.10). Slight erosion of the column bed, between one corner of the flow distributor and the column wall, was seen. A repeat experiment using the re-designed flow distributor produced a single channel in a similar position.

These results suggest that a more suitable design for the flow distributor would be similar to the one in figure 4.11. A flow distributor of this design should distribute the solvent flow more efficiently, thus reducing the linear velocity of the solvent entering the column bed, thereby reducing the incidence of channels. No facilities were available for the production of such a flow distributor, thus it was not possible to test its efficiency.

Radial compression chromatography

A recent development in liquid chromatography has been the introduction of radial compression columns by Waters Associates. Here, a polypropylene column is subjected to a radial compression (by hydraulic rams) greater than the back-pressure of solvent created by the resistance in the column. Any voids that occur in the column are

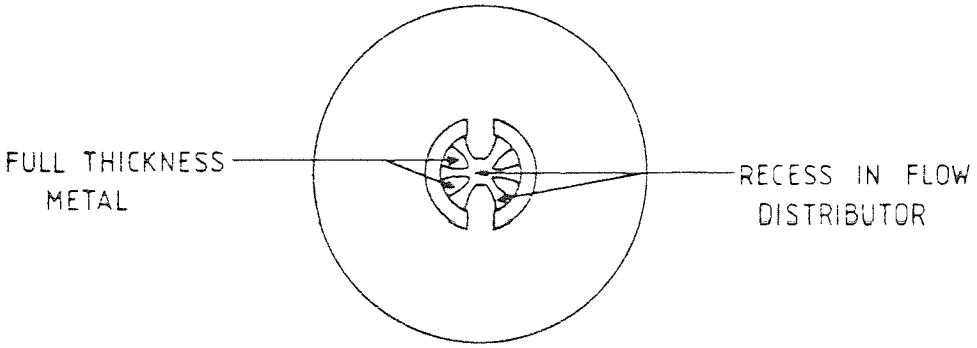


FIGURE 4.11 SUGGESTED DESIGN OF FLOW DISTRIBUTOR

immediately compressed and cease to exist. Waters have shown, by electron micrographs, that the polypropylene column wall moulds itself to the shape of the column packing and claim excellent efficiencies for liquid chromatographic separations.

A radial compression column containing 5 micrometre C-18 reversed-phase packing was used with the Altex 110 pump without a pulse damper. Because of the change from one column packing to another, slight modification of the mobile phase was required to obtain satisfactory separation of penicillin V and phenol (the acetonitrile concentration was increased to 34%). Figure 4.12 contains a typical chromatogram for this system. Continual pumping at 1 ml per minute for 24 hours did not produce any loss in efficiency, suggesting that any channels caused by mechanical damage were instantly sealed.

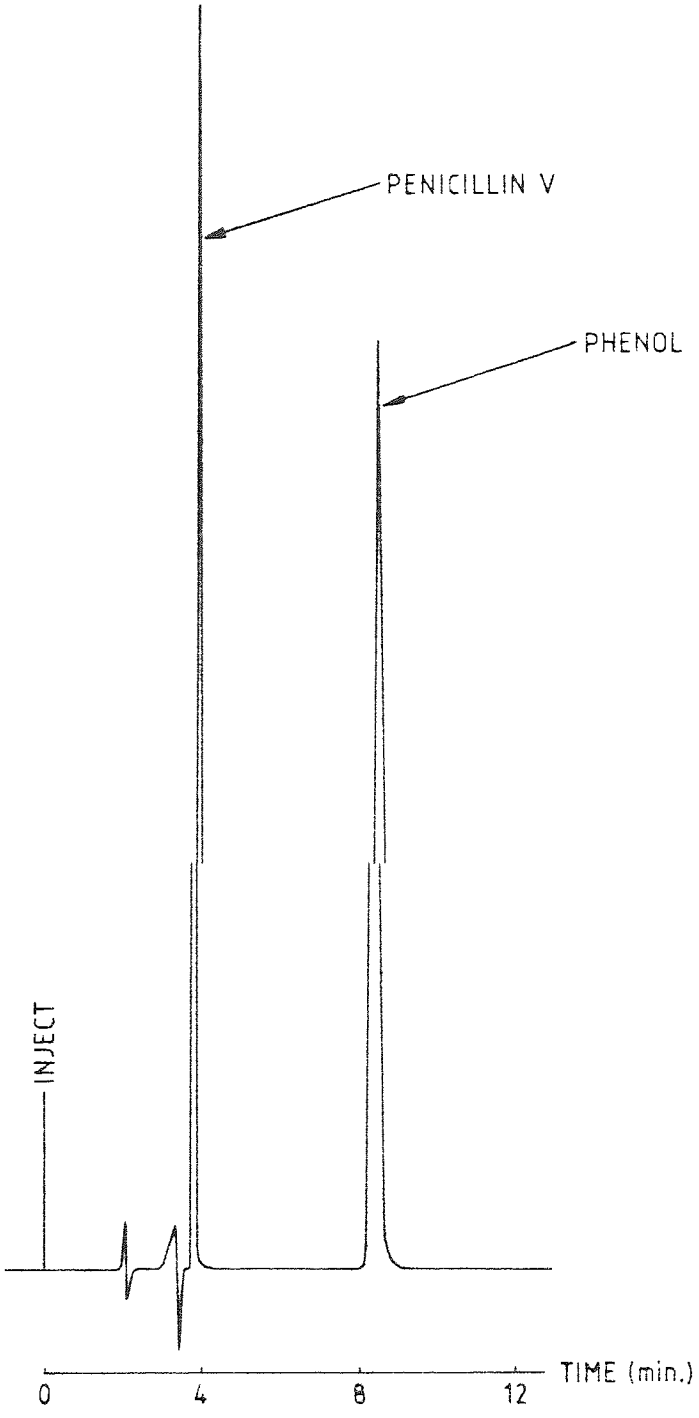


FIGURE 4.12 CHROMATOGRAM OF PENICILLIN V AND PHENOL - RADIALLY COMPRESSED COLUMN

4.4 CONCLUSIONS

The loss in efficiency and occurrence of split peaks, when using self-packed, Shandon design, reversed-phase columns, was attributed to the formation of small channels between the column packing and the column wall. These small channels were caused by mechanical damage due to the high linear velocity of solvent issuing from the narrow bore inlet tubing. Coating the column with SurfaSil prior to packing, and changing the conditioning solvent at the end of packing, had little effect on the formation of these channels.

Re-design of the flow distributor maintained column efficiencies for a slightly longer period. Another flow distributor, designed to reduce linear velocity of the solvent entering the column bed, was proposed but not tested.

Reduction of solvent pulsing in the pump cycle, by the incorporation of a pulse damper and by the use of a two piston pump, resulted in channels being formed at a much slower rate. The only effective way to prevent channel formation at the top of H.P.L.C. columns was by the application of radial compression. Here, channels, if formed, were immediately sealed by the flexible column wall because the columns were operated at a higher radial pressure than back-pressure.

CHAPTER FIVE : NONISOTHERMAL KINETIC ANALYSIS OF PENICILLIN V SOLUTIONS

5.1 INTRODUCTION

Nonisothermal kinetic methods for the prediction of the shelf-life of pharmaceutical preparations are an attractive alternative to traditional isothermal accelerated storage tests. A nonisothermal study involves a temperature change throughout the degradation and enables a full stability-temperature profile to be determined from one experiment. This procedure offers a considerable reduction in effort for the estimation of shelf-life, compared with more traditional isothermal methods, and has received much attention.

Borchardt and Daniels (156) used differential thermal analysis (D.T.A.) to measure the evolution of heat from the decomposition of benzene diazonium chloride (first order reaction) and the reaction between N,N'-dimethylaniline and ethyl iodide (second order reaction or pseudo-first order reaction if one of the reactants is in large excess). The temperature of the water bath containing their reaction vessels was raised with time and the heat evolved during the exothermic reaction (measured by the temperature difference between the reaction flask and a reference flask) monitored. A plot of heat evolved (ΔT) *vs* time (and, therefore, temperature of the water bath, since the temperature was raised with time) produced slope, area and height parameters that were substituted into equation 5.1

$$k = \frac{\left[\frac{K.A.V}{L_o}\right]^{(n+m-1)} \cdot \left[C_p.\frac{d\Delta T}{dt} + K.\Delta T\right]}{\left[K.(A-a)-C_p.\Delta T\right]^n \cdot \left[K\left(\frac{M_o}{L_o}.A-\frac{m}{n}.a\right)-C_p.\Delta T\right]^m \ldots} \qquad \text{equation 5.1}$$

For the reaction :

$$nL + mM + \ldots \longrightarrow \text{products}$$

having the rate expression :

$$\frac{dz}{dt} = (L-z)^n . \left(M - \frac{m}{n} . z \right)^m$$

where z is the number of moles of L reacted in time t

L_o and M_o are the initial number of moles of L and M, resp.

K is the heat transfer coefficient of the reaction and reference cells (these must be matched)

A is the total area under the curve (ΔT vs t)

C_p is the total heat capacity of the reactant solution / reference liquid

$d\Delta T/dt$ is the slope of the curve at the time at which k is evaluated.

T is the height of the curve at the time at which k is evaluated

a is the area of the curve at the time k is evaluated

V is the volume of the reactant solution

From this expression, values of k were calculated at different time (temperature) points. A plot of lnk vs the reciprocal of absolute temperature gave the energy of activation, E_{act}, (from the slope) and the A factor (from the intercept) for the reaction. A linear temperature rise was not required for this treatment to be valid, although the authors chose this particular profile. Energies of activation calculated in this way correlated well with previous literature values.

Davis (157) studied the second order reaction between octatomic sulphur and triphenylphosphine in benzene using U.V. absorption. The temperature of the reaction was raised slowly from 23 to 77°C at about 0.5 degrees per minute (heating rate did not have to be controlled). A plot of the reciprocal of (optical density at time t - optical density at time infinity) against time (temperature) provided a curve from

which the slope at any one point could be determined graphically. The slope was proportional to the rate of reaction, k_t, at that time (temperature). A plot of ln k_t vs 1/T provided a value for E_{act} which was in close agreement with literature values.

The methods of Borchardt and Daniels, and Davis required manual graphic slope determination at different time (temperature) values. This procedure is subjective and inaccurate. The first mathematically precise nonisothermal stability study was reported by Rogers (158), who investigated the first order decomposition of riboflavine and sucrose. The use of a logarithmic temperature programme (equation 5.2) allowed Rogers to utilise the Arrhenius equation (equation 5.3) to derive equation 5.4.

$$1/T_o - 1/T = 2.303.b.\log(1+t) \qquad \text{equation 5.2}$$

where T_o is the initial temperature (at t=0)

T is the temperature at time t

b is the proportionality constant

$$k_t = A.\exp(-E_{act}/R.T) \qquad \text{equation 5.3}$$

where k_t is the rate constant at time t

A is the pre-exponential factor

E_{act} is the energy of activation

R is the gas constant

T is the absolute temperature

$$\log f(C_t) = \log k_o - \log (1 + E_{act}.b/R) + (1 + E_{act}.b/R)\log(1 + t) + \log\left[1 - (k_o/k_t)^{(1 + R/E_{act}.b)}\right] \qquad \text{equation 5.4}$$

where $f(C_t)$ is a concentration function dependent on the order of reaction and k_o is the initial rate constant.

The final term on the right hand side varies with time but it tends to zero as k_t becomes greater than k_o. A plot of log $f(C_t)$ *vs* log(1 + t) provides a straight line with slope $(1 + E_{act}.b/R)$ and intercept log k_o - log $(1 + E_{act}.b/R)$. From these values E_{act} and A can be calculated. The dependence upon a set time/temperature profile reduces the flexibility of the method. Also, the assumption that the final term on the right hand side of equation 5.4 rapidly tends to zero needs consideration. Cole and Leadbeater (159) critically assessed Rogers method, showing that for a first order reaction with an E_{act} of 20.0 kcal.mol^{-1} (96.3 kJ.mol^{-1}), a rate constant at 15°C of 1 x 10^{-4} hours^{-1} and a temperature programme of :

$$1/288.2 - 1/T = 0.001 \log(1 + t)$$

the term still constituted approximately 2% of the value of log $f(C_t)$ after a 10°C rise in temperature. This indicates that data collected during the first 10°C rise in temperature cannot be used in the analysis. This moves the temperature range analysed further away from temperatures at which shelf-lives are likely to be calculated.

Cole and Leadbeater applied Rogers method to the hydrolysis of sucrose and ethyl benzoate, the solvolysis of methyl toluene-p-sulphonate, the decomposition of N-methyl-pyridinium-2-aldoxime methane sulphonate and the activity of cholinesterase (159). They also examined its applicability to the decomposition of solid horse serum cholinesterase (160).

Later, Gober *et. al.* (161) used Rogers nonisothermal method to study the aqueous stability of tetracaine solutions. They compared their results with those obtained isothermally (162) and found good agreement.

Eriksen and Stelmach (163) derived similar equations to those of

Rogers, selecting the temperature function :

$1/T = 1/T_o - a.t$ (where a is a reciprocal heating constant)

and forming the equation :

$$\ln\left[f(C_t)-f(C_{t+\Delta t})\right] = a.E_{act}.t/(R\ ) + \ln\left(\left[\exp(a.E_{act}.\Delta t/R)-1\right]\left[\frac{R.k_o}{a.E_{act}}\right]\right) \qquad \text{equation 5.5}$$

where Δt is a constant increase in time.

A plot of $\ln\left[f(C_t)-f(C_{t+\Delta t})\right]$ vs t gave a straight line with a slope of $(a.E_{act}/R)$. For this treatment to be applied, Δt must be kept constant. The reactions studied by the authors were the hydrolysis of ethyl acetate and p-nitrophenol acetate.

In their discussion, Eriksen and Stelmach claimed that Rogers method was inaccurate for early time intervals if k_o was sufficiently high; and that the last term in Rogers equation would contribute to curvature of the line unless $k_o < k_t$. k_t is always likely to be higher than k_o in nonisothermal studies. Although the Guggenheim type solution of Eriksen and Stelmach's equation does provide a completely linear plot if the order is correct and E_{act} does not change with temperature, their method has inadequacies. They claim that Δt needs to be large to overcome errors in the Guggenheim solution and use Δt=60 minutes in their example for p-nitrophenolacetate. This effectively cancels the first six data points from their plot to determine E_{act}. Also, this approach requires sampling at strict time intervals as well as adherence to a time/temperature program. Eriksen and Stelmach provide a useful discussion on the meaning of the E_{act} value obtained in their experiments.

Recently, Yang (163a) criticised Eriksen and Stelmach's paper, correcting errors in equations 5, 6, 7 and 8 derived by the authors

(equation 5.5 above is the corrected form of equation 8). Although not mentioned by Yang, equations 9 and 10 require a similar modification where $f(C_a,...)$ and $f(C_a^o,...)$ should be interchanged. The errors in equation 8 (equation 5.5) concern the final term in the equation. Although incorrect, it was not used in the plot to obtain E_{act}. Thus the results quoted by Eriksen and Stelmach were correct.

Carstensen *et. al.* (164) noted that the nonisothermal treatment given by Rogers, and Eriksen and Stelmach, was not suitable for equilibrium reactions because it was not possible to derive equations in a manageable analytical form. They also noted that kinetic salt effects could cause a loss of linearity in the graphic plots. However, this effect also exists with traditional isothermal accelerated tests and would cause non-linearity in an Arrhenius plot determined in this way.

Zoglio *et. al.* (165) derived a nonisothermal method that utilised a linear heating program :

$$t = b.T + C$$

where b is the heating rate and C the initial temperature. Their approach was based on the assumption that the arithmetic mean of the rate of change of drug concentration with time was equal to the total degradation of the drug during the experiment, divided by the time span required for the experiment ; i.e.

$$\frac{f(C_o) - f(C_t)}{t_o - t_i} = \frac{k_1 + k_2 + k_3 + \ldots k_i + \ldots k_n}{n} \qquad \text{equation 5.6}$$

Where C_t is the concentration of the reactant at time t_i.

C_o is the initial concentration of the reactant

t_o is the initial time

t_i is the time after i time units

k is the rate constant at any one time

n is the number of rate constants

If n is very large, a discrete plot of all the k values against time /temperature would approach a smooth curve. The required value of n to approach this smooth curve was evaluated by convergence of the expression :

$$n.\left(1 + \sum_{k=1}^{n-1} \exp\left[\frac{E_{act}.C_t}{R} \sum_{i=1}^{k} T_i^{-1}.T_{(i+1)}^{-1}\right]\right)^{-1}$$

Where T_i is the temperature at t_i

T_{i+1} is the temperature at t_{i+1}

k refers to the rate constant number 1,2,3 ....n

The value of n used by the authors was 384, but this was later reduced to 200 (166).

Solving complex equations for k_1 through to k_n at different E_{act} values led to the synthesis of a family of curves. Experimental data was compared with these curves and the curve most closely matching the data selected as the E_{act} of the reaction. The hydrolysis of N-acetyl-p-aminophenol and procainamide hydrochloride were studied.

Maudling and Zoglio (166) extended this approach to flexible heating programs, generating a polynomial function to describe the change in temperature with time. They studied the inversion of sucrose and the hydrolysis of ethyl acetate.

The methods of Zoglio et. al. (165, 166) produced curves that converged towards the start and end of the kinetic run. This made matching experimental data with theoretical curves less accurate and led them to discard these portions of their curves in their later study (166). However, as they stated (165), the theoretical curves provided a useful graphic representation of the effect of temperature on reaction rate at different activation energy levels.

Kay and Simon (167) developed a similar curve matching method to that of Zoglio *et. al.*, but, instead of generating k values by digital computation, they derived equations that could be solved by an analog computer. Computer output directly generated concentration *vs* time/temperature curves. They varied k_o and E_{act}, forcing curves to pass through the initial and last points of experimental nonisothermal data, thereby generating curves that could be matched with the experimental values for fit. Data obtained by Zoglio (165) was used to test their method.

The equations used in the analog program could be adapted for any order of reaction and any heating program, including heating programs that required a polynomial expression to link temperature with time. By experimentally measuring k_o, the rate constant at the start of the experiment, it was possible for them to generate a family of curves by varying E_{act} alone. These curves did not all pass through the final data point, thereby increasing the accuracy of the graphic curve matching method. However, an estimation of k_o can only be made over a long time period, a property that nonisothermal kinetic methods were introduced to overcome.

A completely new approach to nonisothermal estimation of activation energies was made by Madsen *et. al.* (168). They derived equations for zero, first and second order reactions (equations 5.7, 5.8, 5.9 resp.)

$$C_t = C_o - A \int_{t_o}^{t} \exp\left[-E_{act}/R.T(t)\right] .dt \qquad \text{equation 5.7}$$

$$C_t = C_o.\exp\left(-A \int_{t_o}^{t} \exp\left[-E_{act}/R.T(t)\right] .dt \right) \qquad \text{equation 5.8}$$

$$C_t = \left(A \int_{t_o}^{t} \exp\left[-E_{act}/R.T(t)\right] .dt \; + \; C_o^{-1} \right)^{-1} \qquad \text{equation 5.9}$$

Where C_o is the initial concentration of the reactant

C_t is the concentration of the reactant at time t

A is the pre-exponential factor

The integrals in the equations could not be solved exactly and were estimated by the trapezoidal rule.

The concentration data (C_t) were nonlinear with respect to the two unknown parameters A and E_{act}. With initial estimates, an iterative least-squares regression was used to find the best estimates of these parameters, minimising the function :

$$S.S = \sum_{i=1}^{N} (C_{t\ obs} - C_{t\ pred})^2$$

$C_{t\ obs}$ refers to the concentration of reactant measured at time t

$C_{t\ pred}$ refers to the concentration of reactant predicted (from the E_{act} and A values calculated), at time t

Any time/temperature relationship could be used with this method, a seventh order polynomial expression being generated to arithmetically link these parameters.

Details of a computer program that calculated all parameters involved in this approach were presented in their paper. The authors stated that, provided the reaction was followed through at least two half-lives and the data was spread uniformly over this range, only twelve data points were needed to define E_{act} and A sufficiently. One assumption made for this approach was that C_o, the initial

concentration, was known without error. This requirement could be overcome by incorporating three variables in the function minimisation routine, instead of two, but computer processing time was increased dramatically.

The computer program, written in FORTRAN IV, required large computational facilities and a process time of between 2 and 4 minutes. Initial estimates of E_{act} and A had to be close to true values in order to ensure convergence of the minimisation routine within this time. This is a severe disadvantage because good initial estimates are not usually available before the nonisothermal data is analysed. The treatment developed by Madsen et. al. can be used for zero, first and second order reactions where the reactant concentrations are equal. No method for second order reactions where the reactant concentrations are unequal was proposed.

A detailed discussion of the errors involved in the method was presented by the authors.

The reaction studied was the decomposition of riboflavine in alkaline solution.

Zoglio et. al. developed their earlier approach still further in 1975 (169), with a method for nonisothermal-isothermal kinetic analysis. After a flexible nonisothermal program, the reaction continued isothermally at some suitable temperature. From the isothermal section of the analysis the order of the reaction could be determined and this value used, in the analysis of the nonisothermal results, to calculate E_{act} and k_o. Curve matching was replaced by an analytical solution. They derived an expression for relating the activation energy to the average rate constant over the nonisothermal region (k_n), the rate constant for the isothermal region (k_j) and the length of time of each short time segment (Δt) :

$$n\,\frac{\left[f(C_o) - f(C_t)\right]}{(t_o - t_i).k_j} = \frac{n.\bar{k}_n}{\Delta t.k_j}$$

$$= \sum_{i=0}^{n-1} \exp\left[\frac{E_{act}}{R}\cdot\left(\frac{T_i - T_j}{T_i.T_j}\right)\right] = C$$

where C is a constant

T_i is the absolute temperature at t_i

T_j is the absolute temperature for the isothermal region

A series expansion of the exponentials in the above equation resulted in equation 5.10, in which a,b,c ... are the temperature terms and $X = E_{act} / R$.

$$n + (a + b + c + \ldots).X + \frac{(a^2 + b^2 + c^2 + \ldots).X^2}{2!} \qquad \text{equation 5.10}$$

$$+ \frac{(a^3 + b^3 + c^3 + \ldots).X^3}{3!} + \ldots = C$$

Equation 5.10 was evaluated by Newton's method (170). Each set of analytical points could be used to generate equations in the form of equation 5.10 and the mean activation energy calculated from the solutions. Estimates of the error in the calculation of E_{act} were also possible.

Edel and Baltzer (171) used Zoglio's method (166) to evaluate E_{act} and A for a substituted benzazepine. The novelty of their approach was to use a rigid heating program, whereby the temperature was raised 0.3°C every 3 hours. They generated f(c) vs time curves for various E_{act}, in a similar way to that of Zoglio, and compared their experimental data with these curves, selecting the best fit as the best estimate of E_{act}.

They also developed a computer program that calculated predicted concentration values for a particular E_{act}, compared these values with the experimental concentration measurements, then varied E_{act} to minimise :

$$SS = \sum_{i=1}^{N} (C_{t\ obs} - C_{t\ pred})^2$$

Typical computer calculation took approximately 6 hours.

Despite the success of these methods, there was a need for a rapid method of nonisothermal kinetic analysis that did not require large computational facilities, initial estimates for E_{act} and A or a rigid time/temperature program. Thus a treatment, similar to that reported by Davis (157) was developed using a BASIC computer program (NONISO), which could be adapted for use in any microprocessor with 32 K RAM.

5.2 THEORETICAL

The rate of degradation of any drug may be represented by the equation:

$$\frac{-d\,C_t}{dt} = k_T . C_t^{\,n}$$

where C_t is the concentration of the reactant at time t

k_T is the specific rate constant at the temperature T

n is the order of the reaction

For a first order reaction (n=1) this may be written as :

$$C_t = C_o . \exp(-k_T . t)$$

Taking logs and re-arranging,

$$\ln C_t = \ln C_o - k_T . t$$

The slope of the plot of $\ln C_t$ *vs* t is therefore equal to $-k_T$

When the temperature is continually increased throughout the reaction, the degradation rate progressively increases. The isothermal rate constant is now approximated by :

$$k_T = -\left[\frac{\ln C_t - \ln C_{t-\delta t}}{\delta t}\right]$$

where δt is a small increment of time, over which period the temperature may be considered constant. For an infinitesimal increase in time and temperature, the specific rate constant is given by :

$$k_T = \frac{-d\,(\ln C_t)}{dt}$$

Thus the slope of the tangent at any one point for the plot of $\ln C_t$ *vs* t for nonisothermal data yields the specific rate constant at the temperature observed.

If other orders of reaction are followed, the appropriate equations are :

ZERO ORDER $\quad k_T = -d\,(C_t) / dt$

SECOND ORDER (a = b) $\quad k_T = -d\,(1 / C_t) / dt$

SECOND ORDER ($a \neq b$) $$k_T = -d\left(\frac{1}{(B_o-C_o)} \cdot \ln\left[\frac{(B_o-C_o+C_t).C_o}{C_t.B_o}\right]\right) \Big/ dt$$

where C_t is the concentration (mole per litre), at time t, of the monitored reactant of initial concentration C_o

B_o is the initial concentration (mole per litre) of the excess reagent in a second order reaction when the initial concentrations of the two reactants are not identical.

The values of k_T are calculated by firstly fitting a polynomial to the transformed data as a function of time. For a first order reaction this is :

$$\ln C_t = a_o + a_1.t + a_2.t^2 + \ldots a_n.t^n$$

Differentiation at the experimental points yields the corresponding rate constants :

$$\frac{d\ (\ln C_t)}{dt} = -k_T = a_1 + 2.a_2.t + 3.a_3.t^2 + \ldots + n.a_n.t^{n-1}$$

The rate constants at the experimentally measured temperatures are then used to compute the E_{act} and A values in the Arrhenius equation :

$$k_T = A.\ \exp\ (\ -\ E_{act}\ /\ R.T\ )$$

This data enables the calculation of rate constants at storage temperatures and the prediction of shelf-life to be made.

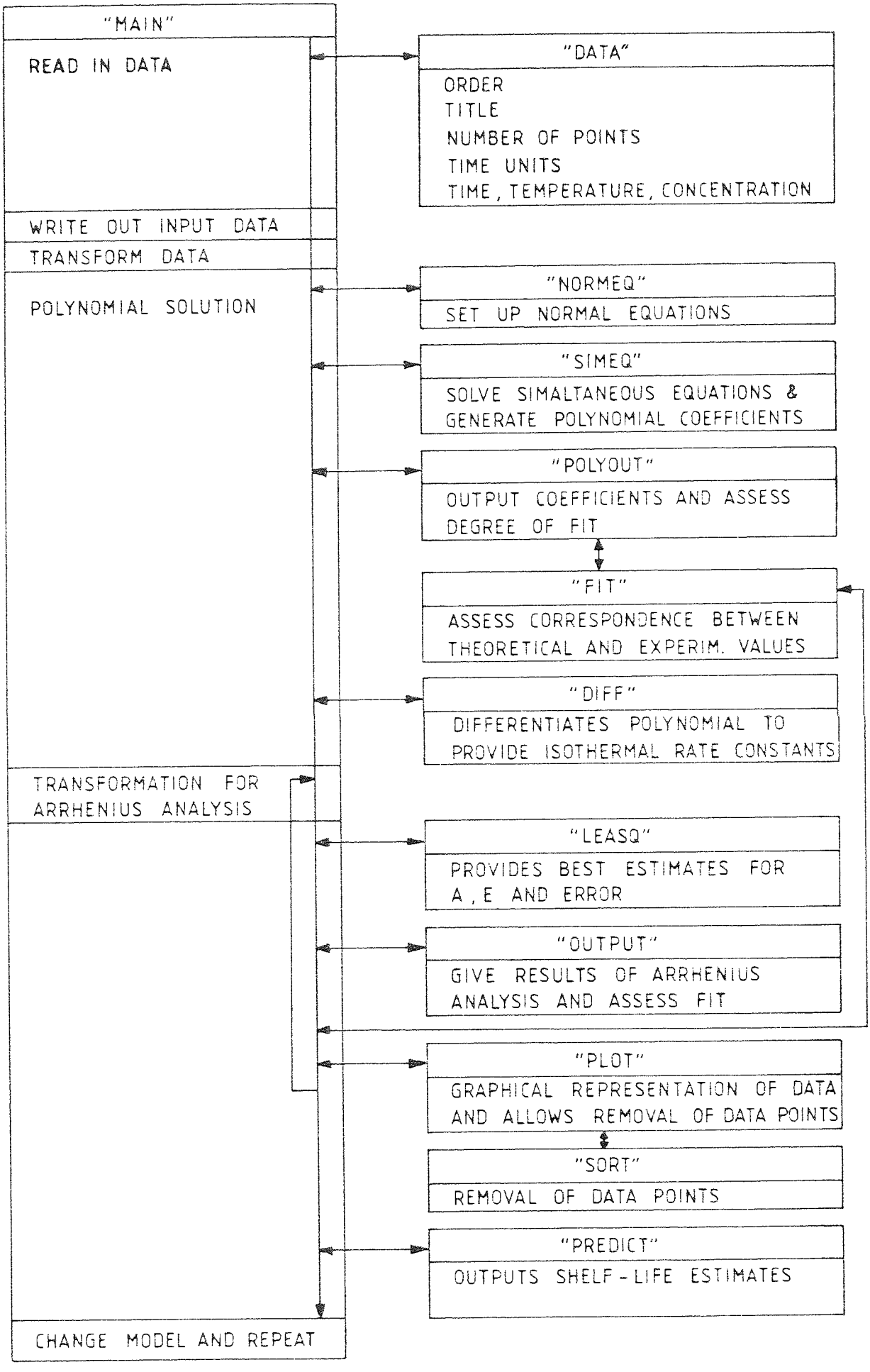


FIGURE 5.1 STRUCTURE OF THE PROGRAM NONISO

NONISO, a basic computer program, was written for implementation on a North Star Horizon Z80 - based Microcomputer with 64 K RAM. Figure 5.1 illustrates the structure of this program. Although BASIC does not allow subroutine names, these are used in the discussion for purposes of clarity.

DATA (lines 3940 - 4300): The data is written into the program before computation. This records the proposed order of reaction, an identifying label for the experiment, the number of data points and the time units used. This is followed by time, temperature (^{o}C) and residual concentration measurements for each experimental point beginning with zero time.

MAIN (lines 470 - 1080): This segment sets dimensions, reads in the input data and transforms the concentration values (cf. table 5.2) so that differentiation of the polynomial yields k_T values. Subsequent calls to the major subroutines follow.

NORMEQ (lines 1150 - 1270): This routine sets up the normal equations for the solution of the transformed concentration data in terms of a time polynomial. The normal equations take the form :

$$\sum_{i=1}^{N}(\ln C_t) = a_o.N + a_1.\sum t_i + a_2.\sum t_i^2 + ... + a_n.\sum t_i^n$$

$$\sum_{i=1}^{N} t_i.(\ln C_t) = a_o.\sum t_i + a_1.\sum t_i^2 + a_2.\sum t_i^3 + ... + a_n.\sum t_i^{n+1}$$

$$\sum_{i=1}^{N} t_i^2.(\ln C_t) = a_o.\sum t_i^2 + a_1.\sum t_i^3 + a_2.\sum t_i^4 + ... + a_n.\sum t_i^{n+2}$$

$$\sum_{i=1}^{N} t_i^n.(\ln C_t) = a_o.\sum t_i^n + a_1.\sum t_i^{n+1} + a_2.\sum t_i^{n+2} + ... + a_n.\sum t_i^{2n}$$

Where n is the order of the polynomial and N is the number of data points; the program is able to generate polynomials up to the tenth order. The maximum available order, however, is dictated by the word-length of the computer used. Values in excess of $\sum t_i^{2n}$ are generated and overflow errors may occur. In practice, a sixth-order polynomial was found satisfactory, with higher-orders giving no improvement.

SIMEQ (lines 1280 - 1709): This subroutine solves up to 10 simultaneous equations by the Gauss Elimination method (172). A typical execution time for 30 points is 90 seconds.

POLYOUT (lines 1710 - 1930): The coefficients for the polynomial are presented and the calculated values are compared with the observed transformations. Subroutine FIT (lines 1940 - 2100) is also called to calculate a determination coefficient, the coefficient of variation and the Chi-squared value.

DIFF (lines 2110 - 2330): The polynomial expression is differentiated here and the result is solved for the experimental points to yield the isothermal rate constants throughout the run. The experimental data are modelled well, but extrapolation beyond this range rapidly introduces unacceptable error. Transformation for the Arrhenius analysis ($k_T \rightarrow \ln k_T$; $T \rightarrow 1/T$) is thus undertaken and the parameters (E_{act}, A) are estimated by subroutine LEASQ (lines 2340 - 2540) which undertakes a linear least squares analysis of the data.

OUTPUT (lines 2550 - 2780): This subroutine prints the parameter estimates for Eact and A and the correlation coefficient.The isothermal rate constants predicted by the model are compared with the experimental values and the degree of correspondence is assessed by subroutine FIT.

PLOT (lines 3000 - 3750): This subroutine displays the theoretical regression line, together with the experimental points, thus allowing a visual assessment of the fit. Options are available to remove wildly

deviating points - in practice, if this is necessary, these are the initial data points when the concentration is changing slowly and is thus subject to more error. The regression calculations are then repeated on the reduced data set.

PREDICT (lines 2790 - 2990): When a satisfactory model is obtained, shelf-lives to 10% and 50% degradation, at typical storage temperatures, are calculated. The model may be changed by the use of the appropriate code [line 3940; 0-zero order, 1-first order, 2-second order (a = b), 3-second order (a $\neq$ b)].

5.4 EXPERIMENTAL

Details of H.P.L.C. analysis can be found in chapter 3, section 3.3.1.5.

5.4.1 Nonisothermal Kinetics

pH 9

A borate buffer (500 ml) containing boric acid (5.17g), sodium hydroxide (1.67g) and hydrochloric acid (5M, 1.9 ml) was placed in a 3-necked round-bottomed flask, suspended in a thermostated water-bath (20 litres capacity; 1 kW). A thermometer, graduated to 0.1^{o}C, was placed into the buffer through one neck; a teflon sampling-tube was fitted through the second neck and a stirrer, rotated at 200 r.p.m., was positioned through the third neck. When the buffer had reached thermal equilibrium, potassium penicillin V (250 mg) was rapidly added to the buffer. When solution was complete (2 minutes), the water-bath heater was turned to 100^{o}C (providing a temperature increase to 80^{o}C in 2 hours). A sample (5 ml) was immediately withdrawn and two 2 ml volumes were accurately measured. To each was added a solution of phenol (2 ml, 0.02% w/v in a phosphate buffer containing 0.9073% KH_2PO_4 adjusted to pH 5) as internal standard. This gave a final solution pH of approximately 7, a value at which the penicillin is more stable (173). The initial penicillin concentration was determined in duplicate by H.P.L.C. analysis through interpolation onto a calibration curve prepared similarly over a penicillin concentration range of 0 - 50 mg per 100 ml ($r = 0.999$). At frequent intervals throughout the run, samples (3 ml) were removed and a 2 ml aliquot assayed. Time and precise temperature were also noted.

pH 6

A citrate buffer (140 ml) containing citric acid (1.76g) and sodium

hydroxide (0.90g) was placed in a medicine bottle fitted with a thermometer and sampling port. When temperature equilibrium was reached, potassium penicillin V (70 mg) was added, samples were removed and the temperature program initiated.

In these experiments , the water-bath was programmed to rise from 60°C to 95°C over a 6 hour period. This was achieved by using a motorised syringe-drive unit to continuously adjust the thermostat. At frequent intervals throughout the run, samples (3 ml) were removed and to an aliquot (2 ml) was added a phenol solution (2 ml, 0.02% w/v in water) as internal standard. H.P.L.C. analysis was undertaken through interpolation onto a calibration curve prepared similarly over a penicillin concentration range of 0 - 50 mg per 100 ml (r = 0.999). Time and precise temperatures were noted for each assay point.

Formulated Products

Syrups were reconstituted as directed by the manufacturer. Typically this involved, in the case of syrup 1, adding 70 ml of water to the product and shaking vigorously to facilitate solution of the granules. The reconstituted product of nominal concentration 62.5 mg per 5 ml and pH 5.8, was then transferred to a 150 ml clear bottle fitted with a sampling tube and thermometer.

The stability of the syrups was monitored from 60°C to 95°C as for the pH 6 samples. H.P.L.C. assay was undertaken by adding phenol solution (2 ml, 0.25% w/v in water) to aliquots (1 ml) which were then diluted to 50 ml with water.

5.4.2 Isothermal Kinetics

Isothermal degradation was undertaken as described for the nonisothermal runs, except that the selected temperature was held constant throughout the experiment. A series of temperatures within the range of those in the corresponding nonisothermal program was used.

5.5 RESULTS AND DISCUSSION

A copy of the BASIC computer program NONISO, together with test data and typical output, can be found in appendix 2 (program 1).

5.5.1 NONISO Analysis of a Typical Set of Nonisothermal Results

Typical results for the nonisothermal degradation of penicillin V are shown in table 5.1.

Time (min.)	Conc. (%)	Temp. (^{o}C)	Time (min.)	Conc. (%)	Temp. (^{o}C)
0	100.0	28.3	73	53.9	68.6
9	99.6	32.8	76	48.8	69.8
19	98.5	39.4	79	43.8	70.8
29	96.4	45.8	82	39.0	71.8
39	92.1	52.0	85	34.4	72.7
44	89.1	54.8	88	30.0	73.6
49	85.2	57.5	91	26.0	74.5
52	82.5	59.0	94	22.0	75.3
55	79.4	60.6	97	18.4	76.0
58	75.9	62.1	100	15.1	76.7
61	71.8	63.6	103	12.5	77.4
64	67.9	64.9	106	10.0	78.0
67	63.4	66.2	109	7.9	78.5
70	58.4	67.5	112	6.4	79.0

Table 5.1 Typical Input Data for NONISO Analysis

On execution of NONISO, transformation of the concentration data (C_t) occurs, depending on the kinetic order model chosen, as in table 5.2.

Order of Reaction	Transform of C_t $f(C_t)$
Zero	C_t
First	$\ln (100.C_t / C_o)$
Second (a = b)	$1 / C_t$
Second ($a \neq b$)	$1 + \left[\frac{B_o}{B_o - C_o}\right] \ln \left[\frac{(B_o - C_o + C_t) . C_o}{C_t . B_o}\right]$

Table 5.2 Transformation of Concentration Data For NONISO

The transformation for the first order and second order ($a \neq b$) data had to be of the form in table 5.2 in order to minimise errors in data handling, mainly in the generation of the polynomial expression linking the transformed data with time, the next operation carried out. Table 5.3 contains the polynomial coefficients for a first order model, based on the data in table 5.1, and Table 5.4 the isothermal rate constants attained by differentiation of this polynomial expression at the experimental points.

$$4.60496 - 6.75179\ E\text{-}05.\ t - 4.09094\ E\text{-}05.\ t^2 + 6.03266\ E\text{-}07.\ t^3 - 1.93521\ E\text{-}08\ t^4 - 1.08408\ E\text{-}10.\ t^5 + 9.47380\ E\text{-}13.\ t^6$$

Table 5.3 NONISO Polynomial Coefficients for a First Order Model

Temp. (°C)	Rate Constant (1/minute)	Temp. (°C)	Rate Constant (1/minutes)
28.3	6.7599728E-05	68.6	3.0118105E-02
32.8	7.1694431E-04	69.8	3.3483762E-02
39.4	1.5562448E-03	70.8	3.7023247E-02
45.8	3.0729157E-03	71.8	4.0720993E-02
52.0	5.8386474E-03	72.7	4.4558119E-02
54.8	7.8519114E-03	73.6	4.8512270E-02
57.5	1.0357407E-02	74.5	5.2557447E-02
59.0	1.2114631E-02	75.3	5.6663843E-02
60.6	1.4070917E-02	76.0	6.0797681E-02
62.1	1.6231218E-02	76.7	6.4921031E-02
63.6	1.8598504E-02	77.4	6.8991669E-02
64.9	2.1173592E-02	78.0	7.2962898E-02
66.2	2.3954983E-02	78.5	7.6783402E-02
67.5	2.6938698E-02	79.0	8.0397000E-02

Table 5.4 NONISO Isothermal Rate Constants for a First Order Model

Table 5.5 contains the initial Arrhenius parameter estimates for this data. The limits of error about the estimates are rather wide and the poor nature of the fit is further revealed by the correlation coefficient (r = 0.984).

