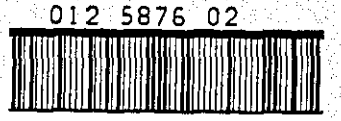

ANALYTICAL CHEMISTRY OF SOME β-LACTAM ANTIBIOTICS

by

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Loughborough University of Technology

To my son

MAAZ

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CONTENTS

ABSTRACT

All cephalosporins studied have been shown to give hydrogen sulphide. The yield of hydrogen sulphide varies widely for different cephalosporins, but is highly reproducible for individual cephalosporins. The hydrogen sulphide formed can be converted into methylene blue directly in the degraded solution, and this procedure has been made the basis of a colorimetric method of determining cephalosporins. Penicillins do not give hydrogen sulphide under these conditions and the method is thus selective for cephalosporins.

The manual visible spectrophotometric method for the determination of cephalosporins by alkaline degradation to sulphide and formation of methylene blue has been adapted for use with an air-segmented Autoanalyser I system. The system has been tested as a method of determining trace amounts of cephalosporins and other sulphide-producing impurities in penicillin G and penicillin V samples. The detection limit was calculated to be 1-2 $\mu g\ g^{-1}$ of cephalosporin in penicillin samples.

Potentiometric titration using standard lead nitrate solution and a sulphide ion-selective indicator electrode, was also carried out. These results confirmed the yields reported previously which were determined colorimetrically.

Alkaline degradation of cephalosporins and penicillin produce ammonia, the amount of ammonia released was determined by the reaction of ammonia with alkaline phenol and sodium hypochlorite to form the indophenol blue colour.

Nitrite can be determined by reduction at a glassy carbon electrode held at +0.3V versus SCE by flow injection voltammetry at concentrations $\geqslant 10^{-6}$M by direct injection of sample solution (25 µl) into an eluent 3.2M in hydrochloric acid and 20% m/V in potassium bromide. Alternatively the nitrite may be pre-reacted with acidic bromide before injection and determined at concentrations $\geqslant 10^{-7}$M.

Excess of nitrite after reaction with aniline, sulphanilic acid, sulphanilamide and cephalexin, cephalonium and penicillin G in presence of potassium bromide is determined by flow injection voltammetry at a glassy carbon electrode by injection of the reaction mixture into 0.3M hydrochloric acid. The reaction is stoichiometric but the signal is not fully rectilinear. For the determination of these amines (1×10^{-6} - 1×10^{-4}M) the use of a 10-90% excess of nitrite is recommended. The amines have also been determined by direct injection of amine (1×10^{-7} - 1×10^{-4}M) in 3.2M hydrochloric acid solution into a neutral eluent 1×10^{-4} or 1×10^{-5}M in nitrite and 20% m/V in potassium bromide. The reaction was incomplete in this direct injection but good reproducibility was obtained.

CHAPTER 1

GENERAL INTRODUCTION

History of Penicillin

It is now more than half a century since Alexander Fleming observed the effects of a penicillium mould on staphylococcus bacterial culture. Fleming's[1] original observation of the production of penicillin after an accidental contamination of his plate on a laboratory bench by penicillium notatum is not easily repeatable. However Fleming cultured the fungus in broth and showed that the culture filtrates, which he called penicillin, were highly active against a variety of gram-positive bacteria. The reason why Fleming considered penicillin might have potential use, quite apart from its effect on bacterial strain in culture were that it was of very low toxicity to animals and to leucocytes and used it in a few cases as alocal antiseptic[2,3].

Attempts to isolate penicillin in the decade following its discovery were soon abandoned, mainly because of difficulties arising from its instability. Apart from some work by Reid[4] in the United States, little appears to have been done until in 1938 during the work of the isolation and elucidation of the chemical structure of penicillin by Chain and Florey[5], it became apparent that penicillin was in fact a mixture of closely related substances.

Despite the outcome of this work, which seemed at the time to be almost miraculous, the production of penicillin in the quantities needed for its general medical use posed a virtually insoluble problem.

Subsequent work in several pharmaceutical companies in the USA was responsible for the addition of corn-steep liquor to the growth medium, the introduction of deep fermentation, and the isolation of higher-yielding strain of penicillium which allowed the large-scale production of penicillin.

During world war II the chemistry of penicillin became the subject of massive and confidential Anglo-American investigation involving many commercial and academic organisations.

Sheehan[6] indicated, in his recent article on the chemistry of penicillin, that most of the important work on penicillin chemistry, remains widely distributed among several investigators. Alicino discovered the hitherto unsuspected presence of sulphur after the best chemists on both sides of the Atlantic had decided that penicillin was composed of only carbon, hydrogen, oxygen and nitrogen. Webb and Trenner performed the potentiometric titrations that eliminated the oxazolone-thiazolidine structure as a possible one for penicillin. Mozingo offered the organic chemists conclusive evidence for the beta-lactam structure with his classic desulphurization experiment, and Hodgkin made the final determination of structure by means of her X-ray crystallography.

The first published argument for the beta-lactam structure was probably that given by Abraham[7] and his group, but even in that presentation, because of the strong opposition of Sir Robert Robinson, the beta-lactam was presented as only one of several possible structures. However, the beta-lactam structure was finally established in 1945.

The observation that the differences in side chain resulted in

differences in biological properties, stimulated interest in preparing other penicillin molecules which might display superior qualities to the naturally-occurring substances. Since then organic chemists have prepared thousands of variations on the original penicillin molecule.

The first attempts to modify the molecule were made by addition of new precursors to the fermentation broth. The only valuable compound that came out was penicillin V, an acid-stable penicillin. When Batchelor[8] and co-workers were able to isolate the penicillin nucleus, 6-aminopenicillanic acid the way was open to prepare new molecules by direct chemical attachment of novel side chain structures at the free 6-aminogroup.

In 1940 the first penicillinase had been discovered in E.Coli by Abraham and Chain[9]. Penicillinase opened the beta-lactam ring of the molecule and by so doing destroyed completely the antibacterial activity. Thus, towards the end of the 1940's the history of penicillin might have been regarded as that of a great medical discovery to which little more would be likely to be added but remarkable developments have been achieved. These were to lead to the production of thousands of new beta-lactam compounds, a number of which were to be responsible for significant advances in chemotherapy.

History of Cephalosporins

The history of the cephalosporins is clearly connected with that of the penicillins. It was when the therapeutic properties of penicillin became known in Sardinia towards the end of world war II, that Brotzu[10] in 1945 isolated a group of antibiotics with a penicillin-like activity

from the broth cultures of a strain of caphalosporium acremonium. Abraham[11] described recently how limited were Brotzu's resources and how hard he first tried to interest the Italian pharmaceutical industry, but without success.

Abraham and Newton started work on Brotzu's fungus at Oxford in 1948. The antibiotic that was found in Oxford in culture fluids of the Sardinian Cephalosporium was penicillin N. Further work by Abraham and Newton[12] with substances produced by cephalosporium acremonium led to the discovery of cephalosporin C.

Cephalosporin C[13] a naturally-occurring substance, had only a weak activity against staphylococci, about 100 times less than penicillin G but was on the other hand much more effective than penicillin G against salmonella, E.Coli and other gram negative bacilli. However the new substance, with the still unknown structure attracted little attention because of its low activity.

The definite structure for cephalosporin C was proposed by Abraham and Newton[14] and confirmed by an X-ray crystallographic analysis by Hodgkin and Maslen[15]. Loder et al[16] were able to isolate the nucleus of the molecule, 7-aminocephalosporanic acid (7-ACA), in low yield by the use of mild acid hydrolysis to remove the side chain. As in the case of penicillin[17], it was quickly appreciated that effectiveness of the antibiotics depended to a large extent upon the nature of the acyl moiety attached to the amino function at C-7. The first available semi-synthetic cephalosporin was cephalothin in 1962[18]. Furthermore it was shown that the acetoxy group at C-3 in cephalosporins C could readily be replaced by a variety of nucleophiles to give compounds with

higher activity. These compounds retained a resistance to hydrolysis by staphylococcal penicillinase, the resistance being due to the nature of the ring system.

The first available semi-synthetic cephalosporins, cephalothin was soon followed by cephaloridine and cefazolin. These parenteral compounds were found to have excellent activity against gram-negative and gram-positive cocci. Because they are stable against penicillinase they also inhibit penicillinase producing staphylococci and they remain active against gram-negative bacilli.

The first cephalosporin which was made to be absorbed orally, cephaloglycin, was found to be only moderately absorbed and partially metabolized, but further derivatives were found to be almost completely absorbed and not metabolized. Such compounds like cephalexin and cephradine have an activity equal to that of cephalothin against gram-negative organisms, but they are less active than cephalothin against gram-positive cocci and H-influenzae. Two oral cephalosporins, cefaclor and cefatrizine display higher activity than for example cephalexin and cephradine.

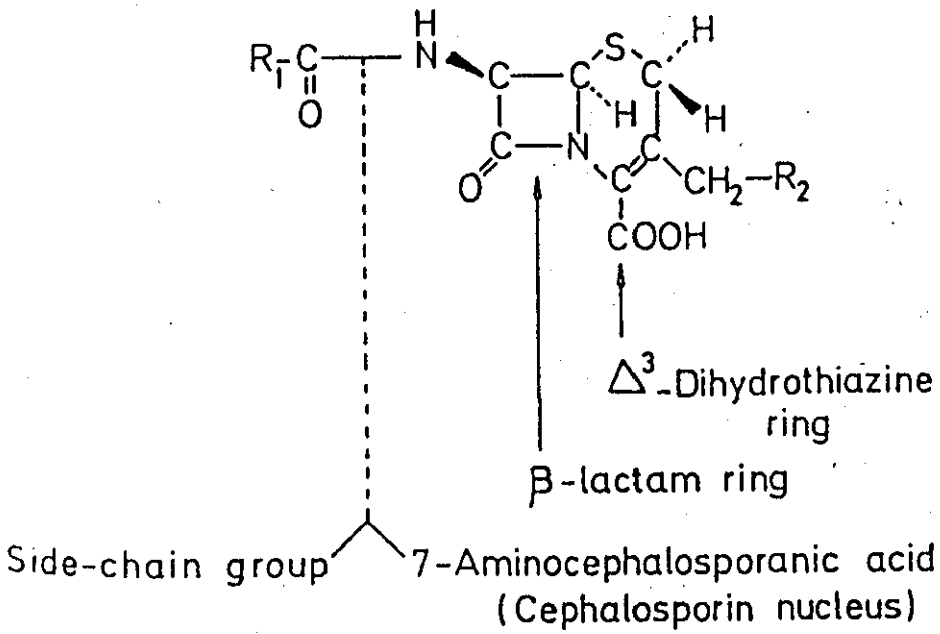
Another objective was the extension of the antibacterial spectrum and the enhancement of the activity against gram-negative bacilli, by the introduction of more potent side chains. A series of structurally related compounds such as cefazaflur, ceforanide, cefamandole and cofotiam, achieved this goal with an extension of the known cephalosporin spectrum to gram-negative bacteria. But the intensive use and misuse of cephalosporins especially in hospitals, had lead to the destruction of the β-lactam ring of the cephalosporins by specific β-lactamases, the cephalosporinases.

In 1971 a new family of β-lactams had been discovered in the fermentation broth of some streptomyces: the cephamycins. The first available cephamycin for clinical use was cefoxitin, with stability to a broad spectrum of β-lactamases, and furthermore good activity against the anaerobes. The cephamycins then are a new class of broad spectrum β-lactam antibiotics which have the attributes of the cephalosporins combined with extended β-lactamase stability which makes them effective against many cephalosporin-resistance gram-negative bacteria and bacteroides fragilis. Soon thereafter the vulnerable β-lactam ring was found to be protected by introducing a methoxy-imino group on the acetamido side chain of the 7-position. The first available derivative was cefuroxime. Finally highly active and β-lactamasestable cephamycin antibiotics were made available and are under clinical investigation[17]. These new cephamycins are reported to have in vitro activities 2 to 8 times higher than cefoxitin[18].

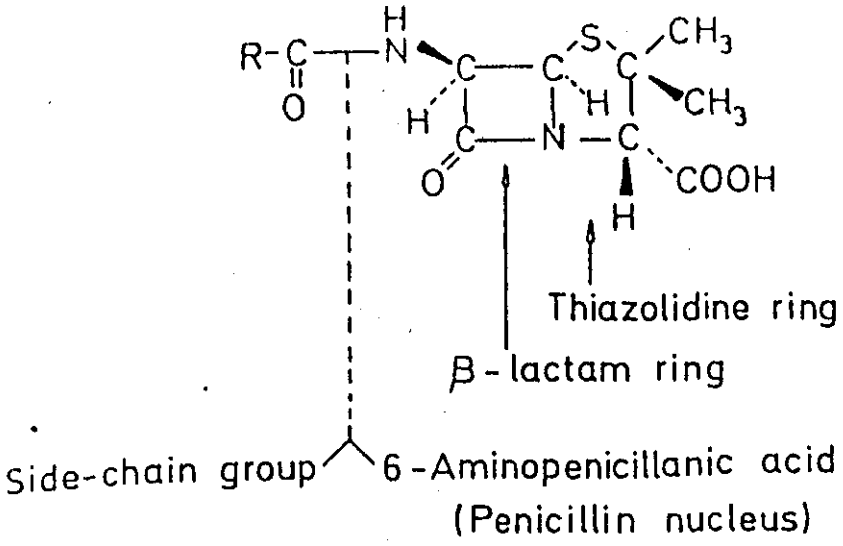
Structure of β-Lactam Antibiotics

Cephalosporins and pencillins constitute one of the most important classes of antibiotics used in antimicrobial chemotherapy. All β-lactam antibiotics are derivatives of a bicyclic ring system. All, with the exception of 6-amino-penicillanic acid (6-APA) and 7-aminocephalosporanic acid (7-ACA), have an acyl group attached as a side chain to the amino group of the β-lactam ring.

The structures of penicillins and cephalosporins are compared in Figures I and II. The active principle, the β-lactam ring, is fused to the five-membered thiazolidine ring in penicillins and they are substituted at C-6, whereas the cephalosporins have the six-membered



BASIC STRUCTURE OF CEPHALOSPORINS



BASIC STRUCTURE OF PENICILLINS

dihydrothiazine ring and are substituted at C-3 and C-7.

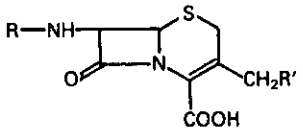
The β-lactam ring is biologically quite rare and chemically difficult to construct. Enzymatic reactions leading to formation of this ring have not been resolved. Nevertheless, the β-lactam compounds are preeminently natural products, and industrial production of all β-lactam antibiotics depends on microbial fermentation. Total chemical synthesis of penicillin and cephalosporin have been successful, but these efforts have not been competitive with microbial synthesis.

The mode of action of penicillins and cephalosporins against bacteria is well reviewed by O'Callaghan et al[19]. However it is known that bacteria protects itself from harmful influences by means of a rigid cell wall. The cell wall protects the bacteria from e.g. osmotic shock. Penicillins and cephalosporins are known to interfere with the synthesis of the rigid component of cell walls. This action results in a disorderly built cell wall structure, and the bacteria increasingly lose their osmotic barrier and the membrane becomes unable to contain the high osmotic pressure of the cell contents and the cell ultimately bursts.

Although most penicillins and cephalosporins investigated so far appear to have similar modes of action, there are large differences in their activities. It is thus evident that the antibacterial effect of a given β-lactam antibiotic may depend on its ability to react with one or more of a variety of different enzymes involved in cell wall synthesis. For example, mecillinam unlike penicillins and cephalosporins with normal N-acyl side chains, has a much higher activity

TABLE I

STRUCTURES OF CEPHALOSPORINS EXAMINED



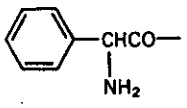
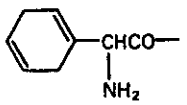
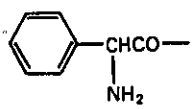
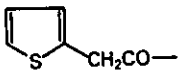
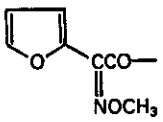
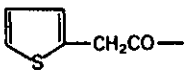
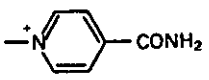
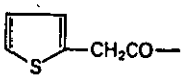
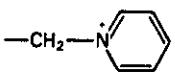
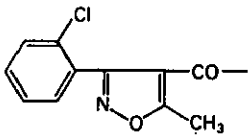
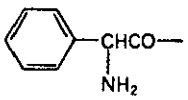
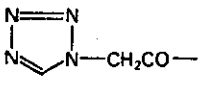
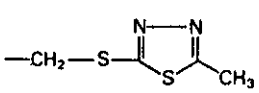
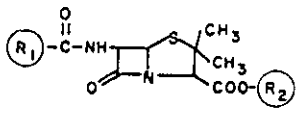
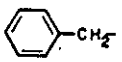
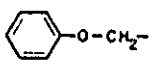
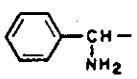
Compound	R	R′
Cephalexin		–H
Cephradine		–H
Cephaloglycin		$-OCOCH_3$
Cephalothin		$-OCOCH_3$
Cefuroxime		$-OOCNH_3$
Cephalonium		
7-ACA	H–	$-OCOCH_3$
7-ADCA	H–	–H
Cephaloridine		
Cephoxazole ..		$-CH_2OCOCH_3$
Cefaclor		–Cl
Cefazolin		

TABLE I... continued
STRUCTURES OF PENICILLINS EXAMINED



R_1	R_2	GENERIC NAME
	K, Na, BENZATHINE PROCAINE	PENICILLIN G. (BENZYL)
	H, K, BENZATHINE	PENICILLIN V.
	H, Na	AMPICILLIN

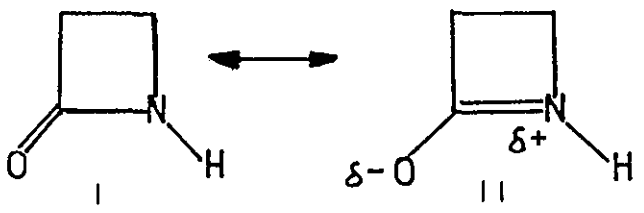
against gram-positive than against gram-negative bacteria and converts gram-negative rods into large osmotically stable round cells[20].

Chemical structures of the cephalosporins and penicillins investgated in this study are given in Table I.

Degradation Reaction of Beta-Lactam Antibiotics

Introduction

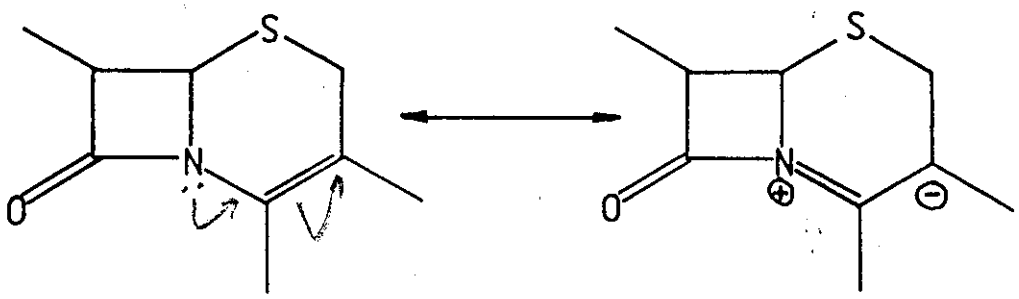
Penicillins and cephalosporins have been known to undergo remarkably facile cleavage of their β-lactam bonds in aqueous solution[21]. The bicyclic β-lactam-thiazolidine structure is more sensitive than the simple β-lactam structure to nucleophiles, electrophiles, oxidizing agents and even water. It has been suggested[22] that isolated β-lactams, due to resonance stabilization, are considerably less reactive than the strained penicillin β-lactam. Simple amides and isolated β-lactams(I) may exist in a planar resonance stabilized configuration (II).



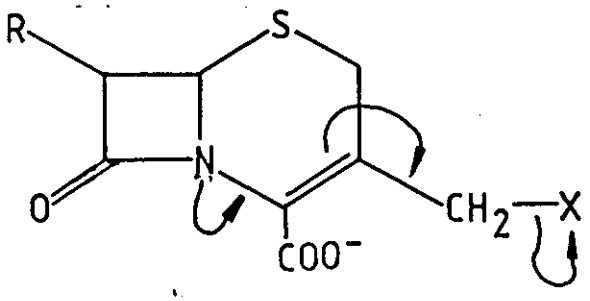
I II

However, in penicillins the nitrogen bond to the carbonyl function cannot become planar because of ring strain. The infrared carbonyl frequencies of isolated β-lactam (1730 cm^{-1}) and penicillins (1775 cm^{-1}) reflect these differences in strain stabilization[23].