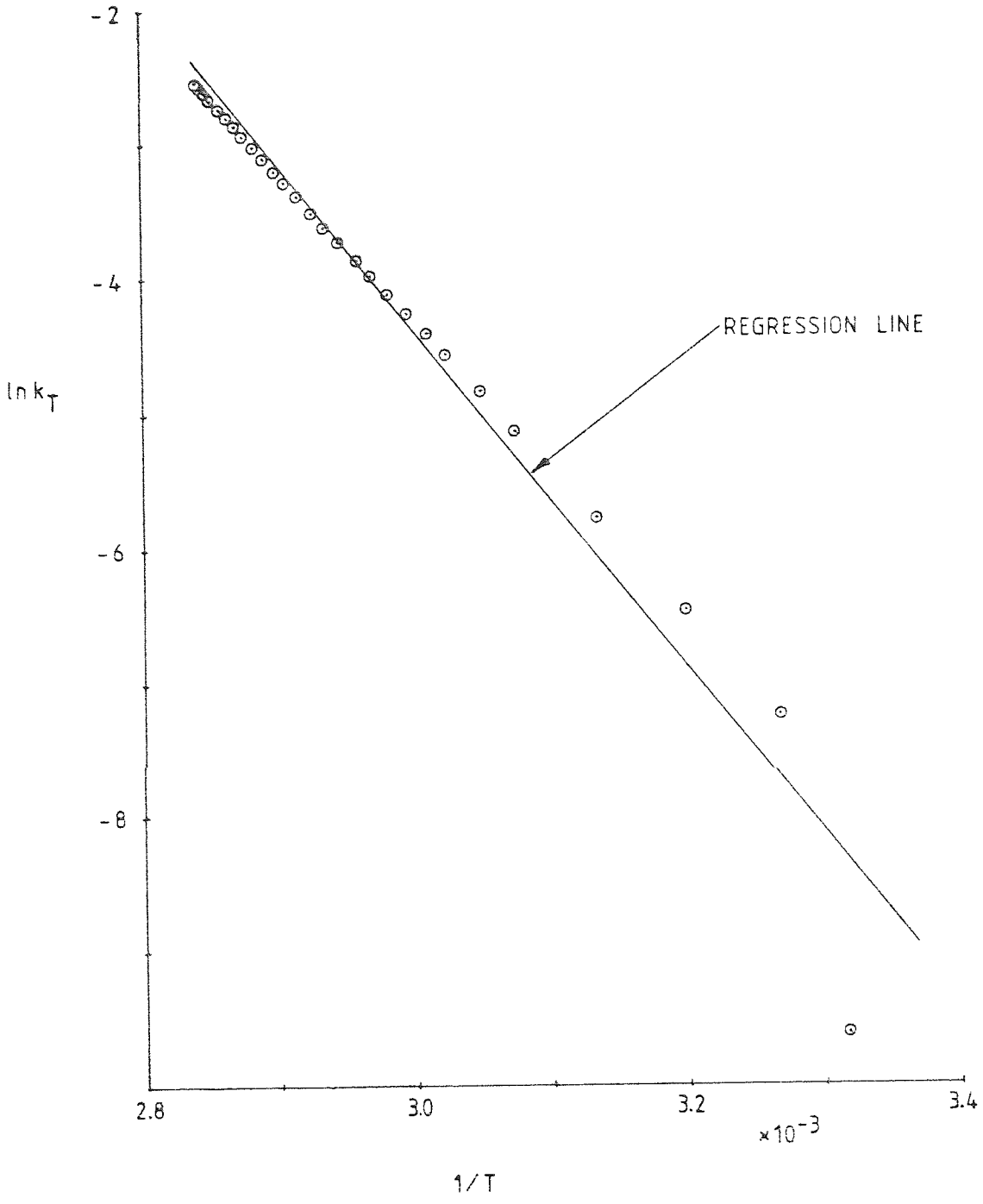


FIGURE 5.2 ARRHENIUS PLOT USING NONISOTHERMAL DATA FOR PENICILLIN V DEGRADATION AT pH 9 - ALL DATA POINTS

Arrhenius Results - All Data Points					
Slope	- 12393.627	+/-	915.66046	(P=95%)	
Intercept	32.770681	+/-	2.7215053	(P=95%)	
	R = 0.98367				
Parameter	Value	Range			Units
E_{act}	103044.33	95431.261		110657.40	$J.Mol^{-1}$
A	1.7065772E+14	1.1225129E+13		2.5945409E+15	$min.^{-1}$

Arrhenius Results - Omission of 28.3°C Point					
Slope	- 10976.566	+/-	48.186273	(P=95%)	
Intercept	28.627614	+/-	0.14256496	(P=95%)	
	R = 0.99994				
Parameter	Value	Range			Units
E_{act}	91262.463	90861.83		91663.093	J.Mol-1
A	2.7090361E+12	2.3490896E+12		3.1241363E+12	$Min.^{-1}$

Table 5.5 Arrhenius Parameter Estimates for the Nonisothermal Degradation of Penicillin V at pH 9

Examination of the plotted data (figure 5.2) reveals that almost all of the variation arises from the first point (at 28.3 °C). This is due to the small amount of degradation which occurs over this initial temperature range. The true polymonial gradient is small and the estimated rate constant is significantly affected by experimental

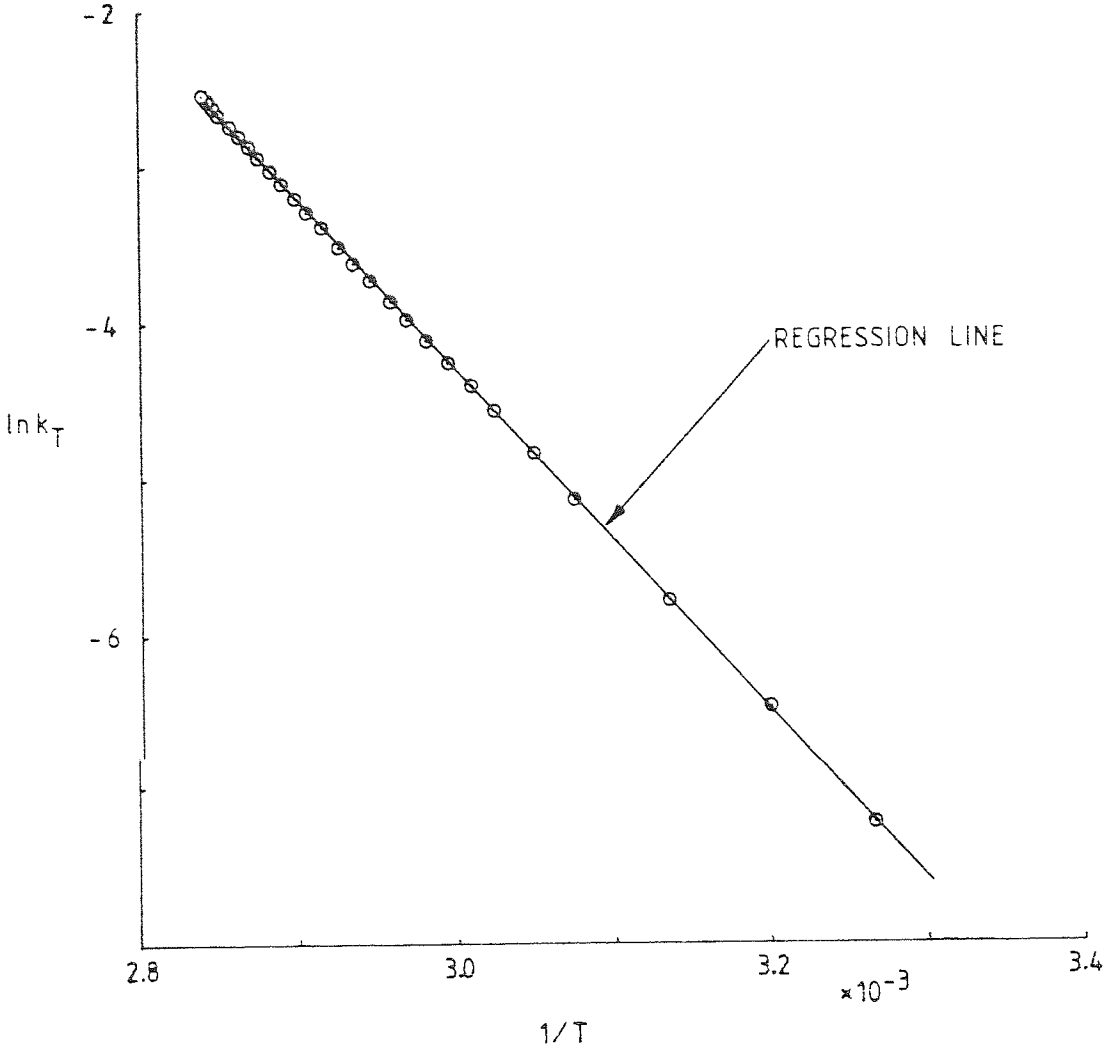


FIGURE 5.3 ARRHENIUS PLOT USING NONISOTHERMAL DATA FOR PENICILLIN V DEGRADATION AT pH 9 - OMITTING 28.3°C POINT

error. The removal of this one point from the data set improves the quality of the fit, estimating the activation energy to a precision of better than ± 0.5% (P=95%), as shown in table 5.5 and figure 5.3. The rate constants calculated from these estimates are held in table 5.6, where they are compared with the experimentally measured rate constants. Predicted shelf-lives are shown in table 5.7.

Temperature (^{o}C)	Experimental Rate Constants (1/minutes x10^{2})	Calculated Rate Constants (1/minutes x10^{2})	Ratio (%)
32.8	0.07172	0.07105	99.07
39.4	0.1554	0.1516	97.50
45.8	0.3074	0.3067	99.81
52.0	0.5839	0.5911	101.23
54.8	0.7853	0.7886	100.42
57.5	1.036	1.036	100.06
59.0	1.211	1.204	99.39
60.6	1.407	1.411	100.26
62.1	1.623	1.634	100.70
63.6	1.860	1.891	101.68
64.9	2.117	2.144	101.25
66.2	2.395	2.428	101.34
67.5	2.694	2.747	101.96
68.6	3.012	3.046	101.16
69.8	3.348	3.409	101.82
70.8	3.702	3.742	101.06

Temperature (°C)	Experimental Rate Constants (l/minutes x10^2)	Calculated Rate Constants (l/minutes x10^2)	Ratio (%)
71.8	4.072	4.104	101.06
72.7	4.456	4.459	100.79
73.6	4.851	4.841	99.80
74.5	5.256	5.255	99.98
75.3	5.667	5.650	99.71
76.0	6.080	6.018	98.99
76.7	6.492	6.409	98.72
77.4	6.899	6.824	98.90
78.0	7.296	7.199	98.66
78.5	7.678	7.526	98.02
79.0	8.039	7.867	97.86

Table 5.6 Comparison of Experimental and Calculated Rate Constants

Shelf - Life Predictions	First Order Model		
Temperature (^{o}C)	t 50%	t 90%	
5	24.440382	3.7150156	Days
10	12.175001	1.8506387	Days
15	149.12272	22.667126	Hours
20	77.87045	11.836555	Hours
25	41.559101	6.3171151	Hours
30	22.644083	3.4419725	Hours

Table 5.7 Shelf-life Predictions for Penicillin V at pH 9

5.5.2 Reproducibility of NONISO

The nonisothermal hydrolysis of penicillin V in pH 9 borate buffer was carried out three times and the data obtained analysed using NONISO. The results can be seen in table 5.8

Temperature Range of Nonisothermal Experiment (^{o}C)	E_{act} ($J.mol^{-1}$)	A ($x10^{-14}.hr^{-1}$)	Predicted k ($x10^{2}.hr^{-1}$)	
			25°C	50°C
28 - 81	91024 (413)*	1.496 (0.205)*	1.697	2.907
31 - 78	90966 (1125)*	1.345 (0.444)*	1.562	2.671
31 - 60	89496 (2305)*	0.809 (0.464)*	1.700	2.776

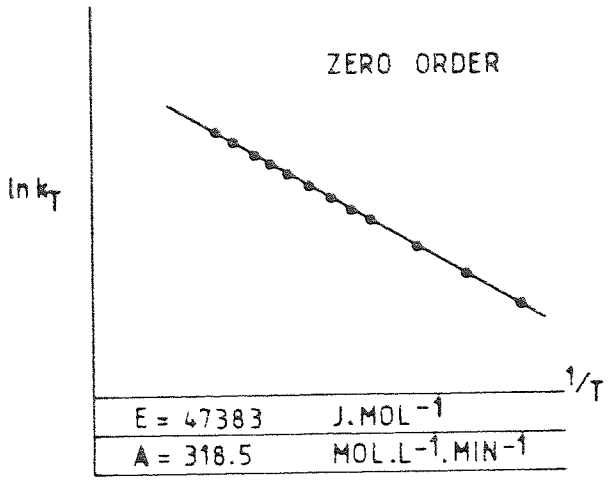
* 95% confidence interval

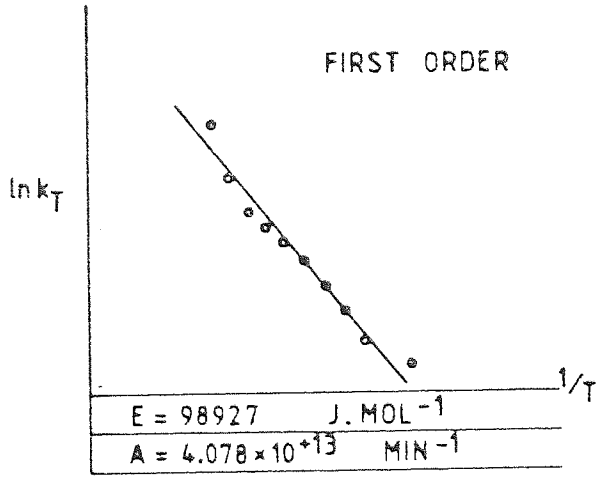
Table 5.8 The Reproducibility of the NONISO Treatment for Penicillin V in pH 9 Borate Buffer

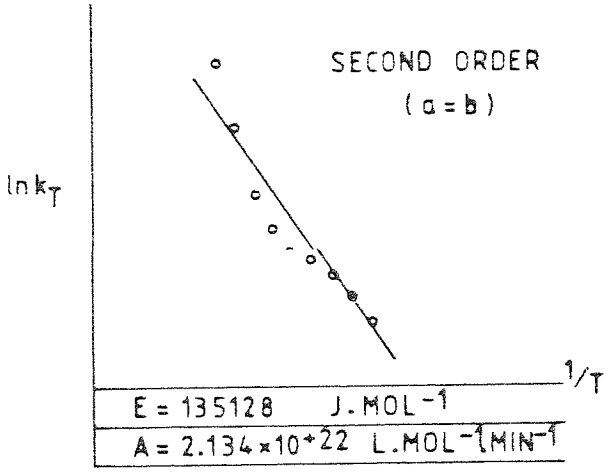
From the results in table 5.8, the reproducibility of NONISO can be seen to be good. The predicted rate constants at 25°C and 50°C for the three experiments, and hence shelf-lives at these temperatures (not shown in table 5.8), are in close agreement.

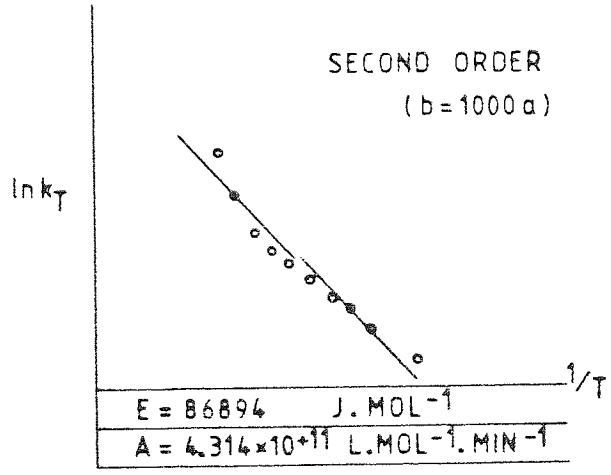
5.5.3 The Effect of Reaction Order Model on the Linearity of the Arrhenius Plot

"Ideal" C_t vs t data sets were generated for zero, first and second









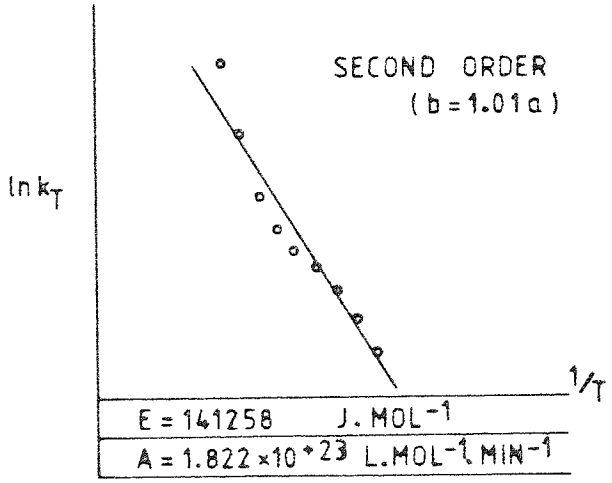


FIGURE 5.4 "IDEAL" NONISOTHERMAL DATA SET FOR ZERO ORDER KINETIC MODEL ANALYSED USING NONISO

order (a = b) reactions, based on the time / temperature profile in table 5.1, using the following approach :

Zero, first and second order (a = b) NONISO treatment of the experimental data in table 5.1 produced calculated $k_T.t$ values for zero, first and second order (a = b) models. These calculated $k_T.t$ values gave perfectly linear Arrhenius plots. Polynomial expressions for $k_T.t$ *vs* t were generated for these three models and then integrated :

$$\int (\text{polynomial}) . dt = f(C_t) + f(C_o) \qquad \text{equation 5.11}$$

where $f(C_t)$ for the different models is given in table 5.2. $f(C_o)$, the transform of the initial concentration, is known. Solving equation 5.11 for different t values provides "ideal" C_t data for the three reaction models.

These "ideal" data sets (for zero, first and second [a = b] order models), were analysed by NONISO, using zero, first, second (a = b) and second ($a \neq b$) order models. The results, graphically illustrated in figures 5.4, 5.5 and 5.6 show that order selection can be made from the linearity of the Arrhenius plots for ln k *vs* 1/T.

5.5.4 Error Analysis

When all experimental points are used in the computation (typically 25 to 30), the major source of error occurs in the calculation of rate constants at the extremes of the temperature range. Inspection of the graphical presentation rapidly reveals any deviation of any point(s) from the bulk of the data and recalculation with the omission of the ill-fitting data increases the validity of the parameter estimates. For this reason, points which correspond to degradation in excess of 95%

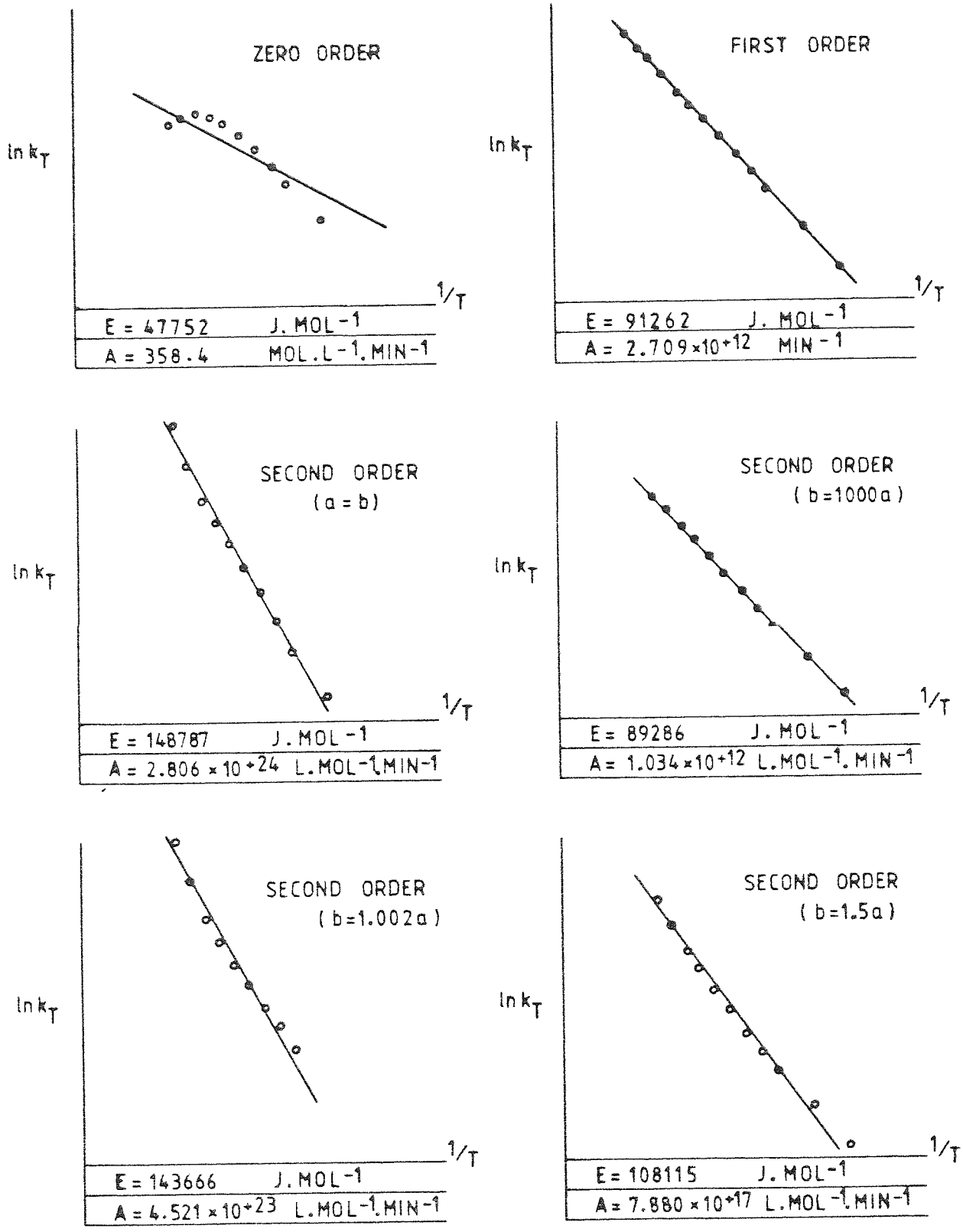


FIGURE 5.5 "IDEAL" NONISOTHERMAL DATA SET FOR FIRST ORDER KINETIC MODEL ANALYSED USING NONISO

for a first order process or in excess of 80% for a zero or second order process, are automatically eliminated from the data set. The elimination of points determined in the initial stages of the degradation is left to the discretion of the operator. The reduced data set may be re-calculated,

(i) in part via the Arrhenius subroutines

(ii) in total through the generation of a new polynomial expression linking $f(C_t)$ with time.

Option (i) is satisfactory if the analytical error is small and has been adopted in all calculations reported in this thesis. This option is selected in NONISO when ill-fitting data is sorted.

The effect of incorporating a ± 2% random error into the concentration values in table 5.1 was investigated. The results of the NONISO analysis of 5 data sets can be seen in table 5.9.

E_{act} ($J.mol^{-1}$) From Data Set					Mean E_{act}	Coefficient
1	2	3	4	5	($J.mol^{-1}$)	of Var.(%)
111674	94420	90161	93714	85814	95157	10.3

Table 5.9 The Effect of Incorporating a ± 2% Random Error in C_t on the Calculation of E_{act} (First Order Treatment, All Data Points)

The large effect of this random error is due to the small concentration differences in the initial data points. Reducing the data set to C_t values of < 90% of C_o decreases the effect of the ± 2% random error (table 5.10).

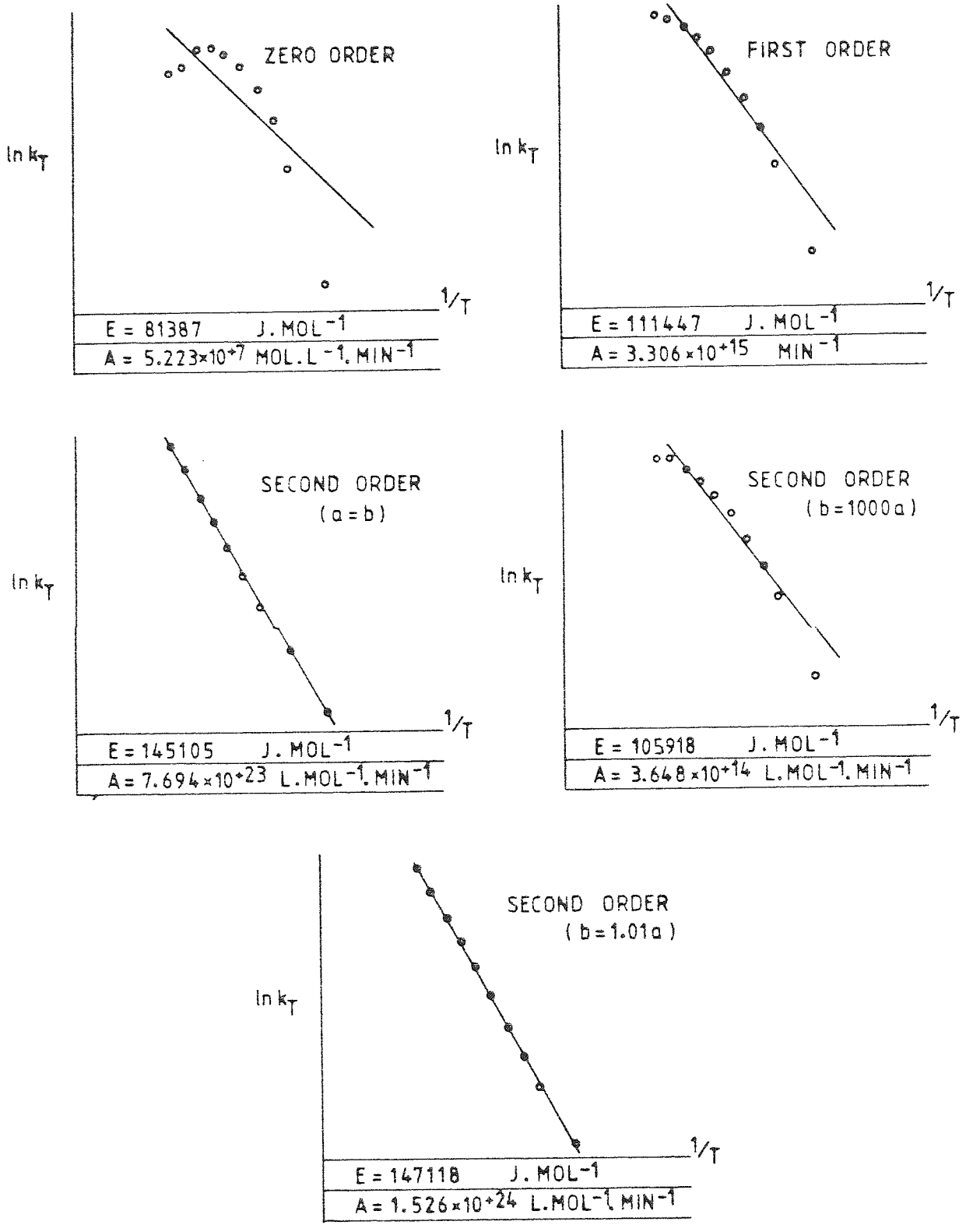


FIGURE 5.6 "IDEAL" NONISOTHERMAL DATA SET FOR SECOND ORDER (a=b) KINETIC MODEL ANALYSED USING NONISO

E_{act} ($J.mol^{-1}$) From Data Set					Mean E_{act} ($J.mol^{-1}$)	Coefficient of Var.(%)
1	2	3	4	5		
91655	86647	90850	88772	88662	89317	2.2

Table 5.10 The Effect of Incorporating a 2% Random Error in C_t on the Calculation of E_{act} (First Order Treatment, Data points < 90% of C_o)

5.5.5 Validation of NONISO

In order to validate NONISO, the data obtained for the nonisothermal hydrolysis of penicillin V in pH 9 borate buffer (three determinations) was analysed using NONISO and the FORTRAN IV program described by Madsen et. al. (168). The results, and those from a traditional isothermal study, are recorded in table 5.11.

EXPERIMENT	TEMPERATURE RANGE (^{o}C)	DIFFERENTIAL (NONISO)				INTEGRAL (MADSEN)			
		E_{act} (J/mol)	A ($x10^{-14}h^{-1}$)	PREDICTED k_T ($x10^{2}h^{-1}$)		E_{act} (J/mol)	A ($x10^{-14}h^{-1}$)	PREDICTED k_T ($x10^{2}h^{-1}$)	
				$25^{o}C$	$50^{o}C$			$25^{o}C$	$50^{o}C$
NONISOTHERMAL Penicillin V pH 9									
1	28 - 81	91024 (413)*	1.496 (0.205)*	1.697	29.07	91209 (43)+	1.588 (o.o24)+	1.673	28.81
2	31 - 78	90966 (1125)*	1.345 (0.444)*	1.562	26.71	90076 (71)+	0.977 (0.025)+	1.625	27.02
3	31 - 60	89496 (2305)*	0.809 (0.464)*	1.700	27.76	94494 (157)+	5.045 (0.028)+	1.412	26.95
ISOTHERMAL Penicillin V pH 9	3 - 72	86485 (2282)*	0.261 (0.152)*	1.848	27.46				

* 95% confidence interval + standard deviation units

Table 5.11 Comparison of Differential (NONISO) and Integral (Madsen's) Calculations of Nonisothermal Data for Penicillin V Degradation at pH 9

Agreement between Madsen's treatment and NONISO treatment of the nonisothermal data is good for this data, as is the agreement between the nonisothermal calculation of E_{act} (by both NONISO and Madsen's method) and the calculation of E_{act} by traditional isothermal means.

To further validate NONISO, data taken from literature sources were re-calculated using NONISO and Madsen's program (table 5.12). Literature sources were :

Roger's paper for the decomposition of riboflavine in 0.05 N NaOH (158)

Zoglio's paper (165) for the nonisothermal degradation of p-acetamidophenol and procainamide.

Madsen's paper for the decomposition of riboflavine in 0.1 N NaOH (168).

Again, agreement between the calculated values of E_{act} was good.

EXPERIMENT	TEMPERATURE RANGE (^{o}C)	DIFFERENTIAL (NONISO)				INTEGRAL (MADSEN'S)			
		E_{act} (J/mol)	A ($x10^{-14} h^{-1}$)	PREDICTED k_T ($x10^{2} h^{-1}$)		E_{act} (J/mol)	A ($x10^{-14} h^{-1}$)	PREDICTED k_T ($x10^{2} h^{-1}$)	
				$25^{o}C$	$50^{o}C$			$25^{o}C$	$50^{o}C$
NONISOTHERMAL Riboflavine (168)	21 - 71	82552 (2420)* 85201#	0.1075 (0.064)* 0.288#	3.718	48.89	84888 (242)+	0.2558 (0.02)+	3.448	48.77
NONISOTHERMAL Riboflavine (158)	19 - 55	76461 (6184)* 74683#	7.368 x10-3)	2.975	32.32	80444 (1481)+	3.453 x10-2	2.796	34.40
NONISOTHERMAL p-Acetamidophenol (165)	35 - 83	67245 (4632)* 71128#	5.282 x10-7	8.745 x10-3	7.131 x10-2	68810 (3284)+	9.232 x10-7	8.129 x10-3	6.961 x10-2
NONISOTHERMAL Procainamide HCl (165)	35 - 83	129513 (9232)* 121336#	4.57 $x10^{3}$	9.328 x10-4	5.311 x10-2	120967 (3396)+	2.338 $x10^{2}$	1.50 x10-3	6.539 x10-2

* 95% Confidence Interval + standard deviation units # literature values

Table 5.12 Comparison of Differential (NONISO) and Integral (Madsen's) Calculation of Nonisothermal Data for Riboflavine, p-Acetamidophenol and Procainamide HCl

5.5.6 The Applicability of NONISO to Formulated Products

To test the applicability of NONISO to formulated products, data from the nonisothermal degradation of two commercially available penicillin V syrups (nominal concentration 62.5 mg per 5 ml) were analysed using NONISO and Madsen's (168) program (table 5.13). Isothermal results for one of the syrups was also included for comparison, as were nonisothermal results for the degradation of penicillin V in pH 6 citrate buffer.

A high degree of similarity was observed between shelf-life estimates from NONISO, Madsen's integral approach and the isothermal method.

All three were applicable to the study of the degradation of penicillin V in penicillin V syrups.

EXPERIMENT	TEMPERATURE RANGE (^{o}C)	DIFFERENTIAL (NONISO)				INTEGRAL (MADSEN'S)			
		E_{act} (J/mol)	A ($x10^{-12} h^{-1}$)	PREDICTED $t_{90\%}$		E_{act} (J/mol)	A ($x10^{-12} h^{-1}$)	PREDICTED $t_{90\%}$	
				$25^{o}C$ (d)	$50^{o}C$ (h)			$25^{o}C$ (d)	$50^{o}C$ (h)
SYRUP 1 :									
NONISOTHERMAL	60 - 94	82717 (967)*	0.251 (0.07)*	5.4	9.8	86951 (789)+	1.043 (0.26)+	7.2	11.5
ISOTHERMAL	25 - 50	80250	0.107	4.7	9.2				
SYRUP 2 :									
NONISOTHERMAL	61 - 95	83945 (1788)*	0.557 (0.25)*	4.0	7.0	83992 (356)+	0.560 (0.07)+	4.0	8.9
		85840 (2169)*	1.115 (0.58)*	4.3	7.1	87091 (254)+	1.684 (0.14)+	4.7	7.5
SOLUTION (pH 6)									
NONISOTHERMAL	60 - 95	92610 (6804)*	2.639 (2.37)*	27.9	37.2	92169 (5470)+	2.251 (3.72)+	27.4	37.0

* 95% confidence interval + standard deviation units

Table 5.13 Nonisothermal Parameter Estimates for Formulated Penicillin V Syrups

5.6 CONCLUSIONS

The advantages of a nonisothermal approach to the study of degradation kinetics include the availability of a full kinetic profile from one single experiment. The assumption is made that E_{act} and the mechanism of reaction do not change over the temperature range chosen.

Equilibrium reactions (non-zero equilibria) have not been dealt with by this approach.

NONISO, a derivative nonisothermal method developed during the study of nonisothermal kinetics, gave similar results to those of Madsen's integral method (168) and isothermal methods, for the degradation of penicillin V, in pH 9 and pH 6 buffers and in formulated syrups.

NONISO provides a rapid, versatile method for the analysis of nonisothermal kinetic parameters. It requires a microcomputer with 32 K RAM for its utilisation.

CHAPTER SIX : RECONSTITUTED ANTIBIOTIC SYRUPS : DOSAGE AND STABILITY

6.1 INTRODUCTION

Oral liquid penicillin formulations are widely prescribed as a means of increasing patient acceptibility. Penicillins are generally too labile for an adequate shelf-life to be assigned to a liquid preparation by a manufacturer. Such preparations are formulated as granules for reconstitution, incorporate a sugar base (usually sucrose) and require the addition of a fixed volume of water. The reconstituted syrup may be in the form of a solution, for example penicillin V, or a suspension, for example ampicillin. A seven or fourteen day expiry date is normal on such preparations.

The stability profile of penicillins (33, 174 - 176) is influenced by pH, buffer salts, buffer concentration, ionic strength (177 - 181), carbohydrates (182 - 186), trace metals (187) and solubility (when suspensions are formulated). The excipients in the granules thus have a profound effect on the stability of the reconstituted preparation, although the shelf-life of well-formulated products is satisfactory (188 - 191). These studies, however, assume that the product is dispensed in accordance with the manufacturers' recommendations. When dilutions of products are required, there is scope for procedural variations. These may lead to an incorrect composition of the vehicle, causing possible stability problems, or, in extreme cases, the antibiotic content of the preparation may vary widely from that required.

To estimate the implications of these problems, the drug content and stability of penicillin V and ampicillin syrups, prepared by different procedures from commercial granules, were examined.

6.2 EXPERIMENTAL

6.2.1 MATERIALS AND METHODS

Antibiotic Granules

Two brands of penicillin V granules (PVA and PVB) and three brands of ampicillin granules (AMC, AMD and AME), all with a labelled content of 125 mg per 5 ml when reconstituted, were purchased. The unreconstituted granules were separated into two fractions of equal weight. Each half was then reconstituted to 100 ml by adding the appropriate diluent. The volume of the diluent was calculated as follows :

a) $x + 0.5 (100 - x)$ ml of water

b) $0.5\ x$ ml of water and 50 ml of Syrup B.P.

c) $x + 0.5 (100 - x)$ ml of Syrup B.P.

where x is the volume of water added to the preparation to produce 100 ml of a full-strength preparation, following the manufacturers' instructions.

Syrups containing 62.5 mg per 5 ml (penicillin V) and 125 mg per 5 ml (ampicillin) were diluted as recommended by the manufacturer and were used as controls.

Chemical Analysis

Chemical analysis of the syrups was undertaken by H.P.L.C. Details of the system and eluent composition can be found in chapter 3, section 3.3.

Approximately 1g of syrup, accurately weighed, was transferred to a volumetric flask (25 ml). Internal standard (5 ml) was pipetted into the flask and water added to volume. 20 microlitre of this solution was

rapidly injected onto the H.P.L.C. column. The internal standards were:

penicillin V	-	phenol (0.5 mg / ml) in water
ampicillin	-	caffeine citrate (0.5 mg / ml) in water

Quantification was achieved by interpolation onto calibration lines prepared from standards of the penicillins.

The stability of the syrups was assessed by storage at 25°C and measuring the residual concentration of penicillin over a period of time. The ampicillin preparations were shaken occassionally to improve the homogeneity of the suspension.

The concentration of ampicillin in solution in the supernatant liquid was also determined. A homogeneous sample was centrifuged at 3000 r.p.m. for 30 minutes, the solution filtered through a millipore filter (0.8 micron) and the filtrate (approximately 1g) assayed as before.

pH values were measured at 25°C using a Radiometer (Copenhagen) PHM 64 Research pH meter.

6.3 CALCULATIONS

Sampling by weight rather than by volume avoided errors associated with pipetting viscous liquids. However, this necessitated calculation of the sample volume before the results obtained with different diluents could be compared. Sample volume is given by :

$$\text{Sample volume} = \frac{\text{Sample weight}}{\text{Specific Gravity (S.G.) of sample}}$$

The specific gravity of the sample can either be measured directly or calculated :

$$\text{S.G. of sample} = \frac{\text{Wt. of granules + Wt. of Syrup BP + Wt. of water}}{\text{Volume of syrup (100 ml)}}$$

$$\text{Wt. of Syrup BP} = \text{S.G. of Syrup BP (1.324)} \times \text{Volume of Syrup BP}$$

First order rate data were analysed using :

$$C_t = C_o.\exp(-k_1.t)$$

$$t_{10\%} = 0.1054 / k_1$$

$$\text{shelf-life} = \frac{\ln (C_o / 11.25)}{k_1}$$

where C_o is the initial concentration (mg / ml) of penicillin

C_t is the concentration at time t (hours)

k_1 is the first order degradation rate ($hours^{-1}$)

Zero order rate data were analysed using :

$$C_t = C_o - k_o.t$$

$$t_{10\%} = C_o / 10.k_o$$

$$\text{shelf-life} = (C_o - 11.25) / k_o$$

where C_o is the initial concentration (mg/ml) of penicillin

C_t is the concentration at time t (h)

k_o is the zero order degradation rate (mg/h)

6.4 RESULTS AND DISCUSSION

Penicillin syrups are normally reconstituted by the addition of a measured volume of water, sufficient to produce 100 ml of the final preparation. Dilutions, to produce a half-strength preparation, are usually made by the addition of equal volumes of Syrup BP to the full-strength product. An alternative procedure is to divide the granules into two lots, by weight or by eye, and to make the half-weight granules up to 100 ml. These half-strength preparations are not made to the manufacturers' specification and are only equivalent to the correctly made dilution if :

a) the granules are homogeneous so that division of the contents produces two identical lots

b) the correct diluent is used. This is not water, which produces a vehicle containing too little sucrose, nor Syrup BP which gives a vehicle containing too much sucrose. The true diluent is a diluted form of Syrup BP, the composition of which depends upon the volume of water needed for reconstitution of the original granules.

6.4.1. Penicillin Content of Diluted Syrups

Tables 6.1 and 6.2 contain typical results for the penicillin V content of diluted syrups. These were prepared from the top and bottom portions of various batches of granules, separated by weight, as may occur in general practice dispensing. All preparations are 1:1 dilutions of 125 mg / 5ml full-strength syrups and the values quoted are those found in a recommended 5 ml dose, nominally 62.5 mg.

Sample Number	Concentration (mg/5ml) of Penicillin V in Syrup Prepared From :		Percentage of Labelled Content (%)
	Top	Bottom	
PVA - 1	61.1	77.7	110.0
PVA - 2	67.3	68.2	108.4
PVA - 3	62.3	70.4	106.2
PVA - 4	56.2	81.4	110.1
PVA - 5	68.4	72.7	112.9
PVA - 6	69.7	73.1	114.2
Mean	64.2	73.9	110.5
*Co.var.(%)	8.09	6.56	2.65
**95% C.L.	5.45	5.09	3.07

*Coefficient of variance **95% confidence limits (+/-)

Table 6.1 Penicillin V Content of Diluted Syrups Prepared From Top and Bottom Fractions of Granules : Product A

Sample Number	Concentration (mg/5ml) of Penicillin V in Syrup Prepared From		Percentage of Labelled Content (%)
	Top	Bottom	
PVB - 1	62.5	63.0	100.4
PVB - 2	61.0	60.2	97.0
Mean	61.8	61.6	98.7
Co.var(%)	1.72	3.21	2.44

Table 6.2 Penicillin V Content of Diluted Syrups Prepared From Top and Bottom Fractions of Granules : Product B

It is apparent that significant segregation of the granule components has occurred. The extreme example is PVA-4 in which the syrup prepared from the top layer has 89.9% of the nominal penicillin content while that from the bottom layer contains 130.2% of the expected level. The Pharmaceutical Codex 1979 refers to the B.P.C. 1973 (36) for a standard concerning penicillin V syrups, in which the content of total penicillins in the preparation should be 100.0 to 125.0 % of the prescribed or stated concentration of penicillin V. Assuming penicillin V is the only penicillin present in the preparation, Syrups PVA-1, PVA-3, PVA-4 (because of segregation of the penicillin in the granules) and PVB-2 (because of low penicillin content) fail this standard. Although the homogeneity of samples PVB-1 and PVB-2 was good, the lack of overage in the samples provides less tolerance of segregation.

Results from ampicillin syrups can be found in tables 6.3, 6.4 and 6.5.

Sample Number	Concentration (mg/5ml) of Ampicillin in Syrup Prepared From		Percentage of Labelled Content (%)
	Top	Bottom	
AMC - 1	52.9	71.7	99.7
AMC - 2	125.1	1.8	101.5
AMC - 3	128.2	<0.5	103.0
AMC - 4	66.3	60.1	101.1
AMC - 5	51.8	69.9	97.4
AMC - 6	<0.5	121.1	97.3
AMC - 7	100.3	21.0	97.0
AMC - 8	58.0	70.0	102.4
AMC - 9	57.0	65.5	98.0
AMC - 10	58.0	70.0	102.4
AMC - 11	55.5	71.5	101.6
AMC - 12	54.5	71.0	100.4
AMC - 13	45.5	75.5	96.8
Mean	65.7	59.2	99.9
Co.var.(%)	52.2	55.9	2.31
95% C.L.	20.7	20.0	1.39

Table 6.3 Ampicillin Content of Diluted Syrups Prepared From Top and Bottom Fractions of Granules : Product C

Sample Number	Concentration (mg/5ml) of Ampicillin in Syrup Prepared From		Percentage of Labelled Content (%)
	Top	Bottom	
AMD - 1	62.4	59.9	97.8
AMD - 2	59.1	60.6	95.8
AMD - 3	60.2	59.5	95.8
AMD - 4	60.5	59.1	95.7
AMD - 5	60.2	59.5	95.8
AMD - 6	60.9	61.7	98.1
AMD - 7	62.9	64.4	101.8
AMD - 8	61.8	61.6	98.7
AMD - 9	60.2	66.0	101.0
Mean	60.9	61.4	97.8
Co.var.(%)	2.00	3.89	2.38
95% C.L.	0.94	1.84	1.79

Table 6.4 Ampicillin Content of Diluted Syrups Prepared From Top and Bottom Fractions of Granules : Product D

Sample Number	Concentration (mg/5ml) of Ampicillin in Syrup Prepared From		Percentage of Labelled Content (%)
	Top	Bottom	
AME - 1	61.1	60.9	97.6
AME - 2	61.1	65.2	101.0
Mean	61.1	63.1	99.3
Co.var.(%)	-	4.82	2.42

Table 6.5 Ampicillin Content of Diluted Syrups Prepared From Top and Bottom Fractions of Granules : Product E

The samples of product C that were analysed in this study show a high degree of segregation of the antibiotic within the granules. This was so marked that in three cases (AMC-2, AMC-3 and AMC-6) almost all of the penicillin was located in one half of the granules. Preparations made by an inappropriate method may thus contain almost no penicillin or up to double the recommended dose.

The Pharmaceutical Codex 1979 refers to the B.P.C. 1973 (36) for a standard concerning ampicillin syrups, in which the content of ampicillin in the preparation should be 90.0 to 120.0 % of the prescribed or stated concentration. Syrups AMC-1, AMC-2, AMC-3, AMC-5, AMC-6, AMC-7, AMC-11, AMC-12 and AMC-13, when prepared by separation of the dry granule before reconstitution, do not conform to this requirement. The total ampicillin content in the whole syrup, however, is well within the required range.

Ampicillin products D and E show no significant segregation of the penicillin and all comply with the official standard.

From the results for penicillin V and ampicillin syrups presented above, it is clear that the practice of separation of the dry granules before reconstitution is to be deprecated. However, one possible solution to this problem might be adequate mixing of the granules followed by sample division. Although this still does not comply with the manufacturers' directions, it would give some improvement in dose uniformity.

6.4.2 Penicillin Stability in Diluted Syrups

The shelf-life is assigned to a reconstituted product on the assumption that reconstitution is in accordance with the manufacturers directions. Diluted products may have a different stability profile due to the changes in concentration of the excipients (buffers, ionic strength, sugars) and the effects may be enhanced by the diluent chosen (water, Syrup BP, diluted Syrup BP).

6.4.2.1 Penicillin V Syrups

Tables 6.6 and 6.7 record stability data for penicillin V syrups prepared using three different diluents; and table 6.8 contains comparative data from an undiluted commercial preparation containing 62.5 mg/5ml of penicillin V.

Sample	Temp. (°C)	First Order Rate Constant ($hours^{-1} x10^{3}$) ± 95% C.L. For Syrup Prepared With : Water	Water / Syrup BP	Syrup BP	* $t_{20\%}$ (days)	** Shelf-life (days)	Initial pH
PVA-1 (Top)	25	0.862 (0.114)			10.8	4.9	5.95
PVA-1 (Bot)	25	0.859 (0.097)			10.8	15.6	5.98
PVA-2 (Top)	25		1.067 (0.190)		8.7	7.2	5.92
PVA-2 (Bot)	25		1.130 (0.192)		8.2	7.1	5.91
PVA-3 (Top)	25			1.061 (0.140)	8.8	4.0	5.85
PVA-3 (Bot)	25			1.044 (0.095)	8.9	9.0	5.83
PVA-4 (Top)	40	3.481 (0.278)			2.7	0	5.55
PVA-4 (Bot)	40	3.458 (0.296)			2.7	4.5	5.54
PVA-5 (Top)	40		5.161 (0.187)		1.8	1.6	5.52
PVA-5 (Bot)	40		4.966 (0.215)		1.9	2.2	5.53
PVA-6 (Top)	40			5.490 (0.293)	1.7	1.6	-
PVA-6 (Bot)	40			5.449 (0.328)	1.7	2.0	-

* time for penicillin V content to fall to 80% of original value

** time for penicillin V content to fall to 90% of labelled claim

Table 6.6 First Order Degradation Rates of Diluted Penicillin V Syrups : Product A

Sample	Temp. (°C)	First Order Rate Constant ($hours^{-1} x10^{3}$) ±95% C.L. For Syrup Prepared With : Water	Water / Syrup BP	Syrup BP	$t_{20\%}$ (days)	Shelf-life (days)	Initial pH
PVB-1 (Top)	25	1.007 (0.057)			9.2	4.4	5.33
PVB-1 (Bot)	25	1.019 (0.093)			9.1	4.6	5.32
PVB-2 (Top)	25		1.203 (0.145)		7.7	2.8	5.20
PVB-2 (Bot)	25		1.183 (0.163)		7.9	2.4	5.16

Table 6.7 First Order Degradation Rates of Diluted Penicillin V Syrups : Product B

Sample	Temp (^{o}C)	First Order Rate Constant ($h^{-1}x10^{3}$) +/- 95% C.L. for Penicillin V Syrups Prepared According Manufact. Instruct.	$t_{20\%}$ (days)	Shelf-life (days)	Initial pH
*PVA-7	25	0.914 (+/- 0.147)	10.2	9.0	5.92
*PVA-8	40	4.612 (+/- 0.277)	2.0	2.9	-
**PVA-9	25	0.911 (+/- 0.061)	10.2	8.6	5.73
**PVA-10	40	4.032 (+/- 0.289)	2.3	2.0	5.73
**PVA-11	50	11.56 (+/- 0.071)	0.8	0.8	5.80

* batch 1 ** batch 2

The rate constants for batch 2 were used to calculate the energy of activation and frequency factor for this syrup :

Activation Energy (E_{act}) = 80.9 $kJ.mol^{-1}$

Frequency Factor (A) = 1.354×10^{11} h^{-1}

Correlation coefficient (r) = - 0.999

Table 6.8 First Order Degradation Rates of Undiluted Penicillin V Syrups (62.5 mg/5ml) : Product A

The standard for penicillin V syrup (36) states that, after seven days storage at $15^{o}C$, the concentration of total penicillins in the stored syrup should not be less than 80% of the concentration found in the freshly prepared syrup. Using the Arrhenius parameters calculated for syrups PVA 9-11, in table 6.8, it is possible to calculate the time taken for the undiluted syrups PVA 9-11 to reach 80% of their initial concentration at $15^{o}C$:

$$\ln k_T = \ln A - \frac{E_{act}}{R.T}$$

At 15°C : $$\ln k_T = 25.6315 - \frac{80900}{8.314 \times 288.2}$$

$$k_T = 2.940 \times 10\text{-}4 \quad h^{-1}$$

$$t_{20\%} = 31.6 \text{ days}$$

This is well within the limits of the standard (7.0 days). Even at 25°C, the standard is complied with ($t_{20\%}$ = 10.2 days - table 6.8). Choice of diluent for the syrups has a significant effect on the degradation rate of penicillin V. Dilution with water (PVA-1) produces a slightly more stable syrup than the undiluted syrup (PVA-7 and 9), but using water/Syrup BP or Syrup BP increases the degradation rate (PVA-2 and 3). This trend is also seen at 40°C (PVA-4 to 6 and PVA-8 and 10). It is likely that this difference in stability is due to the level of sucrose in the diluted syrup. When water is used alone, the sucrose level is lower than that of the normal syrup, and this results in a more stable preparation. As the sucrose level increases, the stability decreases, although there is little difference between the syrups prepared with water/Syrup BP and Syrup BP. Recent work has shown that carbohydrates catalyse penicillin degradation by providing a reactive nucleophilic centre which attacks the beta-lactam ring (175). Syrups prepared from product B (table 6.7) similarly indicate a more stable preparation is obtained if water is used as diluent rather than water/Syrup BP. Here, however, the degradation rates of the diluted syrups are greater than with product A. This is probably explained by the lower pH of the syrups made from product B.

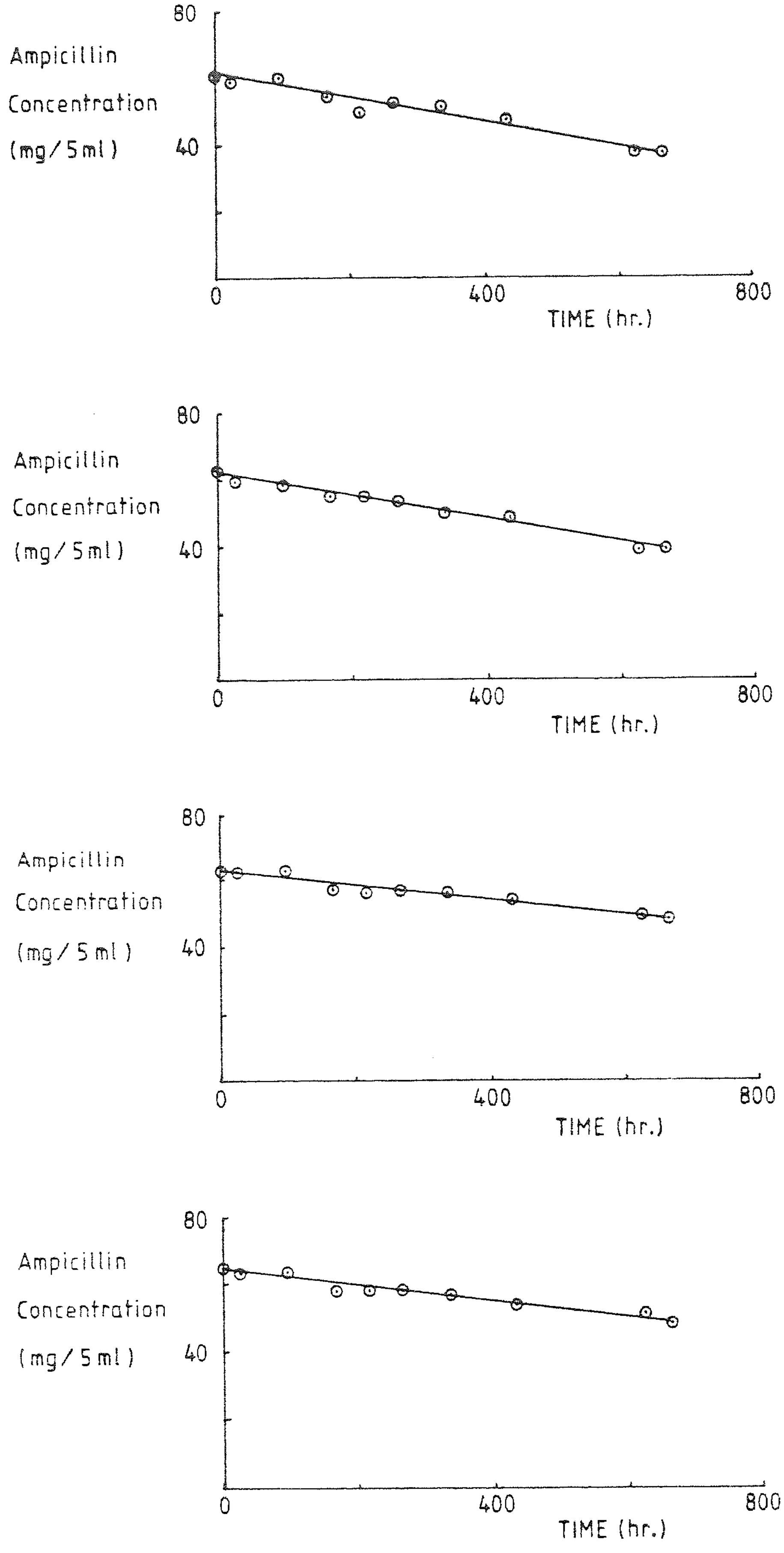


FIGURE 6.1 PSEUDO-ZERO ORDER DEGRADATION PROFILE OF AMPICILLIN SYRUP PRODUCT D

Although diluent selection has been shown to affect the stability of the diluted syrups, none of the diluted syrups, whichever diluent is selected, fail to meet the B.P.C. 1973 standard on stability requirements (36). This is illustrated in tables 6.6 and 6.7, where the $t_{20\%}$ value is >7 days at 25°C for all the diluted syrups.

Segregation of penicillin V in the dry powder had no significant effect on the degradation rate or the initial pH of the diluted syrups, in the samples studied, e.g. PVA-4 (table 6.9).

PVA-4	Initial Conc. (mg/5ml)	Initial pH	First Order Rate Constant ($h^{-1} \times 10^{3}$)
Top	56.2	5.55	3.481
Bottom	81.4	5.54	3.458

Table 6.9 Effect of Penicillin V Segregation on the Initial pH and Degradation Rate Constant for Syrup PVA-4

6.4.2.2 Ampicillin Syrups

Stability data for ampicillin syrup product D produced pseudo-zero order degradation profiles (figure 6.1). Concentration and pH data for these syrups are in tables 6.10 and 6.11.

AMD-6 (Top)		AMD-6 (Bot.)		AMD-7 (Top)		AMD-7 (Bot.)	
Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)
0.0	60.55	0.0	62.20	0.0	63.10	0.0	65.00
22.6	58.85	22.7	59.85	22.7	62.65	22.7	63.85
93.6	60.10	93.3	58.45	93.4	62.90	93.4	64.05
165.5	54.45	165.5	54.90	166.1	57.25	166.1	58.05
211.1	49.75	211.1	55.10	211.2	56.30	211.2	58.30
262.6	52.65	262.7	53.80	264.0	57.25	264.0	58.10
332.2	51.80	332.7	50.00	334.1	56.40	334.1	57.05
428.6	47.85	428.7	48.50	428.7	54.25	428.7	54.20
622.7	37.90	622.7	38.90	622.7	49.40	622.7	51.40
662.2	37.45	662.2	39.10	662.2	48.30	662.2	48.05

Table 6.10 Concentration vs Time Data for Ampicillin Syrup : Product D

AMD-6 (Top)		AMD-6 (Bot.)		AMD-7 (Top)		AMD-7 (Bot.)	
Time(hr)	pH	Time(hr)	pH	Time(hr)	pH	Time(hr)	pH
0.0	4.50	0.0	4.47	0.0	4.58	0.0	4.59
169.3	4.29	169.2	4.29	169.1	4.47	169.0	4.46
308.2	4.26	308.2	4.25	308.1	4.44	308.0	4.42
458.4	4.19	458.3	4.44	458.3	4.44	458.2	4.41
648.0	4.11	648.0	4.09	648.0	4.38	648.0	4.38

Table 6.11 pH vs Time Data for Ampicillin Syrup : Product D

Pseudo-zero order degradation is expected for ampicillin syrups because ampicillin (present as the trihydrate in product D) is not completely soluble in the syrup vehicle. Hence when ampicillin degrades in solution it is replaced by the dissolution of solid ampicillin, maintaining a constant concentration of the penicillin in the vehicle. Degradation rates for product D diluted syrups (table 6.12) show that using water rather than water/Syrup BP as diluent results in a preparation with increased degradation rate and decreased shelf-life.

Sample	Storage Temp. (^{o}C)	Zero Order Rate Constant ($mg.hr^{-1}x10^{3}$) for Syrup Prepared With Diluent : Water	Water/SyrupBP	$t_{10\%}$ (days)	Shelf-life (days)
AMD-6(Top)	25	6.92		7.3	5.6
AMD-6(Bot)	25	6.87		7.5	6.6
AMD-7(Top)	25		4.37	12.0	12.7
AMD-7(Bot)	25		4.65	11.5	14.6

Table 6.12 Zero Order Degradation Rates of Diluted Ampicillin Syrups : Product D

The B.P.C. standard for ampicillin syrups (36) states that, after 7 days storage at $15^{o}C$, the concentration of ampicillin in the stored syrup is not less than 90% of the concentration found in the freshly prepared syrup. All of the diluted syrups in table 6.12 comply with this standard at $25^{o}C$. The shelf-life (the time taken for the syrup to reach 90% of its initial concentration) of the preparations was less

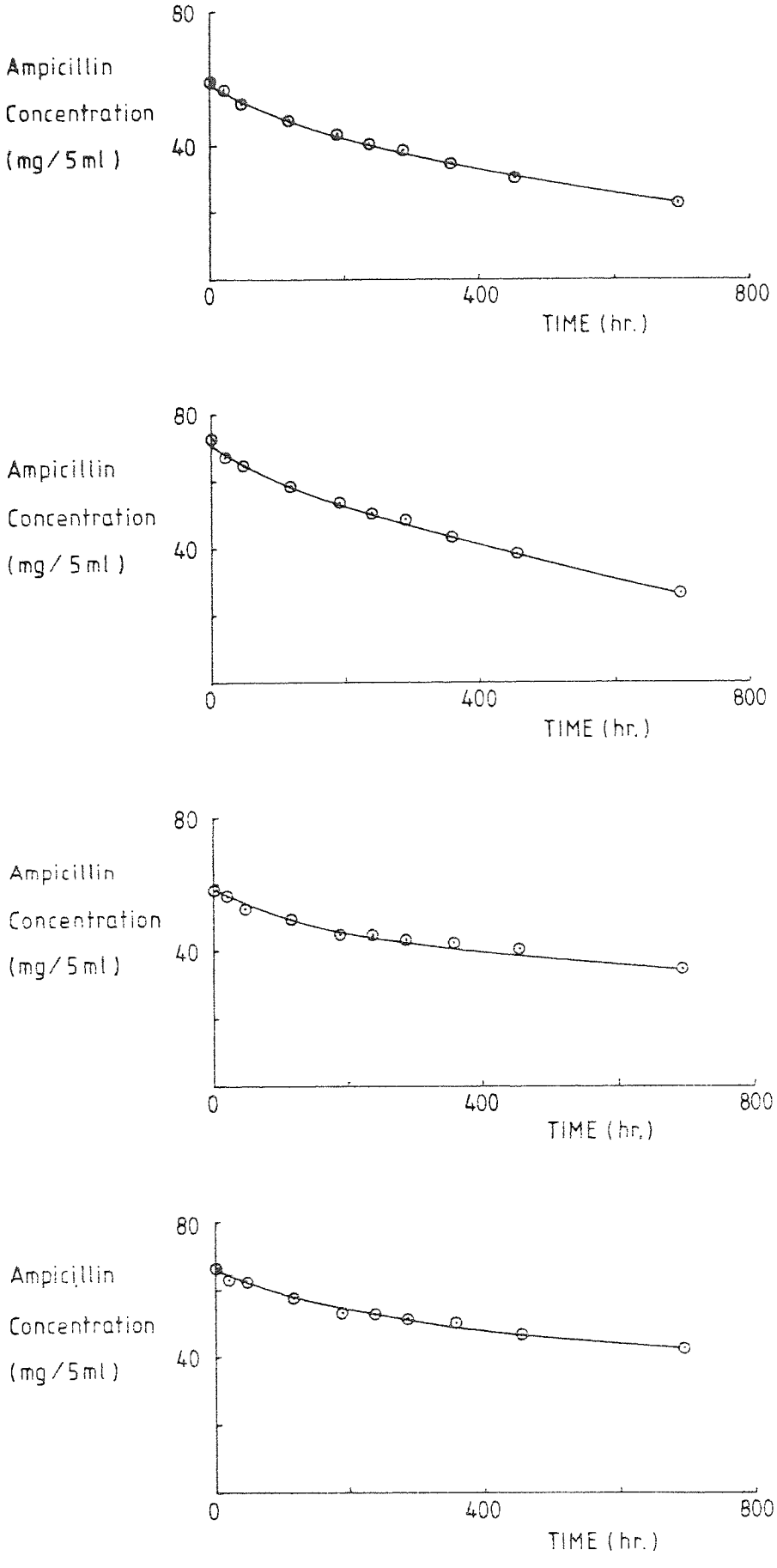


FIGURE 6.2 NON-LINEAR DEGRADATION PROFILE OF AMPICILLIN SYRUP PRODUCT C

than 7 days for the syrups diluted with water. Although the temperature of storage was higher than required in the B.P.C. standard, many medicines are stored at 25°C or higher, despite cautionary labelling on the containers. Thus choice of diluent is important to the syrup's efficacy.

In contrast to product D, degradation profiles produced from product C were not linear (figure 6.2), suggesting that pseudo-zero order degradation did not take place. Concentration and pH data for these syrups can be found in tables 6.13 and 6.14.

AMC-8 (Top)		AMC-8 (Bot)		AMC-9 (Top)		AMC-9 (Bot)		AMC-14	
Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)
0.0	58.45	0.0	72.15	0.0	58.00	0.0	66.10	0.0	130.6
21.9	56.35	21.8	67.05	23.0	56.15	22.9	63.05	23.9	126.5
46.7	52.45	46.7	64.50	46.8	52.65	46.8	62.20	47.8	122.2
117	47.85	116	58.30	116	49.80	116	57.90	119	116.6
190	43.35	190	53.95	189	45.00	189	53.10	190	110.2
236	40.20	236	50.85	235	44.40	235	52.95	237	109.5
285	38.40	285	48.70	287	43.80	287	51.65	288	106.4
357	34.20	357	43.65	357	42.05	357	50.15	358	105.6
454	30.00	454	38.75	453	40.15	453	46.75	454	98.00
693	22.35	693	26.35	692	34.65	693	42.25		

Table 6.13 Concentration vs Time Data for Ampicillin Syrup : Product C

AMC-8 (Top)		AMC-8 (Bot)		AMC-9 (Top)		AMC-9 (Bot)		AMC-14	
Time (hr)	pH	Time (hr)	pH	Time (hr)	pH	Time (hr)	pH	Time (hr)	pH
0.0	5.82	0.0	5.85	0.0	5.95	0.0	5.92	0.0	6.16
195	5.17	195	5.18	194	5.30	194	5.38	195	5.46
334	5.01	334	5.04	333	5.18	333	5.20	334	5.34
485	4.95	484	4.95	484	5.14	483	5.19	484	5.28
674	4.93	674	4.91	674	5.12	674	5.14	674	5.24

Table 6.14 pH vs Time Data for Ampicillin Syrups : Product C

Shelf-life and $t_{10\%}$ measurements were made graphically rather than mathematically since pseudo-zero order degradation was not followed. The results are in table 6.15.

Sample	Diluent	Storage Temp. (°C)	$t_{10\%}$ (days)	Shelf-life (days)
AMC-8 (Top)	Water	25	2.3	0.5
AMC-8 (Bot)	Water	25	2.9	6.5
AMC-9 (Top)	Water/Syrup BP	25	2.0	0.0
AMC-9 (Bot)	Water/Syrup BP	25	2.4	5.4
AMC-14	Control	25	4.5	7.3

Table 6.15 Degradation Rate Estimates for Diluted Ampicillin Syrups : Product C

Syrup AMC - 14 was included as a control in the degradation study. Ideally a syrup of nominal concentration 62.5 mg/5 ml would have been used, but ampicillin syrups of this concentration were not available. Thus a syrup of nominal concentration 125 mg/5ml was reconstituted as recommended by the manufacturer and stored under the same conditions as the diluted syrups.

From table 6.15, the stability of diluted product C was totally inadequate for storage at 25°C, 10% of the original potency being lost in less than 3 days. Stability of the control (AMC-14) was higher although even this product lost 10% of it's original potency in 4.5 days.

There was no significant difference in the degradation rates for ampicillin syrups diluted with water or water/Syrup BP ($t_{10\%}$ values). Shelf-life data was varied because of significant segregation of ampicillin in the dry powder before reconstitution (table 6.13). Despite this obvious segregation, there was no significant difference between the pH of the two halves of the reconstituted syrup (table 6.14). There appears to be a correlation between initial concentration and $t_{10\%}$ values; the higher the initial concentration the higher the $t_{10\%}$ value. Although this would be expected, the limited data does not make this observation significant.

The increased $t_{10\%}$ value for the control compared to the diluted syrups is probably explained by the increased concentration of ampicillin; a greater proportion of the antibiotic being present as solid in the suspension, hence less available for hydrolytic degradation.

In order to further understand the suspension systems, the above experiments were repeated on products C and D with the addition that the concentration of ampicillin in solution was also determined. Results for product D are in tables 6.16, 6.17, 6.18 and 6.19.

AMD-8 (Top)		AMD-8 (Bot)		AMD-9 (Top)		AMD-9 (Bot)		AMD-10	
Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)
0.0	61.50	0.0	61.95	0.0	60.50	0.0	66.00	0.0	124.0
25.5	61.35	25.5	61.10	26.0	60.35	26.0	65.85	25.5	124.0
72.5	58.70	72.5	58.00	72.5	57.50	73.5	63.50	72.5	121.0
95.5	58.13	95.5	57.85	95.5	57.65	96.0	63.30	95.5	121.1
145	56.35	145	56.25	145	56.70	146	62.50	145	119.4
216	53.10	216	53.35	216	54.75	217	60.3	218	117.0
238	53.20	238	53.50	242	55.80	242	60.1	237	116.7

Table 6.16 Whole Sample Concentration vs Time Data for Ampicillin Syrup : Product D

AMD-8 (Top)		AMD-8 (Bot)		AMD-9 (Top)		AMD-9 (Bot)		AMD-10	
Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)
21.0	26.65	21.0	26.60	22.0	20.25	22.0	20.05	22.0	24.20
47.5	26.20	47.5	26.20	48.0	19.40	51.0	18.75	47.5	24.15
92.5	25.85	92.5	26.00	93.5	20.65	93.5	20.90	92.5	-
147	26.25	147	26.00	123	19.85	123	19.70	123	24.25
193	26.90	193	26.75	195	20.40	195	20.05	193	24.95
238	25.55	238	25.85	240	20.75	240	20.60	238	24.00

Table 6.17 Supernatant Concentration vs Time Data for Ampicillin Syrup : Product D

AMD-8 (Top)		AMD-8 (Bot)		AMD-9 (Top)		AMD-9 (Bot)		AMD-10	
Time (hr)	pH	Time (hr)	pH	Time (hr)	pH	Time (hr)	pH	Time (hr)	pH
3.0	4.42	3.0	4.41	3.0	4.59	3.0	4.59	3.0	4.44
28.0	4.42	28.0	4.42	28.0	4.59	28.0	4.60	28.0	4.46
56.0	4.35	56.0	4.34	56.0	4.53	56.0	4.55	56.0	4.39
98.0	4.30	98.0	4.31	98.0	4.51	98.0	4.51	98.0	4.39
148	4.27	148	4.27	148	4.47	148	4.48	148	4.37
198	4.27	198	4.27	198	4.45	198	4.48	198	4.38
247	4.15	247	4.18	247	4.38	247	4.40	247	4.30

Table 6.18 pH vs Time Data for Ampicillin Syrup : Product D

Sample	Storage Temp. (^{o}C)	Zero Order Rate Constant ($mg.hr^{-1}x10^{3}$) for Syrup Prepared With Diluent : Water	Water/Syrup BP	Whole Sample $t_{10\%}$ (days)	Whole Sample Shelf-life (days)
AMD-8(Top)	25	7.57		6.8	6.1
AMD-8(Bot)	25	7.31		7.0	6.1
AMD-9(Top)	25		4.43	11.3	7.4
AMD-9(Bot)	25		5.07	10.8	16.0
AMD-10	25	Control	6.45	16.0	15.0

Table 6.19 Zero Order Degradation Rates of Diluted Ampicillin Syrups : Product D

The pseudo-zero order degradation rate constants and $t_{10\%}$ values for AMD-8 and AMD-9 were similar to those obtained previously for this product (table 6.12). As expected, undiluted product D (AMD-10) had a larger $t_{10\%}$ value than the diluted syrups due to its increased ampicillin concentration. Shelf-life estimates for the two halves of AMD-9 were not the same because of segregation of ampicillin in the dry powder (table 6.4). There was a significant difference between the supernatant concentration in the diluted syrups prepared with water (mean of 26.3 mg/5ml) and water/Syrup BP (mean of 20.1 mg/5ml). This partly explains why AMD-8 was less stable than AMD-9, more ampicillin being in solution, therefore available in a higher concentration for hydrolytic breakdown.

The solubility of ampicillin trihydrate (the form of ampicillin in product D) in water is 35 mg/5ml at 30°C (192) and 40 mg/5ml at 37°C (193). Even allowing for the decreased storage temperature of AMD-8 and AMD-9, these values suggest the ampicillin supernatant concentrations in AMD-8 and AMD-9 were lower than expected. However, it has been shown that buffer salt concentration affects the solubility of ampicillin (179) so the low levels were probably due to excipient effects. The difference in solubility between AMD-8 and AMD-9 was not due to pH (see tables 6.17 and 6.18).

Hou and Poole (33, 179) stated that the stability of ampicillin in buffer solutions was highest at or near the isoelectric point, approximately pH 4.85 (33). Earlier Saccani and Pansera (194) reported the apparent stability of ampicillin in solution at 27°C in buffer solution to be greatest at pH 4.4. Thus the pH of minimum degradation for ampicillin in diluted syrups is probably 4.4 to 4.85. The initial pH of diluted product D ranged from 4.41 to 4.59 (tables 6.11 and 6.18), corresponding to values of maximum stability. During storage, the pH dropped slightly but this did not appear to have a significant

effect on the rate of degradation.

Results for product C are in tables 6.20, 6.21, 6.22 and 6.23.

AMC-12 (Top)		AMC-12 (Bot)		AMC-13 (Top)		AMC-13 (Bot)	
Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)
0.0	54.35	0.0	69.65	0.0	45.55	0.0	75.65
26.5	51.30	26.5	67.35	27.0	43.00	27.0	72.25
73.5	46.65	73.5	61.30	55.5	40.45	55.5	70.65
96.0	44.90	96.0	59.55	75.0	39.10	75.0	67.75
146	41.50	146	54.85	97.0	38.35	97.0	66.35
195	38.45	195	51.60	147	35.90	147	62.95
242	36.80	245	51.40	197	34.10	197	61.95
				217	33.45	218	61.45
				245	33.55	245	12.26

Table 6.20 Whole Sample Concentration vs Time Data for Ampicillin Syrups : Product C

AMC-12 (Top)		AMC-12 (Bot)		AMC-13 (Top)		AMC-13 (Top)	
Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)	Time (hr)	Concent. (mg/5ml)
21.0	43.75	21.0	46.95	25.0	23.40	25.0	25.75
50.0	41.55	50.0	44.00	51.0	22.80	51.0	24.80
93.5	39.35	94.5	41.95	94.5	20.95	94.5	22.75
125	39.10	147	40.60	125	20.60	125	22.15
195	39.10	197	41.55	197	20.10	197	21.40
219	38.05	219	40.40	219	20.00	244	21.70
240	37.65	244	40.45	244	20.80		

Table 6.21 Supernatant Concentration vs Time Data for Ampicillin Syrup : Product C

AMC-12 (Top)		AMC-12 (Bot)		AMC-13 (Top)		AMC-13 (Bot)	
Time (hr)	pH	Time (hr)	pH	Time (hr)	pH	Time (hr)	pH
3	5.98	3	6.00	3	6.04	3	6.13
28	5.86	28	5.89	28	5.95	28	6.05
56	5.65	56	5.70	56	5.80	56	5.89
98	5.41	98	5.47	98	5.59	98	5.71
148	5.26	148	5.31	148	5.42	148	5.43
198	5.17	198	5.22	198	5.32	198	5.43
247	5.11	247	5.13	247	5.24	247	5.35

Table 6.22 pH vs Time Data for Ampicillin Syrup : Product C

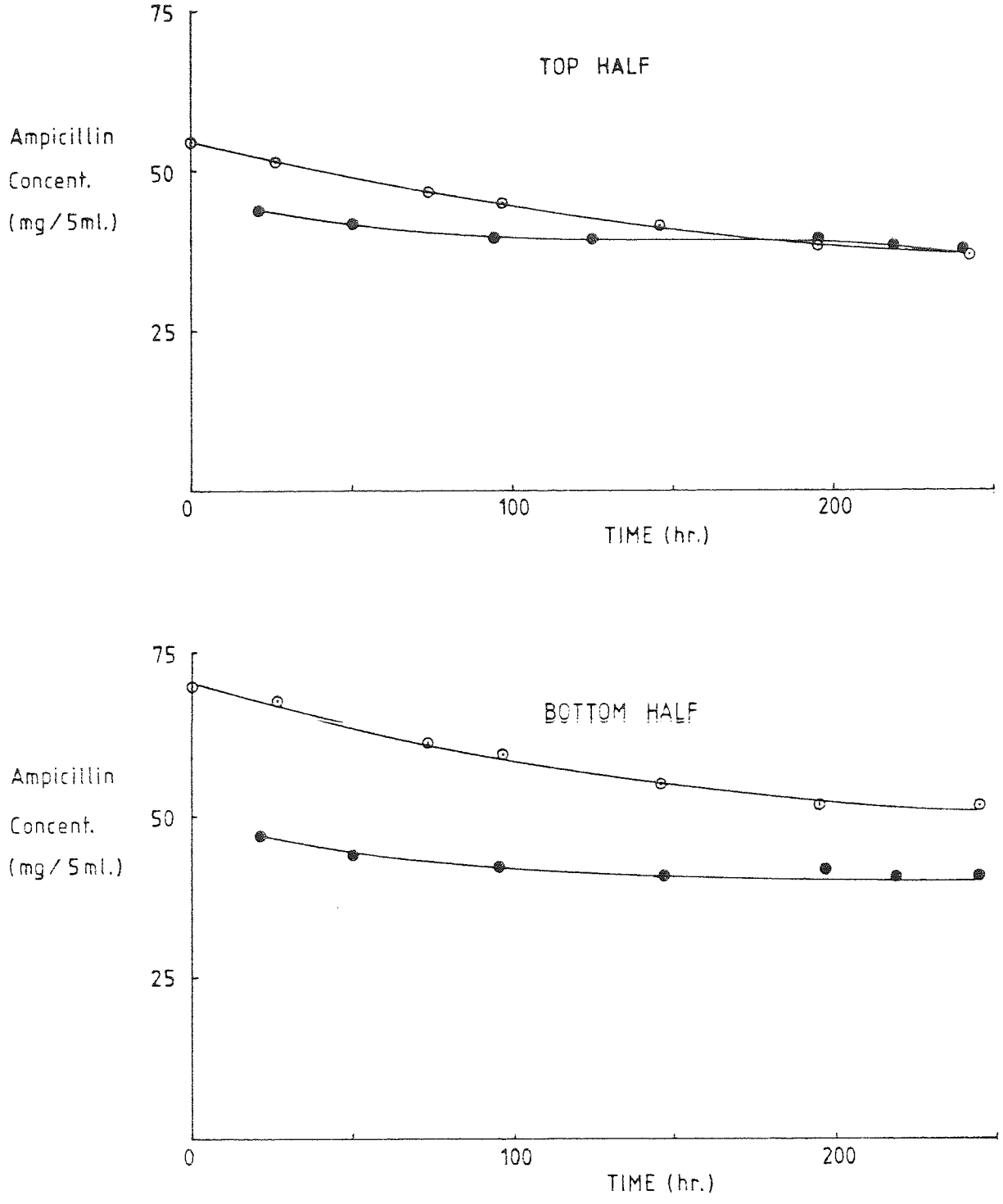


FIGURE 6.3 CHANGE IN AMPICILLIN CONCENTRATION ON STORAGE OF AMC-12 AT 25°C; ⊙ WHOLE SYRUP; ● SUPERNATANT

Sample	Diluent	Storage Temp. (°C)	$t_{10\%}$ (days)	Shelf-life (days)
AMC-12 (Top)	Water	25	2.0	0.0
AMC-12 (Bot)	Water	25	2.1	5.1
AMC-13 (Top)	Water/Syrup BP	25	2.2	0.0
AMC-13 (Bot)	Water/Syrup BP	25	3.2	>10.0

Table 6.23 Degradation Rate Estimates for Diluted Ampicillin Syrups : Product C

Concentration vs time profiles for AMC-12 and AMC-13 (whole sample and supernatant concentration) can be seen in figures 6.3 and 6.4. The $t_{10\%}$ values for AMC-12 and AMC-13 were similar to those obtained previously (table 6.15), the high value for AMC-13 (Bot) being explained by the high ampicillin concentration. Shelf-life data was varied because of significant segregation of ampicillin in the dry powder (table 6.3). The supernatant concentrations in AMC-12 and AMC-13 were higher than in AMD-8 and AMD-9. This was due to three factors :

(i) the form of ampicillin

(ii) pH (to a lesser extent)

(iii) the effect of excipients

Ampicillin is present as the anhydrate in product C. The solubility of the anhydrate is 55 mg/5ml at 30°C (192) and 50 mg/5ml at 37°C (193), significantly higher than the trihydrate (product D). Hou and Poole (179) found the minimum solubility of ampicillin occurred at pH 4.9, it's isoelectric point, although there was little change in solubility between pH 4 and 6. The initial pH of product C was approximately 6.0,

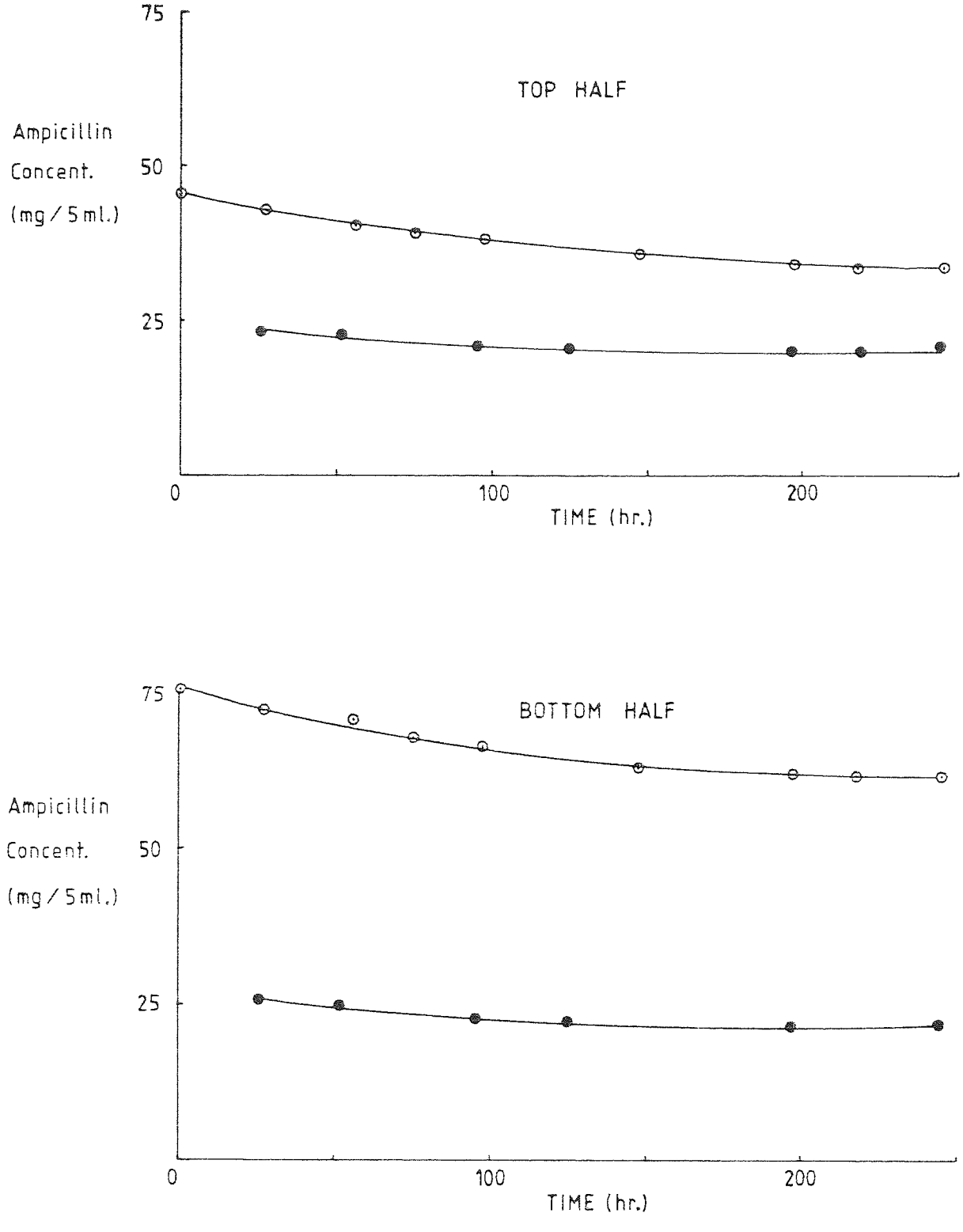


FIGURE 6.4 CHANGE IN AMPICILLIN CONCENTRATION ON STORAGE OF AMC-13 AT 25°C; ⊙ WHOLE SYRUP ; ● SUPERNATANT

further away from the isoelectric point than product D (initial pH approximately 4.4). As with product D, excipients modify the solubility of ampicillin, illustrated by the difference in supernatant concentration between AMC-12 and AMC-13.

Unlike product D, the supernatant concentrations and the degradation rate constants of product C change with time (figures 6.3 and 6.4). This is due to the change in pH of product C with time (table 6.22). As the diluted syrups degrade, the pH approaches the isoelectric point. This causes :

(a) the solubility of ampicillin to decrease

(b) the stability of ampicillin to increase

These two effects combine to produce the observed profiles in figures 6.3 and 6.4.

It is possible to estimate the degradation rate of ampicillin in solution in the different syrup preparations, by assuming the supernatant concentration to be constant during the time of estimation (e.g. time taken to degrade by 10%) and calculating the $t_{10\%}$ value for ampicillin in solution :

$$t10\%(sup.) = \text{time to reach} \left(\text{initial conc.} - \frac{\text{supernatant conc.}}{10} \right)$$

Sample	t10%(supernatant) (days)
AMD - 8 (Top)	2.9
AMD - 8 (Bot.)	3.0
AMD - 9 (Top)	4.1
AMD - 9 (Bot.)	3.3
AMD - 10	3.2
AMC - 12 (Top)	1.6
AMC - 12 (Bot.)	1.7
AMC - 13 (Top)	1.0
AMC - 13 (Bot.)	1.1

Table 6.24 Estimation of Ampicillin Degradation in Solution : Products C and D

From table 6.24, ampicillin in product D appears more stable in solution when the syrup is diluted with water/Syrup BP. The opposite is true for product C. Bundgaard and Larsen (186) have shown that the rate of sucrose accelerated penicillin degradation increases as the pH increases from 6 upwards. Because of their respective initial pH's, product C is more likely than product D to show sucrose catalysis. This could explain the results for AMC-12 and AMC-13 in table 6.24.