In cephalosporins this amide resonance may further be decreased by delocalization of the nitrogen atom's unshared electron pair into the adjacent α, β-unsaturated system (enamine resonance)[24].



Indelicato et al[25] suggested that 3-methylene substituent in cephalosporins may inductively exert pronounced influence on the β-lactam reactivity. An increase in electronegativity at R could help to stabilize the suggested enamine resonance and hence decrease the lactam amide resonance and result in increased reactivity of the β-lactam ring. Hence they proposed that the inductive effect of a 3-substituent is conjugationally transmitted as below, involving a simultaneously proceeding departure of the 3' group and β-lactam ring opening.



Penicillin Degradations

The lability of the penicillin structure has been well known from the time of the earliest investigation[26]. Many chemical conversions are possible, the nature of the end products depending on a variety of factors such as temperature, pH, the nature of solvent and the influence of cations. The β-lactam ring of penicillin is particularly susceptible

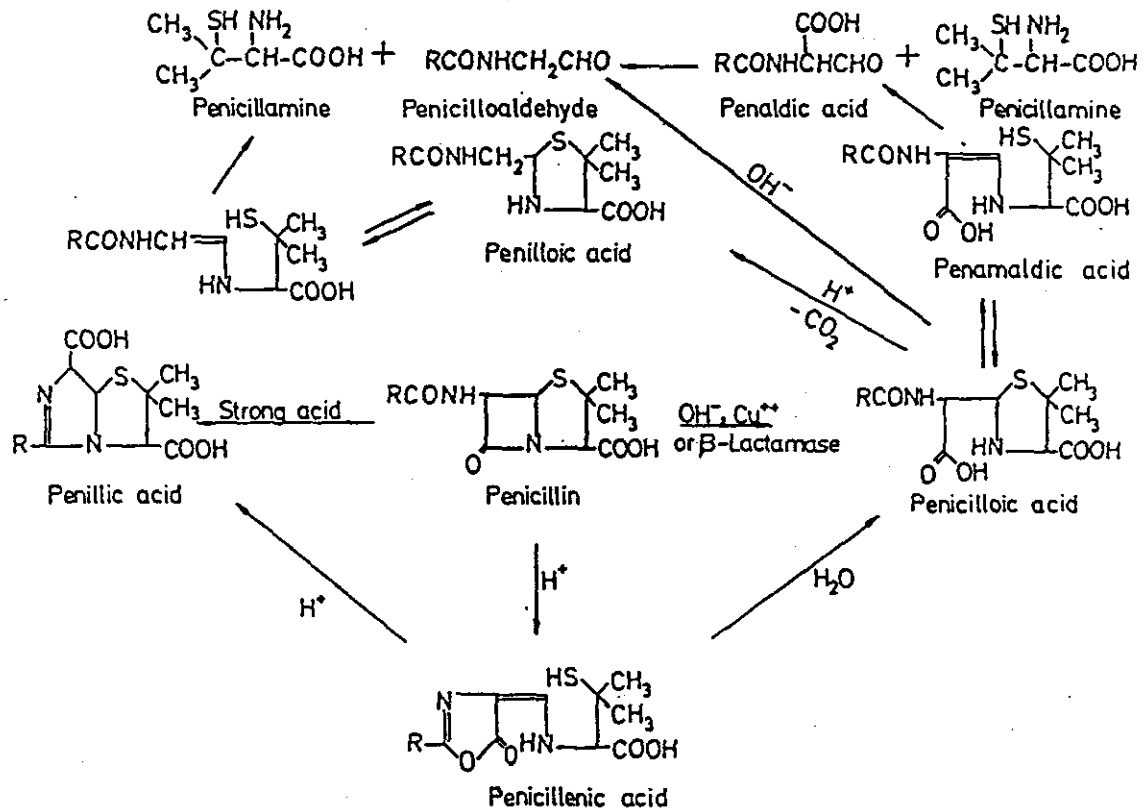
to nucleophilic attack, with the formation of the corresponding penicilloic acid. This acid is obtained as a stable salt in alkaline medium, but on acidification the free acid quickly decarboxylates to the corresponding penilloic acid. In acidic solution penicillin rearranges to penicillenic acid which is unstable (unless stabilized by complexing with cations) and is rapidly converted into penicillenic and penicilloic acids. Reaction of penicillin with mercuric chloride in aqueous solution leads to extensive degradation and formation of fission products such as penaldic acid, penicillamine and, penilloaldehyde. The end hydrolytic degradation products of penicillin are CO_2 andasulphur containing amino acid. The more common penicillin degradation pathways are summarised in scheme (I).

Enzymic Hydrolysis of Penicillins

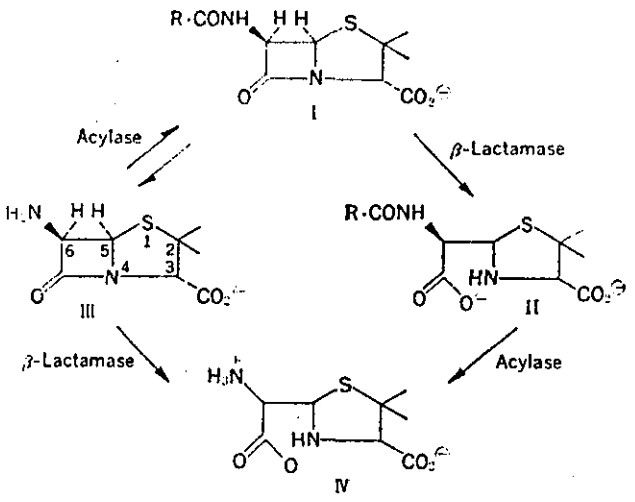
Enzymic hydrolysis of penicillin results in opening of the β-lactam ring of a penicillin (I scheme 2) gives the inactive D-α-penicilloate (II), although the latter may undergo mutarotation with the formation of different diastereoisomers[27].

In 1960 a cell-bound acylase was found in E.Coli and a number of other gram-negative bacteria, which remove the N-acyl side chain of certain penicillins[28]. They not only remove the N-acyl side chains of many penicillins to yield 6-amino-penicillanic acid (III) but also hydrolyse a variety of unrelated N-acyl derivatives.

High activity is shown with substrates containing the N-acyl group RCO, when R is benzyl (as in benzyl penicillin). The activity is much lower when R is phenoxymethyl, or heptyl, or when an amino or carboxy group is substituted on the α-carbon atom, as in ampicillin and carbenicillin respectively and the dimethyoxybenzoyl side chain of methicillin


Penicillamine
Penicilloaldehyde
Penaldic acid
Penicillamine
Penilloic acid
Penamaldic acid
Strong acid
Penillic acid
Penicillin
OH⁻, Cu⁺⁺
or β-Lactamase
Penicilloic acid
Penicillenic acid

scheme 1



Enzymic hydrolysis of penicillin

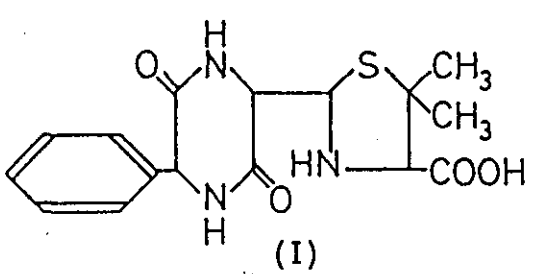
and the δ-(D-α-aminoadipyl) side chain of penicillin N is not hydrolysed.

Degradation Reactions of α-aminopenicillin (Ampicillin)

The α-aminopenicillin, ampicillin is a semi-synthetic penicillin prepared from 6-aminopenicillanic acid. Compared with benzylpenicillin ampicillin shows a broad spectrum of activity against gram-positive and gram-negative bacteria. Unlike benzylpenicillin ampicillin is highly acid stable and is well absorbed when given by mouth.

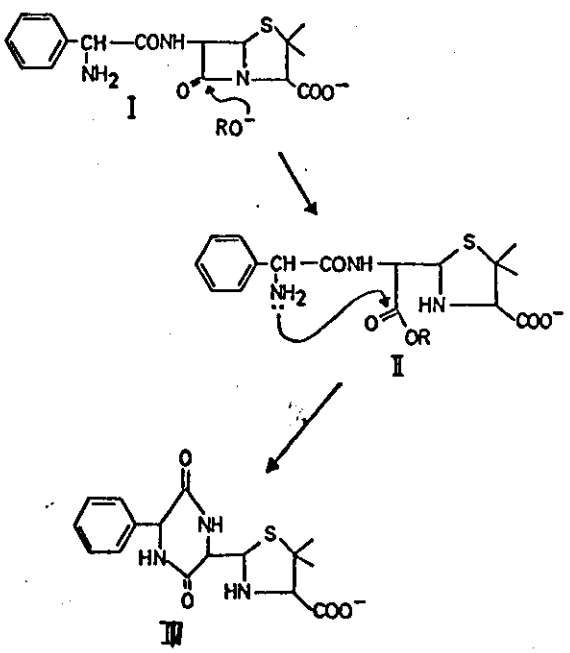
Ampicillin was introduced into clinical practice in 1961. Since its discovery it appears that little work has been done on the mechanism of ampicillin degradation in solution. The first study of the kinetics

and mechanism of ampicillin and other α-aminopenicillins was that of Hou and Poole[29]. They used the iodometric assay in their study and depending on their knowledge of the penicillin degradation, they postulated a degradation pathway for ampicillin similar to that of penicillin G. However Hou and Poole indicated that the α-amino side chain plays a significant role in the rate of the degradation as well as the stability of the compound in acidic solution. Further studies by Jusko[30] lead to the conclusion that β-lactam antibiotics having α-amino group on the side chain yield a highly fluorescent product when they are degraded under certain conditions. Jusko[30], degraded ampicillin in a pH2 solution containing formaldehyde found that the aminobenzyl group and a cleaved β-lactam ring were necessary for the formation of the fluorophore, because benzylpenicillin which lacks the α-amino group of ampicillin gave no fluorescent product. Formaldehyde has been found to accelerate the reaction but the exact mechanism by which the fluorescent product is formed is not known though several reaction pathways have been suggested. Jusko[30] originally proposed that the fluorescent species was the 2,5-diketopiperazine (I).



(I)

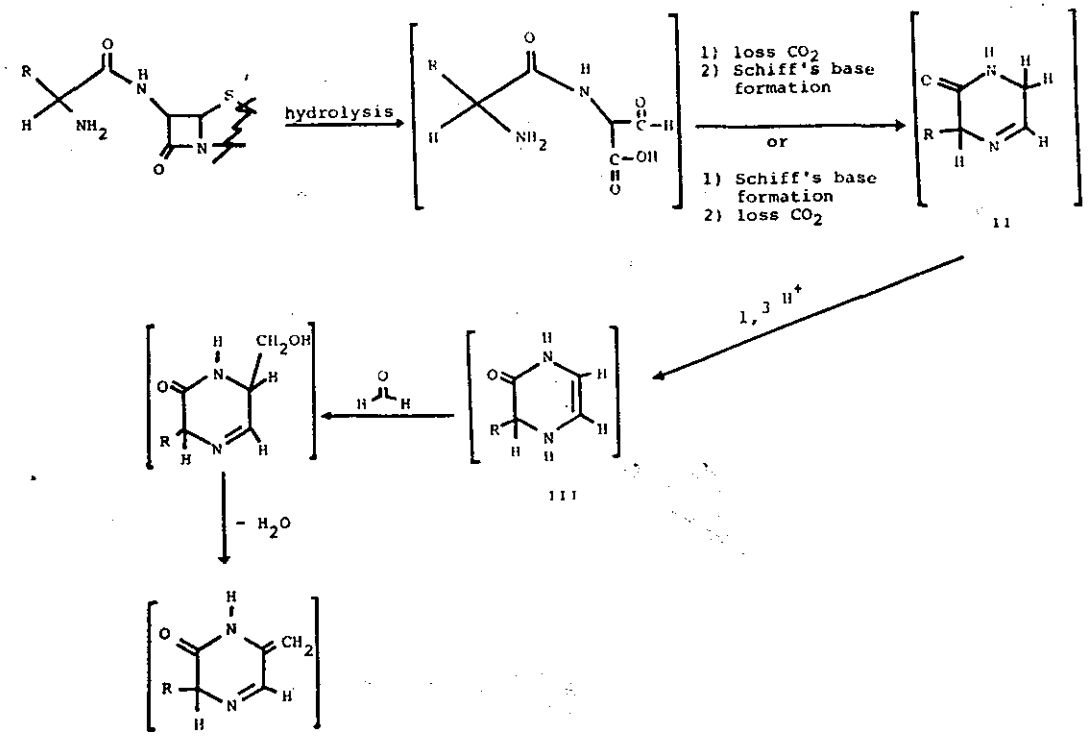
During the early work by Indelicato[31] et al and Roets et al[32] the synthesis of the diketopiperazine from ampicillin was not successful presumably because of steric hindrance. However Bundgaard[33] and Larsen studied the effect of various carbohydrates and polyhydric alcohols on the rate of degradation of ampicillin in weakly alkaline solution. They reported the formation of α-aminobenzylpenicilloyl ester (II) as an initial reaction product. This compound, however, undergoes an intermolecular aminolysis by the side-chain amino group to produce a stable 2,5-diketopiperazine (III) derivative. Bundgaard et al[34] isolated a sample of 2,5-diketopiperazine from a solution of ampicillin degraded in 10% w/v aqueous solution of glucose with the pH adjusted to 9.2 and the temperature of the solution was kept at 22°C for 20 hours, scheme (III)



Scheme 3

The structure of the fluorescent product formed by the degradation of α-aminopenicillin was uncertain until Barbhaiya et al[35] and Lebelle identified it[36]. Barbhaiya et al showed that the alkaline degradation of ampicillin followed by treatment in citrate buffer solution containing formaldehyde yielded a fluorophore whose spectral and chromatographic properties were identical with those of authentic 2-hydroxy-3-phenyl-6-methylpyrazine which was synthesized from α-phenyl glycine amide and methyl glycoxal. Lebelle using similar reaction procedures except that formaldehyde was not used, showed the fluorophore of ampicillin was identical with synthetic 2-hydroxy-3-phenyl pyrazine. Masada et al[37][39] and Uno et al[38] used high performance liquid chromatography (HPLC) in their studies of ampicillin degradation and found two peaks with appreciable fluorescence appearing on the chromatograms of alkaline degradation products of ampicillin. They used both formaldehyde and acetaldehyde in their studies and identified the structure of the two compounds responsible for the two peaks by their isolating and spectral investigation of the two degradation products as 2-hydroxy-3-phenyl-6-methylpyrazine when formaldehyde was used and 2-hydroxy-3-phenyl-penillomethyl-pyrazine when actaldehyde was used instead. Uno et al reported from their studies that previous results could account for only a part of the fluorophore-producing reaction.

Finally the most acceptable mechanism of the ampicillin degradation in solution in the absence of formaldehyde was suggested by Lebelle et al[36] as follows (scheme 4).

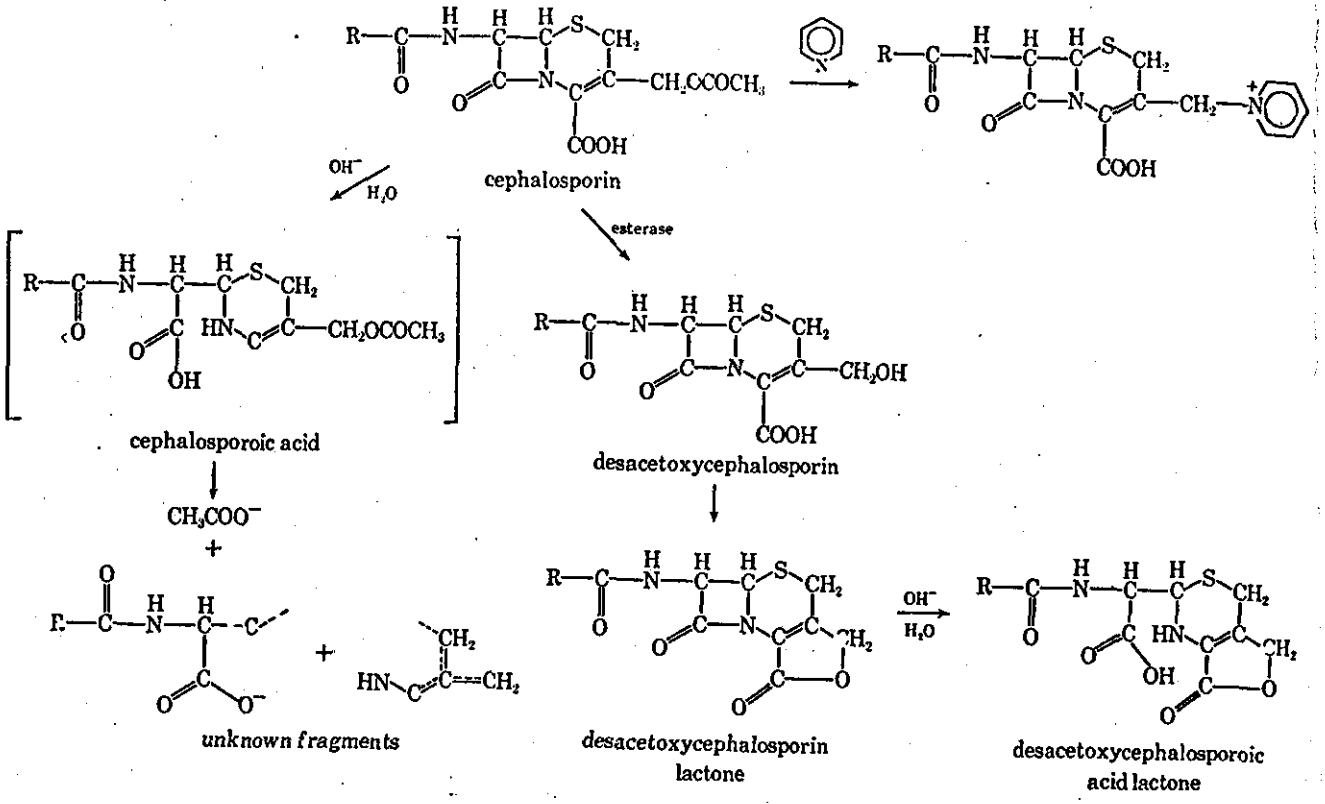

hydrolysis
1) loss CO_2
2) Schiff's base formation
or
1) Schiff's base formation
2) loss CO_2
II
1,3 H^+
III
CH_2OH
$- H_2O$
CH_2

This mechanism however, has been supported by Indelicato[40] who used labelled formaldehyde to determine the position of the formaldehyde carbon and hence suggested a mechanism by which the reaction proceeds. Indelicato found that their findings were in agreement with the mechanisms suggested by Lebelle and showed that the final reaction product proceeded by either condensation with formaldehyde to form Barbhaiyas product, i.e. 2-hydroxy -3-phenyl-6-methyl pyrazine, or upon oxidation in the absence of formaldehyde to produce Lebelle product, 2-hydroxy-3-phenyl pyrazine.

Cephalosporin Degradations

In contrast to the well known chemistry of penicillins, relatively little work has been done on those chemical reactions of cephalosporins to answer questions regarding the acidic, neutral, and alkaline degradations of cephalosporins[41]. However some detailed reports have appeared in recent years on the degradation of cephalosporins[45]. Bundgaard et al[42] reported the degradation studies of some cephalosporins in neutral, alkaline and acidic solutions. Indelicato et al[43], Bundgaard[44] studied the substituent effects of various side chain moieties upon the alkaline hydrolysis of cephalosporin. Yamana et al[45] reported the hydrolysis, and kinetics and suggested some mechanisms of degradation of some therapeutically useful cephalosporins and other semi-synthetic cephalosporins in acidic, neutral and alkaline aqueous solutions.

The degradation studies of cephalosporin C played an important role in the determination of the structure of this compound by Abraham et al[46].

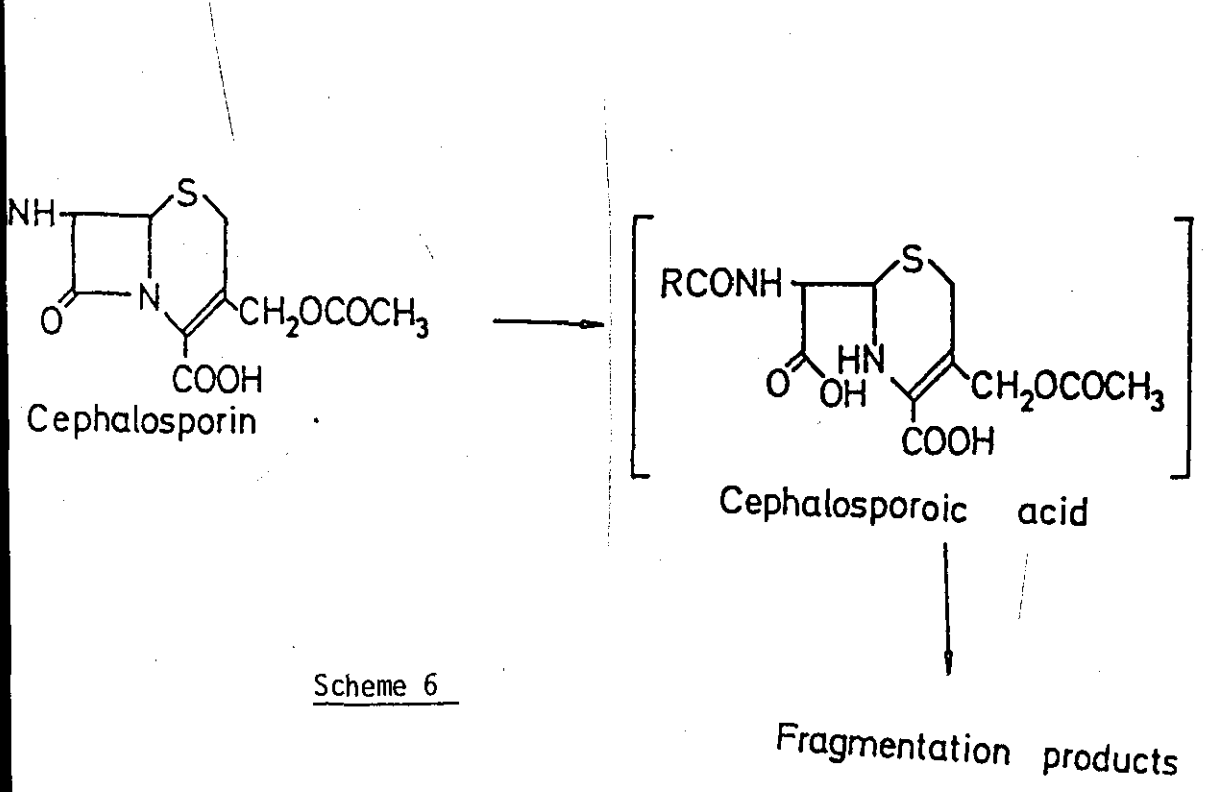

cephalosporin
cephalosporoic acid
desacetoxycephalosporin
unknown fragments
desacetoxycephalosporin lactone
desacetoxycephalosporoic acid lactone
esterase
OH⁻
H₂O
CH₃COO⁻

Scheme 5

Scheme (5) illustrates the general degradation reaction of cephalosporin C. Treatment of cephalosporin C with dilute acid gives not only a small amount of 7-aminocephalosporanic acid but also 7-aminocephalosporanic acid lactone and cephalosporins C lactone (known as cephalosporin Cc). In basic solution and by reacting with aromatic acid chloride, cyclization and esterification take place.

Cephalosporin C absorbs hydrogen; if hydrogenation is carried out in the presence of palladium on charcoal, the product is deacetoxycephalosporin C. In neutral solution, cephalosporin C reacts with pyridine to form cephalosporin CA.

Alkaline hydrolysis of the β-lactam takes place almost as fast or even faster with cephalosporin as with penicillin. The expected hydrolysis product cephalosporanic acid is not stable. It is further fragmented by expelling the acetoxy group as in cephalothin or other substituent at the 3-position as in the case of cephaloridine (Scheme 6).



Scheme 6

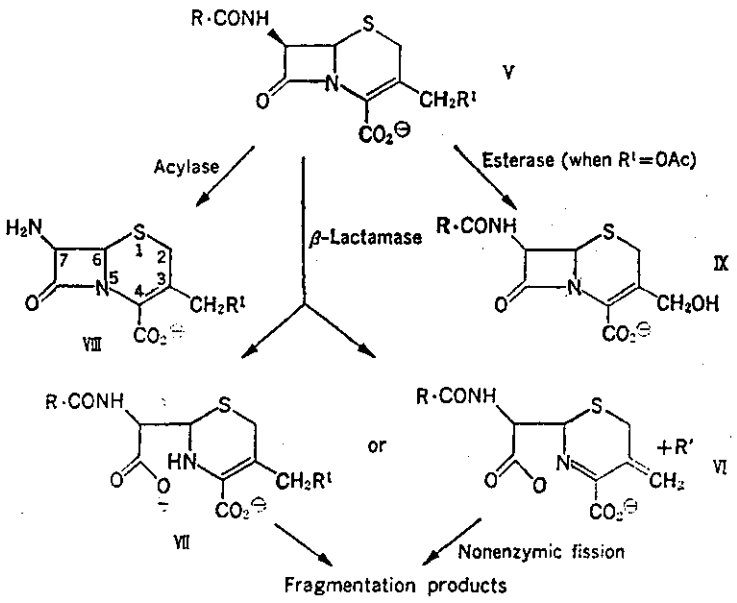
The cephalosporins do not appear to form compounds analogus to penicillanic acid and are relatively stable in acid solution[47]. Furthermore, lacking the gem-dimethyl group in the penicillins,the cephalosporins cannot break down to yield an amino acid analogous to pencillamine.

Bundgaard[44] studied the kinetics of the alkaline hydrolysis of a series of cephalosporins and found that the expulsion of the C-3'pyridinium group of cephaloridine appears to proceed simultaneously with hydrolytic opening of the β-lactam ring. He also found that the rates of hydrolysis of cephaloridine and the rates of formation of free pyridine are identical, which indicated that exactly one mole of pyridine is released per mole of cephaloridine. This clearly demonstrates the quantitative relationship between the hydrolysis of the β-lactam bond and the release of the pyridine from the C-3' position. Yamana et al[48] studied the acidic, neutral and alkaline degradation of some cephalosporins and concluded that the acetyl function of 3-acetoxymethyl cephalosporins were hydrolysed eight times faster than their β-lactam moieties and the reactivity of the cephalosporins in hydroxide-ion-catalysed degradation were influenced significantly by the C-3 methylene substituents. Fogg[49-54] et al have used dpp to study the degradation of cephalosporins in neutral, alkaline and acidic media. They appear to be the first to report the presence of H_2S as a degradation product of some cephalosporins.

Enzymic Hydrolysis of Cephalosporins

Hamilton-Miller et al[55] analysed the change in UV absorption spectra and p.m.r spectra together with a study of the degradation products resulting from enzyme reaction on β-lactam antibiotics, and found that the immediate product of the opening of the β-lactam ring of a cephalosporin depends on the group R' (scheme 6 . They also found that when R' was a leaving group, such as acetoxy as in cephalosporin C or cephalothin, or pyridine as in cephaloridine it was ejected leaving an exocyclic methylene group at C-3 forming a compound (VI) with λ_{max} at 230 nm. This compound, the cephalosporoate was unstable and further fragmented. Of the cephalosporins studied by Hamilton Miller et al only deacetylcephalosporin C lactone yielded a relatively stable cephalosporoate.

In 1960 a cell-bound acylase was found in E.Coli. This enzyme has shown very high activity with substrates containing the N-acyl group RCO, where R is thienyl methyl as in cephalothin and cephaloridine (VIII). while δ(D-α-aminoadipyl) side chain of cephalosporin C is not hydrolysed.[56,57] The enzyme estrase which has been found in a number of bacteria was also shown to act on cephalosporins containing an O-acetyl group as shown in scheme (7).



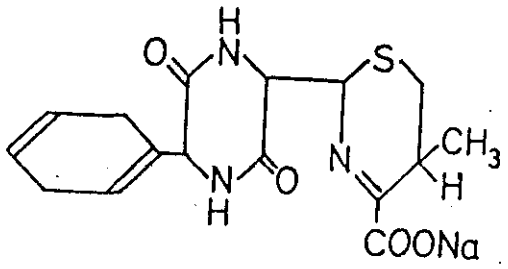
Scheme 7

Enzymic hydrolysis of cephalosporins

Degradation of α-aminobenzyl-cephalosporins in Solution

α-aminobenzyl cephalosporins such as cephalexin, cephradine, cephaloglycin and cefaclor are a group of a very important semi-synthetic cephalosporins which have an α-aminobenzyl group in the 7-position and unsubstituted methyl group in the 7-position as in the case of cephalexin and cephradine and a substituted 3-methyl group in the 3-position as in the case of cephaloglycin and cefaclor. These compounds degrade by means of a different mechanism to other cephalosporins because they are able to undergo intramolecular aminolysis by attack of the α-amino group on the β-lactam ring.

The degradation reaction of α-amino-cephalosporins in solution at different pH values has been investigated extensively in recent years. Indelicato et al[31] suggested that the increase in reactivity of α-aminobenzyl cephalosporins is due to an additional reaction which competes with hydroxide attack on the β-lactam in alkaline solution. They also suggested the formation of a 2,5-diketopiperazine (I) derivatives as a product of the intramolecular α-amino attack on the β-lactam ring, but they were unsuccessful in attempts to isolate the diketopiperazine derivative from aqueous hydrolysis of cephalexin and cephaloglycin, probably because the degradation was performed at pH 10 where the major degradation pathway is β-lactam hydrolysis. They succeeded, however, in isolating the diketopiperazine derivative from heated benzene solution of cephalexin ester and cephaloglycin lactone. Yamana et al[45] studied the stability of several cephalosporins having an α-aminobenzyl group in solution and tentatively suggested that the fluorescent compound they separated from cephalosporin solution by extraction into ethyl acetate, most probably to be the 2,5-diketopiperazine (I) derivative.



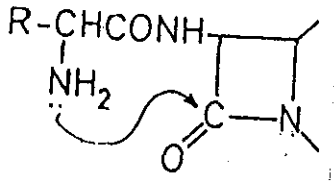
Diketopiperazine derivative

(I)

The 2,5-diketopiperazine derivatives have been isolated by Cohen et al[58] from the reaction solution of cephalosporin esters and cepharadine and he confirmed the structure given above from their spectral studies on the product by NMR.

Bundgaard[59] has shown by primary amino group analysis during degradation of cephalexin at pH 7 and 35°C that a 98% loss of the primary amino group occurs indicating complete degradation of cephalexin via the diketopiperazine derivative formed by intramolecular aminolysis. At higher pH values e.g. above pH 10, primary amine analysis indicated that only 32% of the cephalexin degrades by intramolecular aminolysis of the β-lactam ring. A mechanism of reaction involving the side chain group has been suggested by Yamana et al[45] and by Bundgaard[44]. Three possible mechanisms have been suggested from the shape of the pH rate profile in the neutral pH region, these are:

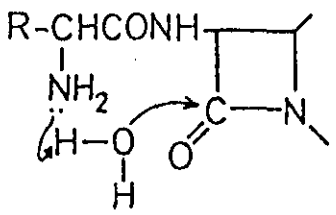
a) Intramolecular nucleophilic attack of the unprotonated side chain amino group on the β-lactam carbonyl moiety.



(A)

Mechanism (a)

b) Intramolecular general base catalysis by the amino group of the attack of a water molecular on the β-lactam bond

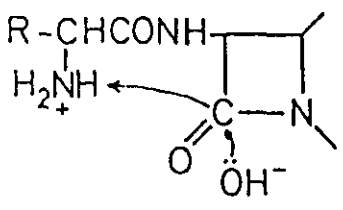


(B)

Mechanism (b)

and

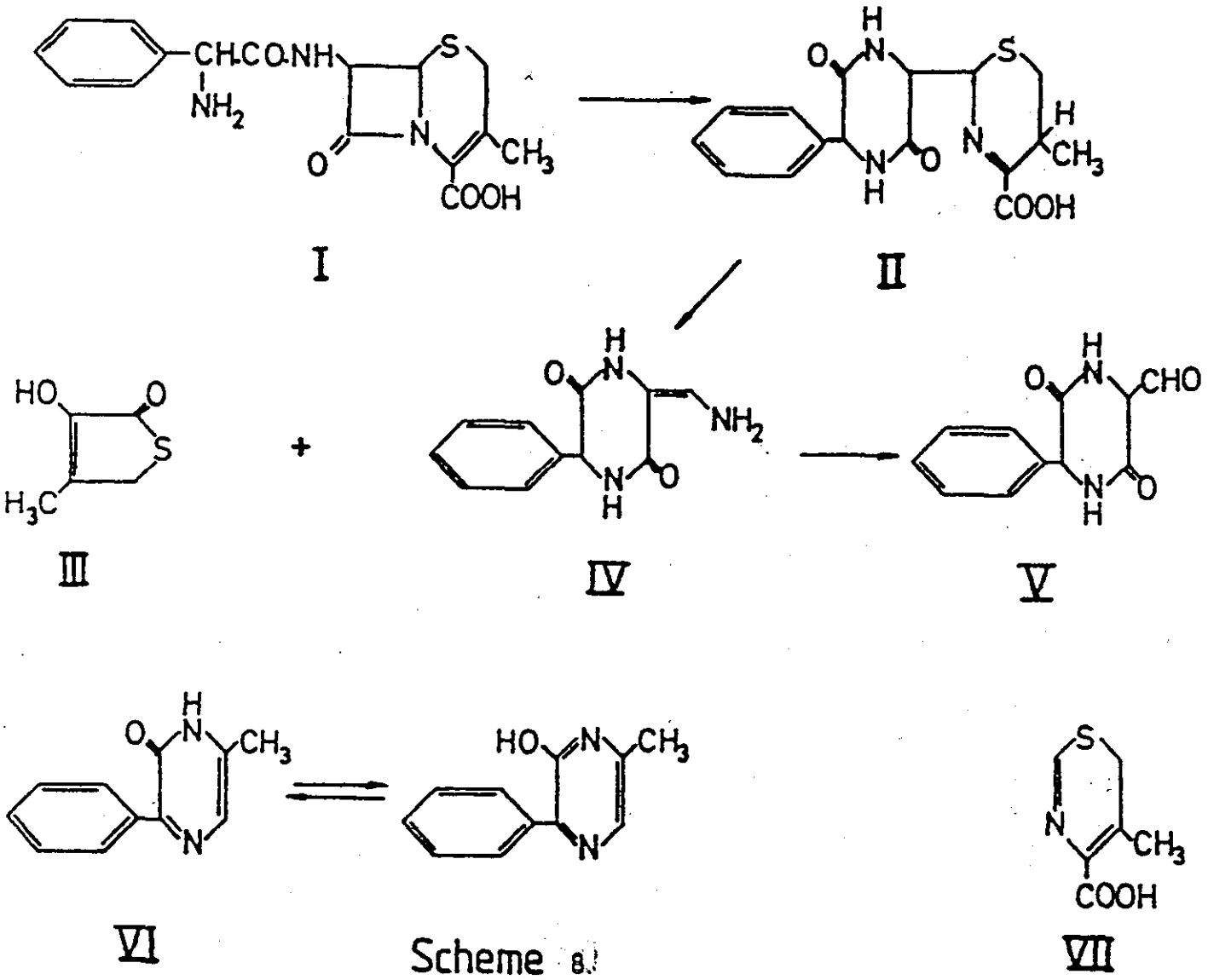
c) Intramolecular general acid catalysis by the protonated amino group of the attack of hydroxide ion on the β-lactam bond.



(C)

Mechanism (c)

Mechanism (a) was found to be the most probable one by Bundgaard because in contrast to mechanisms (b) and (c), mechanism (a) involves the formation of non-hydrolytic reaction products. The isolation of the 2,5-diketopiperazine derivative from cephalexin supported Bundgaard's hypothesis. Additional evidence of the formation of the diketopiperazine derivative as a primary degradation product of cephalexin in neutral and weakly acidic aqueous solution has been reported. Dinner[60] isolated and identified two products of degradation of cephalexin in acidic solution formed via the 2,5-diketopiperazine (II) derivative as 3,-formyl-3,6-dihyro-6-phenyl-2,5 (1H, 4H) pyrazine-dione and 3-hydroxy-4-methyl-2 (5H)-thiophenone (IV). At pH 3 Bundgaard showed that compound (IV) is a precursor of compound (V). Barbhaiya et al[35] have shown that the fluorescent degradation product used to determine several α-amino benzyl cephalosporins by an indirect fluorimetric method is 2-hydroxy-3-phenyl-6-methyl pyrazine (VI). Fogg et al[49-54] have shown by using differential pulse polarography dpp, the formation of diketopiperazine derivative (II) and also appeared to be the first to report the formation of H_2S from α-aminobenzylcephalosporins cephalexin and cephradine as well as from the degradation of the 2,5-diketopiperazine derivative in solution. Recently Indelicato et al have reported the mechanism of the formation of 2-hydroxy-3-phenyl-6methylpyrazine from cephalexin via the method used by Barbhaiya et al employing labelled formaldehyde. They suggested a similar scheme to that of Lebelle but this time α-aminobenzyl cephalosporin were included scheme (8).


CH.CO.NH
NH2
S
N
CH3
O
COOH
I
H
N
O
S
N
H
CH3
COOH
II
HO
O
S
H3C
III
+
NH2
IV
CHO
V
VI
HO
N
CH3
VII

Scheme 8.

Degradation Reaction of β-Lactam Antibiotics in Relation to Penicillin and Cephalosporin Allergy

While allergic reactions have been noted with many drugs, they are observed more often with the penicillins than with any other group of compounds and penicillin allergy has been and remains a major clinical problem. Because of the structural difference between the penicillins and cephalosporins, some hope was offered that the cephalosporins might prove to be non-allergenic or at least not cross-reactive with the penicillins, but these hopes have not proved to be true[61]. It is a generally accepted hypothesis that drug molecules of low molecular weight are non-immunogenic themselves, but must first combine irreversibly with a tissue macromolecule in order to elicit antibody formation. The macromolecule is usually a protein but may be a high molecular weight carbohydrate or lipid.

A complete treatment of the known or potential antigenic determinants of penicillins and cephalosporins must include the degradation products of the drug which can react with protein. These products, for example, may result from instability of drug or reaction with a pharmaceutical adjuvant in dosage form.

It is generally agreed that an irreversible reaction of penicillins occur with a nucleophilic group of a protein in vivo to yield penicilloyl conjugates which are responsible for most of the immunogenicity of these drugs. Thus kinetic studies of the aminolysis reaction of several penicillins and cephalosporins have been reported in the literature. In recent years, several workers[62,63,64] have reported the formation of high molecular weight substances in aqueous solution of ampicillin sodium. These

substances are so called ampicillin polymers, and are formed through a chain process involving nucleophilic attack of the side chain amino group in one ampicillin molecule on the reactive β-lactam moiety of a second molecule. Larsen et al[65] investigated these polymerization reactions using high performance liquid chromatography (HPLC) and found that ampicillin undergoes a self-aminolytic degradation to produce a dimer, followed by the formation of polymers of higher molecular weight. These studies are very important because considerable evidence now exists that the induction of allergy to the penicillins may be mainly or at least partly brought about by contaminants of higher molecular weight which become chemically associated by covalent linking with the degradation products of penicillin. Also reaction of penicillins with various carbohydrates in neutral and alkaline solution have received increasing attention in recent years. Penicillins are commonly infused in dextrose of fructose solutions and these carbohydrates have been shown to accelerate the degradation of penicillin at neutral and alkaline pH[65].

As a conclusion the use of penicillin of high purity is desirable but nevertheless highly purified penicillins may still give rise to allergic reaction. However breakdown products which form covalently linked association with penicillyoil specificity appear to be the most incriminated in allergic reactions and their presence should be kept at a minimum by not allowing solutions of penicillins to stand before injection[66].