6.5 CONCLUSIONS

The penicillin content and stability of diluted antibiotic syrups depended markedly upon the techniques used to prepare the syrups. When granules were portioned before reconstitution, variations in dosage ranging from almost no antibiotic to a double-strength preparation resulted. The stability of the product was also dependent upon the diluent used. Penicillin V preparations became less stable when diluted with solutions containing a higher concentration of sugar. The effect of sugar content on the stability of ampicillin preparations was more complex, due to the system being a suspension rather than a solution. One product (product D) was found to be more stable when diluted with water/Syrup BP rather than water. However, the other product (product C) showed little difference in stability for syrups diluted with water or water/Syrup BP, all of the diluted preparations showing a very high rate of degradation at 25°C.

CHAPTER SEVEN : DISSOLUTION AND STABILITY OF BENZATHINE CLOXACILLIN

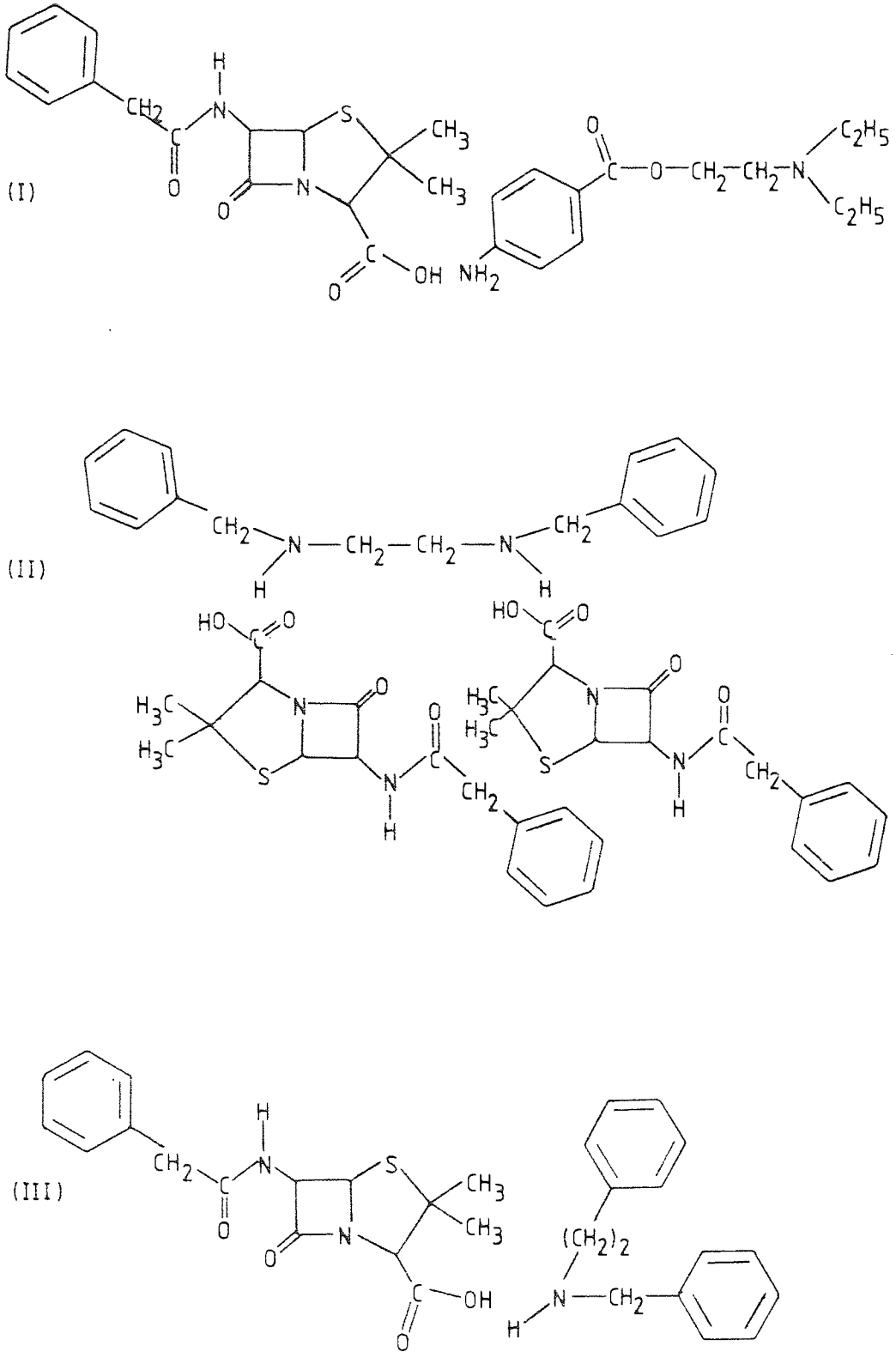


FIGURE 7.1 STRUCTURES OF PROCAINE PENICILLIN G (I); BENZATHINE PENICILLIN G (II) AND BENETHAMINE PENICILLIN G (III)

7.1 INTRODUCTION

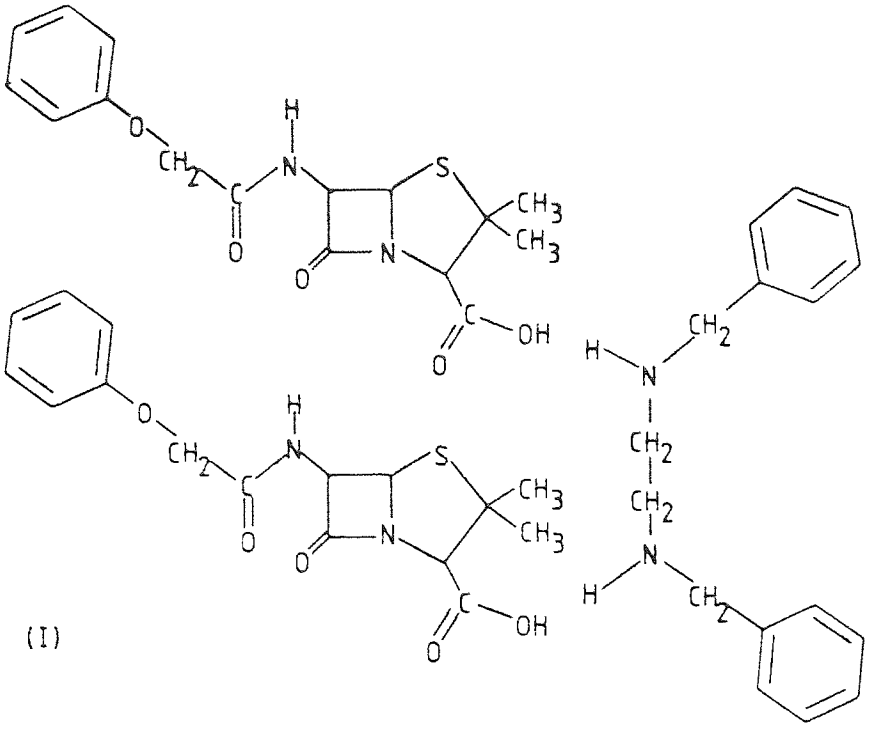
Following its clinical introduction, penicillin G was found to produce rapid, high plasma levels (195). This necessitated frequent injection of the drug in order to maintain an effective antibiotic concentration in the blood. A solution to this problem was provided by the development of depot or repository forms of penicillin G.

The first repository form consisted of a combination of amorphous calcium penicillin in oil and beeswax (196). This soon fell from favour due to adverse reactions, such as the production of sterile abscesses, following intramuscular injection. It was superceded by procaine penicillin G (197, figure 7.1), a salt of penicillin G. The low aqueous solubility (approximately 0.7%) of this salt resulted in slow release of penicillin G and hence sustained blood levels.

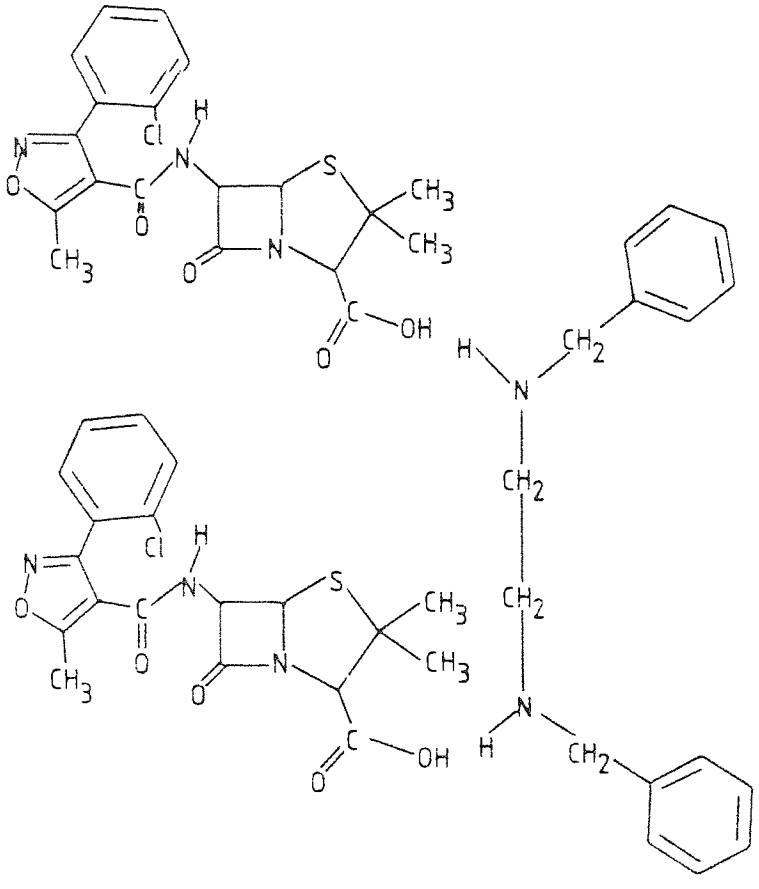
N,N'-dibenzylethylenediamine dipenicillin (benzathine penicillin G, figure 7.1), prepared in 1952 (198), had an even lower aqueous solubility (approximately 0.02%) and provided sustained release for longer periods. Like procaine penicillin G, the amine salt possessed local anaesthetic properties (195), reducing the pain often associated with intramuscular injection. Due to the low solubility of benzathine penicillin G, which retarded degradation of the penicillin when presented as an aqueous suspension, an oral form of this penicillin salt became the first aqueous oral penicillin commercially available (195).

The complexation of penicillins with amines similar to procaine and benzathine resulted in a wide range of depot penicillins such as benethamine penicillin G (199, figure 7.1), benzathine penicillin V (200, figure 7.2) and benzathine cloxacillin (figure 7.2).

An understanding of the physicochemical properties of depot release drugs is necessary to predict and explain *in vivo* release



(I)



(II)

FIGURE 7.2 STRUCTURES OF BENZATHINE PENICILLIN V (I) AND BENZATHINE CLOXACILLIN (II)

characteristics of these salts. However, little information about these properties has appeared in the scientific literature, except for solubility measurements (199, 201 - 203) and degradation rates (202, 198). For this reason, the dissolution and stability of a depot release penicillin, benzathine cloxacillin , was studied. Benzathine cloxacillin is used as an intramammary injection in the treatment of bovine mastitis (204).

7.2 EXPERIMENTAL

7.2.1 MATERIALS

Benzathine cloxacillin, benzathine diacetate, cloxacillin sodium and penicillin V potassium were used as received from Beecham Pharmaceuticals Research Division Laboratories, Worthing, West Sussex. All other materials were of reagent grade. Water was double distilled in an all-glass system.

pH 2 citrate buffer was prepared by dissolving 6.345g of citric acid in 1 litre of water containing 60.4 ml M sodium hydroxide and 69.8 ml M hydrochloric acid.

pH 6 citrate buffer was prepared by dissolving 12.6g of citric acid in 1 litre of water containing 6.4g of sodium hydroxide.

pH 9 borate buffer was prepared by dissolving 10.33g boric acid in 1 litre of water containing 83.5 ml M sodium hydroxide and 16.5 ml M hydrochloric acid.

7.2.2 METHODS

7.2.2.1 Analytical Assay

Details of all H.P.L.C. methods can be found in chapter 3.

7.2.2.2 Dissolution Procedure

A 250 ml three-necked flask, fitted with a glass thermometer and a teflon sampling tube, was placed in a water bath at 34 +/- 0.1^{o}C. A glass stirring rod, driven at 150 r.p.m., was passed through the third neck. The initial volume of dissolution medium was 230 ml. Once this medium had reached 34^{o}C, a weighed amount of benzathine cloxacillin or benzathine penicillin V was added to the flask and the dissolution started. Two to three ml samples were withdrawn from the flask, using a

glass syringe, and filtered (Millipore type AA, 0.8 μm). Samples, where necessary to obtain the correct analytical concentration, were diluted with water immediately. The analytical concentration of benzathine at pH 2 was less than 108 mg per litre and less than 250 mg per litre at pH 6 and 9. Dilution was not necessary at pH 6 and did not exceed 1 ml to 11 ml at pH 2 and 9.

7.2.2.3 Sieve Analysis of Benzathine Cloxacillin

Sieve analysis was carried out using 33 mm diameter brass sieves with phosphor bronze mesh. Mechanical agitation was provided by an "Endrock" shaker. Sieves and shaker were manufactured by Endecotts Ltd. of London.

All sieves were weighed empty before each analysis. 1.0g of benzathine cloxacillin was placed in the top sieve with the remaining sieves underneath, in order of reducing mesh size. The stack of sieves vas then mechanically agitated for seven minutes. Each sieve was re-weighed individually at the end of this period to calculate the weight fraction retained.

7.2.2.4 Solubility Measurements

The solubilities of benzathine and cloxacillin were measured by adding excess material to the appropriate buffer and stirring the solution rapidly. Where necessary, the pH of the resulting solution was adjusted to the original value by addition of either concentrated hydrochloric acid or concentrated sodium hydroxide. The solution was then placed in a 250 ml flask and stirred at 100 r.p.m. for two days (benzathine) or 30 minutes (cloxacillin). After measurement of pH to ensure that it was at the correct value, the solution was diluted to the analytical concentration (approximately 700 mg per litre for cloxacillin and 200 mg per litre for benzathine) and assayed by H.P.L.C. against standards prepared in the same solvent.

7.2.2.5 Determination of the Stability of Benzathine

pH 2 :

36.4 mg benzathine diacetate were dissolved in 230 ml pH 2 citrate buffer at 51°C. The solution was stirred at 150 r.p.m. whilst maintaining the temperature, and sampled at 2, 10, 35 and 245 minutes. The samples were assayed against benzathine diacetate standard freshly prepared in pH 2 buffer, using H.P.L.C.

pH 6 and pH 9 :

120 mg benzathine diacetate were dissolved in 100 ml of the appropriate buffer at 34°C. The solutions were maintained at 34°C in a water bath sampled at 10, 60 and 180 minutes. The samples were assayed against freshly prepared benzathine diacetate standard, dissolved in the appropriate buffer, using H.P.L.C.

7.2.2.6 Determination of the Degradation Rate of Cloxacillin

pH 2 : (without benzathine)

82.2 mg cloxacillin sodium were added to 230 ml of pH 2 citrate buffer at 34°C. The solution was filtered (Millipore type AA 0.8 µm) to remove precipitated degradation products and placed in a 250 ml flask in a water bath at 34°C. The solution was stirred at 150 r.p.m. Samples were withdrawn using a glass syringe, filtered (Millipore type AA 0.8 µm) to remove any precipitate and assayed against cloxacillin sodium standard in water, by H.P.L.C.

pH 2 : (with benzathine)

39.0 mg benzathine and 76.6 mg cloxacillin sodium were dissolved in 230 ml pH 2 buffer at 34° C, filtered (Millipore type AA 0.8 µm), then treated as above.

pH 6 :

72.1 mg and 71.6 mg cloxacillin sodium were dissolved in two 100 ml volumes of pH 6 citrate buffer at 34°C. These solutions were placed in

a water bath at 34°C and assayed after 166 hours against cloxacillin sodium standard dissolved in pH 6 citrate buffer, by H.P.L.C.

pH 9 :

326.1 mg cloxacillin sodium were dissolved in 100 ml of borate buffer at 34°C. The solution was maintained at 34°C and assayed, after dilution (1 to 6) with water, against cloxacillin sodium standard dissolved in the same solvent, using H.P.L.C. Samples were taken at 0, 138 and 336 minutes.

7.2.2.7 Determination of the Degradation Rate of Penicillin V at pH 9

51.3 and 51.8 mg penicillin V potassium were dissolved in two 100 ml volumes of pH 9 borate buffer at 34°C. After 600 minutes at 34°C, these solutions were assayed against freshly prepared penicillin V potassium standard dissolved in pH 9 borate buffer, by H.P.L.C.

7.2.2.8 Reaction Experiments Between Benzathine and Penicillins at pH 9

Benzathine and Cloxacillin

752 mg cloxacillin sodium were dissolved in 230 ml pH 9 borate buffer at 34°C and stirred at 150 r.p.m. After dissolution, 276 mg benzathine diacetate were added. The resulting solution was stirred, maintained at 34°C and sampled periodically. The samples were filtered (Millipore type AA 0.8 µm), diluted 1 ml to 6 ml, by adding 1 ml of sample to 5 ml of water, and assayed by H.P.L.C. against a freshly prepared standard mixture of 30 mg benzathine diacetate and 70 mg cloxacillin sodium in 100 ml sample solvent (30 ml pH 9 borate buffer added to 150 ml water).

Benzathine and Penicillin V

469.2 mg penicillin V potassium were dissolved in 230 ml pH 9 borate buffer at 34°C and stirred at 150 r.p.m. After dissolution, 217.4 mg benzathine diacetate were added. The resulting solution was stirred and kept at 34°C. Samples were filtered, diluted 1 ml to 11 ml with water

and assayed by H.P.L.C. against a freshly prepared standard mixture of 25 mg benzathine diacetate and 50 mg penicillin V in 250 ml sample solvent (50 ml pH 9 borate buffer added to 500 ml water).

7.2.2.9 Preparation of Benzathine Cloxacillin Crystals

Two solutions, one containing 4.6g cloxacillin sodium in 900 ml water and the other 1.8g benzathine diacetate in 100 ml water, were mixed slowly with rapid stirring, at ambient temperature. A fine precipitate immediately appeared. The mixture was left for 42 hours, filtered (Millipore type AA 0.8 μm) and the crystals dried in a vacuum dessiccator. The pH of the supernatant after filtration was 6.0. The yield after drying was 5.4g. Microscopic examination showed the material to be predominantly crystalline.

7.2.2.10 Preparation of Benzathine Penicillin V

3.9g penicillin V potassium were dissolved in 700 ml of water. 1.8g of benzathine diacetate were dissolved in 100 ml water. These solutions were slowly mixed with rapid stirring at ambient temperature. A fine precipitate immediately appeared. The mixture was left for 4 hours, then filtered (Millipore type AA 0.8 μm) and dried in a vacuum des iccator. pH of the supernatant when filtered was 6.2. The yield after drying was 6.9g.

7.2.2.11 Preparation of Reaction Product From Benzathine and Cloxacillin at pH 9

2.5g cloxacillin sodium and 1.8g benzathine diacetate were dissolved in 900 ml and 100 ml, respectively, of pH 9 borate buffer. The two solutions were slowly mixed whilst being rapidly stirred, and then placed in a water bath at 34^{o}C for three days. The precipitate produced was filtered (Millipore type AA 0.8 μm) and dried in a vacuum

dessicator. The yield was 2.92g. H.P.L.C. analysis indicated that this product was >95% pure, impurities being benzathine, cloxacillin and some degradation products of cloxacillin.

7.2.2.12 Reaction Between Benzathine and Degraded Cloxacillin at pH 9

818 mg cloxacillin sodium were dissolved in 250 ml pH 9 borate buffer and left for seven days at 34°C. H.P.L.C. analysis showed this solution contained <0.4% cloxacillin. 280mg benzathine diacetate were added to 230 ml of this solution and the solution stirred at 150 r.p.m. Samples were taken at various time intervals and assayed, after dilution (1 ml to 6 ml with water), against a standard solution of 25 mg benzathine diacetate in 100 ml sample solvent.

7.3 RESULTS AND DISCUSSION

7.3.1 Characterisation of Benzathine Cloxacillin Powder

Microscopic Examination :

The powder appeared as an amorphous material with a wide particle size range.

Sieve Analysis :

Sieve Mesh Size (μm)	Weight Retained By Sieve (mg)			Mean Weight Retained (mg) (+/- 95% C.L.)
	Analysis 1	Analysis 2	Analysis 3	
500	17	17	23	19 (9)
425	22	21	26	23 (7)
355	39	42	51	44 (16)
300	56	71	73	67 (23)
250	79	73	85	79 (15)
212	75	85	89	83 (18)
180	111	137	138	129 (38)
150	155	179	150	161 (38)
125	157	151	143	151 (18)
90	182	160	174	172 (27)
63	70	57	50	59 (24)
53	16	11	8	11 (10)
38	9	6	5	7 (5)
<38	2	2	1	2 (2)

Table 7.1 Sieve Analysis Results for Benzathine Cloxacillin Powder

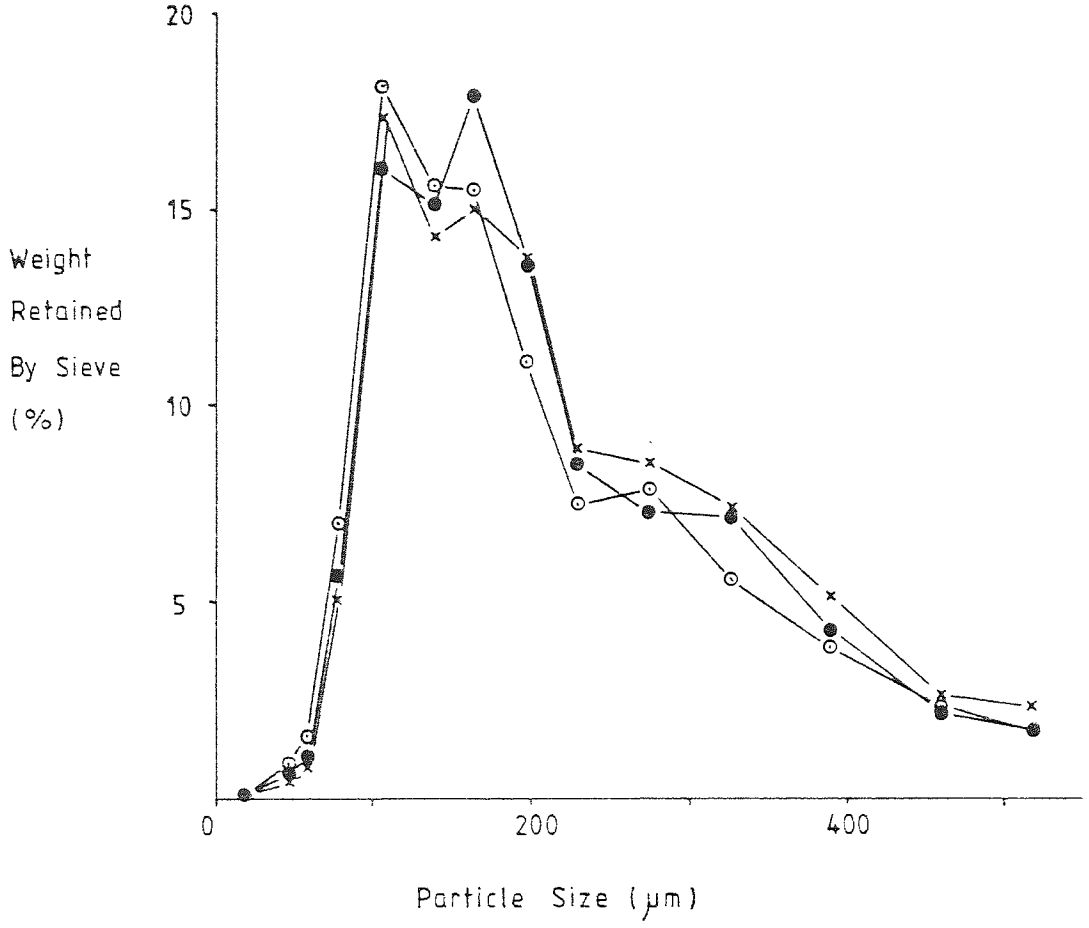


FIGURE 7.3 PARTICLE SIZE DISTRIBUTION OF BENZATHINE CLOXACILLIN POWDER - 3 DETERMINATIONS

Data from table 7.1 are plotted in figure 7.3. Points on the graph represent mid-range values for the size fraction, except for particles >500 μm where a nominal value of 520 μm was chosen. From the results it can be seen that approximately 31% by weight of the material had a particle diameter of 180 μm to 125 μm.

7.3.2. Dissolution of Benzathine Cloxacillin at pH 6

The dissolution profile obtained from the dissolution of 1.0g benzathine cloxacillin, particle size 180 to 125 μm, in 230 ml pH 6 citrate buffer at 34 ^{o}C, is shown in figure 7.4.

Figure 7.4 indicates that a solubility equilibrium was reached by the end of the experiment (300 minutes). It is possible to calculate the solubility of benzathine cloxacillin at pH 6 using either the concentration of cloxacillin or the concentration of benzathine at equilibrium.

The concentration of benzathine at equilibrium was 22.8 mg per 100 ml. Substituting this value in equation 7.1 gives a solubility measurement of 105.6 mg per 100 ml.

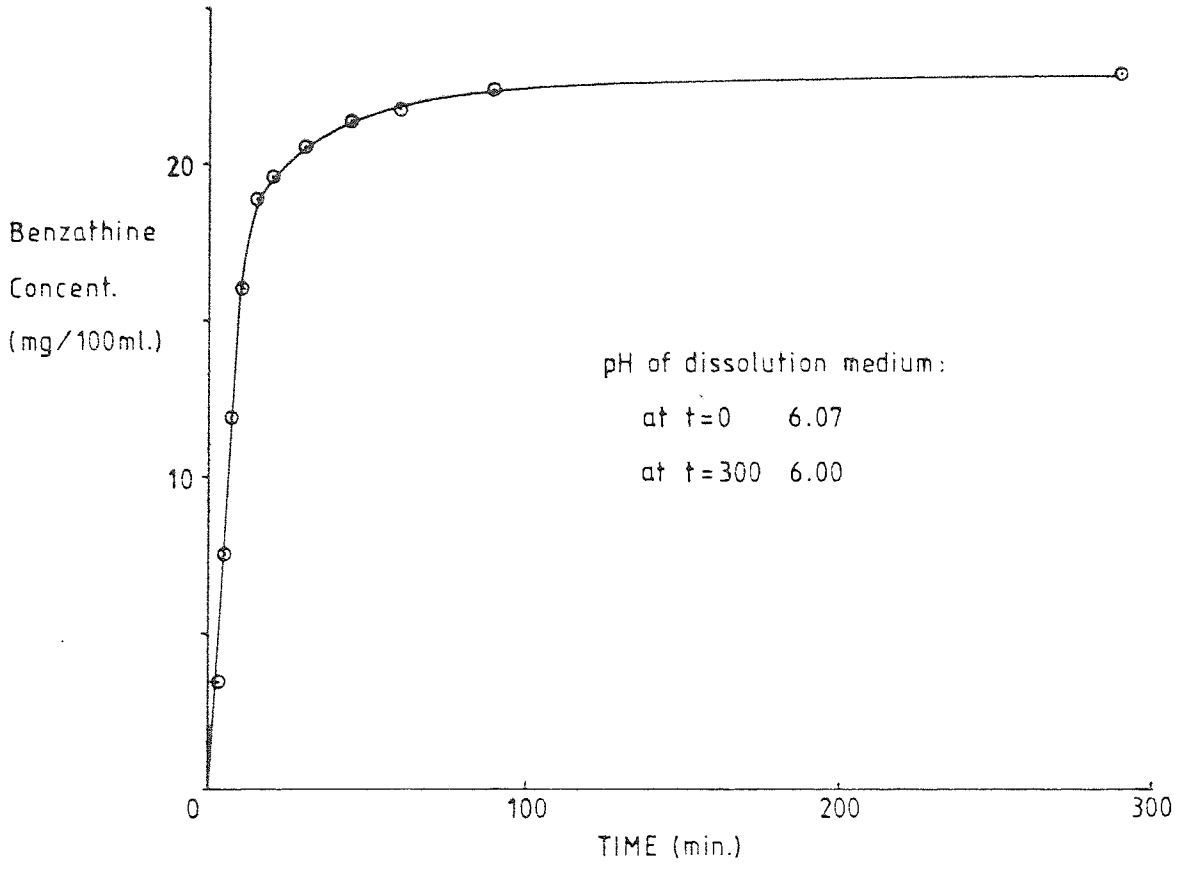
$$\text{Solubility (mg/100ml)} = \frac{[B]_{equil.} \times 1112}{240} \qquad \text{equation 7.1}$$

where $[B]_{equil.}$ is the concentration of benzathine at equilibrium

1112 is the molecular weight of benzathine cloxacillin

240 is the molecular weight of benzathine

The concentration of cloxacillin at equilibrium was 85.0 mg per 100 ml. Substituting this value in equation 7.2 gives a solubility measurement of 108.4 mg per 100 ml.



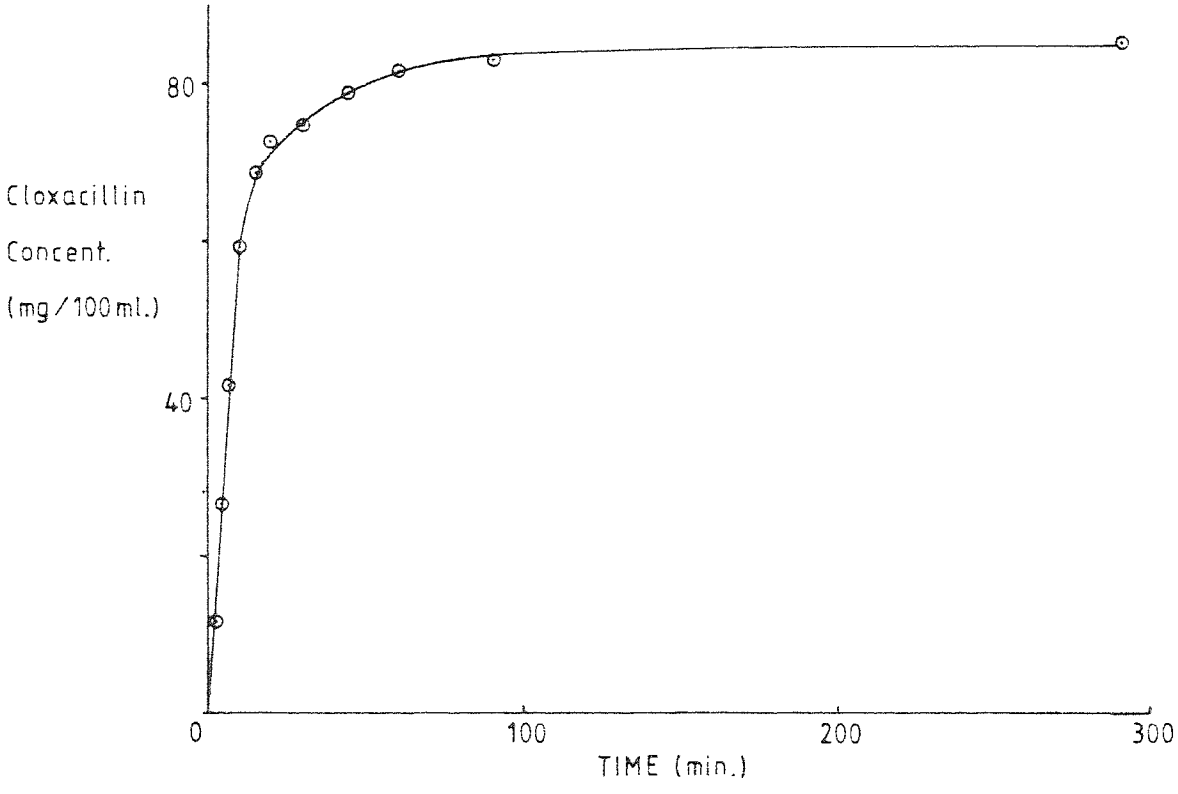


FIGURE 7.4 DISSOLUTION OF BENZATHINE CLOXACILLIN AT pH 6

$$\text{Solubility (mg/100ml)} = \frac{[P]_{equil.} \times 1112}{436 \times 2} \qquad \text{equation 7.2}$$

where $[P]_{equil.}$ is the concentration of cloxacillin at equilibrium

1112 is the molecular weight of benzathine cloxacillin

436 is the molecular weight of cloxacillin

The molecular weight of cloxacillin is doubled in equation 7.2 because there are two molecules of cloxacillin in one molecule of benzathine cloxacillin.

From the solubility values, a mean solubility for benzathine cloxacillin at pH 6 of 107.0 mg per 100 ml was calculated.

Figure 7.4 indicates that no detectable degradation of benzathine or cloxacillin occurred during the dissolution. This finding was supported by independent measurements of these parameters made in pH 6 citrate buffer. Figure 7.5 shows that benzathine was stable at pH 6 for 180 minutes and the measured rate of degradation of cloxacillin at pH 6 was 7.87×10^{-4} hours^{-1}. This rate corresponds to a drop in cloxacillin concentration of 0.4% after 300 minutes.

At equilibrium, 26.4% of solid had dissolved, calculated from equation 7.3.

$$\text{\% Solid Dissoluted} = \frac{\dfrac{\text{Clox. Conc. at Equil.(mg/100ml)}}{\text{Clox. Content of Benz.Clox.(74.0\%)}}}{\dfrac{\text{Volume of Dissolution Medium (230ml)}}{\text{Weight of Benz. Clox. (mg)}}} \qquad \text{equation 7.3}$$

The solubility of benzathine cloxacillin at pH 6 is determined by its solubility product (205, 206) and the degree of ionisation:

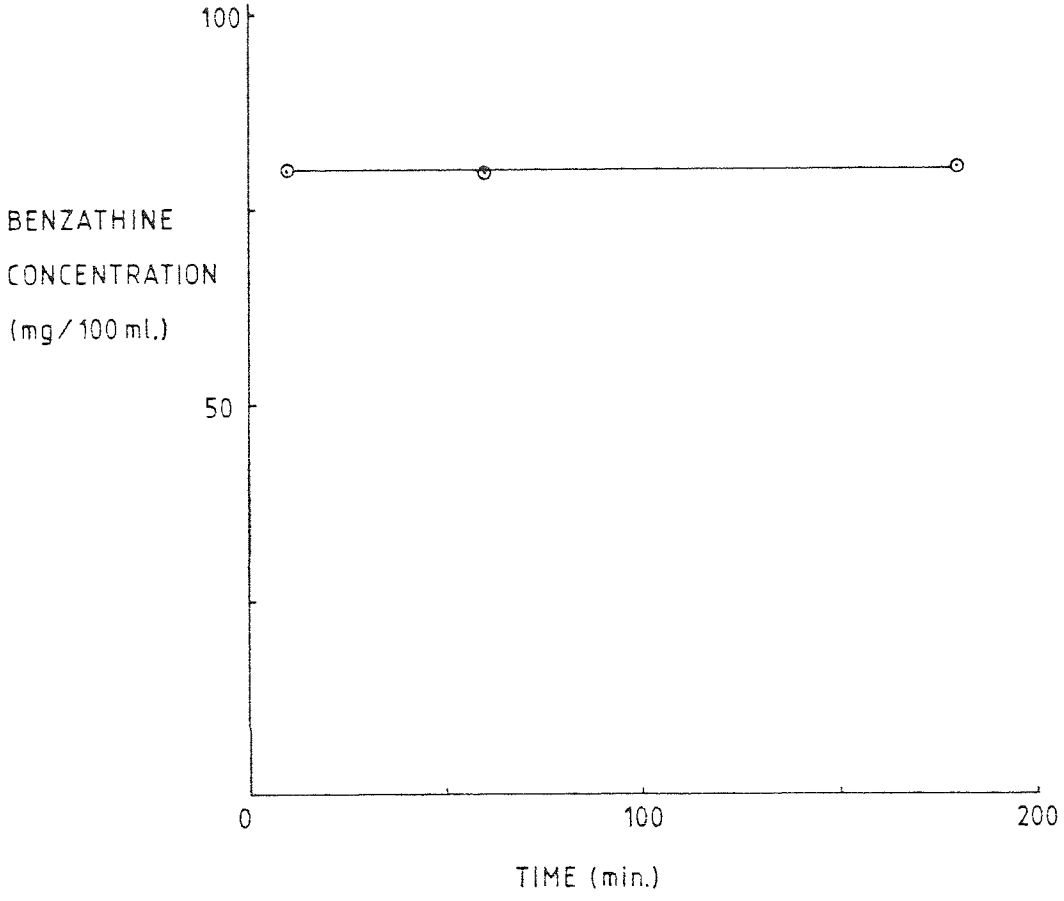


FIGURE 7.5 STABILITY OF BENZATHINE AT pH 6

$$BP_2 \rightleftharpoons B + 2P$$
$$\downarrow\uparrow \quad \downarrow\uparrow$$
$$B^+ \quad P^-$$

$$K_S = a_B \times (a_P)^2$$

where BP_2 represents benzathine cloxacillin

B represents benzathine

P represents cloxacillin

K_S is the solubility product

a_B is the activity of benzathine

a_P is the activity of cloxacillin

The presence of buffer salts can cause "salting in" of the salt by reducing the activity of the ions in solution (207), so the measured solubility of benzathine cloxacillin is only the solubility of the salt under the conditions of the experiment.

Calculation of the Apparent Dissolution Rate Constant

The Noyes-Whitney dissolution rate law is given by equation 7.4 (208).

$$\frac{dA}{dt} = \frac{k.D.S.(A_S - A_t)}{V.h} \qquad \text{equation 7.4}$$

where $\frac{dA}{dt}$ is the rate of increase in A_t, the concentration of drug in solution at time t

k is the proportionality constant

D is the diffusion coefficient of the drug in the solvent

S is the surface area of undissolved drug

V is the volume of solution

h is the thickness of the diffusion layer around a particle

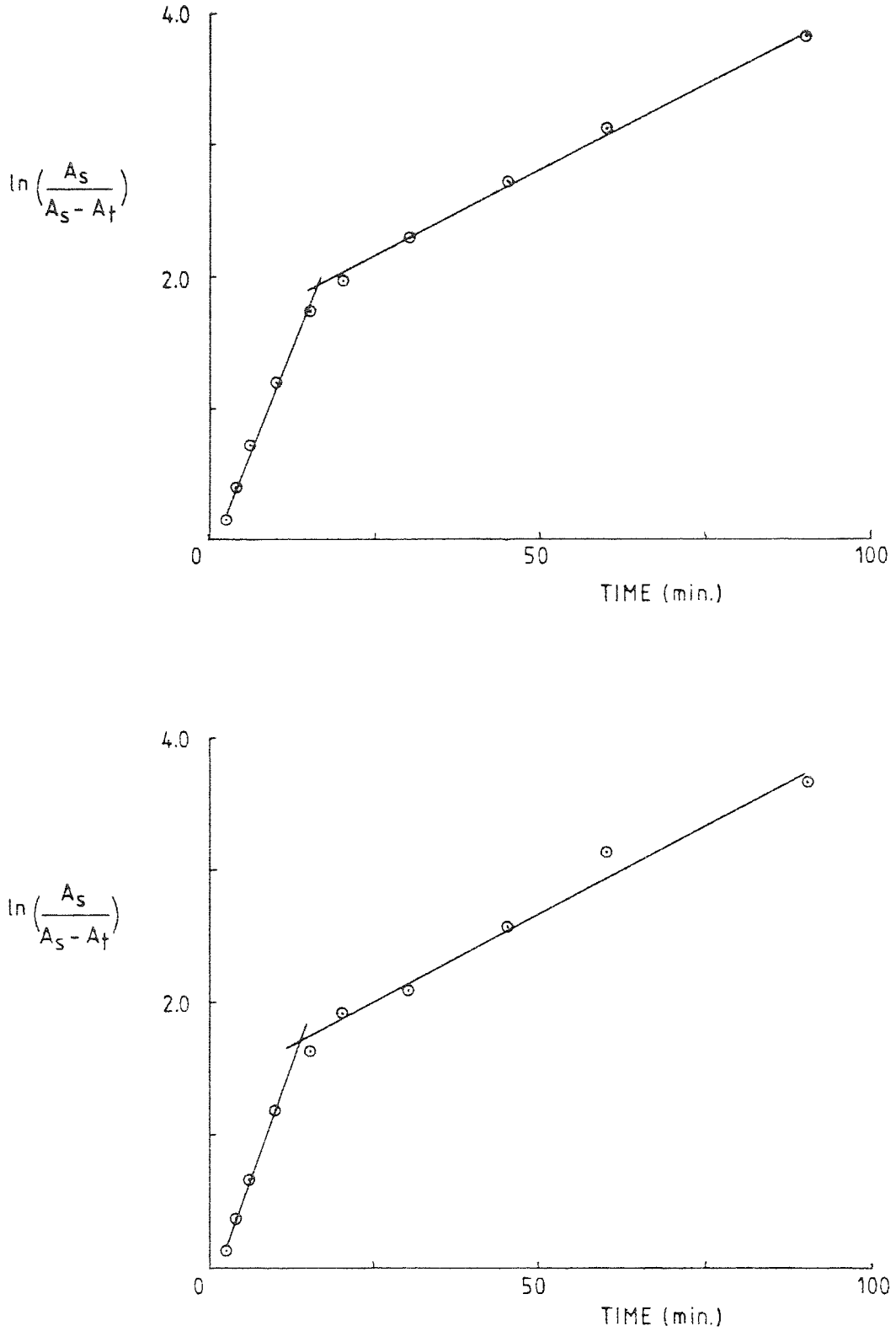


FIGURE 7.6 DISSOLUTION RATE PLOT FOR BENZATHINE CLOXACILLIN AT pH 6

A_s is the solubility of the drug in the solvent

Assuming that D,V and h remain constant, equation 7.4 becomes :

$$\frac{dA}{dt} = k'.S.(A_s - A_t)$$

$$\ln \frac{(A_s)}{(A_s - A_t)} = k'.S.t \qquad \text{equation 7.5}$$

Therefore a plot of $\ln \frac{(A_s)}{(A_s - A_t)}$ against t gives a slope of S.k'

S.k' can be called an apparent dissolution rate constant.