Cephalosporins on the other hand were believed to be non cross-allergenic with the penicillins and it was considered that they could be given to patients who were penicillin sensitive, but it has been found that, while about 90% of penicillin-allergic patients will tolerate

cephaloridine, about 10% react to it. Therefore a significant degree of immunological cross-reactivity must exist. In addition cases of primary allergy to cephalosporins have been reported[63].

CHAPTER 2

ANALYTICAL CHEMISTRY OF PENICILLINS AND CEPHALOSPORINS

Introduction

In this chapter the main aim is to present a critical and exhaustive survey of the literature dealing with physical and chemical methods as well as chromatography, for the quality control of β-lactam antibiotics.

Because of the presence of the β-lactam ring in both cephalosporins and penicillins, the methods of assay of cephalosporins, in many instances, have also been utilised for the determination of penicillins. Two useful reviews have been published recently covering the analytical chemistry of cephalosporins and penicillins[67,68], and a large number of new methods have been published since, these methods can be divided into four sections:

1. microbiological determinations
2. chemical determinations
3. physical-chemical determinations
4. chromatographic determinations.

The chromatographic and physical-chemical methods have been used to provide an estimation of purity as well as methods of identifying the penicillins, cephalosporins and other impurities.

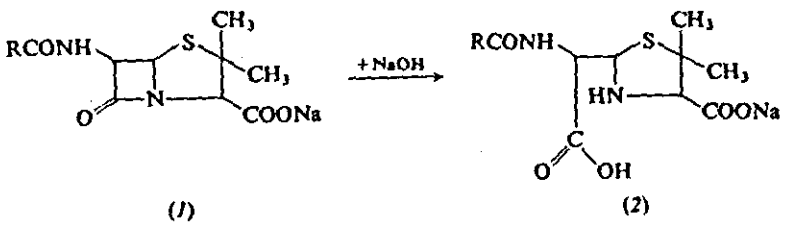
1. Microbiological Determinations

The microbiological assay has been and is still used as a method of preference for the determinations of anitbiotics in (i) pharmaceutical

preparations, (ii) body fluids, and (iii) formulations subjected to long term stability studies. Although routinely other methods such as iodometric or colorimetric assays can be used instead of microbiological methods, in the case of dispute the result of microbiological methods is preferred.

2. Chemical Determinations

Alicino[69] described the iodometric titration for the determination of penicillins. The method is based on the fact that the intact penicillin molecule (I) does not absorb iodine, while the alkaline hydrolysis product, sodium penicilloate (II) does react with iodine.



(1) (2)

The use of a blank containing the penicillin before hydrolysis, allowed for the presence of iodine-absorbing contaminants, including precursors and degradation products. The method compares favourably with the microbiological one in accuracy, and is much more rapid. Numerous modifications were proposed by many workers[70-76] for a variety of applications. Alicino[77] later described solvent extraction procedures for the analysis of synthetic penicillins and 6-aminopenicillanic acid and applied the method to the analysis of cephalosporin C. It has been found that

benzylpenicillin when treated with alkali, consumed nine atoms of iodine per mole of penicillin, whereas one mole of cephalosporin C consumed approximately four atoms of iodine. In practice the extent of iodine consumption varies with hydrolysis time, alkali concentration, temperature, pH of the iodine solution and duration of the iodine uptake reaction.

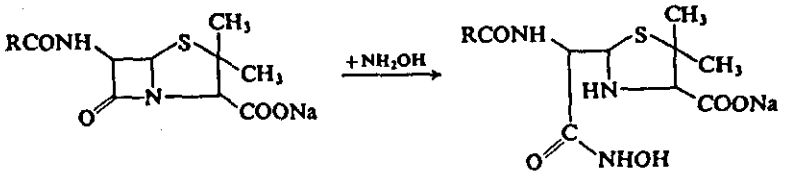
Iodometric methods based on the spectrophotometric assay of iodine, as I_3^-, have been described and have found important applications in automated systems for penicillin and cephalosporin determinations[78,79]. The method was automated and used for the analysis of benzylpenicillin, ampicillin, cephaloridine and cephalosporin C. Bomstein[80] et al described an automated iodometric system which incorporates modifications to eliminate the need for a separate blank determination and to allow for a more direct relationship between colour intensity and the antibiotic concentration. Frantz[81] has recently developed an iodometric assay for cephradine, using the β-lactamase cephradinase, in place of alkali for hydrolysis of the β-lactam ring. The method was described as being relatively straightforward, accurate and precise and to give reproducible results. However the method cannot differentiate cephradine from other α-aminocephalosporins.

The iodometric method has been used in studies of the stability of sodium ampicillin in liquid and solid states[82] and in aqueous suspensions[83], the stability of several semi-synthetic penicillins at various pH values[84], the relative stability of hetacillin and ampicillin in buffered solutions containing phosphate and citrate ions[85], the stability of benzylpenicillin in alcohols[86], the kinetics of complex formation between penicillin and sucrose[87], the kinetics and mechanism of

degradation and pH-solubility profiles of ampicillin and related penicillins[88] and the dissolution behaviour of various forms of ampicillin[89].

Abraham et al[90] observed the inactivation of penicillin by alkali, and that was used by many workers to develop an indicator titration method for penicillin[91]. In this case an alkali is used for hydrolysis and the excess back titrated against acid.

The hydroxylamine procedure was described first by Ford [92]. The method is based on the fact that hydroxylamine cleaves the β-lactam of penicillin (I) to form hydroxamic acid (II) which forms a coloured complex with ferric ion.



(I) (II)

The procedure has also been applied to some cephalosporins. The hydroxylamine method lacks specificity, as many amides, anhydrides, esters, aldehydes and ketones react with hydroxylamine and give rise to erroneous results. Interfering substances can be eliminated by incorporating a blank determination wherein the β-lactam is rendered incapable of forming a hydroxamic derivative.

The hydroxylamine method has been automated by Roudebush[93] and has been utilized for the simultaneous determination of cephalosporin C and penicillin N in fermentation broth. This system provides a rapid monitoring procedure for total cephalosporin β-lactam content in broth samples. Other improved manual and automatic hydroxylamine methods have been described by Mays et al[94]. In this method Nickel (II) was used instead of Ferric(III) as a catalyst and was found to be a significant improvement over current hydroxylamine methods. The method was claimed to provide a rapid reaction between hydroxylamine and the cephalosporin nucleus, and to reach a plateau in 15 minutes for all cephalosporins tested. Furthermore the procedure offers greater sensitivity and much better precision. Another automated procedure based on the hydroxylamine reaction, was described by Niedermeyer et al[95] who considered their single channel method to be superior to automated procedures based on iodometry because it could be used over a much wider range of concentration. In this procedure the chemistry of the method is based on the inactivation of penicillin to penicilloic acid either enzymatically or by alkali. Then the preformed penicilloic acid is separated by means of a continuous dialysis unit in the Autoanalyser and this prevents the penicillin interfering.

Marrelli[96] described a reproducible colorimetric method for the determination of 7-ACA and related compounds. The method is based on the fact that compounds having an α-amino group adjacent to a β-lactam ring react with ninhydrin under controlled conditions to give characteristic chromophores. The method was found to be very good for determining residual amounts of 7-ACA in cephaloglycin and hence provided a convenient means of following the extent of acylation in a synthesis

from 7-ACA. The method can also be used to determine low levels of 7-ADCA in cephalexin. The ninhydrin method has been improved by Papazova et al[97] for the determination of cephalexin. In this method cephalexin was hydrolysed for 5 minutes in sulphuric acid medium on steam-bath and the product of hydrolysis was then reacted with ninhydrin in 25% sulphuric acid to form a colour product. The product was then extracted in chloroform and its absorbance was measured at 520 nm. The method was claimed to be selective and free of interference.

Marelli[98] described another specific colorimetric method for the determination of cephalosporins derivatives having the α-amino group in the 7-position. The method was based on the reaction of these α-amino containing compounds with acetone and sodium hydroxide at 100°C to form characteristic red chromophores. Penicillins having an α-amino side chain responded under the same conditions, but the sensitivity was much less.

Weiss et al[99] reported a bromometric method for penicillins. The method differs from the iodometric method in that no inactivation of penicillin with alkali was required prior to the measurement. Alicino[100] described a method similar to the previous one except that N-bromosuccinimide was used instead of bromine. The reaction of N-bromosuccinimide and penicillin was found to be time dependent and the reagent was found to be light sensitive.

3. Physical-Chemical Methods

i) Ultraviolet absorption

It has been reported by Herriott[101] that under controlled conditions of pH, time and temperature, benzylpenicillin can be converted by acidic degradation into a stable intermediate with an absorption maximum at 322 nm. This method was found to be less practical because of instability of the ultraviolet absorbing penicillenic acid intermediate[102]. The stability was improved by Stock[172] by addition of copper as a means of enhancing and stabilizing the absorption, and the analytical results obtained were in good agreement with results obtained by microbiological methods.

Brandriss et al[103] reported the use of mercury(II) salts instead of copper(II) salts to stabilize penicillenic acids and developed a method on this basis for the determination of penicillins in protein solutions and in human serum. The method was claimed to be sensitive and the reaction to proceed quantitatively. The method was used by Bundgaard and Ilver[104] for the measurement of undecomposed cloxacillin in kinetic studies of the degradation of this penicillin in aqueous solution. Bundgaard[105,106] also found that the conversion of penicillin to penicillenic acid is catalysed by imidazole, and has studied the kinetics of this reaction and discussed its possible implications in the immunogenicity of penicillins. This method has been combined with the mercury(II) ion stabilization of penicillenic acid by Bundgaard et al[107]. They published a rapid and convenient method for the determination of nine-commonly used penicillins. The reaction in this case proceeds quantitatively even at 20°C although the reaction is completed more quickly at 60°C.

The reaction of ampicillin with the imidazole reagent was found to be anomalous, an unstable product with λ_{max} 311 nm being formed rather than the expected mercury(II) mercaptide of α-aminobenzylpenicillenic acid with an expected λ_{max} at 325 nm. Although this unstable product was used as a basis of a quantitative method for ampicillin, an improved procedure has been described involving initially N-acetylation of ampicillin followed by reaction with imidazole and mercury(II) chloride[108]. This procedure overcame the unfavourable behaviour of ampicillin by acetylation of the side-chain amino group with acetic anhydride in weakly basic aqueous solution to yield α-acetamidobenzylpenicillin which behaves as other penicillins in the subsequent treatment with imidazole reagent. The method can be modified to allow the determination of ampicillin in the presence of degradation and polymerization products[109].

Recently Bundgaard[110] has studied the stability of ampicillin in aqueous solution containing various carbohydrates and alcohols and found that it resulted in the formation of a major decomposition product which interfered strongly in the imidazole-assay for ampicillin. The product was identified as 2-(6'-phenylpiperazine-2,5'-dione-3-yl) 5,5-dimethylthiazolidine-4-carboxylic acid. This compound resulted from an intramolecular aminolysis of the penicilloyl esters initially formed from reaction of ampicillin with the hydroxy compounds[111]. Upon the treatment with mercury(II) chloride in neutral aqueous solution the piperazine-2,5-dione derivative was found to be transformed into the corresponding penamaldate structure which is strongly absorbing with λ_{max} 305 nm and hence interferes with the ampicillin determination.

These findings led Bundgaard[112] to develop a method for the determination of ampicillin and other α-aminopenicillins in the presence of the interfering piperazine-2,5-dione. The method was based on the fact that piperazine-2,5-dione was unaffected by the addition of strong alkaline solution while ampicillin was found to hydrolyse completely to α-aminobenzylpenicilloic acid which is non-responsive in the imidazole assay. The final imidazole assay has been conveniently applied to the determination of penicillin and cephalosporins generally as well as in stability indicating tests, in many laboratories.

ii) Fluorometric analysis

It is known that β-lactam antibiotics having an α-amino group on the side chain yield a highly fluorescent product when they are degraded under certain conditions. The reaction has been conveniently utilized for the sensitive fluorometric assay of α-aminopenicillin and α-aminocephalosporins in aqueous solutions and in biological fluids. The reaction which has been used for analytical purposes has been carried out under various conditions of pH, time period, temperature, catalyst and concentration of reactant.

The first report of such fluorometric determinations of both aminopenicillin and aminocephalosporins seems to be that of Jusko[113]. The method was based on the degradation of ampicillin in pH 2 solution containing formaldehyde. Jusko found that the aminobenzyl group and a cleaved β-lactam ring were necessary for the formation of the fluorophore, because Benzylpenicillin which lacks the α-amino group of ampicillin did not give the fluorescent product whilst penicilloic acid obtained

by the hydrolysis of ampicillin with penicillinase gave a product with fluorescence as intense as that of the parent penicillin. The method is reasonably specific for ampicillin, only cephalexin among the β-lactam antibiotics investigated gave any appreciable fluorescence under the reaction conditions. Jusko also found that the addition of formaldehyde accelerated the reaction and concluded incorrectly that the main product of the reaction in his method was probably the 3,6-disubstituted diketopiperazine.

The method has been applied to assay procedures based on the formation of the fluorescent degradation products for the analysis of β-lactam antibiotics containing α-amino group by several workers. Several of these methods have been reported by Barbhaiya et al[114,115] in which they used a method originally developed for ampicillin by Jusko. The addition of formaldehyde to the reaction solution was found to enhance the fluorescence and to produce reproducible assay. However, Barbhaiya et al[116] reported a fluorometric assay for amoxicillin in which no formaldehyde was used. Other methods by Miyazaki et al[117,118], Barbhaiya et al[119] required mild acid hydrolysis of the alkaline degradation product in the presence of mercury(II) chloride. Tsuji et al[120] have suggested a fluorometric method for simultaneous determination of penicillins and penicilloic acids. The method is based on the formation of a fluorescent schiff-base resulting from the reaction between a certain hydrazine and penilloaldehyde which can be produced from the reaction of penicilloic acid and mercury(II) chloride in acidic medium[121]. The method was described as simple, precise and reproducible and capable of measuring simultaneously penicillin and its metabolites

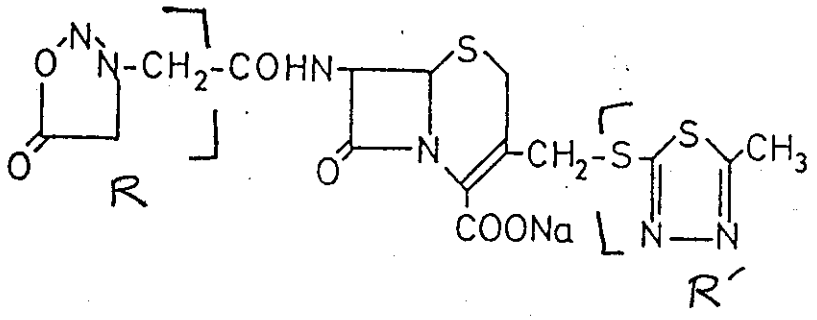
and having advantages over other methods such as microbiological assay procedures.

The fluorescent product formed by the degradation of aminopenicillins and aminocephalosporins under different practical procedures gave essentially the same excitation and emission wavelengths i.e. λ_{ex} = 420-440 nm, λ_{em} = 340-360nm This suggested that the fluorophores derived from different antibiotics might have analogous structure. The structure of the fluorescent derivative has been identified by Barbhaiya[35] et al and Lebelle[36] et al as being the 2-hydroxy-3-phenyl-6-methyl-pyrazine.

iii) Electrochemistry

Jones et al[122] reported the use of d.c. polarography in quantitative and qualitative analysis of cephalosporin C, cephalothin and cephaloridine. The dc wave of cephaloridine was found to be useful for both quantitative and qualitative purposes while the use of the ill-defined wave of cephalosporin C and cephalothin should be restricted to qualitative work.

Several penicillins and some cephalosporins such as cephalothin, cephaloridine and cephalexin in serum have been determined by Benner[123]. Benner used cathode-ray polarography in his method and also found that it was necessary to hydrolyse cephalexin in sodium hydroxide solution at 50°C for 30 minutes prior to the determination. Coulometry and d.c. polarography has been used by Hall[124,145] in a study of the degradation of 3-(5-methyl-1,3,4-thiadiazol-2-yl-thiomethyl)-7-(2-(3-sydnone) acetamido)-3-cephem-4-carboxylic acid sodium salt (I).



He suggested that acid hydrolysis results in the loss of the R'-group at the 3-position and the formation of an α-β-unsaturated lactone, whilst base and enzymatic hydrolysis open the β-lactam ring and simultaneously eliminates the R'-group at the 3-position.

Recently, Rickard et al[126] have used d.c. polarography in analytical control and stability testing of the cephalosporins, cefamandole and cefamandole nafate, while controlled potential coulometry was applied as an absolute measure for purity evaluation of these compounds. A d.c. polarographic method for the determination of cephalexin 10^{-3}M level after hydrolysis in 5M HCl at 80°C has been described by Squella et al[127]. d.c. polarography was also used by Squella et al[128,129] for the determination of ampicillin in capsules and tablets. The method was based on the well-defined polarographic wave at -0.91V resulting from the acidic hydrolysis degradation of ampicillin. The method has the advantages of selectivity, which makes the determination of ampicillin possible without prior separation of the electroactive compound. The determination of amoxycillin after[130] hydrolysis in acidic solution of pH 5 and heating at 100°C for 30 minutes was also described.

Differential pulse polarography (dpp) has been used by Jemal et al[131] to follow the degradation of benzylpenicillin by following the change

in its dpp peaks. Fogg et al[49-54] have used dpp in their study of the degradation reactions of several cephalosporins and penicillins in aqueous solution. They reported the determinations of penicillins and cephalosporins. The method was based on the fact that cephalosporins having a substituted methyl group in 3-position give a dpp peak at about -1V. Although these peaks are near the cathodic limit for the d.m.e, they have been used conveniently for the determination of these cephalosporins, (cephalothin, cephalosporin C, cephaloridine, cephalonium, cefuroxime), 7-ACA and7ADCA and their degradation products[182]. While cephalosporins which have an unsubstituted methyl group in 3-position and no reducible group elsewhere in the molecule do not give dpp peaks at d.m.e, but certain of their degradation products do and these can be used for the determination of these compounds (cephalexin and cephradine), the sensitivity as well as the selectivity of dpp can be used as a possible check on a microbiological assay particularly when a mixture of two dissimilar antibiotics has to be assayed. Without prior separation of the antibiotics, such an analysis would be impossible by the microbiological technique.

iv) Infrared and nuclear magnetic resonance spectroscopy

Because of the specificity of infrared it has been used as an official method for identifying penicillins[133,134]. Several publications suggested the possibility of using infrared techniques for the quantitative analysis of penicillins. Coclers et al[135] used chloroform solutions of the acid form of a number of penicillins for quantitative analysis by determining the absorption at about 1790 cm^{-1} which has been attributed to the β-lactam carbonyl functional group. The method was successfully applied to the determination of active antibiotics in

pharmaceutical preparations, and to a study of degradation in aqueous solutions. It is, however, inapplicable to the zwitterionic penicillins such as ampicillin. Weitkamp[136] and Barth successfully determined ampicillin and other penicillins by using ethylene glycol monomethyl ether/tetrachloroethane (1:4 w/v) as a solvent. They described specific procedures for the assay of formulated products, including mixtures of sodium salts of ampicillin and oxacillin. Results obtained were comparable with those from microbiological analysis but the method was sufficiently specific to allow for differentiation between different penicillins.

The use of NMR in structure determination has been widespread. Recent examples include the structure elucidation of several cephalosporins from streptomyces[137], using ^{1}H NMR, and ^{13}C NMR, studies of penicillins and related sulfoxides[138]. Wilson et al[134] used ^{1}H NMR as an identification method for commercially available penicillins and cephalosporins and developed a specific method for the determination of the diasteroisomer ratio in phenethicillins. NMR has been used by Mangia et al[140] for the quantitative determination of cephalexin. The method was based on the measurement of the methyl resonance (125.6 Hz, 2.09δ) of the dihydrothianyl moiety of cephalexin because the signal is sharp, distant from other proton signals in the molecule, and is specific, being adequately separated from peaks of likely impurities and potential degradation products. NMR was also used by Nelson[141] for the determination of penicillamine in capsules.

4. Chromatography

i) Paper chromatography

Application of paper chromatography to the separation of penicillins was described by Goodal et al[142,143]. A microbiological method of detection was used and quantitative results on crude mixtures of penicillin were obtained by comparing zones of inhibition with authentic standards. Various modifications of this method were published[144], and claims were made for enhanced resolution, reproducibility and accuracy[145], and shorter analysis time[146]. The method and the modifications have been used for the identification of 6-APA and to resolve mixtures of penicillin G, V and K[147].

The well known ability of penicilloic acids to absorb iodine was used by Thomas[148], who used a two stage spray: first, dilute alkali to effect hydrolysis of the β-lactam, then starch-iodine complex to give white spots on a blue background. A modification involved the use of penicillinase instead of alkali as a more specific spray for penicillin sensitive compounds. A quantitative method for 6-APA was developed by Huang et al[149] based on hydrolysis to penicic acid with penicillinase and then paper chromatographic resolution and reaction with ninhydrin.

Paper chromatography has had widespread utility in studies of penicillin breakdown both chemical and microbiological. Thus Hamilton-Miller[150] used paper chromatography in a study of the action of penicillinase on benzylpenicillin.

Paper chromatography proved of value in cephalosporin studies from the first reports of the isolation of cephalosporin C and its differentiation from penicillin N[151].

ii) Thin-layer chromatography

The use of thin layer chromatography as a quality control procedure has been widely investigated by workers of the all Union-Research Institute of Antibiotics (Moscow). For example, Korchagin et al[152] developed systems to resolve degradation products of phenoxymethyl-penicillin from the parent compound, followed by visual comparison with standards as a semi-quantitative purity determination. The method was used for the quality control of phenoxymethyl penicillin isolation and chemical purification, and for purity determination of pharmaceutical dosage forms. Semi-quantitative procedures for estimation of 6-APA in oxacillin and benzyl penicillin in carbenicillin preparation were also described[153]. The same authors applied thin layer chromatography to the separation of degradation products of oxacillin from each other and from the parent antibiotic and to the quality control of carbenicillin isolation and purification and 6-APA production[154,155].

A thin layer chromatography system to resolve cephalosporin C, cephalothin and cephaloridine was described by Buri[156] who suggested its use as an identification procedure. Vandamme and Voets[157] in their study of the resolution of penicillin degradation products also resolved cephalosporin C from 7-ACA. Recently Budd[158] suggested a novel system for the resolution of compounds related to 7-ACA (desacetyl-7-ACA 7-ACAlactone), using silica gel G layers and 0.5M aqueous sodium chloride as mobile phase.

iii) Gas-chromatography

An extensive study was reported by Hishta et al[159] who converted several penicillins to their trimethyl esters and resolved most of them on

a 20% OV.17 column. The method gave quantitative results of good precision and was claimed to be applicable to pharmaceutical preparations using suitable sampling procedures. Ampicillin which was not determined previously has been analysed under similar conditions[160]. While this technique is fast and specific, it is only applicable to these antibiotics which are thermally stable after prior derivatization.

iv) High performance liquid chromatography

A vast number of papers have now appeared describing the application of high performance liquid chromatography to penicillin and cephalosporins[161]. Both normal phase and reversed phase chromatography have been used for the determination of β-lactams[162]. The chemical similarity between the penicillins and the cephalosporins has led to the use of very similar chromatographic systems for their determination. Carrol et al[163] have used reverse phase HPLC to separate and analyse cephalexin and cephradine. HPLC has been successfully applied to the detection and quantitisation of penicillins and cephalosporins in pharmaceutical preparations[164-168] and in biological fluids[169-171].

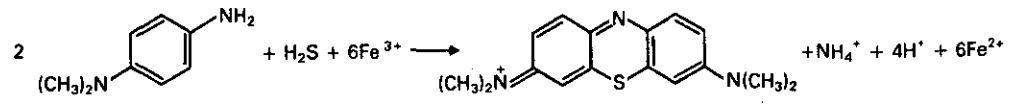
CHAPTER 3

SELECTIVE SPECTROPHOTOMETRIC DETERMINATION OF CEPHALOSPORINS BY ALKALINE DEGRADATION TO HYDROGEN SULPHIDE AND FORMATION OF METHYLENE BLUE

Introduction

Degradation studies of solutions of cephalosporins and penicillins have been made previously in the laboratory of this University, mainly using differential pulse polarography [49-54]. Hydrogen sulphide was shown to be a major degradation product in solutions of pH 7.4 and 8.5 of those cephalosporins such as cephalexin, which have an α-aminobenzyl group at C7, these cephalosporins degrade in solutions of pH 7-8 by intra-molecular aminolysis to give diketopiperazine derivatives [172]. Hydrogen sulphide is not a major degradation product of penicillin or other cephalosporins under these solution conditions. The amount of hydrogen sulphide produced from cephalexin was determined by sweeping the hydrogen sulphide from the degrading solution by means of a stream of nitrogen gas either into sodium hydroxide solution, where it was titrated with silver nitrate solution using a sulphide ion-selective indicator electrode, or into cadmium nitrate, where the excess of cadmium ion was determined polarographically [173].

Hydrogen sulphide, however, can be measured quantitatively as methylene blue. The methylene blue method depends on the formation of the blue colour from the reaction of sulphide with p-amino, N,N-dimethylaniline in the presence of iron(III) chloride. The overall redox reaction may be represented as:



The molar absorptivity of the methylene blue solution was found by Johnson and Nishita[174] to be about 34,000 l mol^{-1} cm^{-1}, very close to that for the maximum sensitivity of colorimetric methods.

The methylene blue method was recommended by Fischer[175], for the identification of hydrogen sulphide. In his method the solution containing hydrogen sulphide was mixed with an acidic solution of N,N-dimethy-p-phenylenediamine, and iron(III) was added. The solution first becomes red but changes to blue as methylene blue is formed.

The method was later used for quantitative determination of sulphide[176] and for the determination of sulphate and other reducible sulphur[177]. Lorant[178] assumes that methylene red, the red colour noted previously, and methylene blue are formed in the proportion 1:50. It is evident that hydrogen sulphide is not quantitatively transformed to methylene blue under the reaction conditions he used. Various methods have been proposed using the colour forming reagent[178]. Sands et al[179] selected the methylene blue method as the basis of an ultrasensitive technique for hydrogen sulphide in gases. This was accomplished by studying the variables affecting the results and recommending a procedure to be followed exactly. The preferred absorbent was 2% acidic zinc acetate solution, and the sulphate of N,N-dimethyl-p-phenylenediamine was preferred over the hydrochloride. Gustafsson[180] investigated the following variables: hydrogen sulphide

losses, acidity of and temperature of reacted and measured solution, and reagent concentrations and found that the method deviates from Beer's law when the concentration of the sulphide is greater than 25 µg/100 ml. Gustafsson[180] also determined the yield of methylene blue obtained from sulphide to be 66.7%. Lorant[181] found that exactly 68.7% of the added sulphide was transformed to methylene blue.

Confusion appears to have arisen as to the yield of methylene blue obtained from the sulphide. Lorant quotes 68.7% and Gustafsson[180] 66.7 ± 0.5%. Zutshi and Mahadevan[182] obtained 65 ± 2.0% based on the apparent molar absorptivity of an undried sample of methylene blue, but obtained 51 ± 2.0% based on the apparent molar absorptivity of a general reagent grade sample that had been dried at 80°C.

Experimental

Equipment;

Visible absorption spectrophotometric measurements were made with a Pye Unicam SP8-100 spectrophotometer. Potentiometric measurements were carried out with an Orion solid-state sulphide ion-selective electrode (Model 94-16) and an expanded scale Radiometer PHM 64 research pH meter. Thin layer chromatography was done on precoated 0.25 mm silica gel F_{254} aluminium sheets (Merck, Germany).

Chemicals and samples:

All chemicals were of analytical reagents grade except where otherwise indicated.

Samples:

Samples of cephalexin, cephalothin, cephaloridine, cephalonium, cefuroxime, cephoxazole 7-ACA and 7-ADCA were obtained from Glaxo Operations (UK) Ltd. Samples of cefaclor, cefazolin and cephaloglycin were obtained from Lilly Research Centre Ltd. Cephradine samples were provided by E R Squibb and Sons Ltd.

Reagents and buffer solutions:

NN-Dimethyl-p-phenylenediamine sulphate solution 0.005M.

Dissolve 0.93g of NN-dimethyl-p-phenylenediamine sulphate in 750 ml of distilled water. Add 186 ml of concentrated sulphuric acid, cool and dilute to 1ℓ with water.

Zinc acetate solution, 0.25M.

Dissolve 27.4g of zinc acetate hexahydrate and 4.10g of sodium acetate in 0.1M sulphuric acid solution and dilute to 500 ml with 0.1M sulphuric acid.

Ammonium iron(III) sulphate solution, 0.25M.

Dissolve 60.8g of ammonium iron(III) sulphate dodecahydrate in 0.5M sulphuric acid and dilute to 500 ml with 0.5M sulphuric acid.

Standard lead nitrate solution, 10^{-2}M.

Preparation of buffer solutions:

Phosphate buffer solution (pH 7.4)

The phosphate buffer was prepared by adjusting a 0.5M disodium hydrogen phosphate solution to pH 7.4 by addition of 0.5M potassium dihydrogen phosphate solution.

Sorensen's citrate buffer solution (pH 5).

A stock solution of Sorensen's citrate buffer solution was prepared by adjusting the pH of 0.1M disodium citrate (21.0g citric acid monohydrate dissolved in 200 ml 1M sodium hydroxide and made up to 1 litre with distilled water) to pH 5 with 0.1 M sodium hydroxide.

Experimental Techniques

1. Effect of hydrolysis time on the formation of hydrogen sulphide during the degradation of twelve cephalosporins and their derivatives.

i) Preparation of stock solutions

Except for 7-aminocephalosporanic acid (7-ACA) and 7-aminodeacetoxycephalosporanic acid (7-ADCA), stock solutions of cephalexin, cephradine, cephaloglycin, cefaclor, cefuroxime, cefoxazole, cephalonium, cephalothin, cephaloridine and cefazolin were prepared in water. Fresh solution were usually used before measurements. 7-ACA and 7-ADCA were dissolved in two ml of 0.5M Na OH and were immediately made up to volume with water to prevent prolonged contact with sodium hydroxide which would cause extensive degradation.

ii) Isolation of a sample of diketopiperazine

A sample of diketopiperazine derivative was isolated from a degraded solution of cephalexin as follows.

A solution of 1g cephalexin in 50 ml of 0.3M aqueous phosphate buffer solution (pH 7.6) was kept at 35°C for 24 hours. The solution was filtered to remove a white ppt and then the solution was brought to pH 2 with 5M hydrochloric acid. The solution was then

flushed with nitrogen gas for 30 minutes. The solution was extracted with 50 ml portions of ethyl acetate. The extract was then washed twice with water and subsequently dried over anhydrous sodium sulphate. The combined ethyl acetate extracts were evaporated under vacuum. The residue of evaporation was suspended in 25 ml of boiling chloroform and filtered off. The residue was dried in vacuum over phosphorus pentoxide for 24 hours. The identity of the product was confirmed by thin layer chromatography and melting point determination as being the piperazine-2,5-dione.

iii) Isolation of a sample of 2-hydroxy-3-phenyl-6-methylpyrazine

A solution of 0.5g cephalexin in distilled water (50 ml) was treated with sodium hydroxide solution (25 ml, 1M) for 10 minutes at room temperature. Hydrochloric acid (25ml 1M) was then added followed by Sørensen's citrate buffer solution (150 ml, pH 5) containing formaldehyde (1% v/v). The mixture was then heated at 100°C for 30 minutes, cooled and extracted repeatedly with ethyl acetate. The combined ethyl acetate extracts were then evaporated, and the residue was crystallised from ethyl acetate. The purity of the prepared compound was checked by comparison of the sample and a sample of pure compound obtained from Fisons Ltd, on thin layer chromatographic plate eluted with acetone-chloroform (1:1 v/v).

Procedure for formation of hydrogen sulphide with heating of the alkaline solution for various cephalosporins and some of their degradation products

Dissolve about 0.1g of the cephalosporin accurately weighed, in water and dilute to 100 ml in a calibrated flask; 7-aminocephalosporanic acid (7-ACA) and 7-aminodeacetoxy cephalosporanic acid (7-ADCA), which do not dissolve in water, were dissolved in, and diluted to 100 ml with 0.5M sodium hydroxide solution. Transfer, by means of a pipette, aliquots (10 ml) of the solutions into stoppered flasks[7-13] and make the solution to 100 ml in such a way that the final concentration of sodium hydroxide is 0.5M. Heat the flasks in a boiling water-bath and remove flask at suitable intervals and then cool.

Add, by pipette, 5 ml of each solution to 50 ml calibrated flasks and add 5 ml of 0.25M zinc acetate solution and mix. Add 5 ml of NN-dimethyl-p-phenylenediamine sulphate solution and 2 ml of ammonium iron(III) sulphate solution and quickly stopper the flask. Shake the flask for 30s, wait 2 min, dilute to 50ml, mix and read the absorbance of the solutions at 667 nm.

2. Determination of the Apparent molar absorptivities of cephalosporins and molar yields of hydrogen sulphides

i) Determination of the apparent molar absorptivity of standard sulphide solution.

In order to determine the molar absorptivity of methylene blue and hence the yield of methylene blue in the reaction of sulphide with the reagents a British Pharmacopeia-grade sample of

methylene blue was used. The monograph for methylene blue in the 1973 Edition of the British Pharmacopeia[185] stated that the sample should contain not less than 96.0% and not more than 101.0% of $C_{16}H_{18}ClN_3S$ calculated with reference to the dried substance, and that when dried at 105°C it should lose not less than 8.0% and not more than 15.0% of its mass. $C_{16}H_{18}ClN_3S$ is the formula of the anhydrous chloride salt, and the assay procedure used is titration with titanium(III).

ii) Preparation of sodium sulphide standard solution

Due to the hygroscopic nature of sodium sulphide, it is not possible to prepare solutions of exact molarity, and it was necessary to standardise these solutions. All solutions used in this study were prepared in tri-distilled water which was boiled for about one hour and cooled under stream of oxygen-free nitrogen. An approximately 0.1M solution of sodium sulphide prepared in 1M sodium hydroxide solution was standardised against 0.1M lead nitrate standard using a sulphide ion-selective electrode.

iii) Procedure for the determination of the apparent molar absorptivity of standard sulphide and the molar yield of methylene blue obtained from standard sulphide

Transfer, by means of a pipette, aliquots (2.0 ml) of the standard sulphide solution into six 50 ml calibrated flasks. Add, by pipette, 5 ml of 0.25M zinc acetate solution and mix. Add 5 ml of NN-dimethyl-p-phenylenediamine sulphate solution and 2 ml of ammonium iron(III) sulphate solution and quickly stopper the flask. Shake the flask for 30s, wait 2 min, dilute to 50 ml, mix and read the absorbance of the solutions at 667 nm. The calculated

apparent molar absorptivity of the methylene blue derived from the standard sulphide was then related to that calculated for the BP grade sample of the methylene blue and the % yield of methylene blue was then calculated.

iv) Determination of the molar yields of hydrogen sulphide from each cephalosporin

Procedure:

Dissolve about 0.1g of the cephalosporin, accurately weighed, in water and dilute to 100 ml in a calibrated flask, 7-aminocephalosporanic acid (7-ADCA), which do not dissolve in water, were dissolved in, and diluted to 100 ml with 0.5M sodium hydroxide solution. Transfer by means of a pipette, aliquots (10 ml) into 6-six 100 ml stoppered flasks and dilute to 100 ml with 0.5M sodium hydroxide solution. Heat the flasks in a boiling water-bath for a length of time appropriate for the particular cephalosporin to produce maximum formation of sulphide (see Table 5) and then cool.

Add by pipette 10 ml of each solution to 50 ml calibrated flasks and add 5 ml of 0.25M zinc acetate solution and mix. Add 5 ml of NN-dimethyl-p-phenylenediamine sulphate solution and 2 ml of ammonium iron(III) sulphate solution and quickly stopper the flask. Shake the flask for 30s, wait 2 min, dilute to 50 ml, mix and read the absorbance of the solutions at 667 nm. The percentage yields of hydrogen sulphide from each cephalosporin was determined by comparing the apparent molar absorptivity of the cephalosporin to that of the standard sulphide. The results from this experiment were also used to study the reproducibility of the hydrogen sulphide obtained from different cephalosporins.

3. Potentiometric determination of hydrogen sulphide formed by alkaline degradation of cephalosporins

For use with the titrimetric procedure approximately 5 x 10^{-3}M solutions of cephalosporins in 1M sodium hydroxide solution containing 20 gl^{-1}, of ascorbic acid were degraded at 100°C for the length of time recommended previously. Aliquots (25 ml) of these degraded solutions were diluted in a water-jacketed titration vessel held at 25°C, with 100 ml of 1M sodium hydroxide solution containing 20 gl^{-1} of ascorbic acid and 10% v/v of glycerol. The solutions were titrated with 10^{-2}M lead nitrate solution using a sulphide ion-selective electrode. The results obtained from this experiment were then compared to those obtained from spectrophotometric studies.

4. Recommended procedure for the colorimetric determination of cephalosporins

Dissolve about 0.1g of the cephalosporins, accurately weighed, in water and dilute to 100 ml in a calibrated flask,dissolve 7-aminocephalosporanic acid (7-ACA) and 7-aminodeacetoxycephalosporanic acid (7-ADCA), which do not dissolve in water, in 0.5M sodium hydroxide solution and dilute to 100 ml with 0.5M sodium hydroxide solution. Transfer by means of a pipette aliquots (0-10 ml) of the solutions into 100 ml calibrated flasks and dilute to volume with 0.5M sodium hydroxide solution. Smaller amounts of cephalosporins (0.01g) can be hydrolysed after being dissolved directly in 100 ml of 0.5 M sodium hydroxide solution. Heat the flasks in a boiling water-bath for a length of time appropriate for the particular cephalosporin to produce maximum formation of sulphide and then cool.

Add by pipette, 5 ml of each solution to 50 ml calibrated flasks and add 5 ml of 0.25 M zinc acetate solution and mix. Add 5 ml of NN-dimethyl-p-phenylenediamine sulphate solution and 2 ml of ammonium iron(III) sulphate solution and quickly stopper the flask. Shake the flask for 30s, wait 2 min, dilute to 50 ml, mix and read the absorbance of the solution at 667 nm. Samples should be treated in a similar manner to the standard and the amount of cephalosporin present can then be calculated by comparison with the appropriate calibration graph.

Results:

The rate of formation of hydrogen sulphide with time of heating of the alkaline solution for various cephalosporins and the diketopiperazine derivative are given in Tables 1 - 3, and Figures 1, 2, 3 and 4. For cephalothin in particular, the sulphide appears to come off in two waves (see Figure 2), which probably indicates that the hydrogen sulphide is formed by two degradation routes. A plateau is eventually reached, i.e. hydrogen sulphide evolution essentially ceases in all instances, and suitable times for heating can be selected for each cephalosporin (see Table 5).