For benzathine cloxacillin at pH 6, A_s is the solubility of benzathine cloxacillin in pH 6 buffer at equilibrium and A_t is the concentration of benzathine or cloxacillin in solution at time t. Figure 7.6 shows the dissolution rate profiles for benzathine and cloxacillin data substituted in equation 7.5.

The slope of the lines in figure 7.6 represents S.k'. Since the lines are not linear throughout, S must change with time. This change appears biphasic and not gradual throughout the dissolution. A suitable explanation for this profile is that the material, although sized to contain only 180 - 125 μm particles, contains a small portion of finer particles. These provide high surface area hence rapid initial dissolution. When they have completely dissoluted, the 180 - 125 μm particles provide relatively constant surface area for the remainder of the dissolution.

The apparent rate constant S.k', calculated from the second phase of the plots in figure 7.6 was 0.027 min^{-1} (benzathine data) and 0.027 min^{-1} (cloxacillin data).

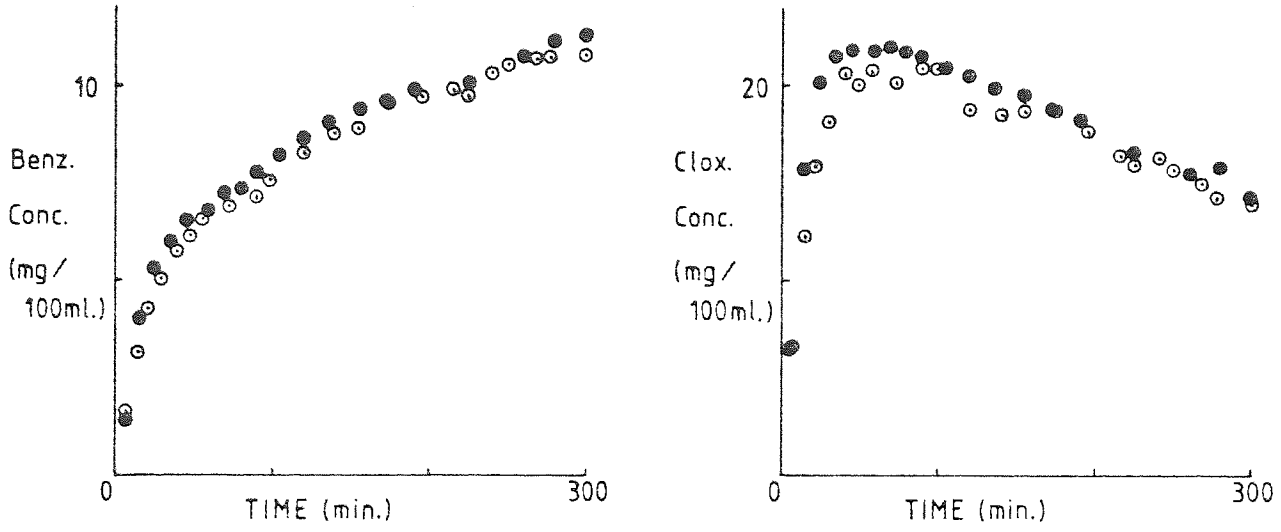


FIGURE 7.7 DISSOLUTION OF 0.2g UNSIZED BENZATHINE CLOXACILLIN AT pH 2. DISSOLUTIONS A (⊙) AND B (●)

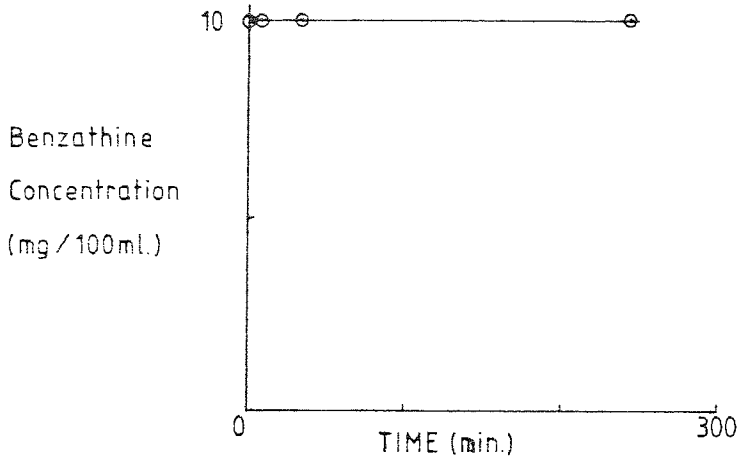


FIGURE 7.8 CHANGE IN BENZATHINE CONCENTRATION WITH TIME, AT pH 2

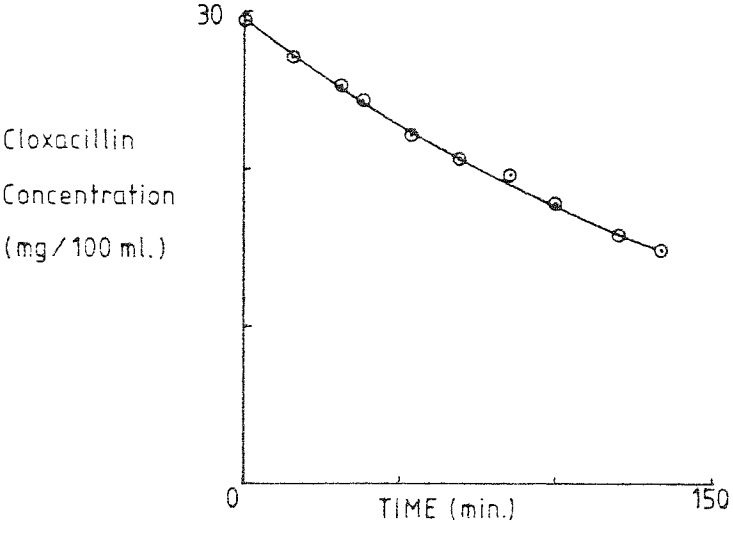


FIGURE 7.9 CHANGE IN CLOXACILLIN CONCENTRATION WITH TIME, AT pH 2

7.3.3 Dissolution of Benzathine Cloxacillin at pH 2

The dissolution of two 0.2g samples of unsized benzathine cloxacillin was carried out in pH 2 citrate buffer (dissolutions A and B, figure 7.7). Benzathine levels rose rapidly at first, then slowly increased with time throughout the experiment. However, after an initial rapid increase, cloxacillin levels decreased with time. To investigate this result, benzathine and cloxacillin were dissolved in pH 2 citrate buffer and their concentrations monitored against time (figures 7.8 and 7.9). A precipitate appeared in the flask containing cloxacillin. The results show that benzathine was stable at pH 2, but cloxacillin degraded with time. This provides an explanation of the dissolution profiles seen in figure 7.7.

Cloxacillin undergoes first order degradation in aqueous buffers between pH 1 and pH 11 (209). A plot of ln[cloxacillin] against t for the data in figure 7.9 gave a straight line with a gradient of -5.13 (+/- 0.23) x 10^{-3}, corresponding to a degradation rate constant of 5.13 (+/- 0.23) x 10^{-3} $minutes^{-1}$. The presence of benzathine in the dissolution medium may influence the degradation rate of cloxacillin. Also, cloxacillin or its degradation products could react with benzathine. These possibilities were tested by repeating the degradation of cloxacillin at pH 2 in the presence of benzathine. Benzathine was found to be stable under these conditions and cloxacillin degraded at 5.07 (+/- 0.12) x 10^{-3} min^{-1}. This value was not significantly different from the rate constant calculated from the degradation of cloxacillin without benzathine. Thus benzathine and cloxacillin were shown not to interact with each other in solution at pH 2.

The effect of particle size on the dissolution profile was investigated by repeating the dissolution using 0.2g of 180 - 125 μm benzathine

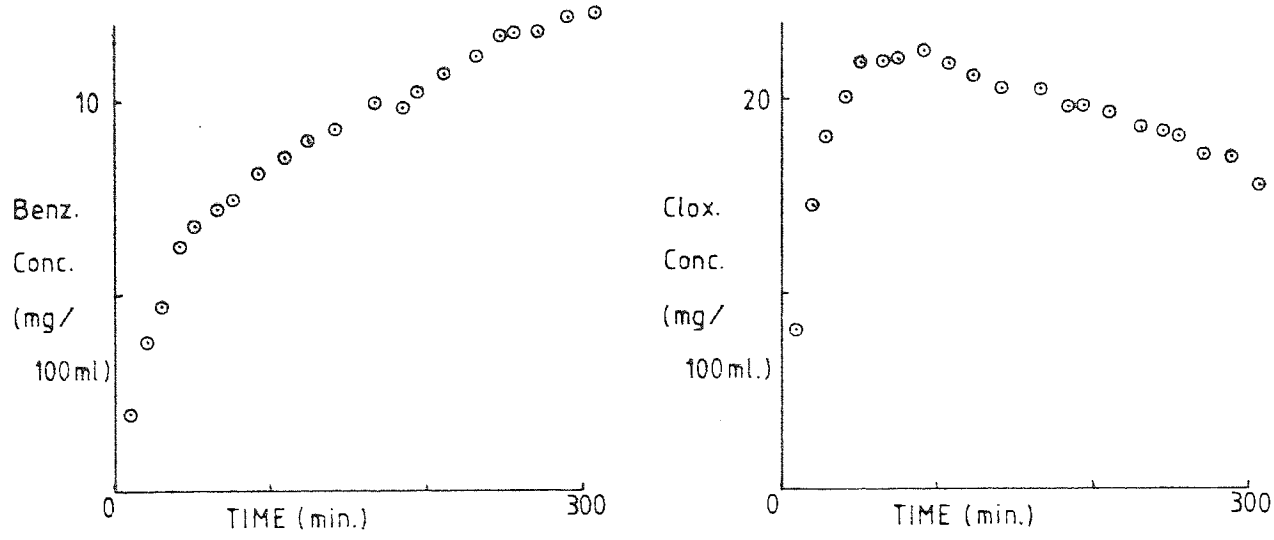


FIGURE 7.10 DISSOLUTION OF 0.2g BENZATHINE CLOXACILLIN (180-125μm) AT pH 2, DISSOLUTION C

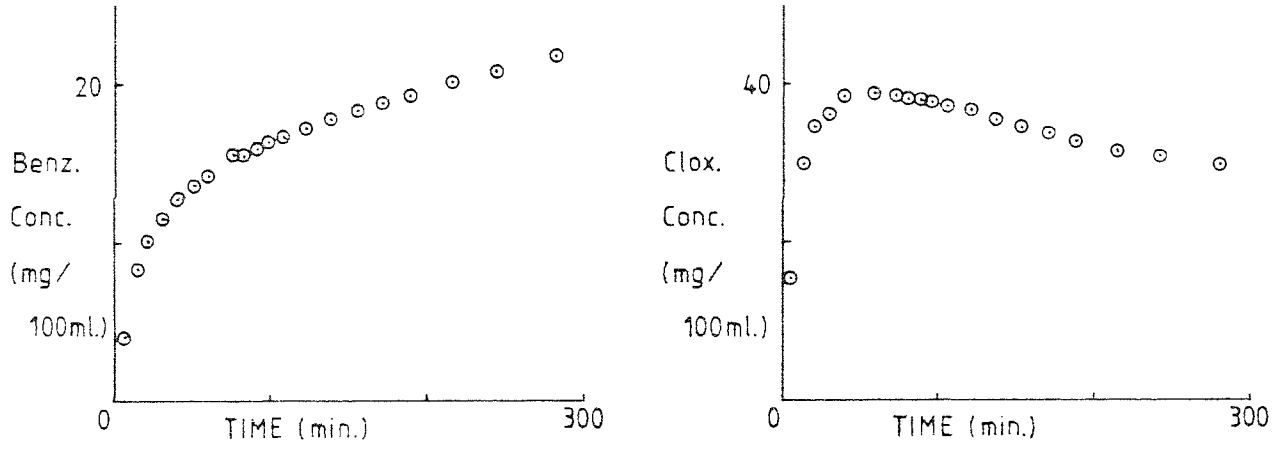


FIGURE 7.11 DISSOLUTION OF 1.0g BENZATHINE CLOXACILLIN (180-125μm) AT pH 2. DISSOLUTION D

– EARLY TIME VALUES

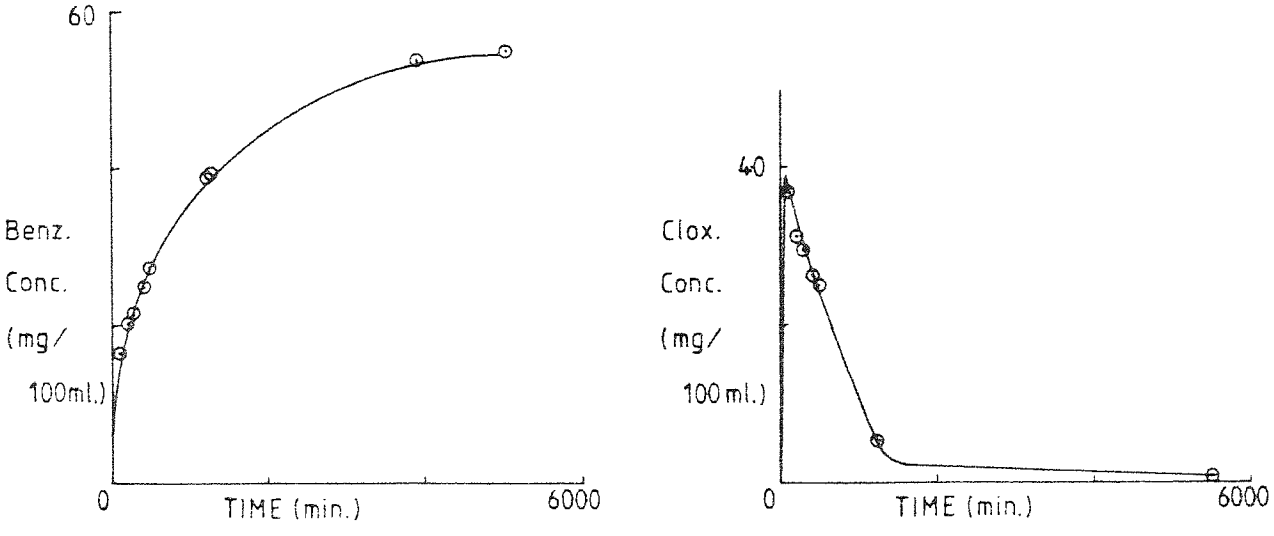


FIGURE 7.12 DISSOLUTION OF 1.0g BENZATHINE CLOXACILLIN (180-125μm) AT pH 2. DISSOLUTION D

– LATE TIME VALUES

cloxacillin (dissolution C, figure 7.10). No significant change in dissolution profile was seen using the sized material.

Since benzathine does not react with cloxacillin at pH 2, the concentration of benzathine at time t, $[B]_t$, can be used to calculate the amount of solid benzathine cloxacillin dissoluted at any time. From figure 7.7, $[B]_t$ at 300 minutes was 10.95 and 11.30 mg per 100 ml, respectively, for dissolutions A and B. These values correspond to 117 and 120 mg, resp., of benzathine cloxacillin per 230 ml, indicating that approximately 40% of the benzathine cloxacillin remained undissolved after 300 minutes. Similarly, values taken from dissolution C indicate that approximately 35% of solid benzathine cloxacillin remained undissolved after 300 minutes. With so much material remaining undissolved after 5 hours, either equilibrium had been reached (the solubility product of benzathine cloxacillin at pH 2 where loss of cloxacillin was balanced by increase in benzathine) or something retarded the dissolution as it progressed. In order to discover which of these explanations was correct, the dissolution was repeated with 1.0g 180-125 μm material, five times the earlier quantity (dissolution D, figures 7.11 and 7.12).

Benzathine and cloxacillin concentrations were much higher in dissolution D compared to dissolutions A, B and C. Because the buffer concentration was identical for all four dissolutions of benzathine cloxacillin at pH 2, the solubility product should be similar in each case, although the concentration of degraded cloxacillin may have some effect, causing "salting in". Thus the increased benzathine and cloxacillin levels in dissolution D suggests equilibrium was not reached in dissolutions A to C and that retardation of dissolution occurred. This was probably caused by precipitated degradation products of cloxacillin coating undissolved benzathine cloxacillin and effectively reducing the available surface area for dissolution.

BP_2 ≡ Benzathine Cloxacillin

B ≡ Benzathine

P ≡ Cloxacillin

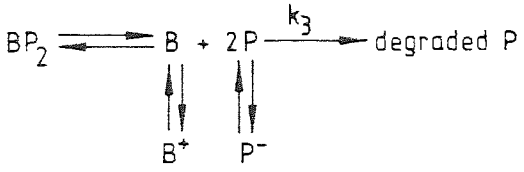


FIGURE 7.13 MECHANISM FOR BENZATHINE CLOXACILLIN DISSOLUTION AT pH2

The mechanism for benzathine cloxacillin dissolution at pH 2 is given in figure 7.13.

According to this mechanism,

$$[P]_t = \frac{\text{Quant. Clox. Dissolved(mg)} - \text{Quant. Clox. Degraded(mg)}}{230}$$

where $[P]_t$ is the concentration of cloxacillin at time t

To confirm the mechanism in figure 7.13, $[P]_t$ was predicted from $[B]_t$ (the concentration of benzathine at time t) and the degradation rate constant for cloxacillin. The resulting values were graphically compared with those obtained experimentally for $[P]_t$.

Prediction of $[P]_t$

$[B]_t$ vs time plots from dissolutions A-D were used to obtain accurate values of $[B]_t$ at ten minute intervals. These were multiplied by a factor $\left(\frac{\text{molecular weight of cloxacillin} \times 2}{\text{molecular weight of benzathine}}\right.$ in mg per 100 ml$\left.\right)$ to convert $[B]_t$ values into $[P]_t$(no deg.). $[P]_t$(no deg.) is the concentration of cloxacillin at time t assuming no degradation.

An eleventh order polynomial expression for $[P]_t$(no deg.) against time was calculated, using a standard BASIC program. This enabled calculation of $[P]_t$(no deg.) for any value of t. For ease of calculation, time was divided into one minute intervals.

The amount of cloxacillin released every minute is then given by :

$$[P]_{t+1}(\text{no deg.}) - [P]_t(\text{no deg.})$$

$[P]_t$ can be calculated from the first order rate expression :

$$\ln C_t = \ln C_o - k.t$$

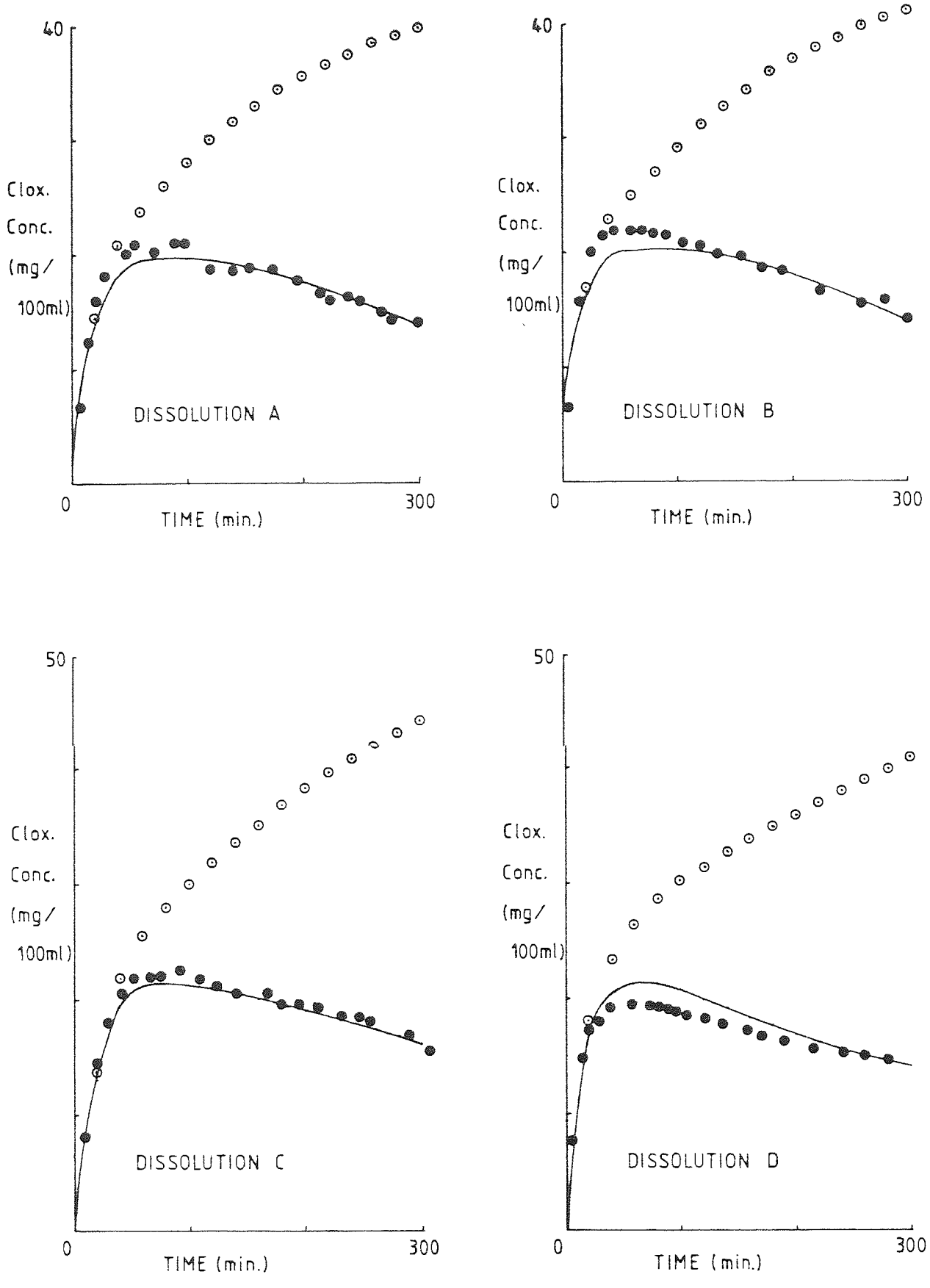


FIGURE 7.14 GRAPHIC FIT BETWEEN CALCULATED AND MEASURED VALUES OF CLOXACILLIN CONCENTRATION FOR BENZATHINE CLOXACILLIN DISSOLUTION AT pH 2.

⊙ NO DEGRADATION (calculated); —— DEGRADATION (calculated); ● DEGRADATION (actual)

where C_t is the concentration of cloxacillin at time t

C_o is the initial concentration of cloxacillin

k is the first order rate constant

In the calculation of $[P]_t$, C_o changes with time and is given by :

$[P]_{t-1}$ + $[P]_t$(no deg.) - $[P]_{t-1}$(no deg.)

C_t is equivalent to $[P]_t$

k is equivalent to k_3 in figure 7.13, the rate of degradation of cloxacillin at pH 2.

Hence :

$$[P]_t = \exp\left(\ln\left[[P]_{t-1} + [P]_t(\text{no deg.}) - [P]_{t-1}(\text{no deg.})\right] - k_3.t\right)$$

$$k_3 = 5.10 \times 10^{-3} \text{ minutes}^{-1}.$$

A FORTRAN IV computer program was written to calculate $[P]_t$ using this expression (program 2 in Appendix 2)

Figure 7.14 shows the graphic comparison between $[P]_t$ calculated as above and $[P]_t$ measured experimentally. The good fit between these values indicates the dissolution mechanism proposed in figure 7.13 is consistent with experimental measurements.

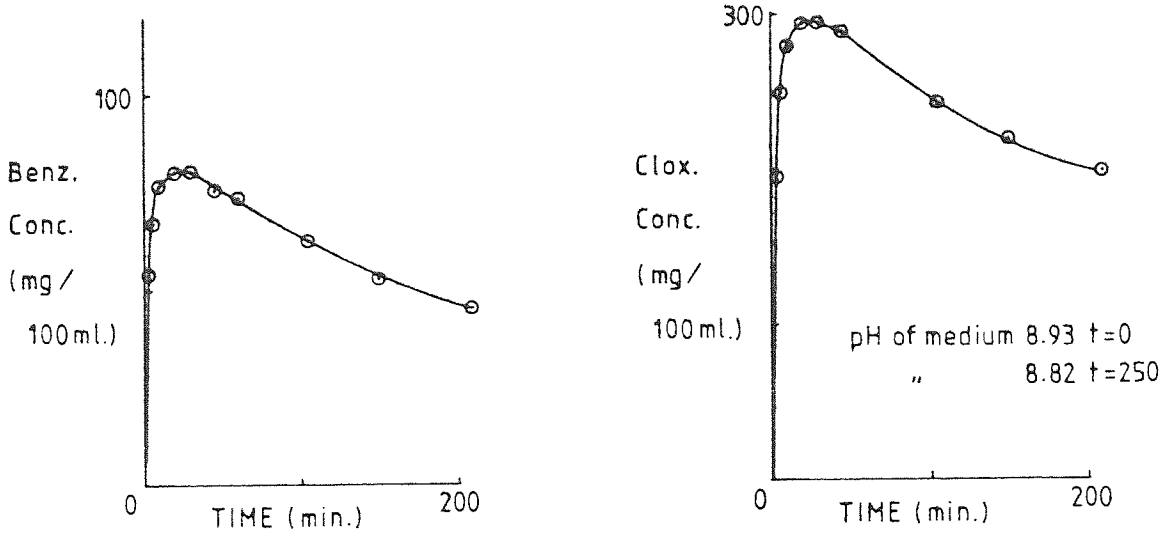


FIGURE 7.15 DISSOLUTION OF 1.0g BENZATHINE CLOXACILLIN (180-125μm) AT pH 9

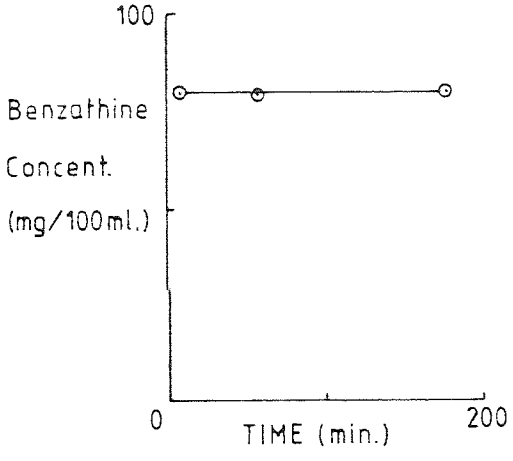


FIGURE 7.16 STABILITY OF BENZATHINE AT pH 9

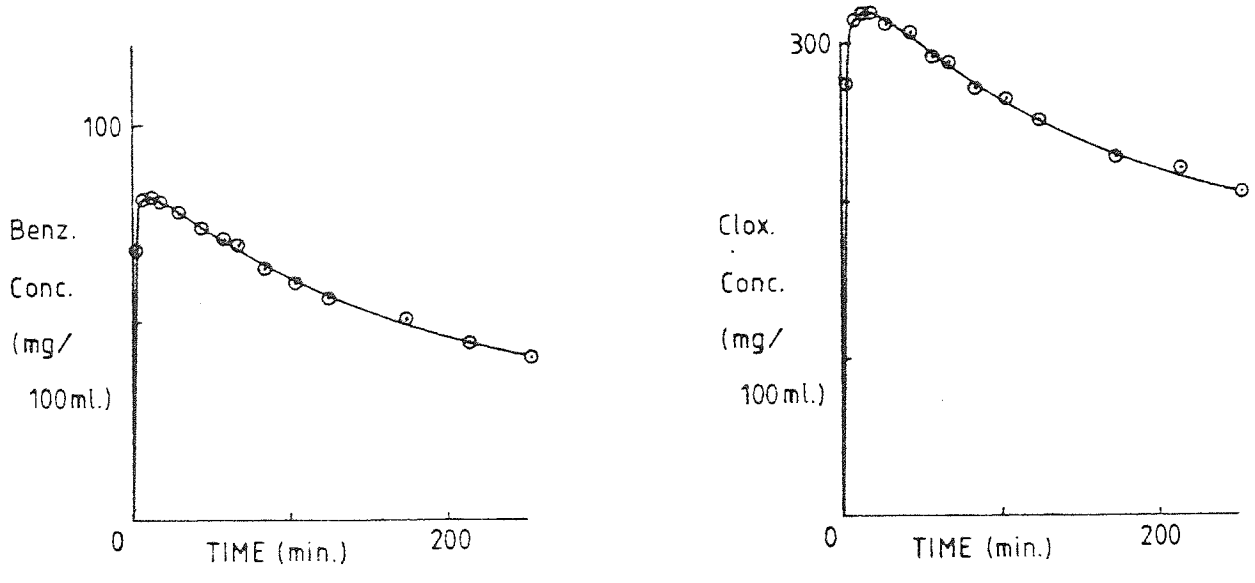
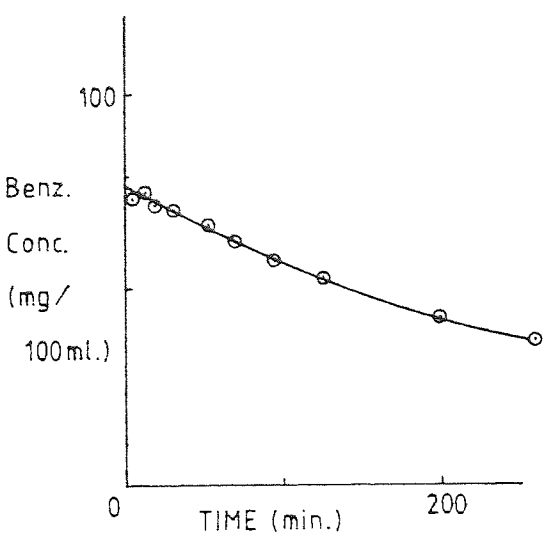


FIGURE 7.17 DISSOLUTION OF 1.0g BENZATHINE CLOXACILLIN CRYSTALS (180-125μm) AT pH 9

7.3.4 Dissolution of Benzathine Cloxacillin at pH 9

The dissolution profile of 1.0g benzathine cloxacillin, particle size range 180-125 μm can be seen in figure 7.15. The solid was immediately wetted at pH 9, noticeably quicker than at pH 6 or pH 2. All of the particles dissolved in less than 60 minutes, leaving a hazy solution. A precipitate was produced later in the dissolution. Maximum concentration levels of cloxacillin and benzathine represent 91.3% and 90.6%, respectively, of solid present at the start of the experiment. Clearly, the profile of this dissolution did not agree with the mechanism proposed in figure 7.13. Cloxacillin degraded in pH 9 borate buffer by first order kinetics at a rate of 5.70×10^{-4} minutes. Due to this degradation, it was expected that cloxacillin levels would initially rise then fall as the rate of degradation exceeded the rate of dissolution. This profile was followed, but the cloxacillin levels dropped more rapidly than expected. Benzathine, stable at pH 9 (figure 7.16), after an initial increase in concentration, unexpectedly disappeared from solution at a higher rate than cloxacillin. This suggests that benzathine and cloxacillin reacted in solution, an explanation supported by H.P.L.C. analysis where an unknown substance eluted after long retention in late dissolution samples. The peak height of this material increased with time.

Another explanation could be the different solubilities of different polymorphic forms of the salt. The benzathine cloxacillin used in the dissolutions was amorphous in character. There have been reports of amorphous material possessing greater solubility than crystalline forms of the same substance (210). It was possible, therefore, that amorphous benzathine cloxacillin initially dissoluted and then re-precipitated as a more stable crystalline polymorph, explaining the dissolution profiles in figure 7.15. Crystalline benzathine cloxacillin was



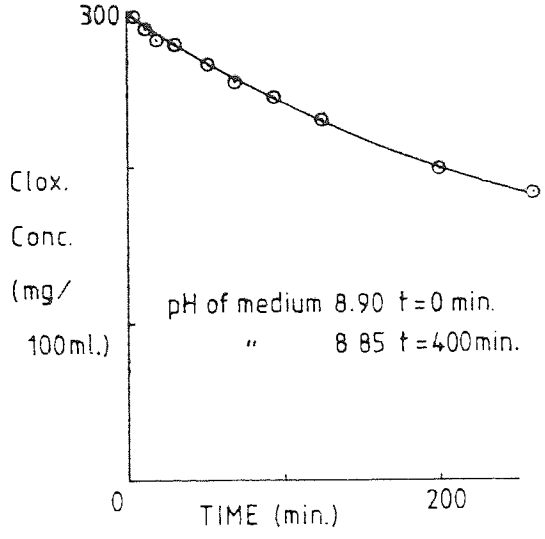
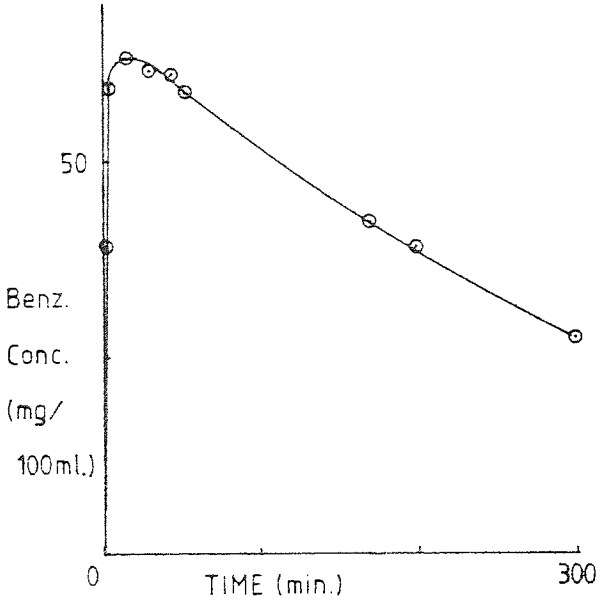


FIGURE 7.18 REDUCTION OF BENZATHINE AND CLOXACILLIN CONCENTRATIONS IN SOLUTION AT pH 9



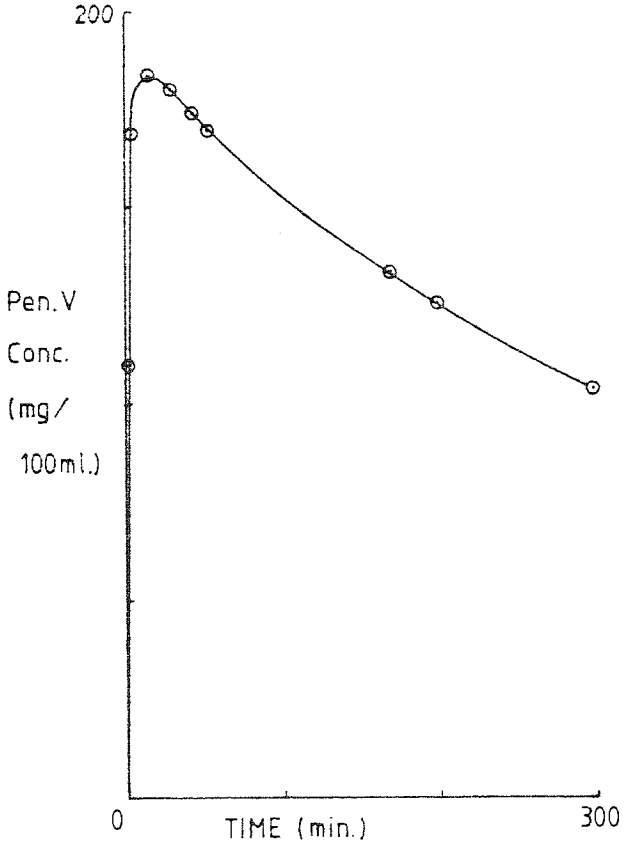


FIGURE 7.19 DISSOLUTION OF 1.0g UNSIZED BENZATHINE PENICILLIN V AT pH 9

prepared and the dissolution of this material at pH 9 carried out. No significant change in dissolution profile was seen (figure 7.17), indicating that different solubilities for the different forms of benzathine cloxacillin was not the cause of benzathine and cloxacillin disappearing from solution at pH 9.

To provide supportive evidence that benzathine and cloxacillin reacted in pH 9 borate buffer, benzathine (as the diacetate salt) and cloxacillin (as the sodium salt) were dissolved in pH 9 buffer and their change in concentration monitored by H.P.L.C. analysis. The levels of benzathine and cloxacillin fell with time (figure 7.18) and the unknown material with long chromatographic retention was again seen.

Investigation Into the Mechanism of Reaction Between Benzathine and Cloxacillin

The behaviour of benzathine in the presence of degraded cloxacillin at pH 9 was monitored to discover if intact cloxacillin was necessary for any reaction to occur. No significant reduction in benzathine concentration occurred after 3 hours, indicating that only intact cloxacillin reacted with the amine.

The most labile moiety in cloxacillin is the beta-lactam ring. Lack of reaction between benzathine and degraded cloxacillin at pH 9 suggests that this ring was involved in the reaction between benzathine and cloxacillin. However, cloxacillin possesses a complex side chain (figure 7.2) which might also have been involved. To investigate the contribution of this side chain, benzathine penicillin V was prepared. Penicillin V possesses a simpler side chain than cloxacillin (figure 7.2).

Dissolution of benzathine penicillin V at pH 9 (figure 7.19) produced similar profiles to the dissolution of benzathine cloxacillin at pH 9

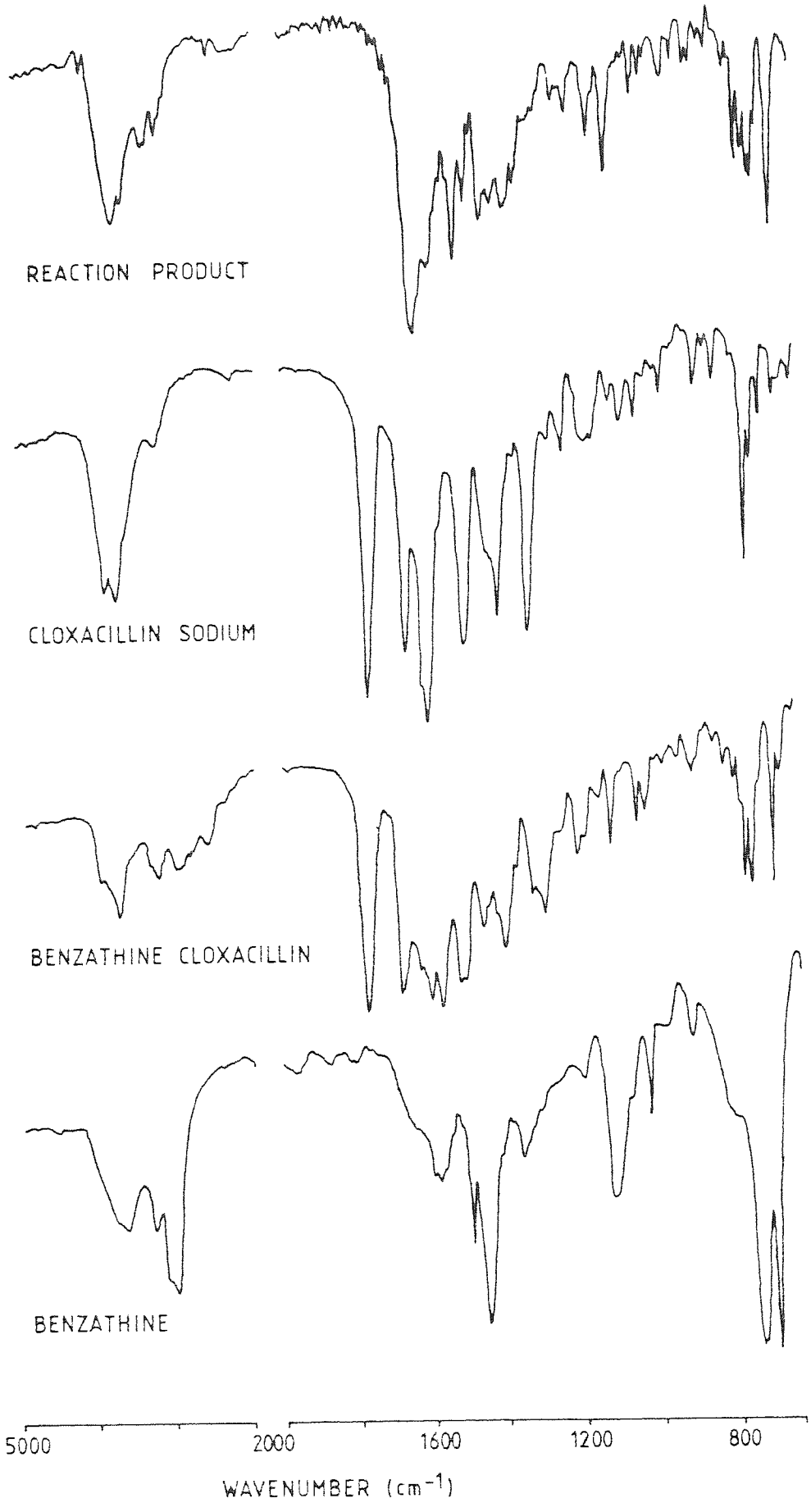


FIGURE 7.20 INFRA-RED SPECTRA OF THE REACTION PRODUCT OF BENZATHINE AND CLOXACILLIN, CLOXACILLIN SODIUM, BENZATHINE CLOXACILLIN AND BENZATHINE

(figures 7.15 and 7.17). H.P.L.C. analysis again showed a long retained peak that increased in size with time. It is likely, therefore, that it is the beta-lactam ring of cloxacillin, and penicillin V, which reacts with benzathine.

Analysis of the Reaction Product of Benzathine and Cloxacillin at pH 9

The reaction product of benzathine and cloxacillin precipitated out of solution at pH 9, but the reaction product of penicillin V and benzathine did not. For this reason, only the reaction product of benzathine and cloxacillin was isolated and analysed.

Melting Point :

175 ^{o}C (compared with 120 - 130 ^{o}C with decomposition for benzathine cloxacillin).

Infra-Red Analysis :

The infra-red (I.R.) spectrum of the reaction product can be seen in figure 7.20. The I.R spectra of cloxacillin sodium, benzathine and benzathine cloxacillin are included for comparison. It can be seen that the absorbance band at 1770 cm^{-1}, due to the beta-lactam ring of cloxacillin sodium and benzathine cloxacillin, has been lost in the reaction product.