TABLE 1:

Effect of time of heating in 0.5M sodium hydroxide solution on the formation of sulphide from cephradine (8.8 ppm) cephalexin (9.1 ppm), cefaclor (18.08 ppm) and cephaloglycin (33 ppm), solutions as indicated by the methylene blue absorbance at 667 nm

Time/min	Absorbance at 667 nm			
	Cephradine	Cephalexin	Cefaclor	Cephaloglycin
10	0.389	0.283	0.372	0.313
20	0.461	0.404	0.568	0.487
30	0.487	0.401	0.620	0.507
40	0.472	0.404	0.647	0.513
50	0.480	0.408	0.660	0.512
60	0.479	0.403	0.660	0.513

TABLE 2:

Effect of time of heating in 0.5M sodium hydroxide solution on the formation of sulphide from, cefuroxime (43 ppm), cephaloridine (74.16 ppm), cephalonium(15.5 ppm) and cephoxazole (69.3 ppm), solutions as indicated by the methylene blue absorbances at 667 nm

Time/min	Cefuroxime	Cephaloridine	Cephalonium	Cephoxazole
10	0.136	0.250	0.125	0.162
20	0.302	0.618	0.180	0.272
30	0.556	0.832	0.250	0.411
40	0.779	0.936	0.342	0.624
50	0.979	1.254	0.404	0.790
60	0.980	1.254	0.406	0.827
70	0.985	1.255	0.406	0.828
80	0.986	1.253	0.408	0.827

TABLE 3:

Effect of time of heating in 0.5M sodium hydroxide solution on the formation of sulphide from cephalothin (28.6 ppm), 7-ADCA (6.3 ppm), 7-ACA (9.2 ppm), Cefazolin (28.9 ppm) and diketopiperazine derivative (45.6 ppm) solutions as indicated by the methylene blue absorbance at 667 nm

Time/min	Absorbance at 667 nm				
	Cephalothin	7-ADCA	7-ACA	Cefazolin	Dikeopiperazine derivative
5	0.209	0.251	0.084	-	-
10	0.241	0.364	0.139	0.386	0.844
15	0.266	0.404	0.149	-	-
20	0.275	0.484	0.208	0.570	0.877
25	0.305	0.475	0.223	-	-
30	0.331	0.486	0.306	0.623	0.875
35	0.368	0.494	0.300	-	-
40	0.404	0.496	0.301	0.627	0.925
45	0.444	-	0.300	-	-
50	0.449	0.499	0.300	0.627	0.920
55	0.461	-	-	-	-
60	0.471	0.499	-	0.630	0.922
65	-	-	-	-	-
70	-	-	-	-	-
75	-	-	-	-	-
80	-	-	-	-	-

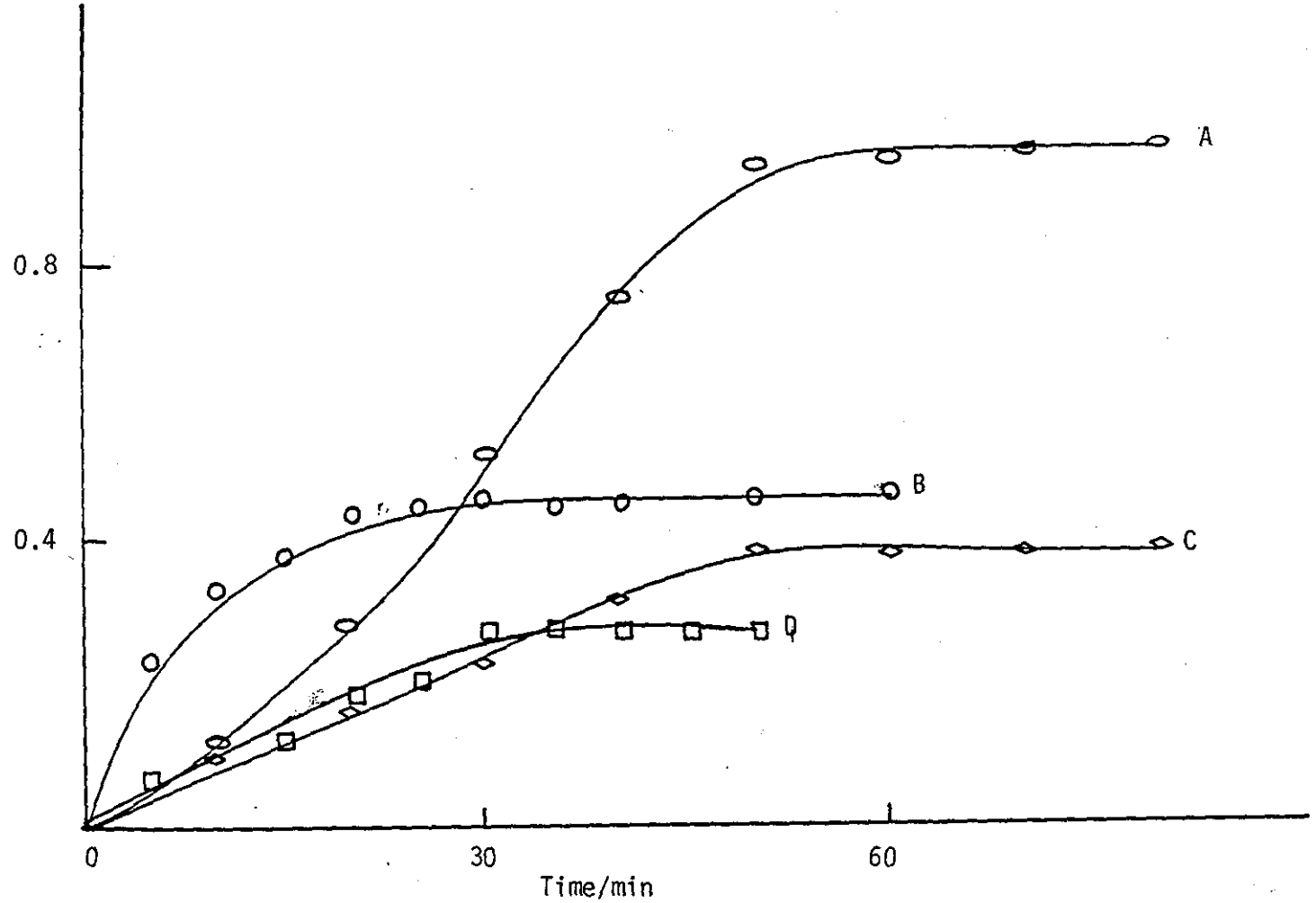


FIGURE 1: Effect of time of heating in 0.5M sodium hydroxide solution on formation of sulphide as indicated by the methylene blue reaction.
A: Cefuroxime (33 ppm); B: 7-ACA (9.2 ppm); C: Cephalonium (15.6 ppm)

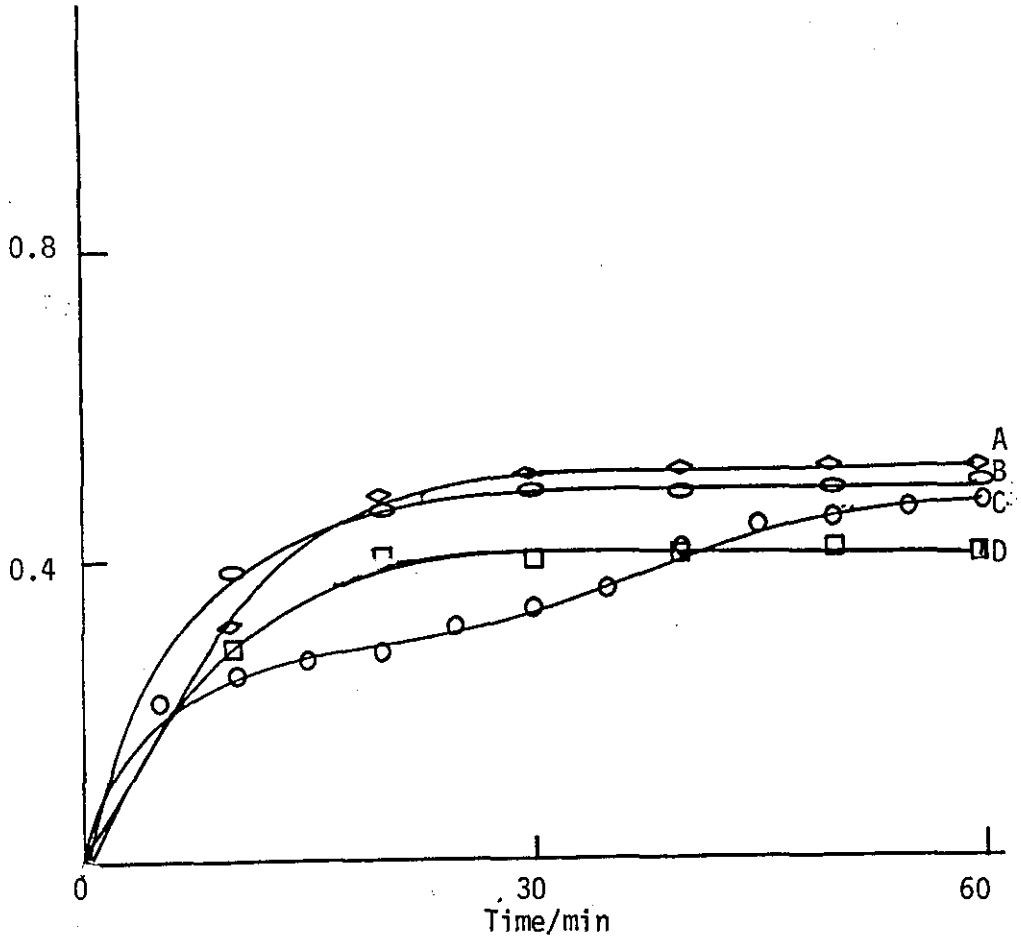


FIGURE 2: Effect of time of heating in 0.5M sodium hydroxide solution on formation of sulphide as indicated by the methylene blue reaction.
A: Cephaloglycin (33 ppm); B: Cephradine (8.8 ppm); C: Cephalothin (28.6 ppm);
D: Cephalexin (9.1 ppm)

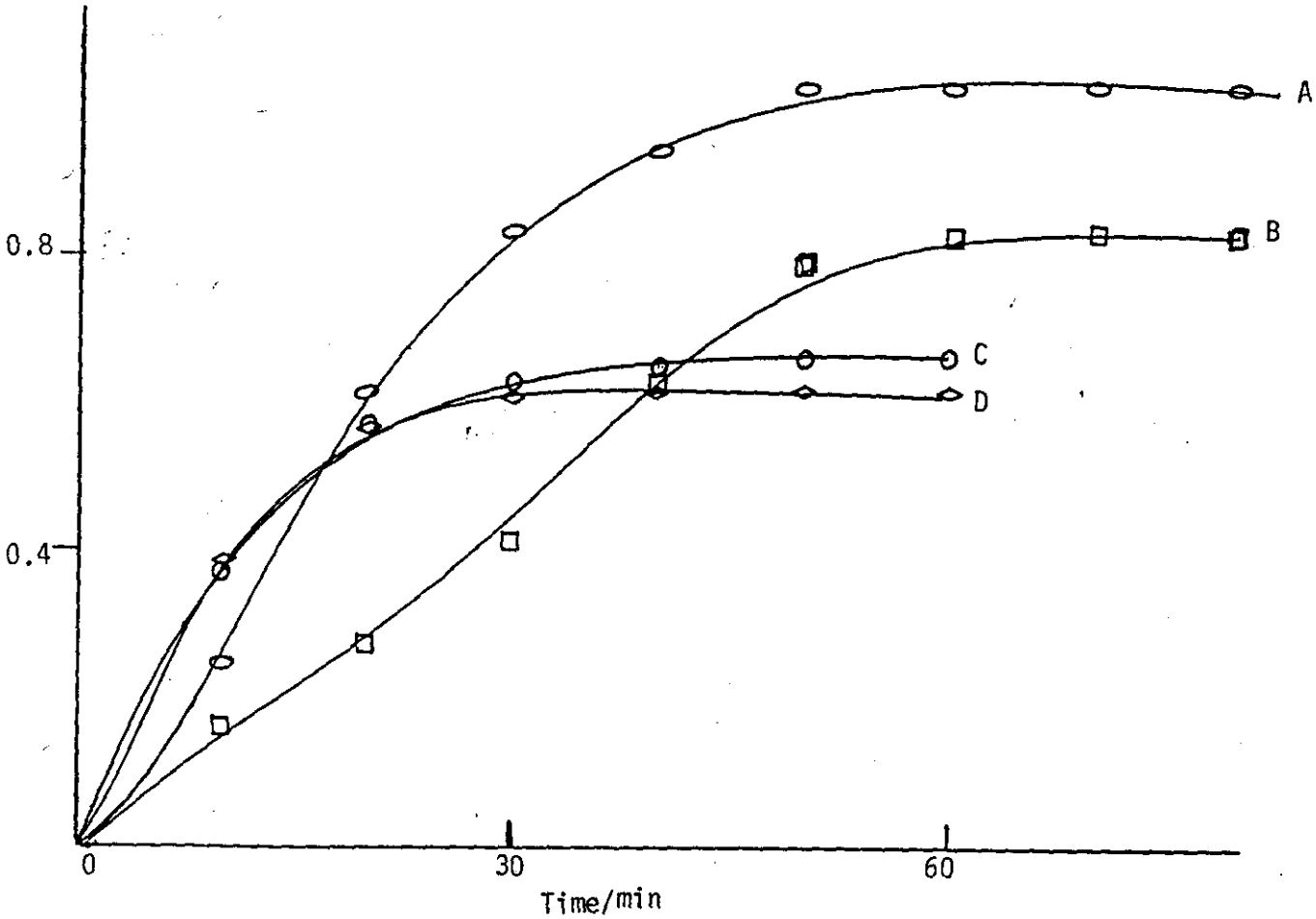


FIGURE 3: Effect of time of heating in 0.5M sodium hydroxide solution on formation of sulphide as indicated by the methylene blue reaction.
A: Cephaloridine (74 ppm); B: Cephoxazole (69 ppm); C: Cefazolin (29 ppm);
D: Cefaclor (8 ppm).

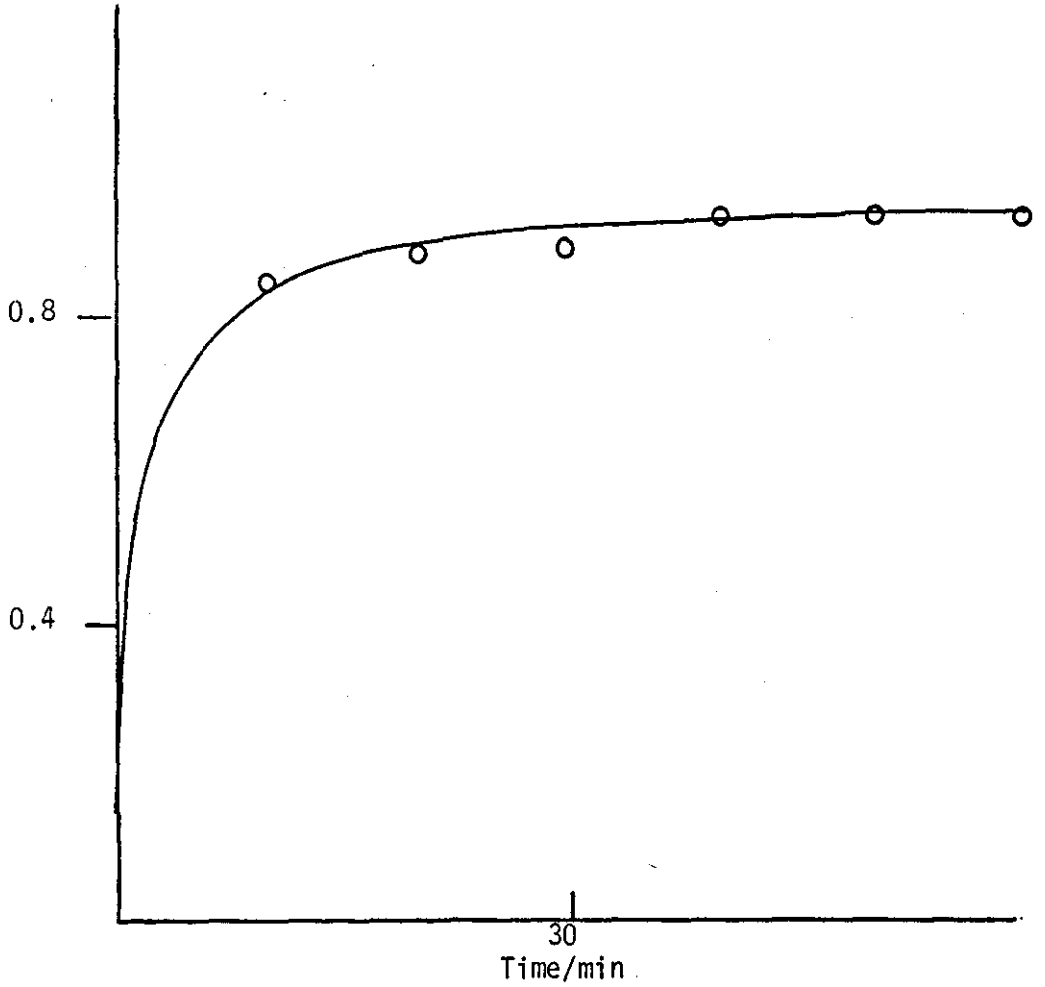


FIGURE 4: Effect of time of heating in 0.5M sodium hydroxide solution on the formation of sulphide as indicated by methylene blue reaction from diketopiperazine derivative

The molar absorptivity of methylene blue at 667 nm under the solution condition of the procedure was found to be $7.10 \times 10^{4}/mol^{-1}\ cm^{-1}$ based on the sample dried at 105°C being 100% $C_{16}H_{18}ClN_3S$.

In order to determine the molar absorptivity of methylene blue derived from standard sulphide solution, sodium sulphide solution of about 0.01M was titrated against 10^{-2}M lead nitrate standard solution as shown in Table 4.

TABLE 4:

Standardization of about 0.01M sodium sulphide (10 ml) with 0.01M lead nitrate solution using a sulphide ion-selective electrode

Volume of 0.01M lead nitrate added (ml)	Potential (mV)	Volume of 0.01M lead nitrate added (ml)	Potential (mV)
0.6	-770.8	3.6	-759.3
0.8	-770.2	3.8	-758.0
1.0	-769.6	4.0	-756.7
1.5	-768.1	4.5	-752.3
2.0	-766.5	5.0	-746.0
2.5	-764.6	5.5	-732.5
3.0	-762.3	5.6	-321.2
3.2	-761.3	5.7	-025.1
3.4	-760.5		

From the plot of the dE/dV versus the volume of lead nitrate, the end point was obtained at 5.70 ml of lead nitrate. The molarity of sodium sulphide solution is 5.70×10^{-3}M.

By using a solution of sodium sulphide standardized by lead nitrate the yield of methylene blue in the reaction was found to be 52.5% and the apparent molar absorptivity of methylene blue derived from the standard

sulphide was found to be 3.71×10^4 l mol^{-1} cm^{-1}.

The apparent molar absorptivities at 667 nm based on the concentration of reacted cephalosporins in the measured solutions and the final yield of hydrogen sulphide from each cephalosporin, are given in Table 5. Different cephalosporins were shown to give different but reproducible yields of sulphide. The yield varied from 14.0% for cefuroxime to 64.4% for cephalexin. These yields were calculated assuming that the yield of methylene blue in the reaction of sulphide with NN-dimethyl-p-phenylenediamine and iron(III) as oxidizing agent, which had been determined as 52.2% using standard sulphide solutions, was unchanged in the presence of the cephalosporin degradation products and that the degradation products did not react with the cyclisation reagents.

Potentiometric titration of sulphide formed by degradation of various cephalosporins in 1M sodium hydroxide solution containing 20gl^{-1} of scorbic acid, against a standard lead nitrate using a sulphide ion-selective electrode was used. The end point was calculated from the plot of dE/dV versus volume of lead nitrate. The results are shown in Tables 6-17. The yields of sulphide determined by potentiometric titration for all of the cephalosporins are given in Table 18. This has confirmed the yields reported previously, which were determined spectrophotometrically. Clearly close agreement was obtained.

Lead nitrate is being used increasingly in place of silver nitrate for the titration of sulphide[183] mainly because there is less adsorption of sulphide ion on lead sulphide than on silver sulphide and this gives more accurate results. The addition of glycerol has been advocated recently as increasing the stability of the potential readings[184], and this was confirmed in our study. The degraded cephalosporin solution

TABLE 5:

Apparent molar absorptivities of cephalosporins and molar yields of hydrogen sulphide

	Apparent Molar Absorptivity x 10^4/$lmol^{-1}cm^{-1}$	Molar Yield of Hydrogen Sulphide	Recommended hydrolysis time/min	Coefficient of Variation %
Sodium sulphide	3.71	100.0	-	1.1
Cephalexin	2.39	64.4	30	2.0
Cephradine	2.36	63.7	30	0.8
Cephalo-glycin	0.70	18.9	40	0.9
Cephalo-thin	0.74	19.8	45	1.4
Cefuroxime	0.52	14.0	50	0.2
Cephalonium	0.59	16.0	60	0.4
Cephaloridine	0.68	18.9	60	0.2
Cephoxazole	0.55	15.7	60	1.5
Cefaclor	1.60	43.1	40	1.7
Cefazolin	1.00	26.9	40	1.0
Diketopiper-azine deri-vative	0.69	18.7	40	1.1
7-ACA	1.76	47.4	40	0.4
7-ADCA	2.15	57.9	35	0.8
Pyrazine compound	-	-	-	-

TABLE 6:

Potentiometric titration of sulphide formed from cephalexin (5.15×10^{-3}M) 25 ml against 10^{-2}M lead nitrate solution

Volume of 0.01M lead nitrate added (ml)	Potential (mV)	Volume of 0.01M lead nitrate added	Potential (mV)
0.00	-771.0	5.0	-751.2
0.5	-770.0	5.5	-748.4
1.0	-769.1	6.0	-746.0
1.5	-767.2	6.5	-741.3
2.0	-765.7	7.0	-737.0
2.5	-763.6	7.5	-730.0
3.0	-761.9	8.0	-712.0
3.5	-759.9	8.5	-710.1
4.0	-758.7	9.0	-501.2
4.5	-756.5	9.2	-212.1

End point = 8.50 ml
Calculated molarity of sulphide = 3.4×10^{-3}M

TABLE 7:

Potentiometric titration of sulphide formed from cephaloglycin (6.64×10^{-3}M) 50 ml against 10^{-2}M lead nitrate solution

Volume of 0.01M lead nitrate added (ml)	Potential (mV)	Volume of 0.01M lead nitrate added (ml)	Potential (mV)
0.00	-791.0	4.50	-767.2
0.50	-787.0	5.00	-762.0
1.00	-785.5	5.50	-757.1
1.50	-783.0	6.00	-749.2
2.00	-780.5	6.50	-734.1
2.50	-778.0	7.00	-630.0
3.00	-776.4	7.20	-412.1
3.50	-774.2	7.40	-201.2
4.00	-771.0		

End point = 7.00 ml
Calculated molarity of sulphide = 1.3×10^{-3}M

TABLE 8:

Potentiometric titration of sulphide formed from cephalothin (5.56×10^{-3}M) 50 ml against 10^{-2}M lead nitrate solution

Volume of 0.01M lead nitrate added (ml)	Potential (mV)	Volume of 0.01M lead nitrate added	Potential (mV)
0.00	-771.0	3.50	-748.0
0.50	-769.0	4.00	-743.2
1.00	-765.6	4.50	-734.5
1.50	-763.5	5.00	-724.0
2.00	-760.0	5.50	-670.0
2.50	-756.0	6.00	-400.0
3.00	-752.5	6.20	-212.0

End point = 5.60 ml
Calculated molarity of sulphide = 1.1×10^{-3}M

TABLE 9:

Potentiometric titration of sulphide formed from cephaloridine (6.6×10^{-3}M) 50 ml against 10^{-2}M lead nitrate solution

Volume of 0.01M lead nitrate added (ml)	Potential	Volume of 0.01M lead nitrate added (ml)	Potential (mV)
0.00	-760.1	3.50	-743.3
0.50	-758.5	4.00	-740.0
1.00	-756.8	4.50	-734.0
1.50	-755.0	5.00	-723.0
2.00	-752.6	5.50	-680.0
2.50	-750.0	6.00	-410.0
3.00	-747.0	6.20	-211.0

End point = 6.00 ml
Calculated molarity of sulphide = 1.1×10^{-3}M

TABLE 10:

Potentiometric titration of sulphide formed from cephalonium (3.4×10^{-3}M) 50 ml against 10^{-2}M lead nitrate solution

Volume of 0.01M lead nitrate added (ml)	Potential (mV)
0.00	-737.5
0.50	-737.0
1.00	-730.1
1.50	-723.5
2.00	-671.5
2.50	-400.0
2.70	-200.1

End point = 2.50 ml
Calculated molarity of sulphide = 5.0×10^{-4}M

TABLE 11:

Potentiometric titration of sulphide formed from cephoxazole (1.01×10^{-2}M) 50 ml against 10^{-2}M lead nitrate solution

Volume of 0.01M lead nitrate added (ml)	Potential (mV)	Volume of 0.01M lead nitrate added (ml)	Potential (mV)
0.00	-762.0	5.00	-742.3
0.50	-761.2	5.50	-739.0
1.00	-759.8	6.00	-735.5
1.50	-758.0	6.50	-730.0
2.00	-756.4	7.00	-722.5
2.50	-745.5	7.50	-709.0
3.00	-752.5	8.00	-570.0
3.50	-750.0	8.20	-302.0
4.00	-748.3	8.50	-201.1
4.50	-746.5		

End point = 7.50 ml
Calculated molarity = 2.51×10^{-3}M

TABLE 12:

Potentiometric titration of sulphide formed from t-ACA (4.57×10^{-3}M, 50ml) against 10^{-2}M lead nitrate solution

Volume of 0.01M lead nitrate added (ml)	Potential (mV)	Volume of 0.01M lead nitrate added (ml)	Potential (mV)
0.00	-760.2	4.00	-740.2
0.50	-758.0	4.50	-736.0
1.00	-755.7	5.00	-730.0
1.50	-754.0	5.50	-718.5
2.00	-751.5	6.00	-690.0
2.50	-749.6	6.50	-500.0
3.00	-746.9	6.70	-300.0
3.50	-743.9	7.00	-101.2

End point = 6.2 ml
Calculated molarity of sulphide = 1.2×10^{-3}M

TABLE 13:

Potentiometric titration of sulphide formed from 7-ADCA (8.02×10^{-3}M, 25ml) against 10^{-2}M lead nitrate solution

Volume of 0.01M lead nitrate added (ml)	Potential (mV)	Volume of 0.01M lead nitrate added (ml)	Potential (mV)
0.00	-771.8	6.50	-754.9
0.50	-770.3	7.00	-753.2
1.00	-769.2	7.50	-751.2
1.50	-768.2	8.00	-749.5
2.00	-766.6	8.50	-747.8
2.50	-765.0	9.00	-745.0
3.00	-763.7	9.50	-742.9
3.50	-762.5	10.00	-739.3
4.00	-761.5	10.50	-735.5
4.50	-760.0	11.00	-729.5
5.00	-759.0	11.50	-721.5
5.50	-757.0	12.00	-709.0
5.00	-756.2	12.30	-400.0

End point = 12.1 ml
Calculated molarity of sulphide = 4.8×10^{-3}M

TABLE 14:

Potentiometric titration of sulphide formed from cefuroxime (4.22×10^{-3}M, 50 ml) against 10^{-2}M lead nitrate solution

Volume of 0.01M lead nitrate added (ml)	Potential (mV)
0.00	-746.0
0.50	-743.0
1.00	-740.1
1.50	-736.2
2.00	-730.5
2.50	-723.5
3.00	-700.0
3.50	-400.0

End point = 3.00 ml
Calculated molarity of sulphide = 0.60×10^{-3}M

TABLE 15:

Potentiometric titration of sulphide formed from cefaclor (6.92×10^{-3}M, 25 ml) against 10^{-2}M lead nitrate solution

Volume of 0.01M lead nitrate added (ml)	Potential (mV)	Volume of 0.01M lead nitrate added (ml)	Potential (mV)
0.00	-774.5	4.50	-750.5
0.50	-773.9	5.00	-754.0
1.00	-773.3	5.50	-748.3
1.50	-772.2	6.00	-740.0
2.00	-771.0	6.50	-727.5
2.50	-769.8	7.00	-670.0
3.00	-766.3	7.50	-460.0
3.50	-763.9	7.70	-300.0
4.00	-760.0	8.00	-120.0

End points = 7.50 ml
Calculated molarity of sulphide = 3.0×10^{-3}M

TABLE 16:

Potentiometric titration of sulphide formed from cefazolin (5.57×10^{-3}M, 25 ml) against 10^{-2}M lead nitrate solution

Volume of 0.01M lead nitrate added (ml)	Potential (mV)
0.00	-760.0
0.50	-756.2
1.00	-752.8
1.50	-749.0
2.00	-745.0
2.50	-740.0
3.00	-732.0
3.50	-715.0
3.70	-507.0

End point = 3.50 ml
Calculated molarity of sulphide = 1.4×10^{-3}M

TABLE 17:

Potentiometric titration of sulphide formed from cephradine (2.88×10^{-3}M, 25 ml) against 10^{-2}M lead nitrate solution

Volume of 0.01M lead nitrate added (ml)	Potential (mV)
0.00	-754.5
0.50	-748.0
1.00	-746.0
1.50	-744.0
2.00	-737.0
2.50	-732.0
3.00	-727.0
3.50	-718.0
4.00	-710.0
4.50	-694.0
4.70	-321.0
5.00	-200.0

End point = 4.50 ml
Calculated molarity of sulphide = 1.8×10^{-3}M

gave similar titration curves to those of standard sulphide solutions of the same sulphide concentration. A slight instability of the potential readings was observed near the end-point but a sharp end-point jump, usually in excess of 200 mV, was observed.

The opportunity was also taken to check that the loss of sulphide by atmospheric oxidation during the degradation and the colorimetric or titrimetric procedures is negligible. The addition, before the degradation, of ascorbic acid as antioxidant causes the degraded solutions of most cephalosporins to become more strongly coloured, but the yield of sulphide is unchanged. Solutions of the α-aminobenzylcephalosporins, cephalexin, cefaclor and cephaloglycin showed a strongly yellow colour that was unchanged in the presence of ascorbic acid; the same occurred for solutions of 7-ACA and 7-ADCA, although these solutions were paler in colour. The solutions of the other cephalosporins were colourless in the absence of ascorbic acid but were coloured when it was present. Cefuroxime and cephalonium solutions containing ascorbic acid were yellow, whereas those of cephoxazole and cefazolin were brown. Those of cephaloridine took on a slight green colour initially but this became red-brown. Those of cephalothin went through green and red to give a strong red-brown colour when cool.

Calibration graphs obtained to determine individual cephalosporins were found to be rectilinear and coefficients of variation were typically less than 2% see Table 19. Typical calibration graphs obtained from cephalexin samples are shown in Figures 5 and 6.

Pencillin G and ampicillin were shown not to give the reaction and not to interfere in the determination of cephalosporins when

TABLE 18:

Molar yield of hydrogen sulphite formed in 0.5 and 1.0M sodium hydroxide solution as determined spectrophotometrically and by titration with lead nitrate solution

	Molar Yield, %	
	Spectrophotometrically	Titrimetrically
Sodium Sulphide	100.0	100.0
Cephalexin	64.4	65.8
Cephradine	63.7	61.9
Cephaloglycin	18.9	19.5
Cephalothin	19.8	19.7
Cefuroxime	14.0	14.2
Cephalonium	16.0	17.4
7-ACA	47.4	48.1
7-ADCA	57.9	57.3
Cephaloridine	18.9	18.1
Cephoxazole	15.7	14.8
Cefaclor	43.1	43.5
Cefazolin	26.9	25.1

TABLE 19:

Calibration graphs for determination of cephalosporins in standard solutions

Cephalosporin	Concentration Range µg/ml	Coefficient of Variation %
Sodium sulphide	10 - 40	1.4
Cephalexin	10 - 40	2.0
Cephradine	10 - 40	0.8
7-ACA	10 - 40	0.7
7-ADCA	10 - 40	0.6
Cephaloglycin	20 - 80	0.8
Cefuoxime	20 - 80	0.6
Cephoxazole	20 - 80	0.7
Cephaloridine	20 - 80	0.6
Cephalothin	20 - 80	0.8
Cephalonium	20 - 80	0.9
Cefaclor	10 - 40	0.9
Cefazolin	20 - 80	1.1

TABLE 20:

Effect of penicillins on cephalexin calibration graphs

Cephalexin Standards x 10^{-5}M	Absorbance of Cephalexin Standard	Absorbance of Cephalexin Standard 5×10^{-5}M penicillin V	Absorbance of Cephalexin Standard + Penicillin G 5×10^{-5}M
0.00	0.007	0.007	0.007
1.45	0.348	0.345	0.345
2.90	0.700	0.702	0.704
4.35	1.033	1.040	1.039
5.80	1.390	1.387	1.379

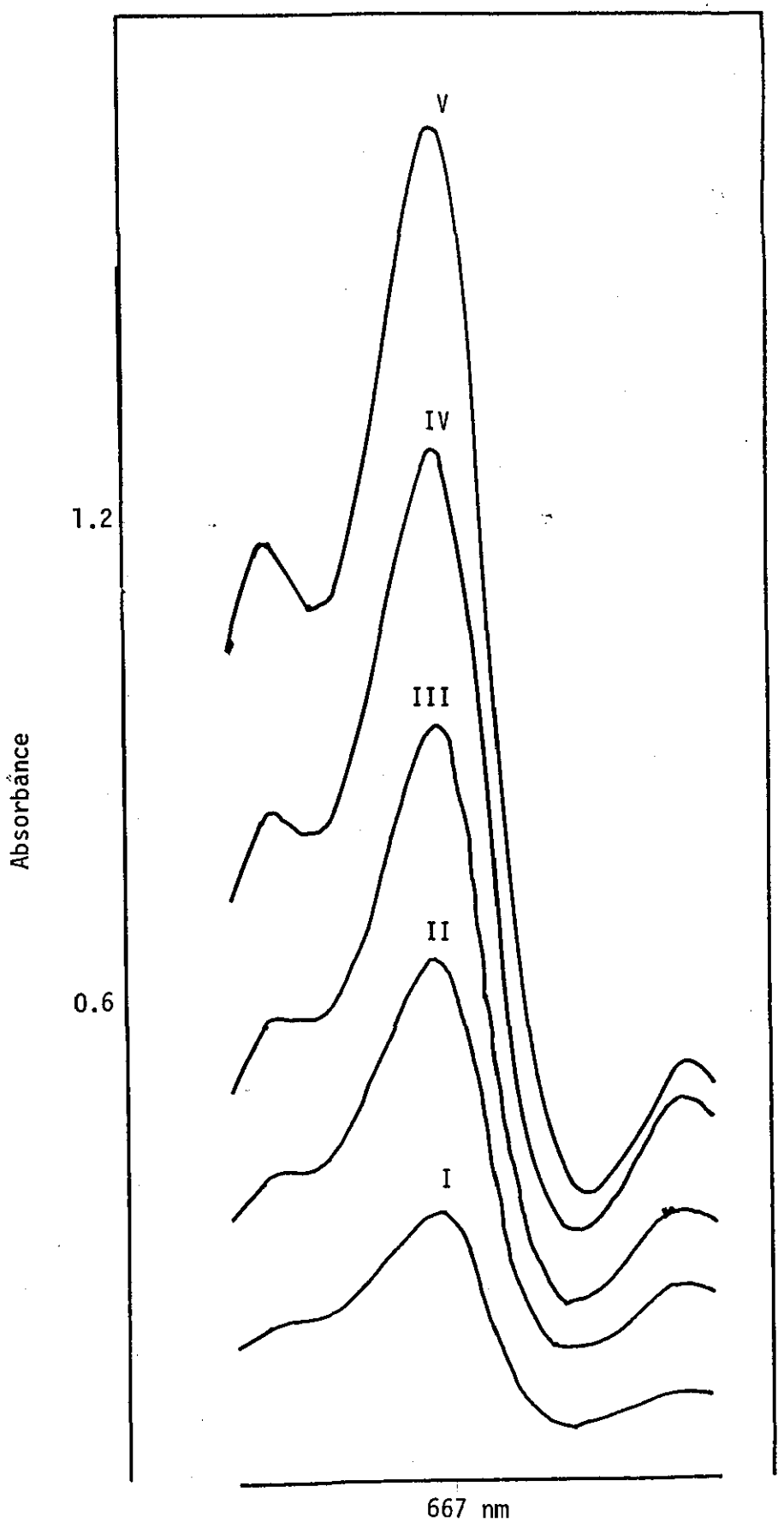


FIGURE 5: Calibration graph for cephalexin by the methylene blue method at 667 nm for the concentration range (I) 10 µg, (II) 20 µg, (III) 30 µg, (IV) 40 µg, (V) 50 µg/ml

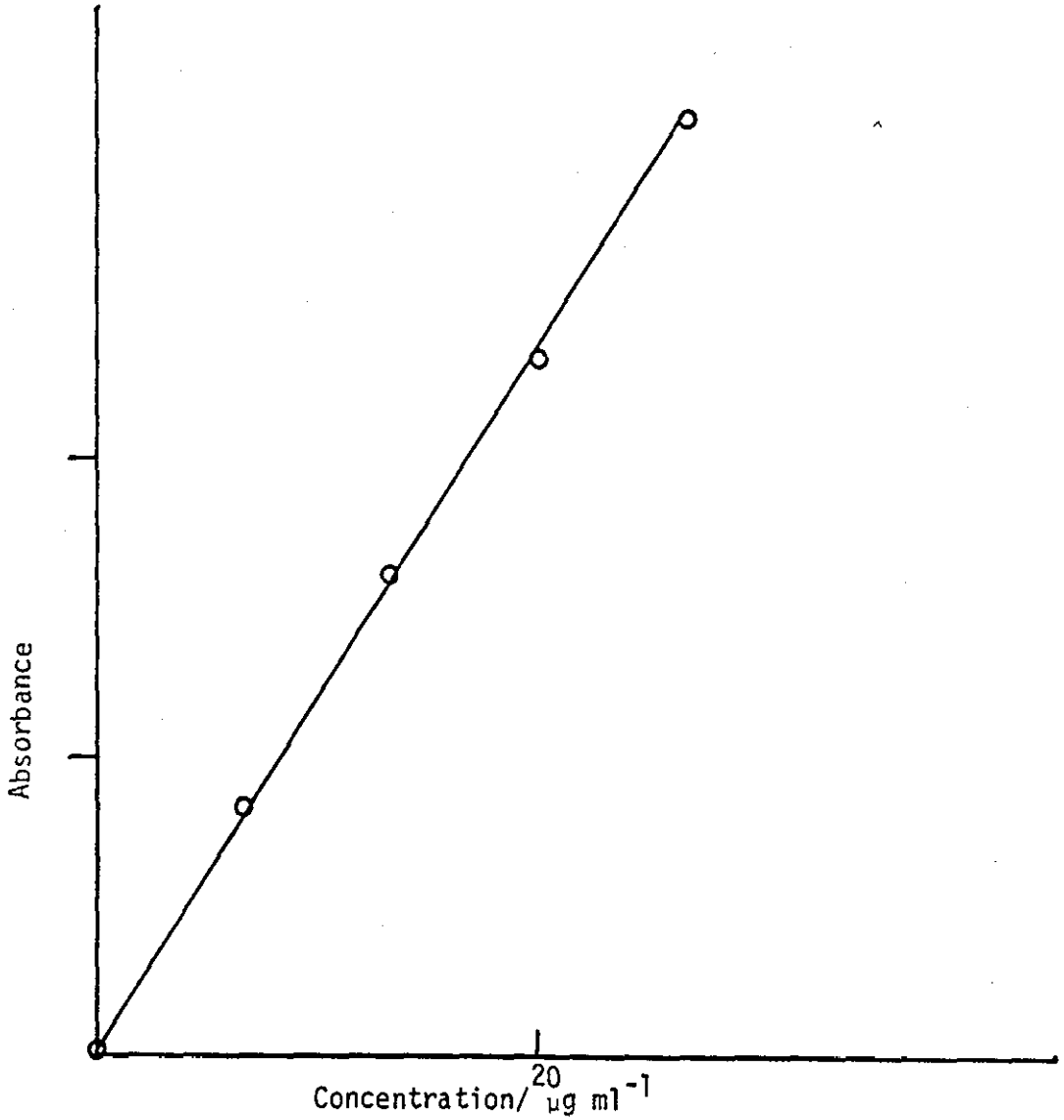


FIGURE 6: Calibration graphs obtained for cephalexin

present in similar amounts, see Table 20.

Discussion

The recommended procedure is very simple and compares favourably with the ultraviolet spectrophotometric method based on the reaction of cephalosporins and penicillins with imidazole and mercury(II) which is favoured currently in many laboratories[172]. This method reported in this chapter has the advantage of using the visible region and of being selective for cephalosporins in the presence of penicillins. However, it has the disadvantage that it is unlikely to be stability indicating as some degradation products could also give hydrogen sulphide for example the diketopiperazine derivative.

The values determined spectrophotometrically and titrimetrically for the yields of sulphide obtained when cephalosporins are degraded in sodium hydroxide solution are similar. The degradation products of the cephalosporins would not be expected to interfere in the titrimetric method, thiols if present would give a separate potential jump at a less negative potential. Thus, it is probable that other degradation products neither react colorimetrically with the cyclisation reagents nor interfere with the formation of methylene blue from sulphide.