Nuclear Magnetic Resonance

The only solvent which dissolved the reaction product was deuterated tri-fluoro acetic acid. However, the high reactivity of this acid soon degraded the reaction product and resolution in the spectrum waspoor. It was, therefore, not possible to use this form of analysis.

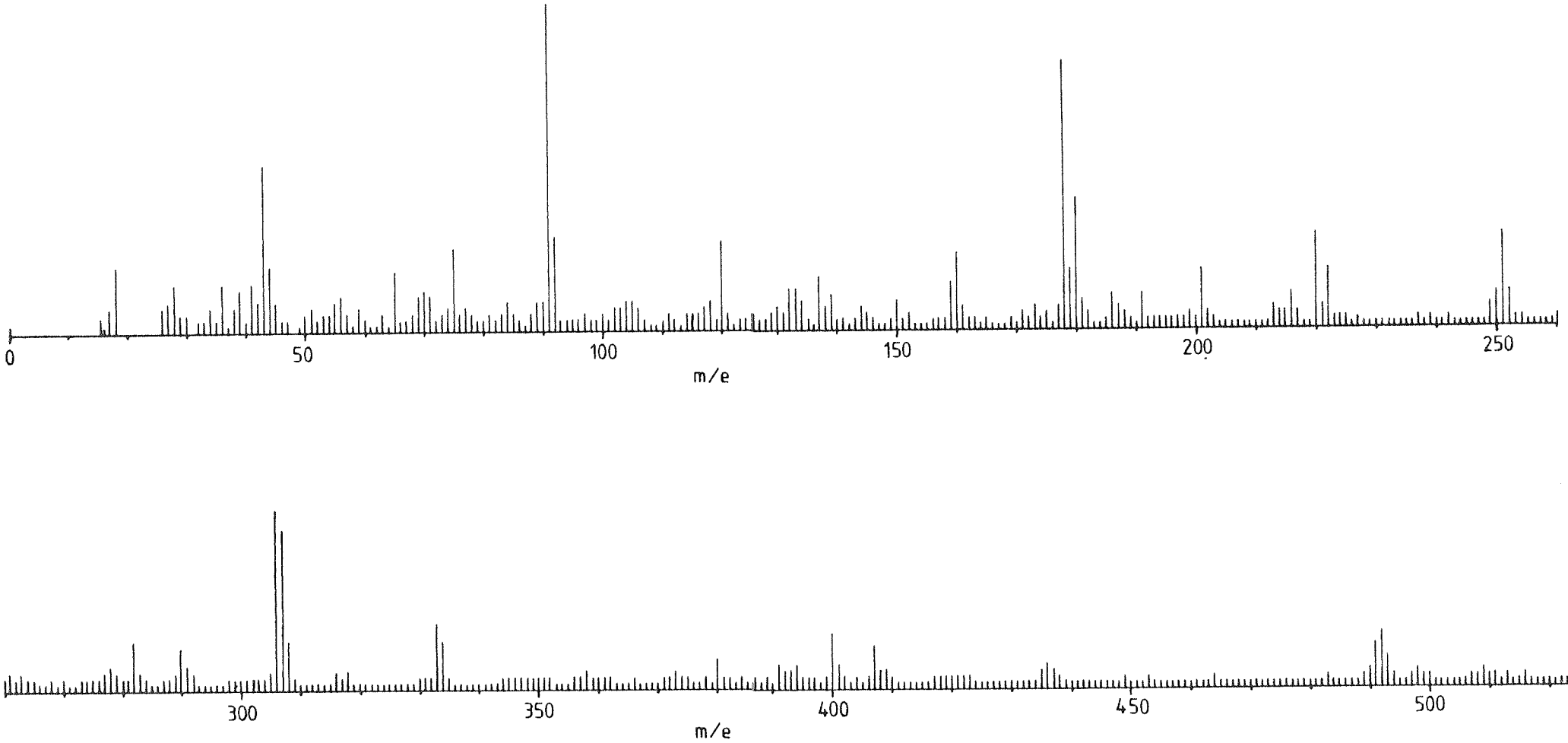
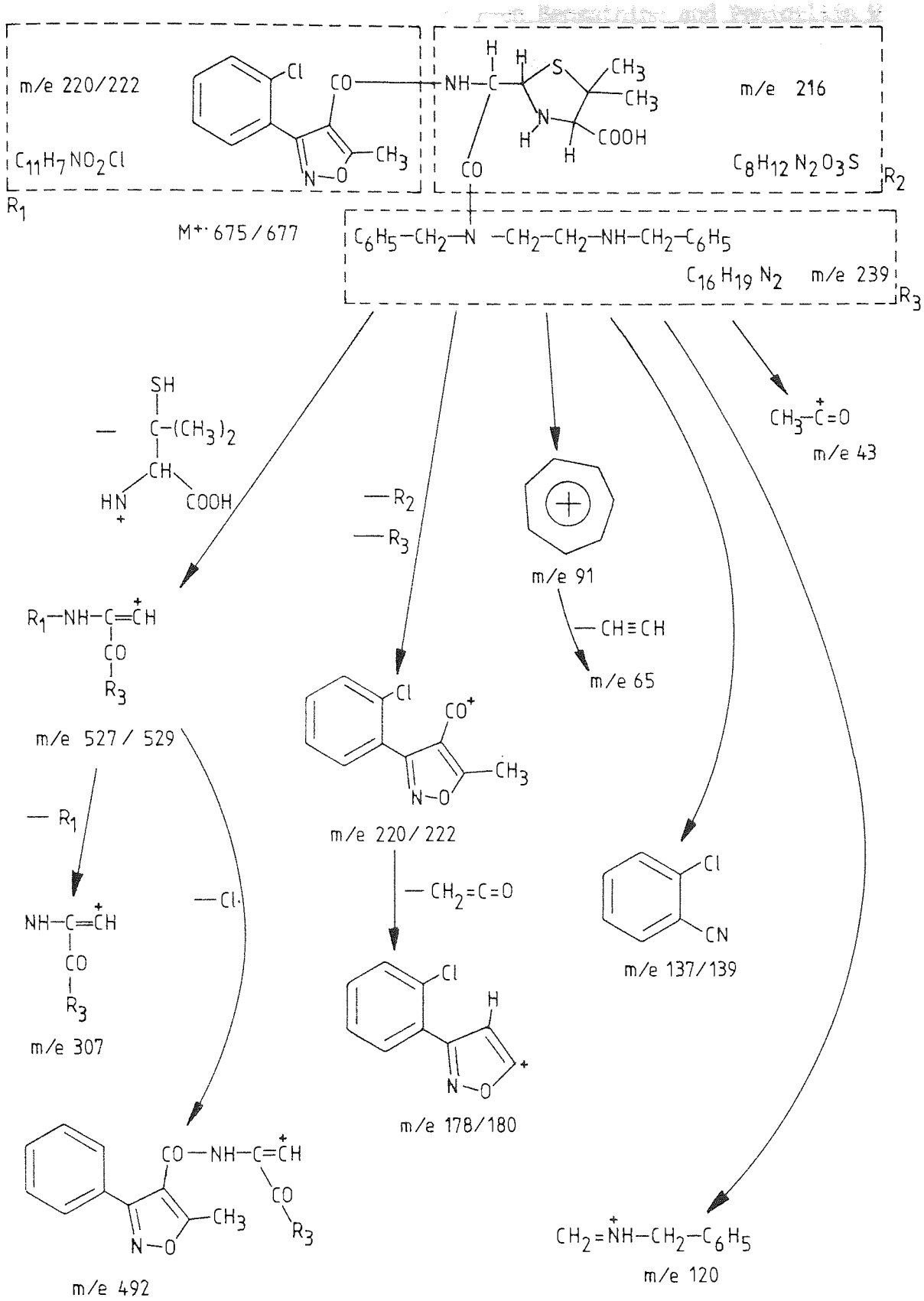


FIGURE 7.21 MASS SPECTRUM OF THE REACTION PRODUCT OF BENZATHINE AND CLOXACILLIN AT pH 9

Mass Spectrum

The mass spectrum of the reaction product (figure 7.21) provided sufficient information to support the proposed structure for the reaction product of benzathine and cloxacillin at pH 9 (figure 7.22). A diagrammatic explanation of some of the peaks seen in the mass spectrum is shown below.

The reaction product of benzathine and penicillin V at pH 9 probably had an analogous structure to the reaction product of benzathine and cloxacillin in figure 7.22.



DIAGRAMMATIC EXPLANATION OF THE MASS SPECTRUM OF THE REACTION PRODUCT

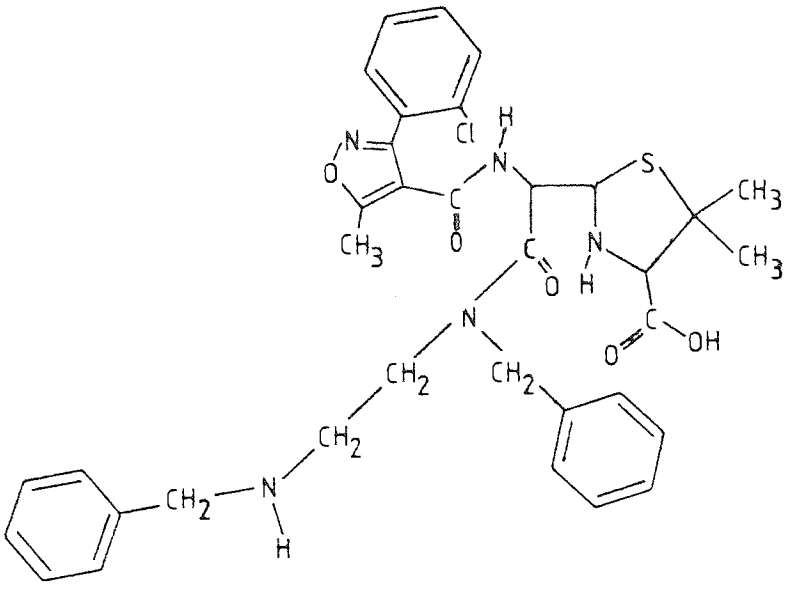


FIGURE 7.22 PROPOSED STRUCTURE FOR THE REACTION PRODUCT OF BENZATHINE AND CLOXACILLIN AT pH 9

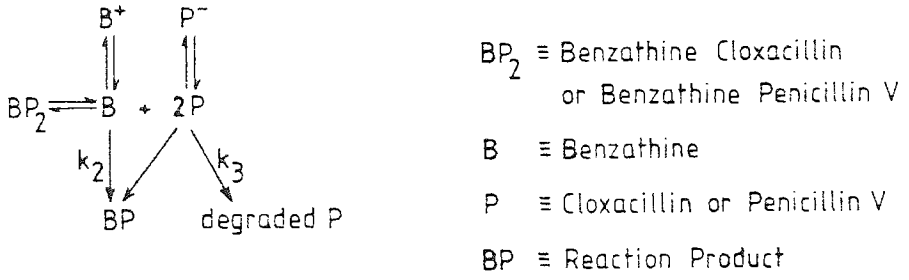


FIGURE 7.23 MECHANISM FOR BENZATHINE CLOXACILLIN AND BENZATHINE PENICILLIN V DISSOLUTION AT pH 9

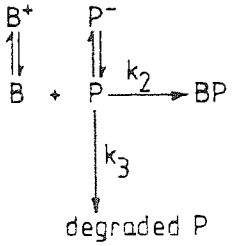


FIGURE 7.24 REACTIONS IN SOLUTION OF BENZATHINE AND CLOXACILLIN OR BENZATHINE AND PENICILLIN V, AT pH 9

Kinetic Analysis of the Reaction Between Benzathine and Penicillin V or Cloxacillin at pH 9

The mechanism for benzathine cloxacillin and benzathine penicillin V dissolution at pH 9 is given in figure 7.23. In order to confirm this mechanism, kinetic analysis of reactions in solution between benzathine and cloxacillin or penicillin V at pH 9 were studied. Starting with the reactants in solution, the mechanism in figure 7.23 can be simplified to that shown in figure 7.24. From this :

$$-\frac{d\,P}{d\,t} = k_2.[P].[B] + k_3.P \qquad \text{equation 7.6}$$

$$-\frac{d\,B}{d\,t} = k_2.[P].[B] \qquad \text{equation 7.7}$$

$$\frac{d\,BP}{d\,t} = k_2.[P].[B] \qquad \text{equation 7.8}$$

The production of BP is a second order process where the reactant concentrations are not equal. Despite its simultaneous degradation the concentration of penicillin is in excess of the concentration of benzathine throughout the experiment. Thus k_2 can be represented as (211) :

$$k_2.t = \frac{1}{[P]_o - [B]_o}.\ln\left[\frac{[B]_o.\,(\,[P]_o - [BP]_t\,)}{[P]_o.\,(\,[B]_o - [BP]_t\,)}\right] \qquad \text{equation 7.9}$$

when [BP] is measured against time

Substituting for $[BP]_t = [B]_o - [B]_t$ in equation 7.9 :

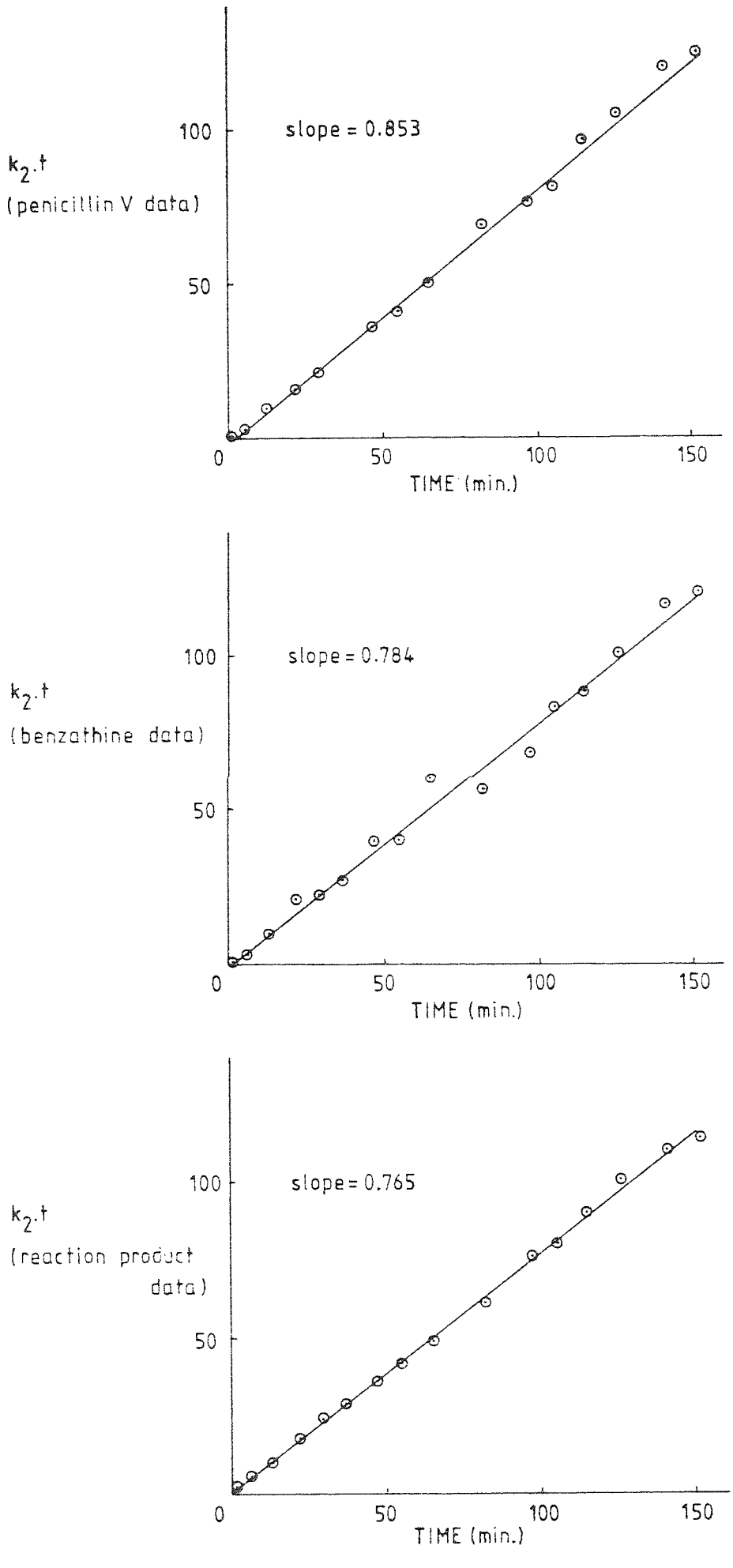


FIGURE 7.25 KINETIC RATE PLOTS FOR THE REACTION BETWEEN BENZATHINE AND PENICILLIN V AT pH 9. $[P]_o = [P]_m$

$$k_2.t = \frac{1}{[P]_o - [B]_o} .\ln \left[\frac{[B]_o . ([P]_o - [B]_o + [B]_t)}{[P]_o . [B]_t} \right] \quad \text{equation 7.10}$$

when [B] is measured against time

Substituting for $[BP]_t = [P]_o - [P]_t$ in equation 7.9 :

$$k_2.t = \frac{1}{[P]_o - [B]_o} .\ln \left[\frac{[B]_o . [P]_o}{[P]_o . ([B]_o - [P]_o + [P]_t)} \right] \quad \text{equation 7.11}$$

when [P] is measured against time

However, these equations do not account for the simultaneous first order degradation of P given by equation 7.12.

$$k_2.t = \ln [P]_o - \ln [P]_t \quad \text{equation 7.12}$$

Since $[P]_o$ is the only term affected by this, three modified expressionscan be derived by placing $[P]_o$ in equations 7.9, 7.10 and 7.11 by $[P]_m$ where $[P]_m$ is equivalent to $[P]_t$ in equation 7.12. A BASIC computer program was written (program 3 in Appendix 2) to calculate values of $k_2.t$ from the measurement of [BP], [B] and [P]

Reaction of Benzathine and Penicillin V

The first order degradation rate constant (k_3) for penicillin V in pH 9 borate buffer at 34°C was 8.50×10^{-4} minutes^{-1}. In the reaction experiment, [B], [P] and the peak height of BP were measured. In order to estimate [BP] from the peak height of BP (no standard preparation was available), the equation $[BP]_t = [B]_o - [B]_t$ was used.

$[B]_o = 2.87 \times 10^{-3}$ M and $[B]_t$ at 141 minutes was measured from the plot of $[B]_t$ vs time as 1.81×10^{-3} M.

Therefore, $[BP]_{t=141} = (2.87 - 1.81) \times 10^{-3}$ M $= 1.06 \times 10^{-3}$ M

The peak height of BP at 141 minutes was 63.5 mm

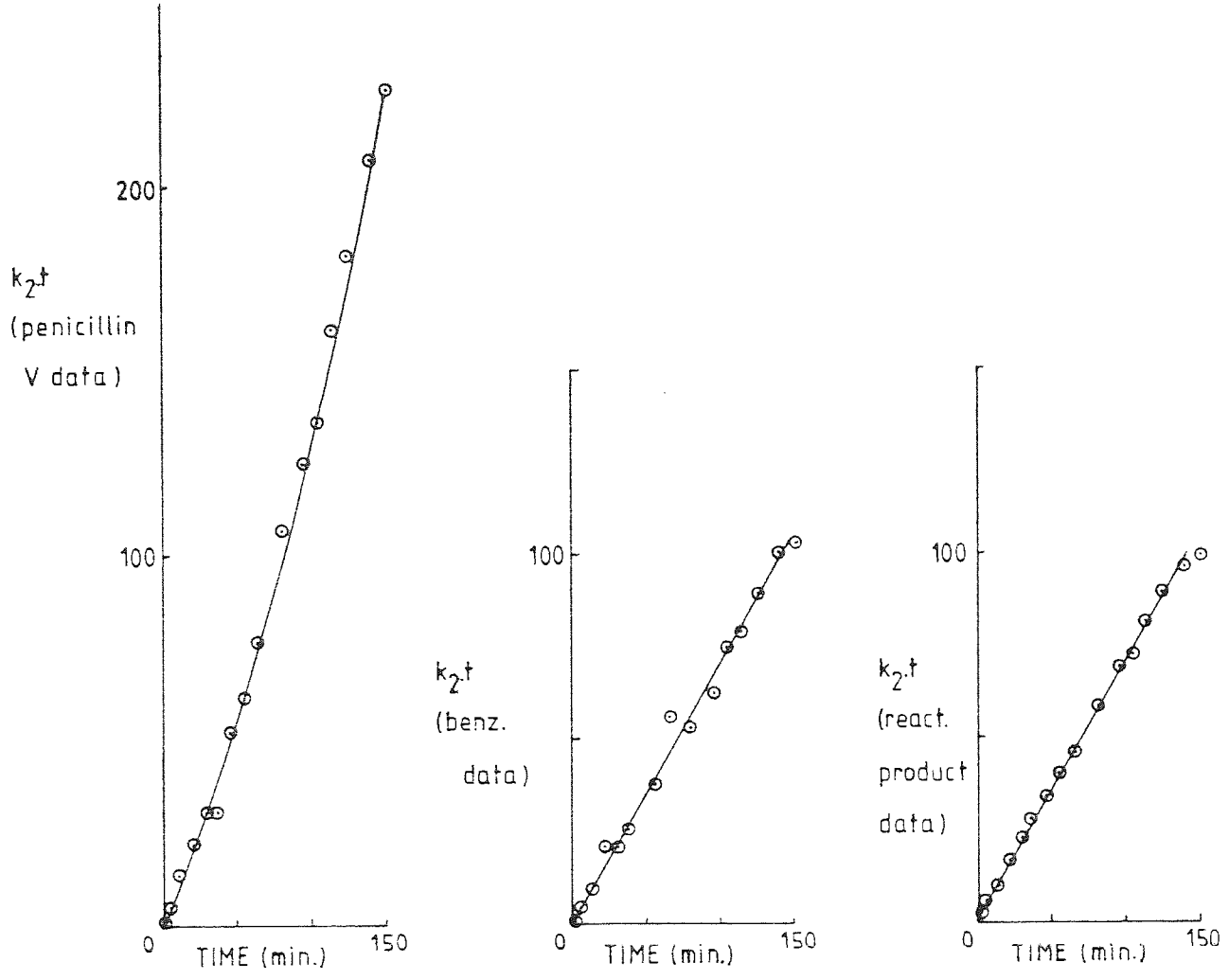


FIGURE 7.26 KINETIC RATE PLOTS FOR THE REACTION BETWEEN BENZATHINE AND PENICILLIN V AT pH 9. $[P]_0 = [P]_0$

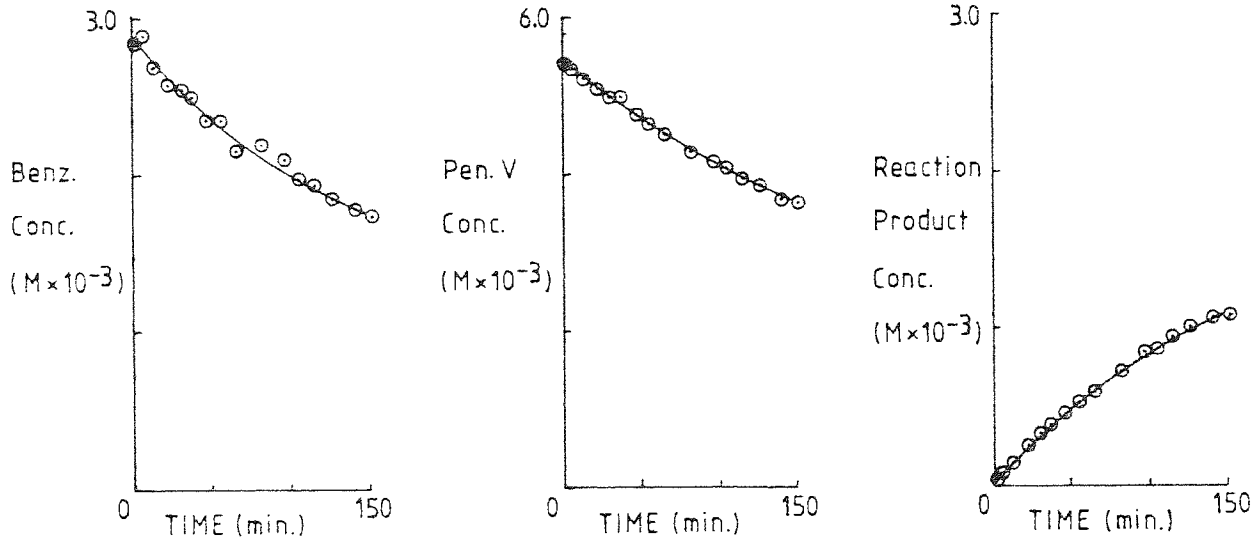


FIGURE 7.27 CHANGE IN BENZATHINE, PENICILLIN V AND REACTION PRODUCT CONCENTRATIONS IN SOLUTION AT pH 9

Therefore, $[BP]_t = \frac{(\text{peak height BP})_t \times 1.06 \times 10^{-3}\ M}{63.5}$

Figure 7.25 contains plots of $k_2.t$ vs t, calculated from equations 7.9, 7.10 and 7.11 where $[P]_o = [P]_m$. For comparison, figure 7.26 contains plots of $k_2.t$ vs t, calculated from equations 7.9, 7.10 and 7.11 where $[P]_o = [P]_o$. Figure 7.27 contains plots of [B], [P] and [BP] vs time. The need to modify equations 7.9, 7.10 and 7.11 ($[P]_o = [P]_m$) in order to obtain straight line plots fot $k_2.t$ vs t is clearly seen.

The slopes of $k_2.t$ vs t ($[P]_o = [P]_m$) provide a value for k_2 :

equation 7.9	0.765	$mol^{-1}.minute^{-1}$
equation 7.10	0.853	" "
equation 7.11	0.784	" "
mean	0.801	" "

The agreement between these values provides support for the mechanism in figure 7.24.

Reaction of Benzathine and Cloxacillin

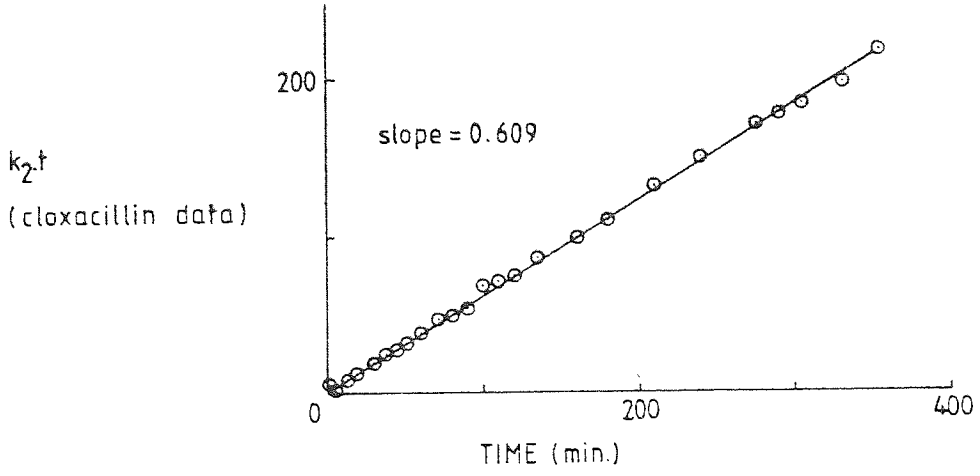
The first order degradation rate constant for cloxacillin in pH 9 borate buffer was 5.70×10^{-4} $minutes^{-1}$. In a similar way to that used for the reaction between benzathine and penicillin V, [BP] was estimated using $[BP]_t = [B]_o - [B]_t$, but, in this case, BP precipitates from solution thereby complicating the calculation of $[BP]_t$. It was assumed that no precipitation occurred during the first 120 minutes, an assumption supported by experimental observations where opacity was first noticed after 130 minutes.

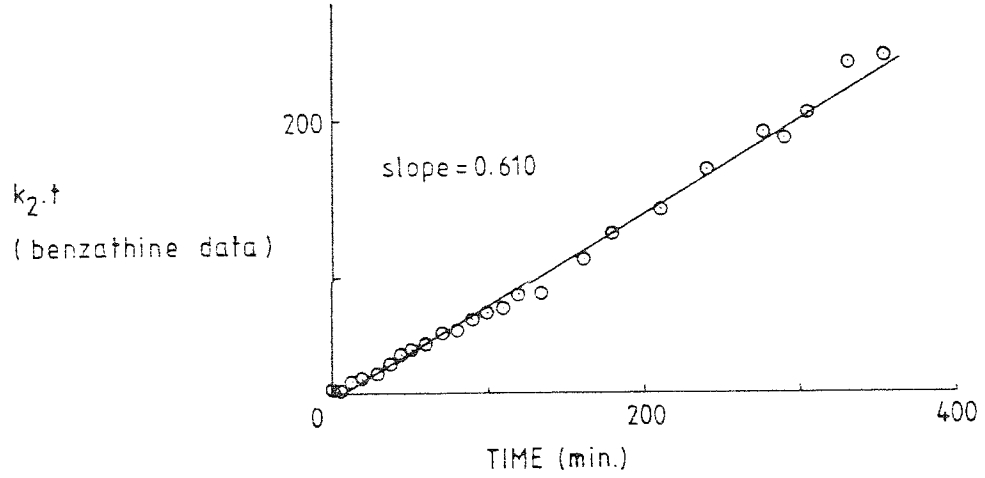
$[B]_o = 3.33 \times 10^{-3}$ M

$[B]_t = 2.27 \times 10^{-3}$ M at 120 minutes

Therefore, $[BP]_t = 1.06 \times 10^{-3}$ M at 120 minutes

The peak height of BP at 120 minutes was 131.5





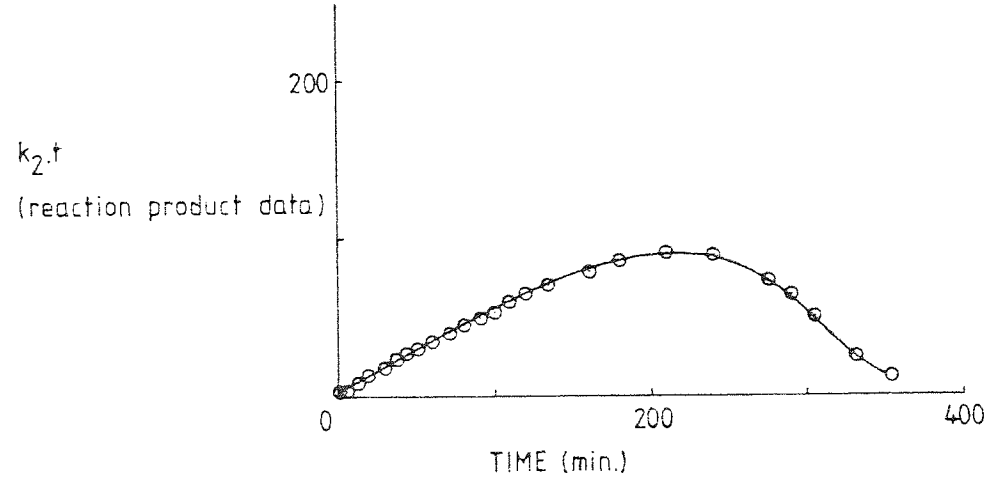


FIGURE 7.28 KINETIC RATE PLOTS FOR THE REACTION BETWEEN BENZATHINE AND CLOXACILLIN AT pH 9. $[P]_o = [P]_m$

Therefore, $[BP]_t = \frac{(\text{peak height BP})_t \times 1.06 \times 10^{-3}\ M}{131.5}$

Plots of $k_2.t$ vs t for equations 7.9, 7.10 and 7.11 ($[P]_o = [P]_t$ and $[P]_o = [P]_o$) can be seen in figures 7.28, 7.29 and 7.30. The slopes of the plots of $k_2.t$ vs t were :

equation 7.10	0.609	$mol^{-1}.minute^{-1}$
equation 7.11	0.610	" "
mean	0.610	" "

No slope was calculated from equation 7.9 (non-linear - precipitation). As before, the good agreement between these values provides support for the dissolution mechanism in figure 7.23.

The results obtained for benzathine reacting with cloxacillin and penicillin V can also be analysed using an analog computer. In this case, the degradation rate (k_3) does not have to be known. Lines generated according to equations 7.6, 7.7 and 7.8 are matched with experimentally obtained plots for [B], [P] and [BP] against time. The circuit diagram for analog computation can be seen below. Results obtained using this method were in general agreement with those obtained above.

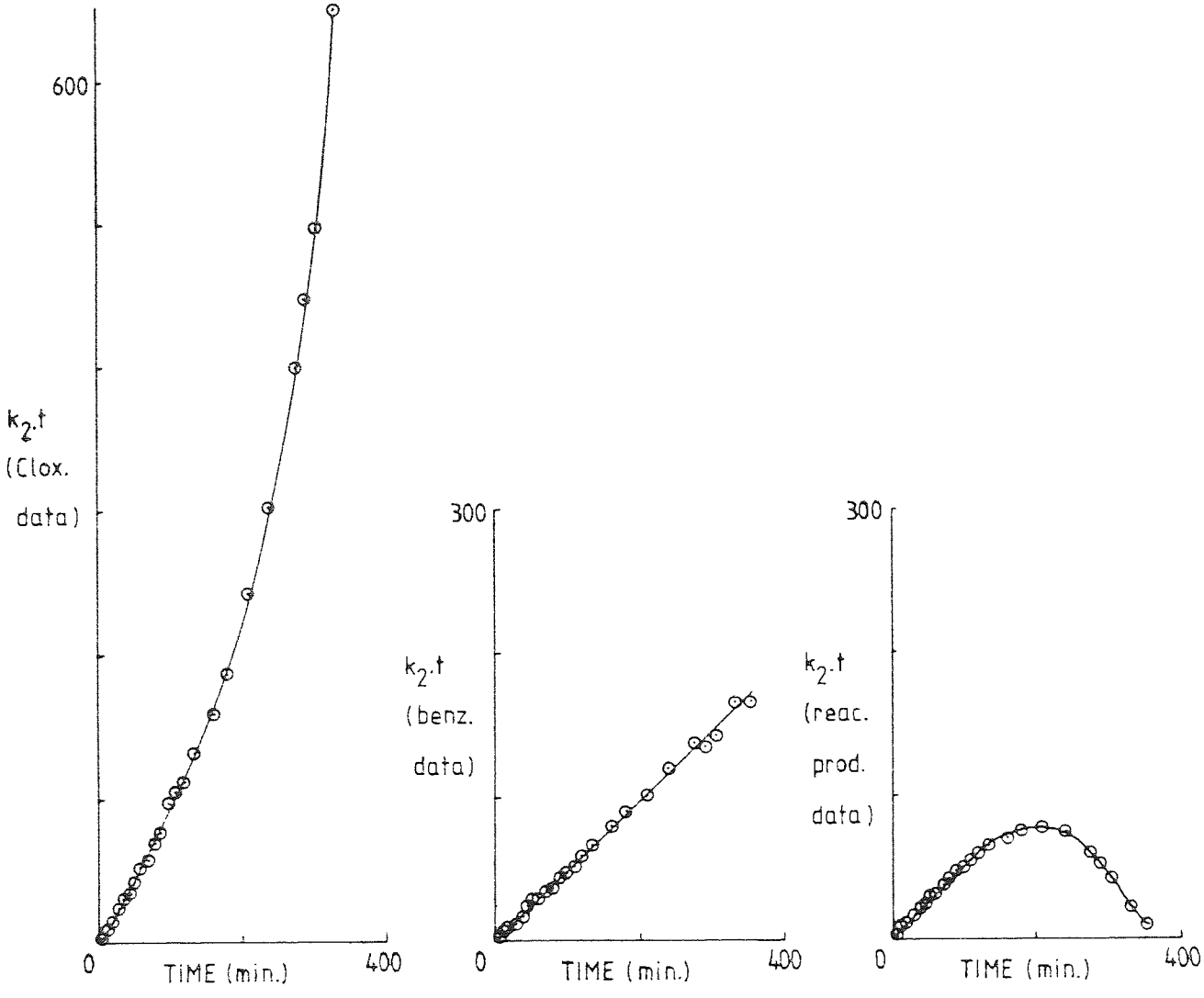


FIGURE 7.29 KINETIC RATE PLOTS FOR THE REACTION BETWEEN BENZATHINE AND CLOXACILLIN AT pH 9. $[P]_0 = [P]_0$

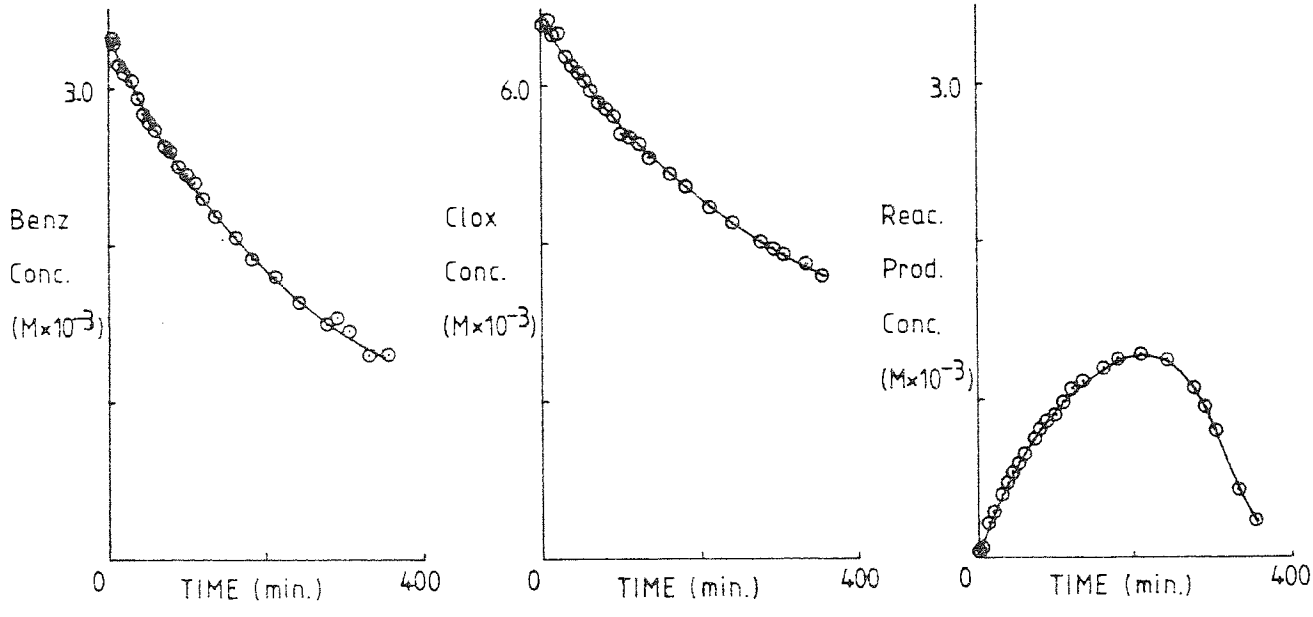
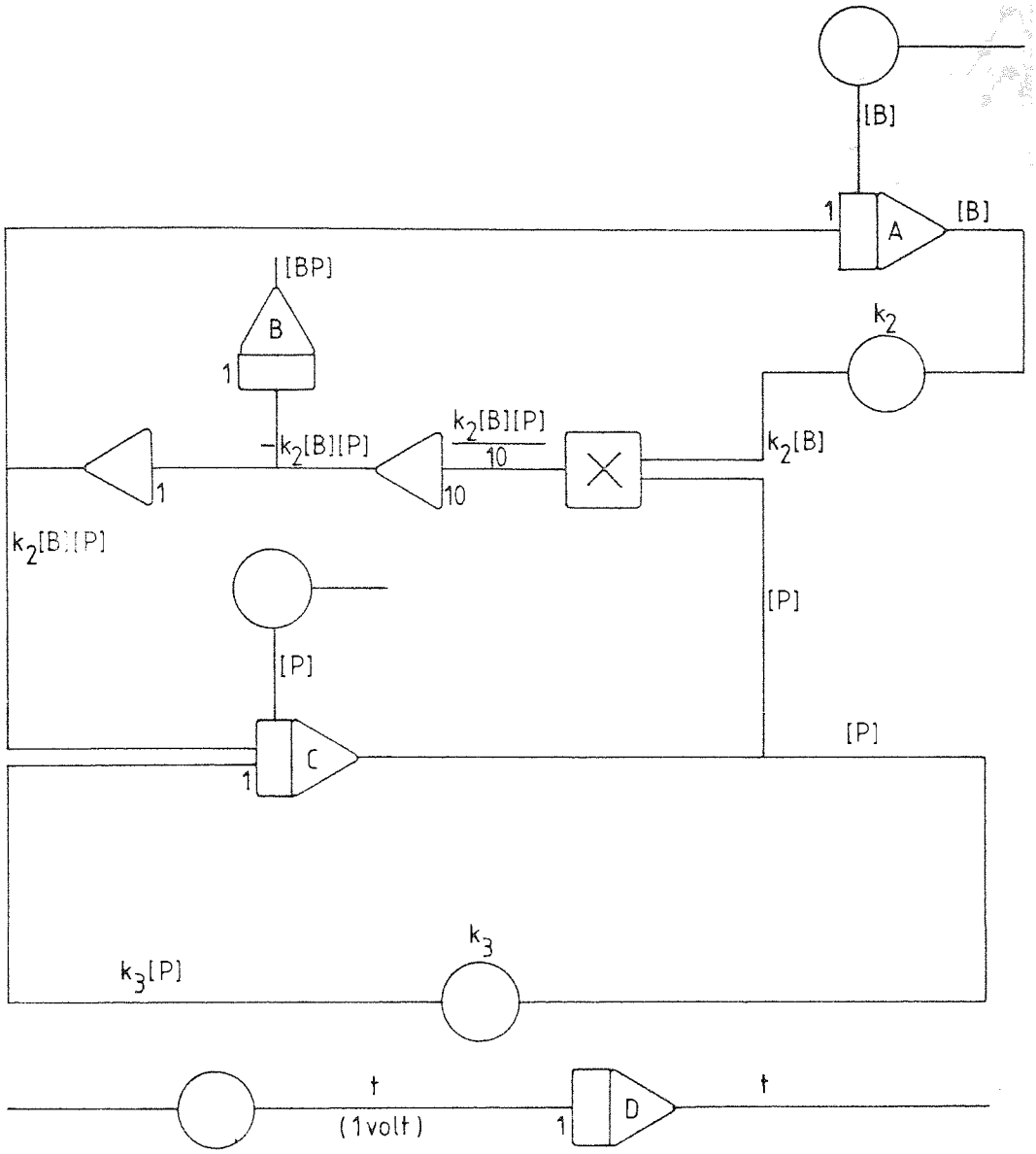


FIGURE 7.30 CHANGE IN BENZATHINE, CLOXACILLIN AND REACTION PRODUCT CONCENTRATIONS IN SOLUTION AT pH 9



Potentiometer

Multiplier

Integrator

Operational Amplifier

$[B]_t$ was measured as output from integrator A

$[P]_t$ " " " " C

$[BP]_t$ " " " " B

t " " " " D

ANALOG COMPUTER CIRCUIT FOR THE ANALYSIS OF THE REACTION BETWEEN BENZATHINE AND PENICILLIN V OR CLOXACILLIN

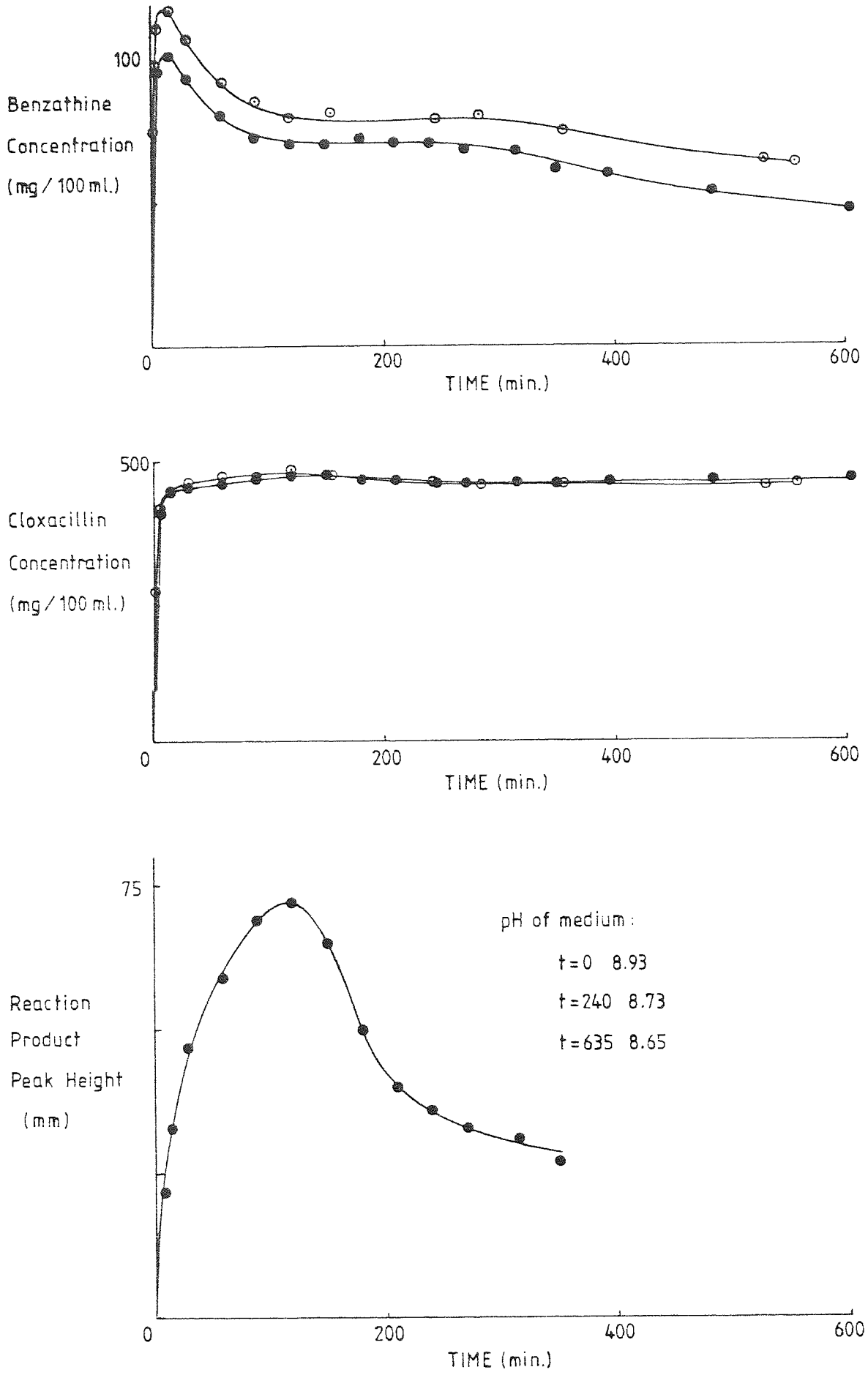


FIGURE 7.31 DISSOLUTION OF 5.0g BENZATHINE CLOXACILLIN AT pH 9

Dissolution of 5g Benzathine Cloxacillin at pH 9

Dissolution experiments using 1g of benzathine cloxacillin in 230 ml dissolution medium resulted in all the material dissolving in approximately 45 minutes, without saturated levels being reached. Repeating the experiment with 5g of material resulted in excess benzathine cloxacillin being present after ten hours. Figure 7.31 shows the profiles obtained in two independent dissolutions. The peak height of BP was only measured in the second of them.

The profiles cannot be fully explained. It would appear that [P] is constant throughout the experiment and this determines the solubility. However, the solubility of cloxacillin sodium in pH 9 borate buffer was >5% w/v, much higher than the levels measured in the dissolutions. The dissolution mechanism in figure 7.23 requires that $[B].[P]^2$ is constant, provided excess solid is present and the dissolution rapid. Obviously this is not the case here, where [P] is constant and [B] falls after 15 minutes. It is possible to calculate the expected loss of benzathine from the amount of cloxacillin in solution, but more benzathine is lost than can be explained by this mechanism. One possible explanation is that benzathine becomes associated with (by dissolving in or attaching to) the particles of benzathine cloxacillin remaining. Benzathine is less ionic at pH 9 than at pH 6 or pH 2, supporting this theory.

When BP starts precipitating out of solution, after approximately 120 minutes, it may coat the particles, preventing any more benzathine association with solid benzathine cloxacillin. This could explain the plateau for [B] between 100 and 250 minutes. It is possible that stirrer agitation then breaks down the precipitated layer of BP coating the benzathine cloxacillin, allowing more benzathine to be adsorbed. However, no evidence for this model was found. Also, there is no

explanation for the constant [P], which would be expected to rise in order to compensate for loss of [B], if the mechanism in figure 7.23 was correct.

7.4 CONCLUSIONS

Dissolution mechanisms for the dissolution of benzathine cloxacillin in pH 2 citrate, pH 6 citrate and pH 9 borate buffers were proposed. At pH 6, equilibrium solubility was achieved, providing a measure for the solubility of benzathine cloxacillin of 107.0 mg per 100 ml. Equilibrium solubility was not reached at pH 2 or pH 9.

Degradation of cloxacillin occurred at pH 2 and precipitation of its degradation products onto the remaining benzathine cloxacillin retarded further dissolution.

Degradation of cloxacillin and reaction between benzathine and cloxacillin to produce an amide occurred at pH 9. It is possible that similar reaction products could be formed in formulated medicines containing amines and penicillins. The presence of such products would reduce the efficacy of the medicine and could increase its toxicity.

SUGGESTIONS FOR FURTHER WORK

Nonisothermal kinetic analysis, using NONISO or alternative methods, could be applied to other formulated systems in order to further validate the treatment. It would be interesting to discover how many drug molecules in solution undergo a change in degradation reaction mechanism with a corresponding increase in temperature. This is likely to produce a non-linear plot for ln k_T *vs* t(T) and a temperature dependent E_{act}.

Degradation reactions in pharmaceutical suspensions and solids could be investigated by nonisothermal methods, although reaction mechanisms would be more complicated than in solution. With suspensions, there is normally an increase in solubility with a corresponding increase in temperature. This would increase the degradation rate disproportionately, producing a non-linear plot for ln k_T *vs* t(T) even if the degradation reaction mechanism did not alter with temperature. Measurement of time, temperature, concentration in solution and concentration in the whole sample would have to be made, and a subroutine written into NONISO to adjust the results for changing solubility with temperature.

Nonisothermal degradation of solids often illustrates a changing mechanism of reaction with temperature because,

a) the temperature range is often larger

b) many degradation reactions in solids involve hydrolytic reactions at the surface of the solid. The mechanism of this type of reaction is usually very dependent upon physical parameters such as the moisture content of the air and the rate of diffusion of water vapour through pores in the solid. These are greatly affected by a change in temperature.

Thus the study of the degradation of pharmaceutical solids by nonisothermal kinetic analysis would require careful interpretation of the results.

The formation of an amide by reaction between benzathine and cloxacillin or penicillin V in solution at pH9 suggests that such a reaction might occur in formulated products, albeit at a much slower rate. The presence of these products in formulated medicines could be investigated and the mutagenic and toxicological properties of such amides determined.

APPENDIX 1

ASSAY DATA FOR CHAPTER TWO

B.P. 1973 METHOD

STANDARD					
Concentration (µg/ml)	U.V. Absorbance				
33.3	1.086	1.133	1.103	1.113	1.103
26.7	0.860	0.828	0.907	0.827	0.856
20.0	0.631	0.622	0.636	0.624	0.633
13.3	0.385	0.417	0.402	0.428	0.411
6.67	0.187	0.194	0.192	0.186	0.186

SAMPLES						
Sample	Concentration (µg/ml)	U.V. Absorbance				
Amp. Sod.	20.0	0.636	0.623	0.629	0.654	0.647
Deg. Amp. Sod.	20.0	0.311	0.309	0.312	0.306	0.298
1:1 Mixture Amp. Sod./Clox.Sod.	20.0	0.298	0.276	0.270	0.280	0.282
Deg.1:1 Mixt. Amp. Sod./Clox. Sod.	20.0	0.213	0.209	0.207	0.211	0.216

Ampicillin (B.subtilis) Microbiological 5x5 Method

STANDARD					
Concentration (µg/ml)	Antilog. (Area)				
5.13	40.20	37.40	41.10	37.90	43.10
4.10	36.30	31.30	35.10	33.00	44.90
2.91	23.20	19.50	23.00	20.80	29.00
1.51	11.80	12.70	12.70	11.50	12.80
0.98	7.80	11.00	8.40	7.60	7.90

SAMPLES						
Sample	Concentration (µg/ml)	Antilog. (Area)				
Amp. Sod.	2.95	24.40	19.40	20.20	22.80	22.00
Deg. Amp. Sod.	8.78	25.20	20.40	21.70	22.30	23.80

Cloxacillin (B.subtilis) Microbiological 5x5 Method

STANDARD					
Concentration (µg/ml)	Antilog. (Area)				
19.20	25.70	23.20	23.50	39.90	28.00
17.10	22.40	21.50	18.60	27.50	26.40
13.00	14.40	17.70	13.20	14.40	14.60
10.40	11.10	16.30	13.00	9.40	10.70
6.70	7.10	7.30	8.40	5.10	6.40

SAMPLES						
Sample	Concentration (µg/ml)	Antilog. (Area)				
Clox. Sod.	13.20	19.50	14.90	16.50	15.30	17.20
Deg. Clox. Sod.	14.90	17.80	20.70	15.90	19.60	13.70
1:1 Mixt. Amp. Sod./Clox. Sod.	26.70	12.60	13.60	12.70	13.50	8.20
Deg.1:1 Mixt.Amp. Sod./Clox. Sod.	34.40	14.20	13.30	16.40	13.00	9.50

Ampicillin (S.lutea) Microbiological 5x5 Method

STANDARD					
Concentration (µg/ml)	Antilog. (Area)				
0.080	40.60	41.80	35.70	37.60	38.90
0.0656	34.00	29.80	31.10	30.30	33.00
0.0512	26.80	33.80	28.90	25.90	27.90
0.0360	15.50	21.20	20.90	22.50	18.70
0.0224	11.80	10.70	11.90	12.10	9.30

SAMPLES

Samples	Concentration (µg/ml)	Antilog. (Area)				
1:1 Mixt. Amp. Sod./Clox.Sod.	0.109	30.10	27.20	29.00	29.40	31.40
Deg.1:1 Mixt.Amp. Sod./Clox.Sod.	0.142	29.40	31.70	27.90	27.30	31.10

Ampicillin Imidazole Method (Procedure 1)

STANDARD					
Concentration (mg/ml)	U.V. Absorbance				
0.10	0.542	0.542	0.534	0.524	0.515
0.08	0.418	0.433	0.425	0.432	0.422
0.06	0.334	0.336	0.326	0.317	0.328
0.04	0.222	0.220	0.214	0.217	0.216
0.02	0.112	0.115	0.106	0.104	0.111

SAMPLES						
Sample	Concentration (mg/ml)	U.V. Absorbance				
Amp. Sod.	0.06	0.325	0.319	0.329	0.318	0.316
Deg.Amp.Sod.	0.06	0.167	0.166	0.163	0.161	0.158

Ampicillin Imidazole Method (Procedure 2)

STANDARD					
Concentration (mg/ml)	U.V. Absorbance				
2.00	0.690	0.671	0.688	0.678	0.665
1.60	0.524	0.548	0.532	0.543	0.554
1.20	0.408	0.419	0.415	0.424	0.418
0.80	0.259	0.273	0.267	0.282	0.276
0.40	0.142	0.147	0.142	0.136	0.139

SAMPLES						
Sample	Concentration (mg/ml)	U.V. Absorbance				
Amp. Sod.	1.20	0.416	0.410	0.401	0.413	0.436
Deg. Amp. Sod.	2.00	0.258	0.248	0.265	0.263	0.264

Cloxacillin Imidazole Method

STANDARD					
Concentration (mg/ml)	U.V. Absorbance				
0.100	0.965	0.981	0.971	0.991	0.958
0.080	0.768	0.781	0.783	0.795	0.774
0.060	0.580	0.576	0.576	0.589	0.573
0.040	0.380	0.389	0.384	0.378	0.383
0.020	0.185	0.193	0.195	0.196	0.192

SAMPLES						
Sample	Concentration (mg/ml)	U.V. Absorbance				
Clox. Sod.	0.06	0.581	0.585	0.581	0.573	0.589
Deg. Clox. Sod.	0.06	0.527	0.523	0.521	0.534	0.512

Ampicillin H.P.L.C. Method (No Internal Standard)

STANDARD					
Concentration (mg/ml)	Peak Height (mm)				
1.60	166.7	166.1	167.2	169.5	169.8
1.30	137.7	136.9	135.9	137.6	139.4
1.00	103.2	105.8	105.6	107.2	109.0
0.70	72.8	72.7	70.2	73.4	77.3
0.40	41.1	42.7	41.3	42.1	43.1

SAMPLES

Sample	Concentration (mg/ml)	Peak Height (mm)				
Amp. Sod.	1.00	105.3	105.3	105.9	105.5	109.7
Deg. Amp. Sod.	2.00	74.9	74.8	75.2	77.7	77.8
1:1 Mixt. Amp. Sod./Clox.Sod.	2.00	99.7	98.7	101.1	98.7	103.4
Deg.1:1 Mixt.Amp. Sod./Clox. Sod.	2.00	74.6	75.0	76.8	76.3	78.4

Ampicillin H.P.L.C. Method (Internal Standard)

STANDARD					
Concentration (mg/ml)	Peak Ratio				
1.60	1.338	1.341	1.320	1.306	1.318
1.30	1.087	1.093	1.079	1.079	1.082
1.00	0.847	0.848	0.838	0.839	0.819
0.70	0.598	0.586	0.553	0.578	0.574
0.40	0.336	0.337	0.326	0.336	0.325

SAMPLES						
Sample	Concentration (mg/ml)	Peak Ratio				
Amp. Sod.	1.0	0.830	0.832	0.836	0.839	0.856
Deg. Amp. Sod.	2.0	0.604	0.605	0.595	0.594	0.594
1:1 Mixt.Amp. Sod./Clox.Sod.	2.0	0.802	0.777	0.792	0.771	0.780
Deg.1:1 Mixt.Amp. Sod./Clox. Sod.	2.0	0.585	0.591	0.600	0.601	0.608

Cloxacillin H.P.L.C. Method (No Internal Standard)

STANDARD					
Concentration (mg/ml)	Peak Height (mm)				
1.40	163.3	163.9	163.8	164.6	165.3
1.14	135.8	136.1	136.9	137.0	140.7
0.88	105.9	107.1	104.0	106.5	106.9
0.62	75.0	75.7	76.9	77.3	76.6
0.36	44.7	45.9	46.0	44.9	45.1

SAMPLES						
Sample	Concentration (mg/ml)	Peak Height (mm)				
Clox. Sod.	0.88	105.4	105.3	106.9	106.9	105.5
Deg. Clox. Sod.	1.00	106.9	107.3	106.6	106.5	106.9
1:1 Mixt. Amp. Sod./Clox.Sod.	1.76	105.3	106.1	106.1	107.5	106.7
Deg.1:1 Mixt.Amp. Sod./Clox. Sod.	1.76	88.1	89.3	88.7	89.0	89.3

Cloxacillin H.P.L.C. Method (Internal Standard)

STANDARD					
Concentration (mg/ml)	Peak Ratio				
1.40	1.678	1.700	1.728	1.736	1.729
1.14	1.396	1.418	1.425	1.454	1.486
0.88	1.094	1.112	1.112	1.133	1.134
0.62	0.775	0.789	0.804	0.822	0.819
0.36	0.463	0.474	0.483	0.477	0.480

SAMPLES

Sample	Concentration (mg/ml)	Peak Ratio				
Clox. Sod.	0.88	1.084	1.111	1.116	1.132	1.122
Deg.Clox.Sod.	1.00	1.093	1.107	1.109	1.101	1.125
1:1 Mixt.Amp. Sod./Clox.Sod.	1.76	1.091	1.099	1.125	1.145	1.135
Deg.1:1 Mixt.Amp. Sod./Clox.Sod.	1.76	0.927	0.936	0.943	0.953	0.951

Ampicillin Direct U.V. Method

STANDARD					
Concentration (mg/ml)	U.V. Absorbance (difference)				
1.60	0.227	0.219	0.220	0.223	0.221
1.28	0.190	0.194	0.182	0.185	0.186
0.96	0.125	0.135	0.129	0.128	0.130
0.64	0.098	0.093	0.088	0.090	0.098
0.32	0.052	0.045	0.053	0.051	0.047

SAMPLES						
Sample	Concentration (mg/ml)	U.V. Absorbance (diff.)				
Amp. Sod.	1.28	0.176	0.186	0.188	0.185	0.188
Deg. Amp. Sod.	1.28	0.109	0.113	0.122	0.116	0.117
1:1 Mixt.Amp.Sod. Clox.Sod.	2.56	0.168	0.174	0.168	0.175	0.182
Deg.1:1 Mixt.Amp. Sod./Clox.Sod.	2.56	0.113	0.123	0.128	0.129	0.125

Cloxacillin Direct U.V. Method

STANDARD					
Concentration (mg/ml)	U.V. Absorbance				
1.60	0.769	0.757	0.749	0.761	0.765
1.28	0.607	0.620	0.609	0.610	0.614
0.96	0.442	0.442	0.430	0.434	0.449
0.64	0.300	0.298	0.295	0.304	0.302
0.32	0.149	0.144	0.143	0.156	0.152

SAMPLES						
Sample	Concentration (mg/ml)	U.V. Absorbance				
Clox. Sod.	1.28	0.606	0.595	0.600	0.610	0.594
Deg. Clox. Sod.	1.28	0.676	0.681	0.678	0.680	0.685
1:1 Mixt.Amp. Sod./Clox.Sod.	2.56	0.593	0.600	0.608	0.588	0.590
Deg.1:1 Mixt.Amp. Sod./Clox.Sod.	2.56	0.552	0.593	0.580	0.576	0.581

APPENDIX 2

PROGRAM ONE

```
10 REM*************************************************************************
20 REM********************** N  O  N  I  S  O **********************
30 REM
40 REM     A PROGRAM TO CALCULATE NONISOTHERMAL KINETIC PARAMETERS
50 REM
60 REM           WRITTEN BY W.J.IRWIN AND J.M.HEMPENSTALL
70 REM
80 REM    DEPARTMENT OF PHARMACY - ASTON UNIVERSITY ,BIRMINGHAM ,ENGLAND
90 REM
100 REM************************************************************************
110 REM************************************************************************
120 INPUT "ARE INSTRUCTIONS ON DATA ENTRY REQUIRED ?  (Y OR N)",P$\ !\ !
130 IF P$="N" THEN 460
140 !#Z\ !#Z\ !#Z\ !#Z\ !#Z \ !#Z,"DATA ENTRY:"\ !#Z
150 !#Z," LINE 3940 - 0 FOR ZERO ORDER TREATMENT OF DATA"
160 !#Z,"              1 FOR FIRST ORDER TREATMENT OF DATA"
170 !#Z,"              2 FOR SECOND ORDER (A=B) TREATMENT OF DATA"
180 !#Z,"              3 FOR SECOND ORDER (A#B) TREATMENT OF DATA"\ !#Z
190 !#Z," LINE 3950 - TITLE OF EXPERIMENT (UP TO 72 CHARACTERS)"\ !#Z
200 !#Z," LINE 3960 - NUMBER OF DATA POINTS (UP TO 100)"\!#Z
210 !#Z," LINE 3970 - TIME UNITS AS MINS OR HRS (IN INVERTED COMMAS)"\ !#Z
220 !#Z," LINE 3980 - EXPERIMENTAL DATA (AS TIME,TEMPERATURE,CONCENTRATION)"
230 !#Z,"  ONWARDS                                (CENTIGRADE) (MOLES/LITRE)"
240 !#Z,"                STARTING WITH TIME=0"\ !#Z\ !#Z
250 !#Z," THE POLYNOMIAL ORDER CAN BE CHANGED AT LINE 1190"\ !#Z\ !#Z\ !#Z
260 REM - ASSIGNMENT OF VARIABLES -
270 REM      T1 = TIME (MIN OR HRS)                T2 = TEMPERATURE (CENTIGRADE)
280 REM      C1 = CONCENTRATION(MOLES/LITRE)  K1 = ISOTHERMAL RATE CONSTANTS
290 REM      X1,Y1 AND X2,Y2 = ARRAYS USED FOR CALCULATIONS INVOLVING
300 REM                              TRANSFORMED DATA
310 REM      R1 = MATRIX STORING POSITIONS FOR ARRHENIUS PLOT
320 REM      B  = CONC. OF EXCESS REAGENT IN SECOND ORDER(A#B) REACTION
330 REM      A$ = TITLE                          B$ = TIME UNITS
340 REM      C$ = REACTION ORDER                 D$ = TIME UNITS
350 REM      X$ = CONCENTRATION UNITS            W$ = RATE CONSTANT TERM
360 REM      C  = INITIAL CONCENTRATION OF REACTANT
370 REM      N1 = NUMBER OF DATA POINTS          N2 = REDUCED NUMBER OF POINTS
380 REM      N3 = POLYNOMIAL ORDER               P  = POLYNOMIAL COEFFICIENTS
390 REM      O1 = ORDER REACTION CODE            E  = ACTIVATION ENERGY
400 REM      A  = PRE-EXPONENTIAL FACTOR         T3 = HALF LIFE
410 REM     T4 = TIME OF 10% DEGRADATION
420 REM     A AND B WERE USED TO CALCULATE POLYNOMIAL COEFFICIENTS AND AS
430 REM                 DUMMY VARIABLES IN THE SORT ROUTINE
440 REM     Z WAS USED TO DIRECT OUTPUT (0=V.D.U.,1=LINE PRINTER)
450 REM     U WAS USED TO CONVERT RATE CONSTANTS TO 1/HRS
460 INPUT "ENTER DEVICE NUMBER (0 FOR V.D.U.,1 FOR LINE PRINTER)",Z\ !\ !
470 REM************************************************************************
480 REM********************** M  A  I  N **********************
490 REM************************************************************************
500 GOSUB 1100\!#Z TAB(15),"NON-ISOTHERMAL KINETICS"\GOSUB 1100
510 DIM T1(100),T2(100),C1(100),X1(100),Y1(100),X2(100),Y2(100)
520 DIM K1(100),P(11,11),A(100),B(100),A$(72),B$(3),C$(12),D$(10)
530 DIM R1(101,3),C(100),T(15)
540 FOR I=1TO 15\READ T(I)\NEXT I
550 REM READ ORDER OF REACTION
560 READ O1\IF O1 <0 OR O1 >3 THEN 4310
570 O1=O1+1\ON O1 GOTO 580,590,600,610
580 C$="ZERO"\GOTO 650
590 C$="FIRST"\GOTO 650
600 C$="SECOND (A=B)"\GOTO 650
610 C$="SECOND (A#B)"
620 !\ !
630 INPUT "GIVE INITIAL CONC. OF EXCESS REACTANT (IN MOL./LITRE) ",B\ !
640 W3=B
650 REM "GIVE TITLE OF EXPERIMENT"
660 READ A$
670 REM "HOW MANY DATA POINTS"
680 READ N1
690 REM "TIME UNITS-MINUTES OR HOURS"
700 U=1\READ B$\IF B$="MIN" THEN 710 ELSE GOTO 720
710 U=60
720 REM GIVE DATA POINTS STARTING AT TIME=0
730 REM TIME,TEMPERATURE,CONCENTRATION
740 FOR I=1 TO N1\READ T1(I),T2(I),C1(I)\ NEXT I
750 IF O1=2 THEN 760\ GOTO 770
```

```
760 U4=100/C1(1)\ FOR I=1 TO N1\ C1(I)=C1(I)*U4\ NEXT I
770 GOSUB 1100\!#Z A$\GOSUB 1100
780 IF O1=2 THEN 790\ X$="(MOLES/L)"\ GOTO 800
790 X$="(%)"
800 !#Z"INPUT DATA:"\!#ZTAB(12),"TIME(",B$,")",TAB(30),
810 !#Z,"TEMPERATURE(CENT.)",TAB(52),"CONCENTRATION",X$
820 FOR I=1 TO N1\!#ZTAB(14), T1(I),TAB(35),T2(I),TAB(56),C1(I)\NEXT I
830 REM TRANSFORMATIONS
840 J=0\C=C1(1)
850 U4=1
860 IF O1<4 THEN 880
870 U4=2
880 FOR I=U4 TO N1
890 REM ELIMINATE FINAL SECTION OF CURVE
900 IF O1=2 THEN 920
910 IF C1(I)<C*0.2 THEN 1000\ GOTO 930
920  IF C1(I)<C*0.05 THEN 1000
930 J=J+1\C1(J)=C1(I)\T1(J)=T1(I)\T2(J)=T2(I)\X1(J)=T1(I)
940 IF X1(1)=0 THEN X1(1)=0.001
950 ON O1 GOTO 960,970,980,990
960 Y1(J)=C1(I)\GOTO 1000
970 Y1(J)=LOG(C1(I))\GOTO 1000
980 Y1(J)=1/C1(I)\ GOTO 1000
990 Y1(J)=1+(B/(B-C))*LOG(((B-C+C1(I))*C)/(C1(I)*B))
1000 NEXT I
1010 N2=J
1020 GOSUB 1160\GOSUB 1290\GOSUB 1720\GOSUB 2120
1030 REM TRANSFORM FOR ARRHENIUS PLOT
1040 FOR I=1 TO N2\Y2(I)=LOG(K1(I))\X2(I)=1/(T2(I)+273.15)\NEXT I
1050 GOSUB 2350\GOSUB 2560\GOSUB 1950\GOSUB 3010\IF D9>1 THEN 1030
1060 GOSUB 2800
1070 GOSUB 1100
1080 STOP
1090 REM*****************************************************************************
1100 REM********************  D   R   A   W  ********************************
1110 REM  SUBROUTINE TO DRAW ASTERISKS
1120 REM*****************************************************************************
1130 !#Z\FOR I=1 TO 72\!#Z"*",
1140 NEXT I\!#Z\RETURN
1150 REM*****************************************************************************
1160 REM********************  N   O   R   M   E   Q  ************************
1170 REM SUBROUTINE TO SET UP NORMAL EQUATIONS FOR POLYNOMIAL
1180 REM*****************************************************************************
1190 N3=7
1200 N4=N3+1
1210 FOR I=1 TO N4\FOR J=1 TO N4\P(I,J)=0\NEXT J\NEXT I
1220 FOR I=1 TO N3\FOR J=1 TO N3\FOR K=1 TO N2
1230 P(I,J)=P(I,J)+X1(K)^(I+J-2)
1240 NEXT K\NEXT J\NEXT I
1250 FOR I=1 TO N3\FOR K=1 TO N2
1260 P(I,N4)=P(I,N4)+Y1(K)*X1(K)^(I-1)
1270 NEXT K\NEXT I\RETURN
1280 REM*****************************************************************************
1290 REM********************  S   I   M   E   Q  ************************
1300 REM SUBROUTINE TO SOLVE SIMULTANEOUS EQUATIONS BY ELIMINATION
1310 REM*****************************************************************************
1320 N5=N4+1
1330 FOR I=1 TO N4
1340 A(I)=I\B(I)=I\NEXT I
1350 FOR I=1 TO N3-1\IF P(I,I)<>0 THEN 1490
1360 FOR J=I TO N3\FOR Q=I TO N3
1370 IF P(Q,J)<>0 THEN 1410
1380 NEXT Q\NEXT J
1390 !#Z "SMALL DIAGONAL ELEMENT-NO POLYNOMIAL SOLUTION POSSIBLE"
1400 STOP
1410 IF J<=I THEN 1450
1420 B(N4)=B(I)\B(I)=B(J)\B(J)=B(N4)
1430 FOR L=1 TO N3\P(L,N5)=P(L,I)
1440 P(L,I)=P(L,J)\P(L,J)=P(L,N5)\NEXT L
1450 IF Q<=I THEN 1490
1460 FOR N=1 TO N4\P(N4,N)=P(I,N)
1470 P(I,N)=P(Q,N)\P(Q,N)=P(N4,N)\NEXT N
1480 A(N4)=A(I)\A(I)=A(Q)\A(Q)=A(N4)
1490 S=P(I,I)\I1=I+1
1500 FOR J=I1 TO N3\T=P(J,I)
```

```
1510 IF T=0 THEN 1540
1520 FOR R=1 TO N4\P(J,R)=P(J,R)-(T*(P(I,R)/S))
1530 NEXT R
1540 NEXT J
1550 NEXT I
1560 IF P(N3,N3)<>0 THEN 1590
1570 !#Z"PIVOT FAILURE - NO POLYNOMIAL SOLUTION POSSIBLE"
1580 STOP
1590 P(N3,N4)=P(N3,N4)/P(N3,N3)\L=N4
1600 FOR J=1 TO N3-1\L=L-1\M=L-1\E1=0
1610 FOR I=L TO N3\E1=E1+P(M,I)*P(I,N4)\NEXT I
1620 P(M,N4)=(P(M,N4)-E1)/P(M,M)\NEXT J
1630 FOR K1=1 TO N3\IF B(K1)=K1 THEN 1690
1640 FOR J=1 TO N3\IF B(J,I)<>K1 THEN 1680
1650 P(N5,N4)=P(K1,N4)\P(K1,N4)=P(J1,N4)\P(J1,N4)=P(N5,N4)
1660 B(N4)=B(K1)\B(K1)=B(J1)\B(J1)=B(N4)
1670 GOTO 1700
1680 NEXT J1
1690 NEXT K1
1700 RETURN
1710 REM*************************************************************************
1720 REM********************  P   O   L   Y   O   U   T  ********************
1730 REM SUBROUTINE TO PRINT POLYNOMIAL RESULTS
1740 REM*************************************************************************
1750 GOSUB 1100\!#Z A$\GOSUB 1100
1760 IF O1<4 THEN 1790
1770 !#Z"THIS CALCULATION USES A ",C$," ORDER MODEL , B= ",W3,"  MOLES"
1780 GOTO 1800
1790 !#Z"THIS CALCULATION USES A ",C$," ORDER MODEL"
1800 GOSUB 1100\!#Z "POLYNOMIAL RESULTS"\!#Z
1810 FOR I=1 TO N2\Y2(I)=0
1820 FOR J=1 TO N3\Y2(I)=Y2(I)+P(J,N4)*X1(I)^(J-1)
1830 NEXT J\NEXT I
1840 !#Z TAB (15),"COEFFICIENT",TAB(45),"POWER"
1850 FOR I=1 TO N3\!#Z TAB(15),P(I,N4),TAB(45),I-1\NEXT I\!#Z
1860 !#Z "TIME(",B$,")",TAB(14),"CONC.",X$,TAB(30),"TRANSFORM",
1870 !#Z,TAB(45),"CALC.",TAB(60),"RATIO(%)"
1880 FOR I=1 TO N2
1890 !#Z T1(I),TAB(15),C1(I),TAB(30),Y1(I),TAB(45),Y2(I),
1900 !#Z,TAB(60),Y2(I)/Y1(I)*100
1910 NEXT I
1920 GOSUB 1950
1930 RETURN
1940 REM*************************************************************************
1950 REM********************  F   I   T  ********************
1960 REM SUBROUTINE TO CALCULATE CORRESPONDENCE BETWEEN THEORETICAL
1970 REM                  AND OBSERVED VALUES
1980 REM*************************************************************************
1990 S1=0\S2=0\S3=0\S4=0
2000 FOR I=1 TO N2\S1=S1+Y1(I)\S2=S2+Y1(I)^2\S3=S3+(Y1(I)-Y2(I))^2
2010 S4=S4+(Y1(I)-Y2(I))^2/Y2(I)\NEXT I\Y3=S1/N2
2020 V1=SQRT(S3/(N2-N3))\V2=100*V1/Y3
2030 R=1-(S3/(S2-Y3))\IF R>0.9999 THEN R=1
2040 !#Z\!#Z
2050 !#Z"DETERMINATION",TAB(20),"COEFFICIENT OF",TAB(40)," CHI-",TAB(60),
2060 !#Z,"DEGREES OF "
2070 !#Z"COEFFICIENT",TAB(20),"VARIATION (%)",TAB(40),"SQUARED",TAB(60),
2080 !#Z,"FREEDOM"
2090 !#Z\!#Z R,TAB(20),V2,TAB(40),S4,TAB(63),N2-N3-1
2100 !#Z\RETURN
2110 REM*************************************************************************
2120 REM********************  D   I   F   F  ********************
2130 REM SUBROUTINE TO DIFFERENTIATE POLYNOMIAL TO GENERATE
2140 REM              ISOTHERMAL RATE CONSTANTS
2150 REM*************************************************************************
2160 FOR I=1 TO N2\K1(I)=0
2170 FOR J=2 TO N3\K1(I)=K1(I)+P(J,N4)*(J-1)*X1(I)^(J-2)\NEXT J
2180 K1(I)=ABS(K1(I))\NEXTI
2190 GOSUB 1100\!#Z A$\GOSUB 1100
2200 !#Z"ISOTHERMAL RATE CONSTANTS"\!#Z
2210 ON O1 GOTO 2220,2230,2240,2240
2220 W$="(MOLE/L/"\ GOTO 2250
2230 W$="(1/"\ GOTO 2250
2240 W$="(1/MOLES/"
2250 !#Z "TIME(",B$,")",TAB(17),"CONC.",X$,TAB(34),"TEMP.(CENT.)",TAB(49),
```

```
2260 !#Z,"RATE CONSTANT",W$,B$,")"
2270 FOR I=1 TO N2
2280 IF O1<4 THEN 2300
2290 K1(I)=K1(I)/W3
2300 !#Z T1(I),TAB(17),C1(I),TAB(34),T2(I),TAB(51),K1(I)\ NEXT I
2310 REM TRANSFORM FOR ARRHENIUS PLOT
2320 FOR I=1 TO N2\Y2(I)=LOG(K1(I))\X2(I)=1/(T2(I)+273.15)\NEXT I
2330 RETURN
2340 REM*****************************************************************
2350 REM********************  L   E   A   S   Q  **************************
2360 REM LINEAR LEAST SQUARES SUBROUTINE
2370 REM*****************************************************************
2380 S1=0\S2=0\S3=0\S4=0\S5=0\N6=N2-2
2390 FOR I=1 TO N2\S1=S1+X2(I)\S2=S2+Y2(I)\S3=S3+X2(I)*X2(I)
2400 S4=S4+X2(I)*Y2(I)\S5=S5+Y2(I)*Y2(I)\NEXT I
2410 S6=S1*S1\S7=S2*S2\X3=S1/N2\Y3=S2/N2
2420 D0=S4-S1*S2/N2\D1=S3-S6/N2\D2=S5-S7/N2
2430 B=D0/D1\A=Y3-(B*X3)\R=D0/SQRT(D1*D2)
2440 REM VARIANCE ABOUT Y
2450 V1=(S5-(N2*Y3*Y3)-B*B*(S3-N2*X3*X3))/N6
2460 V2=V1/D1*N2\V3=V2*S3/N2
2470 REM ASSIGN T-VALUES
2480 IF N6>15 THEN 2500
2490 T=T(N6)\GOTO 2510
2500 T=2.27567-1.24641E-02*N6+1.8006E-04*N6^2-8.09E-07*N6^3
2510 E2=SQRT(V2/N2)
2520 IF E2=0 THEN E2=1E-06
2530 E3=SQRT(V3/N2)*T\F1=(B/E2)^2\E2=E2*T
2540 RETURN
2550 REM*****************************************************************
2560 REM********************  O   U   T   P   U   T  *********************
2570 REM SUBROUTINE TO PRINT OUT RESULTS
2580 REM*****************************************************************
2590 GOSUB 1100\!#Z A$\GOSUB 1100
2600 ON O1 GOTO 2610,2620,2630,2630
2610 Y$="MOLES/L/"\ GOTO 2640
2620 Y$="/"\ GOTO 2640
2630 Y$="1/MOLES/"
2640 !#Z"ARRHENIUS RESULTS"\!#Z
2650 !#Z "SLOPE",TAB(10),B,TAB(25),"+/-",TAB(40),E2,TAB(60),"(P=95%)"
2660 !#Z"INTERCEPT",TAB(10),A,TAB(25), "+/-",TAB(40),E3,TAB(60),"(P=95%)"
2670 !#Z\!#Z"R= ",R
2680 B1=-B*8.3143\B2=-8.3143*(B-E2)\B3=-8.3143*(B+E2)
2690 A1=EXP(A)\A2=EXP(A+E3)\A3=EXP(A-E3)
2700 !#Z "PARAMETER",TAB(15),"VALUE",TAB(43),"RANGE",TAB(66),"UNITS"\!#Z
2710 !#Z " E",TAB(12),B1,TAB(30),B3,TAB(45),":",B2,TAB(66),"J/MOL"
2720 !#Z " A",TAB(12),A1,TAB(30),A3,TAB(45),":",A2,TAB(66),Y$,B$\!#Z
2730 !#Z\GOSUB 1100\!#Z\!#Z "PREDICTED RATE CONSTANTS",W$,B$,")",\ !#Z
2740 !#Z "TEMP.",TAB(20),"RATE",TAB(40),"CALC.",TAB(60),"RATIO (%)"\!#Z
2750 FOR I=1 TO N2
2760 Y2(I)=A1*EXP(-B1/(8.3143*(T2(I)+273.15)))
2770 !#Z T2(I),TAB(20),K1(I),TAB(40),Y2(I),TAB(60),Y2(I)/K1(I)*100
2780 Y1(I)=K1(I)\NEXT I\N3=1\RETURN
2790 REM*****************************************************************
2800 REM********************  P   R   E   D   I   C   T  *****************
2810 REM SUBROUTINE TO PREDICT SHELF LIVES
2820 REM*****************************************************************
2830 !#Z\GOSUB 1100\!#Z
2840 !#Z"SHELF-LIFE PREDICTIONS",TAB(40),C$," ORDER MODEL"
2850 !#Z\!#Z"TEMPERATURE",TAB(25),"T-50%",TAB(45),"T-90%"\!#Z
2860 FOR I=5 TO 30 STEP 5
2870 K1=A1*EXP(-B1/(8.3143*(273.15+I)))
2880 ON O1 GOTO 2890,2900,2910,2920
2890 T3=C/(2*K1*U*168)\ T4=T3/5\ GOTO 2940
2900 T3=.69315/(K1*U*168)\ T4=T3/6.57881\ GOTO 2940
2910 T3=1/(K1*U*168*C)\ T4=T3/9\ GOTO 2940
2920 T3=1/((K1*U*168)*(W3-C))*LOG(((W3-C/2)*C)/(C/2*W3))
2930 T4=1/((K1*U*168)*(W3-C))*LOG(((W3-0.1*C)*C)/(0.9*C*W3))
2940 D$="WEEKS"\ IF T4>1 THEN 2980
2950 D$="DAYS"\ T3=T3*7\ T4=T4*7
2960 IF T4>1 THEN 2980
2970 D$="HOURS"\ T3=T3*24\ T4=T4*24
2980 !#Z I,TAB(20),T3,TAB(40),T4,TAB(60),D$
2990 NEXT I\GOSUB 1100\RETURN
```

```
3000 REM*************************************************************************
3010 REM********************  P   L   O   T  ******************************
3020 REM SUBROUTINE TO PLOT ARRHENIUS RESULTS
3030 REM*************************************************************************
3040 GOSUB 1100\IF Z=1 THEN  !#Z CHR$(12),
3050 !#ZTAB(25),"ARRHENIUS PLOT OF LOG (K) VS 1/T"
3060 FOR I=1TON2\Y1(I)=LOG(K1(I))\NEXTI
3070 A4=A+B*X2(N2)\A5=Y1(1)\FOR I=1TON2\IF Y1(I)>A4 THEN A4=Y1(I)
3080 IF Y1(I)<A5 THEN A5=Y1(I)
3090 NEXTI
3100 REM H1 FIXES NUMBER OF HORIZONTAL POINTS.V1 THE VERTICAL.
3110 H1=50\V1=20
3120 H=INT(H1/10)\H1=H*10\V1=INT(V1/10)*10
3130 REM  Z=0 FOR V.D.U. , Z=1 FOR LINEPRINTER
3140 H1=H1+H1*Z\V1=V1+(V1+10)*Z
3150 !#Z A4,
3160 S1=(V1-1)/(A4-A5)\S2=(H1-1)/(X2(1)-X2(N2))
3170 REM SET UP MAP OF POINTS IN R1
3180 FOR I1=1TON2\I=N2-I1+1\P1=(A4-Y1(I))*S1\P1=INT(P1)+1
3190 P2=(X2(I)-X2(N2)) *S2\P2=INT(P2)+1
3200 R1(I1,1)=P1\R1(I1,2)=P2\R1(I1,3)=I\NEXT I1
3210 REM Y-AXIS DATA FOR PLOT
3220 FOR I=1TON2\A(I)=R1(I,1)\B(I)=R1(I,2)\C(I)=R1(I,3)\NEXTI
3230 N8=N2\GOSUB 3770
3240 FOR I=1TON2\R1(I,1)=A(I)\R1(I,2)=B(I)\R1(I,3)=C(I)\NEXTI
3250 I1=1\I2=1
3260 IF I1>V1 OR I2>N2 THEN 3510
3270 IF I1=R1(I2,1) THEN 3330
3280 P3=A4-(I1)/S1\P3=-(A-P3)/B\P3=(P3-X2(N2))*S2\P3=INT(P3)+1
3290 IF P3<1 THEN 3310
3300 IF P3<= H1 THEN 3320
3310 I1=I1+1\!#ZTAB(15),"I"\GOTO 3260
3320 !#ZTAB(15),"I",TAB(P3+15),"."\I1=I1+1\GOTO 3260
3330 N8=0
3340 N8=N8+1\J=N8+I2\IFJ>N2 THEN 3360
3350 IFR1(I2,1)=R1(J,1)THEN3340
3360 IFN8=1 THEN 3420
3370 FOR I3=1TON8\I=I2+I3-1\A(I3)=R1(I,2)\B(I3)=R1(I,1)\C(I3)=R1(I,3)
3380 NEXT I3
3390 GOSUB 3770
3400 FOR I3=1TON8\I=I2+I3-1\R1(I,1)=B(I3)\R1(I,2)=A(I3)\R1(I,3)=C(I3)
3410 NEXT I3
3420 !#ZTAB(15),"I",\I3=0
3430 I3=I3+1\I=I2+I3-1\!#ZTAB(R1(I,2)+15),"*",
3440 IF I3=N8 THEN 3480
3450 IF R1(I,2)=R1(I+1,2) THEN 3470
3460 GOTO 3430
3470 I3=I3+1\I=I+1\GOTO 3440
3480 FOR I3=1TON8\I=I2+I3-1\!#ZR1(I,3),\NEXTI3
3490 !#Z\I1=I1+1\I2=I2+N8
3500 GOTO3260
3510 D$="+---------"\!#ZA5,TAB(15),"+",\FOR I=1TOH+H*Z
3520 !#ZD$,\NEXTI\!#Z"+"
3530 !#ZTAB(10),X2(N2),TAB(H1+10),X2(1)\!#Z
3540 !\ !"1 TO CONTINUE; 2 TO OMIT FIRST POINT;",
3550 !" 3 TO SPECIFY OMITTED POINTS  ",\INPUT D9
3560 IF D9<0 OR D9>3THEN3540
3570 ON D9 GOTO 3580,3590,3620
3580 RETURN
3590 !#Z\ !#Z\ !#Z\ !#Z,"FIRST POINT OMITTED"\ !#Z\ !#Z
3600 N2=N2-1\FOR I=1TON2\T1(I)=T1(I+1)\T2(I)=T2(I+1)
3610 C1(I)=C1(I+1)\K1(I)=K1(I+1)\NEXTI\RETURN
3620 I=0
3630 !\ !\ !"IT IS SUGGESTED ONLY EARLY POINTS ARE OMITTED"\ !
3640 !"DATA POINT 0 TERMINATES CHOICE"\ !\ !
3650 I=I+1\ INPUT " ENTER DATA POINT TO BE OMITTED - ",A(I)
3660 IF A(I)=0 THEN 3670\ !#Z\ !#Z\ !#Z,"DATA POINT  ",A(I),"  OMITTED"
3670 IFI>=N2-4THEN3690
3680 IF A(I)>0 AND A(I)<=N2 THEN 3650
3690 I=I-1\N2=N2-I\N8=I\GOSUB 3770
3700 I=1\ I1=1\ FOR J=1 TO (N2+N8)
3710 IF J=A(I) THEN 3740
3720 T1(I1)=T1(J)\ T2(I1)=T2(J)\ C1(I1)=C1(J)\ K1(I1)=K1(J)
3730 I1=I1+1\ GOTO 3750
3740 I=I+1
3750 NEXT J\ RETURN
```

```
3760 REM*************************************************************************
3770 REM******************** S   O   R   T ********************************
3780 REM          SORT SUBROUTINE
3790 REM*************************************************************************
3800 FORJ=1TON8\X=A(J)\Y=B(J)\Z1=C(J)\K=J
3810 FOR I=JTON8\IFA(I)>=X THEN 3830
3820 X=A(I)\Y=B(I)\Z1=C(I)\K=I
3830 NEXTI\A(K)=A(J)\B(K)=B(J)\C(K)=C(J)
3840 A(J)=X\B(J)=Y\C(J)=Z1
3850 NEXT J\RETURN
3860 REM*************************************************************************
3870 REM                    T-VALUES HELD HERE
3880 REM*************************************************************************
3890 DATA 12.71,4.3,3.18,2.78,2.57,2.45,2.37,2.31
3900 DATA 2.26,2.23,2.2,2.18,2.16,2.14,2.13
3910 REM*************************************************************************
3920 REM              TEST DATA HELD HERE
3930 REM*************************************************************************
3940 DATA 1
3950 DATA "JMH PENICILLIN HYDROLYSIS 8:1:81"
3960 DATA 33
3970 DATA "MIN"
3980 DATA 0,28.3,1.287E-03
3990 DATA 9,32.8,1.2819E-03
4000 DATA 19,39.4,1.2677E-03
4010 DATA 29,45.8,1.2407E-03
4020 DATA 39,52,1.1853E-03
4030 DATA 44,54.8,1.1467E-03
4040 DATA 49,57.5,1.0965E-03
4050 DATA 52,59,1.0618E-03
4060 DATA 55,60.6,1.0219E-03
4070 DATA 58,62.1,.9768E-03
4080 DATA 61,63.6,.9241E-03
4090 DATA 64,64.9,.8739E-03
4100 DATA 67,66.2,.816E-03
4110 DATA 70,67.5,.7516E-03
4120 DATA 73,68.6,.6937E-03
4130 DATA 76,69.8,.6281E-03
4140 DATA 79,70.8,.5637E-03
4150 DATA 82,71.8,.5019E-03
4160 DATA 85,72.7,.4427E-03
4170 DATA 88,73.6,.3861E-03
4180 DATA 91,74.5,.3346E-03
4190 DATA 94,75.3,.2831E-03
4200 DATA 97,76,.2368E-03
4210 DATA 100,76.7,.1943E-03
4220 DATA 103,77.4,.1609E-03
4230 DATA 106,78,.1287E-03
4240 DATA 109,78.5,.1017E-03
4250 DATA 112,79,.0824E-03
4260 DATA 115,79.5,.0631E-03
4270 DATA 118,80,.0489E-03
4280 DATA 121,80.5,.036E-03
4290 DATA 125,81,.0245E-03
4300 DATA 129,81.4,.0154E-03
4310 END
```

****

NON-ISOTHERMAL KINETICS

****

****

JMH PENICILLIN HYDROLYSIS 8:1:81

****

INPUT DATA:

TIME(MIN)	TEMPERATURE(CENT.)	CONCENTRATION(%)
0	28.3	100
9	32.8	99.60373
19	39.4	98.500389
29	45.8	96.402487
39	52	92.097902
44	54.8	89.098679
49	57.5	85.198136
52	59	82.501943
55	60.6	79.40171
58	62.1	75.897436
61	63.6	71.802642
64	64.9	67.902098
67	66.2	63.403264
70	67.5	58.399379
73	68.6	53.900544
76	69.8	48.803419
79	70.8	43.799534
82	71.8	38.997669
85	72.7	34.397825
88	73.6	30
91	74.5	25.998446
94	75.3	21.996892
97	76	18.399378
100	76.7	15.097125
103	77.4	12.501943
106	78	10
109	78.5	7.9020979
112	79	6.4024864
115	79.5	4.9028749
118	80	3.7995338
121	80.5	2.7972028
125	81	1.9036519
129	81.4	1.1965812

****

JMH PENICILLIN HYDROLYSIS 8:1:81

****

THIS CALCULATION USES A FIRST ORDER MODEL

****

POLYNOMIAL RESULTS

COEFFICIENT	POWER
4.6049818	0
-7.8626558E-05	1
-3.9919227E-05	2
5.6817475E-07	3
-1.876541E-08	4
-1.1302847E-10	5
9.6117987E-13	6

TIME(MIN)	CONC.(%)	TRANSFORM	CALC.	RATIO(%)
0	100	4.6051702	4.6049817	99.995907
9	99.60373	4.6011996	4.6013256	100.00274
19	98.500389	4.5900604	4.590294	100.00509
29	96.402487	4.5685319	4.5679677	99.98765
39	92.097902	4.5228521	4.5246734	100.04027
44	89.098679	4.4897446	4.4906378	100.01989
49	85.198136	4.4449796	4.4453256	100.00778
52	82.501943	4.4128219	4.4116652	99.973788
55	79.40171	4.3745198	4.3724371	99.95239
58	75.897436	4.3293829	4.3270361	99.945794
61	71.802642	4.2739212	4.2748451	100.02162
64	67.902098	4.218067	4.2152412	99.933007
67	63.403264	4.1495153	4.1476024	99.953901
70	58.399379	4.0673052	4.071315	100.09859
73	53.900544	3.9871405	3.9857808	99.965898
76	48.803419	3.8878003	3.8904256	100.06753
79	43.799534	3.7796232	3.7847083	100.13454
82	38.997669	3.6635018	3.6681294	100.12632
85	34.397825	3.5379934	3.5402415	100.06354
88	30	3.4011975	3.4006591	99.98417
91	25.998446	3.2580368	3.2490689	99.724745
94	21.996892	3.0909011	3.0852426	99.81693
97	18.399378	2.9123168	2.9090465	99.887708
100	15.097125	2.7145045	2.7204558	100.21924
103	12.501943	2.525884	2.5195659	99.749866
106	10	2.3025851	2.3066064	100.17464
109	7.9020979	2.0671283	2.0819551	100.71727
112	6.4024864	1.8566865	1.8461504	99.432532

DETERMINATION COEFFICIENT	COEFFICIENT OF VARIATION (%)	CHI-SQUARED	DEGREES OF FREEDOM
1	.1490241	2.6734327E-04	20

****

JMH PENICILLIN HYDROLYSIS 8:1:81

****

ISOTHERMAL RATE CONSTANTS

TIME(MIN)	CONC.(%)	TEMP.(CENT.)	RATE CONSTANT(1/MIN)
0	100	28.3	7.8706394E-05
9	99.60373	32.8	7.1719348E-04
19	98.500389	39.4	1.5544417E-03
29	96.402487	45.8	3.0725403E-03
39	92.097902	52	5.8394196E-03
44	89.098679	54.8	7.8527332E-03
49	85.198136	57.5	1.0357951E-02
52	82.501943	59	1.2114896E-02
55	79.40171	60.6	1.4070861E-02
58	75.897436	62.1	1.6230833E-02
61	71.802642	63.6	.01859782
64	67.902098	64.9	2.1172676E-02
67	63.403264	66.2	2.3953931E-02
70	58.399379	67.5	.02693763
73	53.900544	68.6	3.0117159E-02
76	48.803419	69.8	3.3483078E-02
79	43.799534	70.8	3.7022957E-02
82	38.997669	71.8	.0407212
85	34.397825	72.7	4.4558884E-02
88	30	73.6	4.8513588E-02
91	25.998446	74.5	5.2559222E-02
94	21.996892	75.3	5.6665865E-02
97	18.399378	76	.0607996
100	15.097125	76.7	.06492231
103	12.501943	77.4	.06899158
106	10	78	.07296047
109	7.9020979	78.5	.07677736
112	6.4024864	79	.08038579

****

JMH PENICILLIN HYDROLYSIS 8:1:81

****

ARRHENIUS RESULTS

SLOPE	-12275.223	+/-	839.18789	(P=95%)
INTERCEPT	32.424479	+/-	2.4942155	(P=95%)

R= -.98597074

PARAMETER	VALUE	RANGE	UNITS
E	102059.89	95082.626 : 109037.15	J/MOL
A	1.2071808E+14	9.9666238E+12 : 1.4621656E+15	/MIN

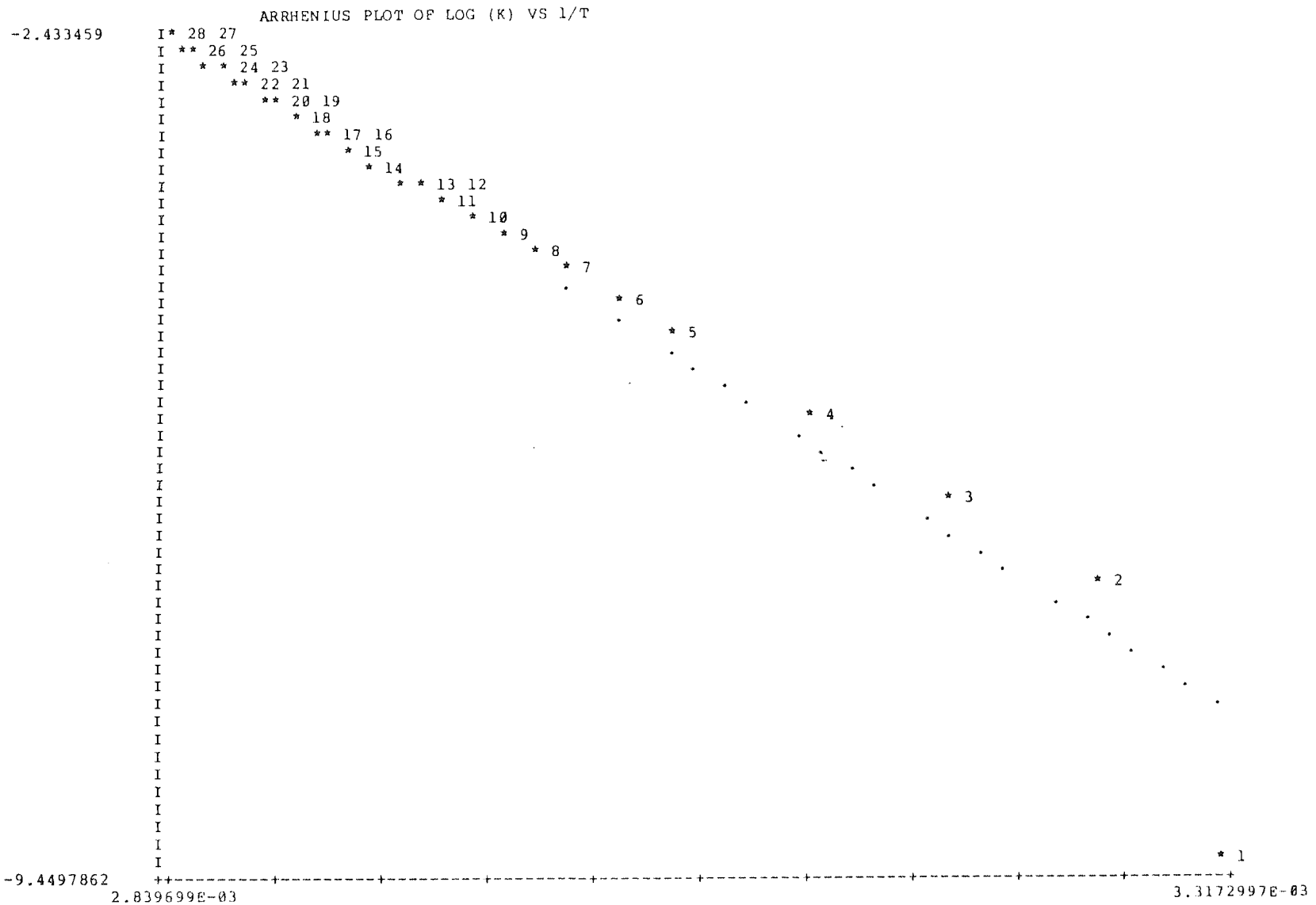
****

PREDICTED RATE CONSTANTS(1/MIN)

TEMP.	RATE	CALC.	RATIO (%)
28.3	7.8706394E-05	2.4948371E-04	316.98023
32.8	7.1719348E-04	4.5410327E-04	63.316704
39.4	1.5544417E-03	1.0595076E-03	68.160009
45.8	3.0725403E-03	2.3300234E-03	75.833778
52	5.8394196E-03	4.8536906E-03	83.119401
54.8	7.8527332E-03	6.699731E-03	85.317186
57.5	1.0357951E-02	9.094877E-03	87.805754
59	1.2114896E-02	1.0754952E-02	88.774613
60.6	1.4070861E-02	1.2839653E-02	91.249946
62.1	1.6230833E-02	1.5136383E-02	93.25697
63.6	.01859782	1.7817788E-02	95.805783
64.9	2.1172676E-02	2.0499004E-02	96.818221
66.2	2.3953931E-02	2.3558396E-02	98.348768
67.5	.02693763	2.7045653E-02	100.40101
68.6	3.0117159E-02	3.0371702E-02	100.84518
69.8	3.3483078E-02	3.4439126E-02	102.85532
70.8	3.7022957E-02	3.8216154E-02	103.22286
71.8	.0407212	4.2381813E-02	104.07801
72.7	4.4558884E-02	4.6494002E-02	104.34283
73.6	4.8513588E-02	5.0980607E-02	105.08521
74.5	5.2559222E-02	.05587362	106.30603
75.3	5.6665865E-02	6.0591667E-02	106.92798
76	.0607996	6.5025816E-02	106.95106
76.7	.06492231	.06976485	107.45898
77.4	.06899158	7.4828159E-02	108.45984
78	.07296047	7.9441927E-02	108.88352
78.5	.07677736	.08349037	108.74348
79	.08038579	8.7732666E-02	109.13952

DETERMINATION COEFFICIENT	COEFFICIENT OF VARIATION (%)	CHI-SQUARED	DEGREES OF FREEDOM
.9825567	9.4820748	5.2754407E-03	26

****


ARRHENIUS PLOT OF LOG (K) VS 1/T
-2.433459
-9.4497862
2.839699E-03
3.3172997E-03

FIRST POINT OMITTED

****

JMH PENICILLIN HYDROLYSIS 8:1:81

****

ARRHENIUS RESULTS

SLOPE	-10976.972	+/-	49.787218	(P=95%)
INTERCEPT	28.628778	+/-	.14730156	(P=95%)

R= -.99993954

PARAMETER	VALUE	RANGE	UNITS
E	91265.838	90851.894 : 91679.782	J/MOL
A	2.7121912E+12	2.3407113E+12 : 3.1426263E+12	/MIN

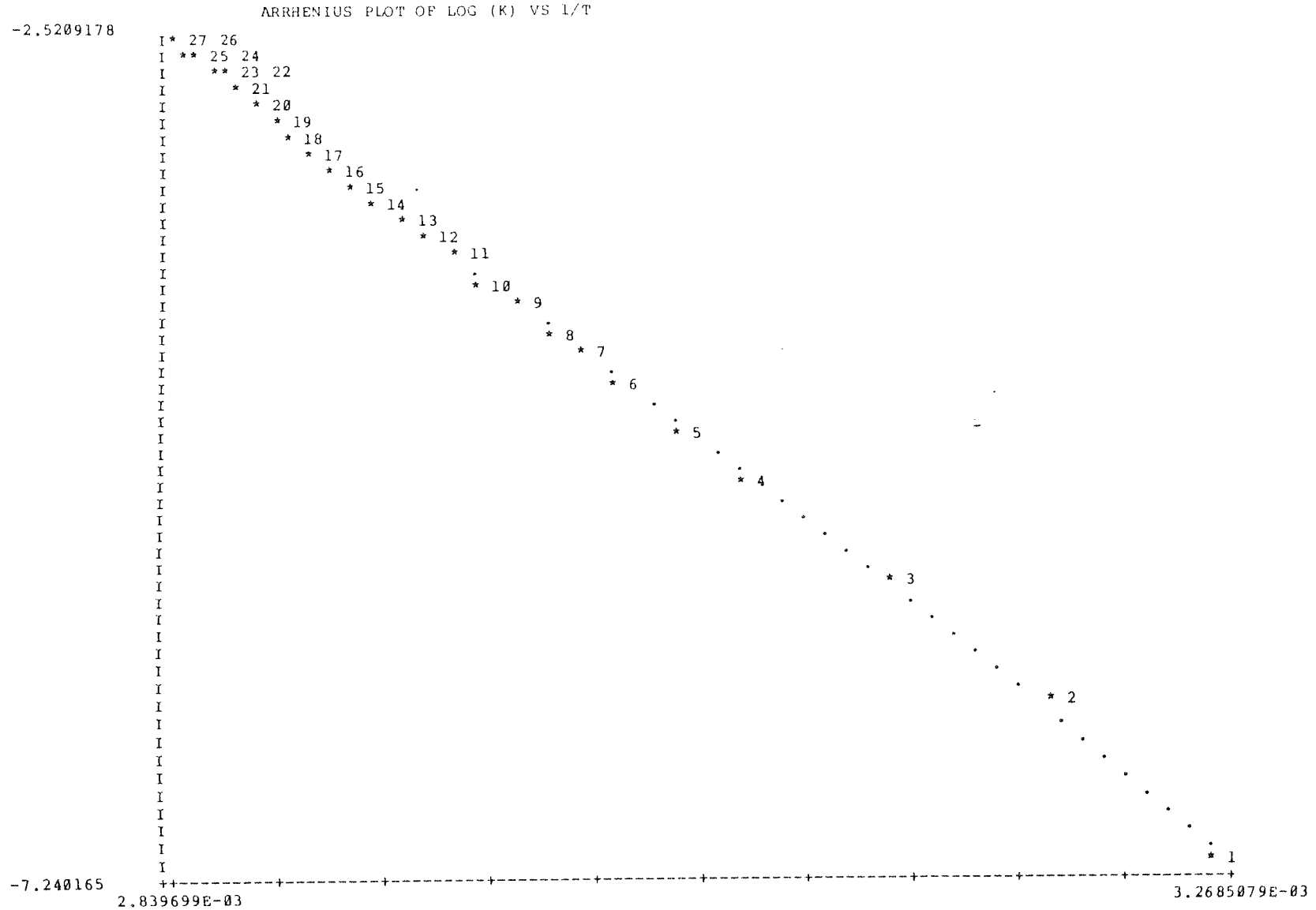
****

PREDICTED RATE CONSTANTS(1/MIN)

TEMP.	RATE	CALC.	RATIO (%)
32.8	7.1719348E-04	7.1049974E-04	99.066676
39.4	1.5544417E-03	1.5156469E-03	97.504261
45.8	3.0725403E-03	3.0665965E-03	99.806551
52	5.8394196E-03	5.9110016E-03	101.22584
54.8	7.8527332E-03	7.8857179E-03	100.42004
57.5	1.0357951E-02	1.0364352E-02	100.0618
59	1.2114896E-02	1.2040733E-02	99.387836
60.6	1.4070861E-02	1.4107821E-02	100.26267
62.1	1.6230833E-02	1.6344448E-02	100.69999
63.6	.01859782	.01891082	101.68299
64.9	2.1172676E-02	2.1436354E-02	101.24537
66.2	2.3953931E-02	2.4275831E-02	101.34383
67.5	.02693763	2.7465351E-02	101.95905
68.6	3.0117159E-02	3.0467004E-02	101.16161
69.8	3.3483078E-02	3.4091028E-02	101.81569
70.8	3.7022957E-02	3.7415771E-02	101.061
71.8	.0407212	4.1042629E-02	100.78934
72.7	4.4558884E-02	4.4586061E-02	100.06099
73.6	4.8513588E-02	4.8414544E-02	99.795843
74.5	5.2559222E-02	5.2549431E-02	99.981371
75.3	5.6665865E-02	5.6500261E-02	99.707754
76	.0607996	6.0183805E-02	98.987173
76.7	.06492231	.06409134	98.720055
77.4	.06899158	6.8235386E-02	98.903933
78	.07296047	7.1985685E-02	98.663955
78.5	.07677736	.07525748	98.020406
79	.08038579	7.8667987E-02	97.863051

DETERMINATION COEFFICIENT	COEFFICIENT OF VARIATION (%)	CHI-SQUARED	DEGREES OF FREEDOM
.999349	1.72686	1.5531836E-04	25

****


ARRHENIUS PLOT OF LOG (K) VS 1/T
-2.5209178
-7.240165
2.839699E-03
3.2685079E-03

****

SHELF-LIFE PREDICTIONS FIRST ORDER MODEL

TEMPERATURE	T-50%	T-90%	
5	24.447616	3.7161153	DAYS
10	12.178277	1.8511368	DAYS
15	149.15926	22.67268	HOURS
20	77.887666	11.839172	HOURS
25	41.567292	6.3183605	HOURS
30	22.648046	3.4425749	HOURS

****

****

PROGRAM TWO

```
      REAL*8 SUMA,SUMB,SUMC,SUMD,XX
      INTEGER I
      DIMENSION SUMA(300),SUMC(300)
      XX=0.0
      SUMD=0.0
      DO 99 I=1,300
      XX=XX+1.0
      SUMA(I)=2.2021307E-02+7.5635064E-01*XX+2.7416652E-03*(XX**2.0)
     1-4.6314698E-04*(XX**3.0)+1.006476E-05*(XX**4.0)
     2-1.1295633E-7*(XX**5.0)+7.6366051E-10*(XX**6.0)
     3-3.2230805E-12*(XX**7.0)+8.3212063E-15*(XX**8.0)
     4-1.2033944E-17*(XX**9.0)+7.4724365E-21*(XX**10.0)
      IF(I.EQ.1)GOTO 95
      SUMB=SUMA(I)-SUMA(I-1)
90    SUMC(I)=SUMD+SUMB
      SUMD=DEXP(DLOG(SUMC(I))-0.0051)
99    CONTINUE
      IF(I.GT.2)GOTO 92
95    SUMB=SUMA(I)
      GOTO 90
92    WRITE(1,30)
      WRITE(1,40)
      WRITE(1,60)
      WRITE(1,10)SUMA
      WRITE(1,30)
      WRITE(1,50)
      WRITE(1,60)
      WRITE(1,10)SUMC
      STOP
10    FORMAT(1X,10F6.2)
30    FORMAT(///////35H CLOXACILLIN CONCENTRATION LEVELS -)
40    FORMAT(/19H      NO DEGRADATION///)
50    FORMAT(/21H      WITH DEGRADATION///)
60    FORMAT(//38H (MG PER 100ML - ONE MINUTE INTERVALS)//////)
      CALL EXIT
      END
```

CLOXACILLIN CONCENTRATION LEVELS -

NO DEGRADATION

(MG PER 100ML - ONE MINUTE INTERVALS)

0.78	1.54	2.30	3.06	3.82	4.57	5.31	6.05	6.77	7.49
8.19	8.88	9.55	10.21	10.85	11.48	12.09	12.69	13.27	13.83
14.38	14.91	15.43	15.92	16.40	16.87	17.32	17.75	18.17	18.57
18.96	19.34	19.70	20.05	20.38	20.70	21.01	21.31	21.60	21.87
22.14	22.39	22.64	22.87	23.10	23.32	23.53	23.74	23.93	24.12
24.31	24.48	24.66	24.82	24.98	25.14	25.29	25.44	25.58	25.73
25.86	26.00	26.13	26.26	26.38	26.51	26.63	26.75	26.87	26.98
27.10	27.21	27.32	27.44	27.55	27.66	27.76	27.87	27.98	28.08
28.19	28.30	28.40	28.50	28.61	28.71	28.81	28.92	29.02	29.12
29.22	29.32	29.42	29.52	29.62	29.72	29.82	29.92	30.02	30.12
30.21	30.31	30.41	30.51	30.60	30.70	30.79	30.89	30.98	31.07
31.17	31.26	31.35	31.44	31.53	31.62	31.71	31.80	31.89	31.98
32.07	32.16	32.24	32.33	32.41	32.50	32.58	32.67	32.75	32.84
32.92	33.00	33.08	33.17	33.25	33.33	33.41	33.49	33.57	33.65
33.73	33.81	33.89	33.97	34.05	34.13	34.20	34.28	34.36	34.44
34.52	34.59	34.67	34.75	34.83	34.91	34.98	35.06	35.14	35.22
35.30	35.37	35.45	35.53	35.61	35.68	35.76	35.84	35.92	36.00
36.08	36.15	36.23	36.31	36.39	36.47	36.54	36.62	36.70	36.78
36.86	36.93	37.01	37.09	37.17	37.24	37.32	37.40	37.47	37.55
37.63	37.70	37.78	37.86	37.93	38.01	38.08	38.15	38.23	38.30
38.38	38.45	38.52	38.59	38.67	38.74	38.81	38.88	38.95	39.02
39.09	39.16	39.23	39.30	39.36	39.43	39.50	39.57	39.63	39.70
39.76	39.83	39.90	39.96	40.03	40.09	40.16	40.22	40.29	40.35
40.41	40.48	40.54	40.61	40.67	40.73	40.80	40.86	40.92	40.99
41.05	41.12	41.18	41.24	41.31	41.37	41.44	41.50	41.57	41.63
41.70	41.76	41.83	41.89	41.96	42.02	42.09	42.15	42.22	42.28
42.34	42.41	42.47	42.54	42.60	42.66	42.72	42.78	42.85	42.91
42.97	43.02	43.08	43.14	43.20	43.25	43.31	43.36	43.41	43.46
43.51	43.56	43.61	43.66	43.71	43.75	43.80	43.84	43.89	43.93
43.98	44.02	44.07	44.11	44.16	44.21	44.26	44.32	44.37	44.43

CLOXACILLIN CONCENTRATION LEVELS -

WITH DEGRADATION

(MG PER 100ML - ONE MINUTE INTERVALS)

0.78	1.54	2.29	3.04	3.78	4.51	5.23	5.94	6.64	7.31
7.98	8.63	9.26	9.87	10.46	11.04	11.59	12.13	12.65	13.15
13.63	14.09	14.53	14.96	15.36	15.75	16.12	16.47	16.80	17.12
17.42	17.71	17.98	18.24	18.48	18.71	18.92	19.12	19.31	19.49
19.65	19.81	19.95	20.09	20.21	20.33	20.44	20.54	20.63	20.72
20.79	20.87	20.93	20.99	21.05	21.10	21.14	21.18	21.22	21.25
21.28	21.31	21.33	21.35	21.37	21.38	21.39	21.41	21.42	21.42
21.43	21.43	21.44	21.44	21.44	21.44	21.44	21.44	21.44	21.43
21.43	21.43	21.42	21.42	21.41	21.41	21.40	21.39	21.39	21.38
21.37	21.36	21.36	21.35	21.34	21.33	21.32	21.31	21.30	21.29
21.28	21.27	21.26	21.25	21.23	21.22	21.21	21.20	21.18	21.17
21.15	21.14	21.12	21.11	21.09	21.07	21.06	21.04	21.02	21.00
20.98	20.96	20.94	20.92	20.90	20.88	20.86	20.84	20.82	20.79
20.77	20.75	20.72	20.70	20.68	20.65	20.63	20.60	20.58	20.55
20.53	20.50	20.48	20.45	20.43	20.40	20.38	20.35	20.33	20.30
20.28	20.25	20.23	20.20	20.18	20.15	20.13	20.10	20.08	20.05
20.03	20.01	19.98	19.96	19.94	19.91	19.89	19.87	19.84	19.82
19.80	19.77	19.75	19.73	19.71	19.69	19.66	19.64	19.62	19.60
19.58	19.55	19.53	19.51	19.49	19.47	19.44	19.42	19.40	19.38
19.36	19.33	19.31	19.29	19.27	19.24	19.22	19.20	19.17	19.15
19.12	19.10	19.08	19.05	19.03	19.00	18.98	18.95	18.92	18.90
18.87	18.84	18.82	18.79	18.76	18.73	18.71	18.68	18.65	18.62
18.59	18.56	18.54	18.51	18.48	18.45	18.42	18.39	18.36	18.33
18.30	18.27	18.24	18.22	18.19	18.16	18.13	18.10	18.07	18.05
18.02	17.99	17.96	17.94	17.91	17.88	17.85	17.83	17.80	17.78
17.75	17.73	17.70	17.68	17.65	17.63	17.60	17.58	17.55	17.53
17.50	17.48	17.45	17.43	17.40	17.37	17.35	17.32	17.29	17.27
17.24	17.21	17.18	17.15	17.12	17.09	17.06	17.02	16.99	16.95
16.92	16.88	16.84	16.81	16.77	16.73	16.69	16.65	16.61	16.57
16.53	16.49	16.45	16.41	16.38	16.34	16.31	16.28	16.26	16.24

****ST

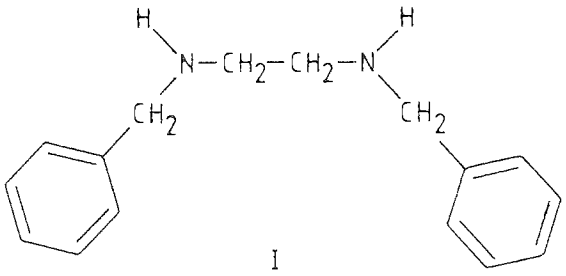
OK, LO

```
3 INPUT "DEVICE NUMBER (0,1)",Z
5 !#Z,"TIME(MIN)",TAB(12),"TIME*RATE",TAB(28),"RATE"
6 !#Z,TAB(24),"(1/MOLE/MIN)"
7 !#Z\ !#Z
9 REM B=CLOX. INIT.,K1=DEG.RATE,N=NO.POINTS,A=BENZ.INIT.
10 READ A,B,K1,N
20 FOR I=1 TO N
25 REM T=TIME,B2=CONC.PEN.V. AT T
30 READ T,B2
40 B1=EXP(LOG(B)-K1*T)
60 K3=1/(B1-A)*LOG((A*B2)/(B1*(A-B1+B2)))
70 K2=K3/T
80 !#Z,T,TAB(10),K3,TAB(24),K2
90 NEXT I
100 DATA 3.33E-03,6.88E-03,5.5E-04,25
110 DATA 2,6.77E-03
120 DATA 6,6.83E-03
130 DATA 14,6.65E-03
140 DATA 20,6.56E-03
150 DATA 30,6.36E-03
160 DATA 38,6.25E-03
170 DATA 45,6.17E-03
180 DATA 52,6.07E-03
190 DATA 60,5.93E-03
200 DATA 72,5.78E-03
210 DATA 80,5.7E-03
220 DATA 90,5.61E-03
230 DATA 100,5.38E-03
240 DATA 110,5.33E-03
250 DATA 120,5.26E-03
260 DATA 135,5.09E-03
270 DATA 161,4.89E-03
280 DATA 180,4.73E-03
290 DATA 210,4.46E-03
300 DATA 240,4.25E-03
310 DATA 277,4.02E-03
320 DATA 290,3.94E-03
330 DATA 305,3.87E-03
340 DATA 332,3.73E-03
350 DATA 354,3.58E-03
```

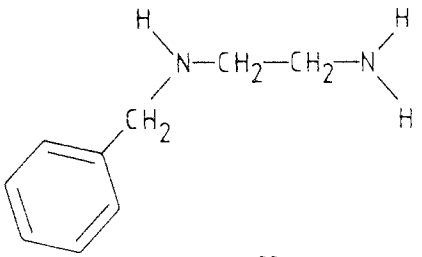
PROGRAM THREE

TIME (MIN)	TIME*RATE	RATE (1/MOLE/MIN)
2	4.5807542	2.2903771
6	1.2043276	.20072127
14	8.117322	.57980871
20	11.427193	.57135965
30	19.902246	.6634082
38	24.421606	.64267384
45	27.621231	.61380513
52	32.149897	.61826725
60	39.253901	.65423168
72	46.671747	.64821871
80	50.494633	.63118291
90	54.747314	.60830349
100	70.529215	.70529215
110	72.18238	.65620345
120	75.640765	.63033971
135	87.221969	.64608866
161	99.830478	.62006508
180	111.59299	.61996106
210	134.98809	.64280043
240	153.56934	.63987225
277	175.266	.63272924
290	183.57273	.6330094
305	189.53102	.62141318
332	203.80654	.61387512
354	224.41522	.6339413

APPENDIX 3

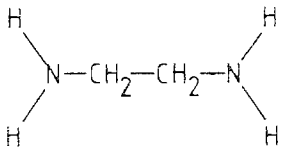


I



II

	pKa_1	pKa_2
20°C	9.80	6.70
25°C	9.41	6.48



III

	pKa_1	pKa_2
20°C	10.08	6.99
0°C	10.71	7.56

FIGURE A.3.1 STRUCTURES OF N,N' DIBENZYLETHYLENEDIAMINE (BENZATHINE I), N BENZYLETHYLENEDIAMINE (II) AND ETHYLENEDIAMINE (III)

THE CALCULATION OF THE pKa VALUES FOR BENZATHINE

(N,N' DIBENZYL ETHYLENE DIAMINE)

The structures of N,N'dibenzylethylene diamine (I), N benzylethylene diamine (II) and ethylene diamine (III) can be seen in figure A.3.1. The pKa values for II and III have been measured (150) and are included in the figure.

It is possible to calculate the pKa values for I in two ways (150a) :

a) using base weakening effects

b) the Hammett equation

a) Base Weakening Effects

The pKa of a typical secondary amine (I contains two secondary amine functions) is 11.15.

First ionization :

base weakening effect of $-C_6H_5$ on carbon next to the nitrogen group = - 1.4

base weakening effect of -NHR two carbons away from the nitrogen group = - 0.9

Thus pKa_1 = 11.15 - 1.4 - 0.9 = 8.85

Second ionization :

base weakening effect of $-C_6H_5$ on carbon next to the nitrogen group = - 1.4

base weakening effect of $-\overset{+}{N}H_2R$ two carbons away from the nitrogen group = - 3.6

Thus pKa_2 = 11.15 - 1.4 - 3.6 = 6.15

In order to check their predictive nature, similar calculations were carried out for II and III, and these values compared with the observed

pKa values (figure A.3.1).

For II,

The pKa of a typical primary amine is 10.77. Assuming that nitrogen A ionises first :

First ionization :

base weakening effect of $-\ddot{N}HR$ two carbon atoms away from the nitrogen group = -0.9

Thus pKa_1 = 10.77 - 0.9 = 9.87

Second ionization :

base weakening effect of $-C_6H_5$ on carbon next to the nitrogen group = -1.4

base weakening effect of $-\overset{+}{N}H_3$ two carbon atoms away from the nitrogen group = -3.6

Thus pKa_2 = 11.15 - 1.4 - 3.6 = 6.15

For III,

First ionization :

base weakening effect of $-\ddot{N}H_2$ two carbons away from the nitrogen group = -0.8

Thus pKa_1 = 10.77 - 0.8 = 9.97

Second ionization :

base weakening effect of $-\overset{+}{N}H_3$ two carbon atoms away from the nitrogen group = -3.6

Thus pKa_2 = 10.77 - 3.6 = 7.17

The calculated pKa values for II and III are similar to those observed experimentally, validating this method of calculation.

b) Hammett Equation

The Hammett equation calculates the contribution of substituents

towards the pKa of the molecule :

$$pKa = \begin{matrix}(\text{typical value for amine type}) \\ - (\text{constant for amine type}).\sum(\text{substituent effects})\end{matrix}$$

σ^{-}* = substituent effect

for a primary amine, $pKa = 13.23 - 3.14 \sum \sigma^{-}*$

for a secondary amine, $pKa = 12.13 - 3.23 \sum \sigma^{-}*$

For the substituents in I, II, III, σ^{-}* values could only be found for H and $-CH_2-Ph$ (0.49 and 0.21, resp.). However, knowing the pKa values for II and III, it is possible to derive σ^{-}* values for the remaining substituents <u>via</u> the Hammett equation.

Considering II,

$$pKa_1 = 13.23 - 3.14 \sum 0.49 + 0.49 + \sigma^{-}_1*$$

where σ^{-}_1* is the substituent effect for $-CH_2-CH_2-NH-CH_2-Ph$

Using $pKa_1 = 9.80$, $\sigma^{-}_1* = 0.11$

$$pKa_2 = 12.13 - 3.23 \sum 0.49 + 0.21 + \sigma^{-}_2*$$

where σ^{-}_2* is the substituent effect for $-CH_2-CH_2-\overset{+}{N}H_3$

Using $pKa_2 = 6.70$, $\sigma^{-}_2* = 0.98$

Considering III,

$$pKa_1 = 13.23 - 3.14 \sum 0.49 + 0.49 + \sigma^{-}_3*$$

where σ^{-}_3* is the substituent effect for $-CH_2-CH_2-NH_2$

Using $pKa_1 = 10.08$, $\sigma^{-}_3* = 0.02$

$$pKa_2 = 13.23 - 3.14 \sum 0.49 + 0.49 + \sigma^{-}_2*$$

Using $pKa_2 = 6.99$, $\sigma^{-}_2* = 1.01$

The only substituent effect that could not be calculated in this way was σ^{-}_4*, for $-CH_2-CH_2-\overset{+}{N}H_2-CH_2-Ph$. This is likely to be similar to σ^{-}_2*, thus a good estimate would be :

$$\sigma^-_4{}^* = \sigma^-_2{}^* + \sigma^-_1{}^* - \sigma^-_3{}^* = 1.09$$

Putting these calculated values in tabular form :

Substituent	$\sigma^-{}^*$
$-CH_2-CH_2-NH_2$	0.02
$-CH_2-CH_2-\overset{+}{N}H_3$	0.98, 1.01
$-CH_2-CH_2-NH-CH_2-Ph$	0.11
$-CH_2-CH_2-\overset{+}{N}H_2-CH_2-Ph$	1.09

The pKa values for benzathine can now be calculated :

First ionization :

$$pKa_1 = 12.13 - 3.23\sum 0.49 + 0.11 + 0.21 = 9.51$$

Second ionization :

$$pKa_2 = 12.13 - 3.23\sum 0.49 + 1.09 + 0.21 = 6.35$$

Combining the calculated pKa values from a) and b) :

pKa_1 = 8.85, 9.51 mean 9.2

pKa_2 = 6.15, 6.35 mean 6.3

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