Clearly the titrimetric method can be used to determine cephalosporins but the spectrophotometric method is probably simpler. The spectrophotometric method has been automated using an air-segmented flow system, and this procedure is given in the following chapter.

The yield of hydrogen sulphide from cephalexin in the recommended procedure is similar to that obtained after about 25h at 80°C in pH 8.5 phosphate buffer[54]. After 25h at 80°C in pH 7.4 phosphate buffer,

hydrogen sulphide evolution slows to a very low rate and the yield is only about 32%[50]. The different yields are probably associated with different degradation routes under different conditions of acidity or alkalinity.

The yields of sulphide as determined spectrophotometrically are the same when 0.5 or 1M sodium hydroxide solution is used. The use of the lower sodium hydroxide concentration is more convenient in the spectrophotometric method as the degraded solution has to be acidified, whereas sulphide is usually measured potentiometrically in 1M sodium hydroxide solution. Better potentiometric end-points were obtained at the higher concentrations of the cephalosporins, but similar yields of sulphide were obtained at lower levels.

CHAPTER 4

CONTINUOUS-FLOW ANALYSIS

Introduction

Continuous flow analysis was introduced by Skeggs in 1957[185]. This technique, more than any other single advancement, has been responsible for the great impact of automation on the modern analytical laboratory.

Continuous flow analysis is carried out with a series of connected components: a sampler, a multichannel peristaltic pump, one or more mixing/reaction coils, a dialyser (sometimes) and a recording colorimeter or other detector. A typical continuous flow analysis system is shown in Figure 1. Samples and intersample wash fluid are intermittently aspirated at the sampler; the resulting stream is segmented with air bubbles and is combined with a diluent A from the pump. The stream is passed through a mixing coil and then through the dialyser. Dialyzable analyte diffuses through the dialyzer membrane into a second segmented stream B, is combined with a reagent C, passes through a thermostatted reaction coil where a chromogenic reaction takes place, and is finally fed to a colorimeter. In this way it is possible for a series of separate steps in an analytical procedure to be carried out in a controlled reproducible and fully automatic fashion.

Fundamental Aspects of Continuous-Flow Analysis

The quality of the shape of the signal produced by the flow-through detector in continuous flow analysis is influenced by the following

variables: (1) sample dispersion, (2) mixing, and (3) flow stability.

1. Sample dispersion

From the plot of the analyte concentration versus time (see Figure 2), the left hand curve is a rectangular input function, corresponding to the successive aspiration of samples and analyte-free wash fluid. The height of these sample curves reflect the analyte concentration in each sample. At the end of continuous flow analysis, some rounding of the initial input curves, owing to axial dispersion of analyte molecules along the flowing stream is seen. The extent of this dispersion depends upon the experimental conditions used in the particular continuous flow analysis and is markedly reduced by the segmenting air bubbles which serve as a physical barrier to dispersion. If the dispersion is not excessive, the flat region for each sample curve will reflect the analyte concentration of that sample. On the other hand the effect of excessive dispersion will result in sample carry over or interaction, so that one sample overlaps the flat region of an adjacent sample, thus altering its apparent concentration. This effect can be countered by increasing intersample wash time at the expense of sampling time.

The main contribution to sample dispersion in well-designed continuous-flow systems arises from the flow of the sample slug through the continuous-flow network. The physical basis of this dispersion is shown in Figure 3, which shows a graphical representation of a flowing stream segmented by air-bubbles. If a small amount of sample is introduced into one of these liquid segments, and the liquid stream plus sample then flows through a length of tubing, the sample originally in

segment 0 will gradually be dispersed into following segments 1, 2 etc. The reason for this is that the liquid wets the tubing so that a thin film of liquid lies under each air-bubble. Liquid from segment 0 is deposited on the tube wall in the form of this film, and is picked up by advancing segment 1. In this way, sample is transferred from segment 0 to 1 to 2 etc.

More recently[186,187], this model has been expanded to allow the prediction of dispersion as a function of experimental conditions. The amount of dispersion, expressed as the variance σ_t^2 of the sample curve, can be related to tube internal diameter dt, liquid flow rate F_l, bubble segmentation rate n, liquid viscosity η and surface tension γ, time t in the flow network, and sample mass transfer coefficient D_{w25}. Dispersion is mainly a function of F_l, dt, n, η and t, with dispersion being less for smaller values of t, dt and η.

2. Mixing

Mixing of sample and diluent is required in all continuous-flow systems, and usually several such mixing operations take place as successive reagent additions are made. Complete mixing must occur in each liquid segment, and the incomplete mixing will result in a noisy detector output, which is in fact indicative of unmixed solutions. Mixing in continuous-flow takes advantage of the natural fluid motions that occur within short segments of liquid enclosed at each end by the gas-liquid interface. The pattern of fluid motion, so called bolus-flow, occurs naturally in any segmented-flow system (see Figure 3). An important advantage of mixing in continuous-flow is that no external

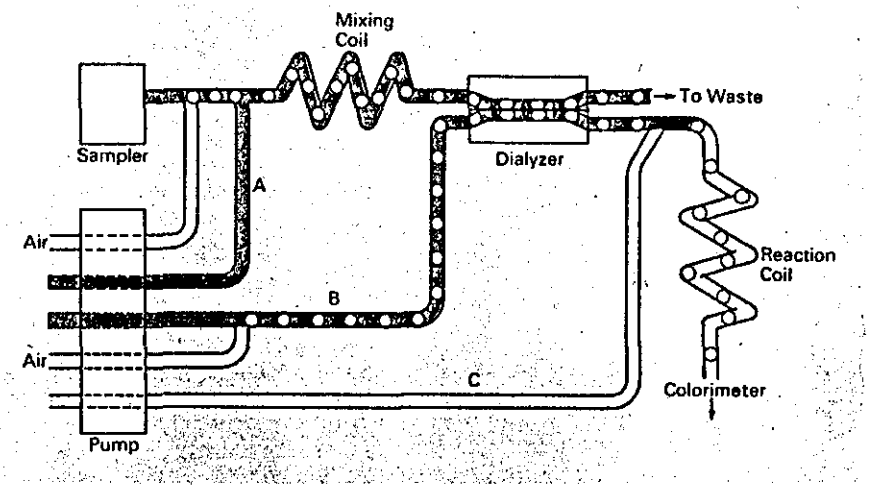


Figure 1. Example of continuous-flow analysis scheme
A, B: diluents; C: reagent stream

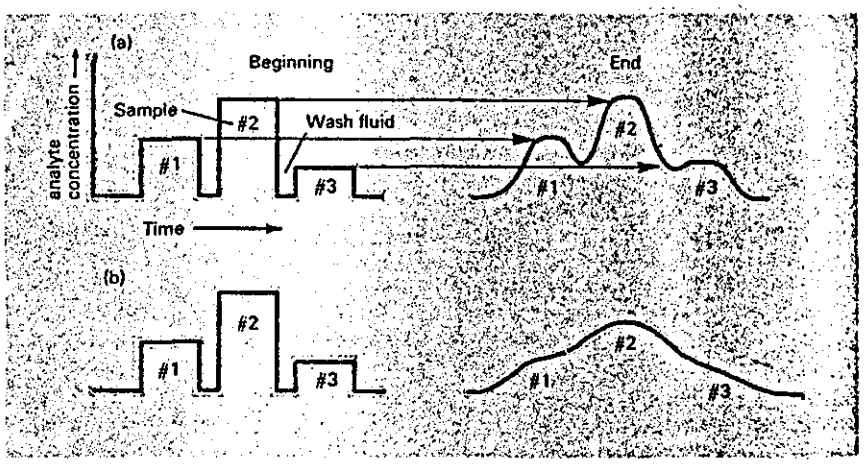
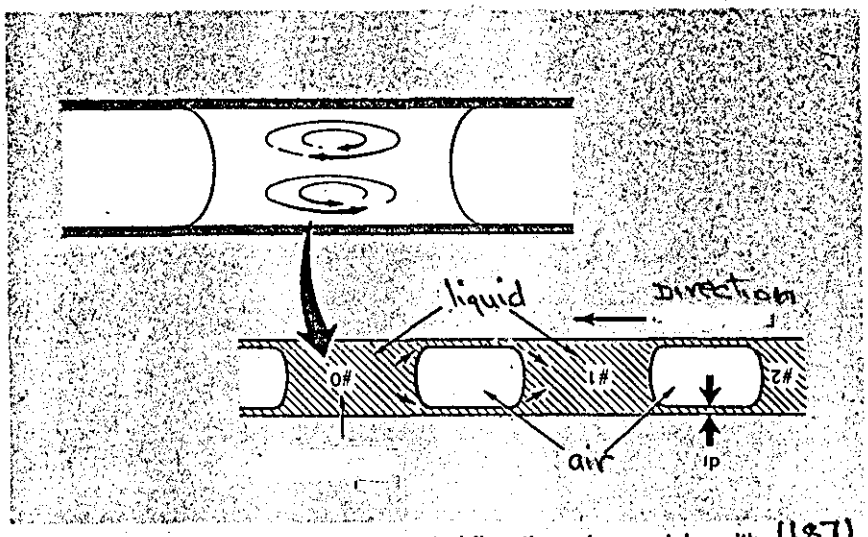


Figure 2. Effect of dispersion during continuous-flow analysis on sample concentration curve shape
(a) Acceptable dispersion; analyte and analyte-chromogen concentrations plotted on molar basis; (b) unacceptable dispersion



(187)

Figure 3. (a) Sample dispersion during segmented flow through open tube with marker-dye sample injected into segment #0. Film of liquid phase (d_f = film thickness) from segment #0 will be overtaken and mixed into segment #1, allowing dye to move from #0 to #1; dye built up within segment #1 can also be transferred to #2. Dye will become dispersed over many following segments. (b) Bolus circulation within moving liquid segment occurs naturally in any segmented-flow system

means are necessary. Only sufficient length of tubing is required.

In a straight length of tubing, however, radial mass transfer occurs mainly by molecular diffusion, which is slow in liquids. This can be overcome by creating convective radial mixing simply by substituting a helical coil for the straight tube. Helical coils also permit a more compact physical arrangement of the final manifold.

Studies of mixing efficiency show that both liquid physical properties and tube geometry are important in determining the total time required for complete mixing. Liquid viscosity, density, and flow rate all affect mixing, with viscosity being most significant. Tube internal diameter, helix coil diameter, and segment length are other major parameters.

3. Flow stability

A stable liquid-air stream in a continuous-flow manifold is one in which the proportions of sample to reagents are constant for all segments, from one sampling cycle to the next. If proportioning varies during this period, analysis precision is adversely affected. Several events can occur to create an incorrectly proportioned stream: varying liquid or air flow rates, intersample air compression, a short sample, sample probe or manifold-tube blockage. Flow stability can be achieved by using pulse-free pumps or simply by synchronising the pulsation of all sample and reagent streams.

Flow stability is also affected by many other variables, whose impact can be blunted in well-designed continuous-flow systems. These are sudden injection of air-bubbles, changes in temperature along the flow network, and by the use of liquids that do not wet the tubing walls.

Applications of Flow Analysis in Pharmaceutical Chemistry

The uses to which automatic analysers are now being put cover every field of analytical chemistry. The unique requirements of clinical laboratories, i.e. a large number of determinations for a small number of sample constituents with a relatively wide tolerance on accuracy and precision, together with the introduction of the Technicon Autoanalyser were initially responsible for the widespread application in this field. Subsequent improvement in instrumentation and development of a greater variety of methods resulted in automatic analyser being used also in other areas particularly in pharmaceutical quality control.

Two symposia[188,189] and a review[190] have dealt with the use of automated techniques in pharmaceutical analysis. Some of the special requirements of pharmaceutical quality control include the need to determine one or more active components in a single determination, the ability to handle solid samples such as tablets and the use of turbidimetric procedures for biological assays.

The construction and operation of an automatic dispensing analyser for the assay of individual tablets has been described by Beyer and Smith[191]. It incorporates a Technicon pump and continuous filter. The simultaneous determination of several components in a single tablet has been described by Urbanyi and O'Connell[192]. Reserpine, hydralazine hydrochloride and hydrochlorothiazide were assayed respectively by fluorometric, colorimetric and ultraviolet spectrophotometric measurements. Murfin[193] has reported Autoanalyser methods for the commercial analgesic tablet formulations. Urbanyi and Lin[194] used an infrared spectrophotometer coupled to an Autoanalyser with solid sampler to

determine methylphenidate hydrochloride in tablet after extraction.

Penicillin determination by a colorimetric Autoanalyser method has been described recently by Holm. A high degree of sensitivity, accuracy and precision is claimed. The assay of a variety of commercial penicillin preparations has been reported by Mills[195]. An automated apparatus for the turbidimetric determination of antibiotics has been constructed by Kuzel and Kavanagh[196,197]. The calculation of potencies may be performed by computer[198]. More recently a similar apparatus has been built by Rippere and Arret[199] from commercially available components. Several other procedures for automated microbiological analysis have been described[200,201].

Experimental

Air-segmented continuous-flow analysis was carried out with an AI Autoanalyser manifold using a two-speed utoanalyser peristaltic pump and a Newton Instruments' automatic sampler. Visible spectrophotometric measurements were made with a Pye Unicam SP600 spectrophotometer fitted with a Hellma flow-cell (80 µl capacity) and connected to a Pye Unicam AR55 recorder.

Reagents

N,N-Dimethyl-p-phenylenediamine sulphate solution, 0.005M

Dissolve 0.93g of N,N-dimethyl-p-phenylenediamine sulphate in 750 ml of distilled water. Add 186 ml of concentrated sulphuric acid, cool and dilute to 1 litre with water.

Ammonium iron(III) sulphate solution, 0.25M

Dissolve 60.8g of ammonium iron(III) sulphate dodecahydrate in 0.5M sulphuric acid and dilute to 500 ml with 0.5M sulphuric acid.

Sodium hydroxide solution, 1M, with detergent

Dissolve 40g of sodium hydroxide in water, add 100 ml of Triton X-100 and dilute to 1 litre.

Sodium hydroxide solution, 0.5M, with detergent

Prepare as above but using only 20g of sodium hydroxide.

Samples

A sample of cephradine was kindly provided by E R Squibb and Sons Ltd, and a sample of ampicillin by Beecham Pharmaceuticals, samples of cephaloglycin, cefaclor and cefazolin by Lilly Research Centre Ltd and samples of cephalexin, cephaloridine, cephalothin, cephalonium, cefuroxime, cephoxazole, 7-ACA, 7-ADCA, penicillin G and penicillin V by Glaxo Operations (UK) Ltd.

Automatic System

The schematic diagram of Figure 1 indicates the keys of the flow diagram, the automated equipment used for the analytical procedure, and the special tubing where necessary.

Experimental Techniques

1. Preparation of stock solutions

Cephalosporins were dissolved in water with the exception of 7-aminodeacetoxycephalosporanic acid (7-ADCA) and 7-aminocephalosporanic acid (7-ACA) which were dissolved in 2 ml of 0.5M sodium hydroxide solution and then diluted rapidly before extensive hydrolysis could occur. Stock standard solutions of cephaloglycin, cefuroxime, cephoxazole, cephalonium, cephaloridine, cephalothin, cefaclor and cefazolin were prepared at 0.02% w/v, those of 7-ADCA and 7-ACA at 0.01% and those of cephalexin and cephradine at 0.008%.

Stock solutions of 1g amount of penicillin V, penicillin G were dissolved in water and diluted to 5 ml.

2. Optimisation of the Autoanalyser system

When the Autoanalyser system was first set up, and prehydrolysed samples were pumped (without using the delay coil and the water bath) straight line response was observed.

Unhydrolysed samples of cephalexin containing 0, 10, 20, 30, 40 µg/ml were tried through the system to obtain a calibration graph at different bath temperatures, starting at 50°C and this was then raised by 10°C increments until it reached 90°C. The optimum temperature was then chosen.

3. Construction of a calibration graph for the determination of standard cephalosporins in solution

Transfer, by means of a pipette, aliquots (0-20 ml) of the stock

solutions into 50 ml calibrated flasks and dilute to volume with distilled water. These solutions were presented to the Autoanalyser system by the use of the autosampler.

4. Calibration graphs for the determination of cephalosporins in the presence of high concentrations of penicillin V, penicillin G and ampicillin

Into five clean 25 ml calibrated flasks add 5g amounts of the penicillin sample and dissolve them in small amounts of water. Add by means of pipette 0, 5, 10, 15, 20 ml of standard cephalosporin solution, and then dilute the solutions to volume with distilled water. These solutions were presented to the Autonanalyser system.

Triton X-100 (10% w/v) was incorporated in the 1M sodium hydroxide solution, and the 0.5M sodium hydroxide wash solution.

Results

An Autoanalyser I system for carrying out the colour reaction on prehydrolysed cephalexin solutions was shown to give good rectilinear calibration graphs. When the delay coil contained in a water bath was incorporated in the system in order to effect the hydrolysis step on-line the optimum temperature of the water bath was found to be 80^{o}C although unsatisfactory pulsing was observed in the system when temperatures above 70^{o}C were used. This problem was overcome by resampling after the hydrolysis step (at debubbler I) and by incorporating a small glass bulb (to act as a pulse suppressor) in the air line segmenting the sample solutions before the hydrolysis stage, and by debubbling and resampling the hydrolysed sample solution automatically

after passing through the water bath. The recommended system is shown in Figure 1.

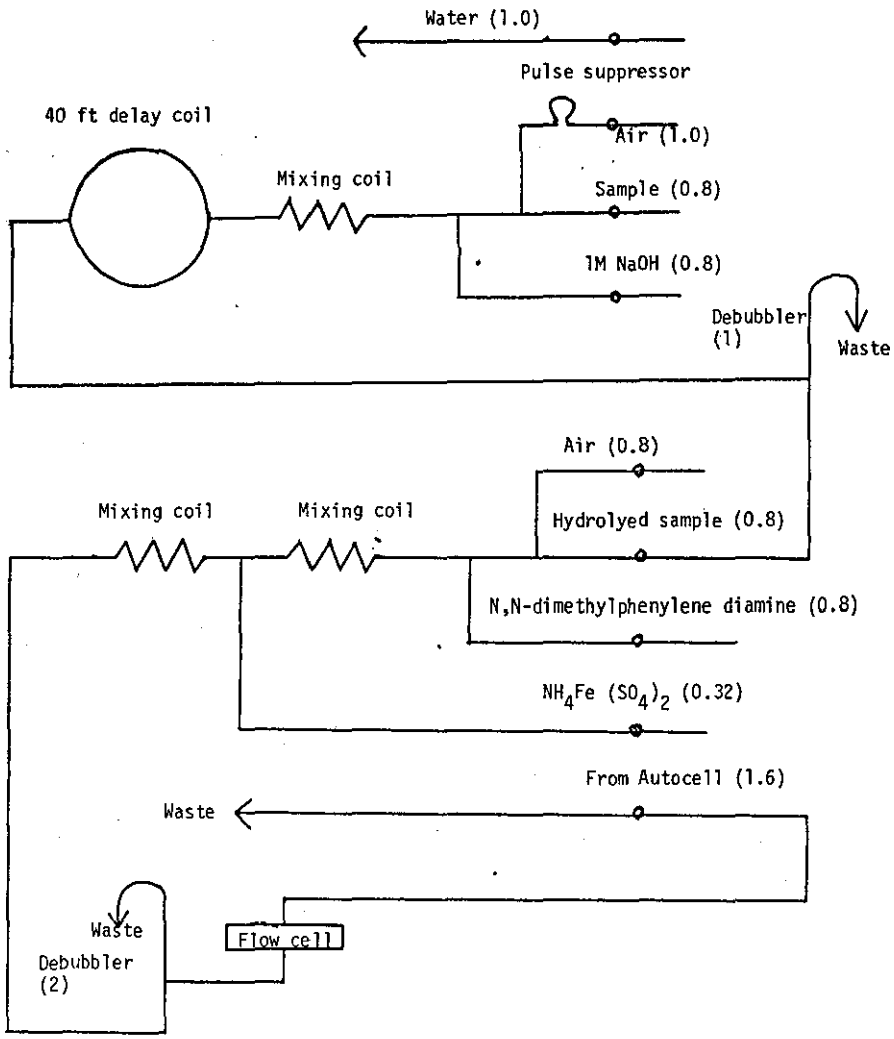
Data obtained for calibration graphs for cephalexin at various water bath temperatures is given in Table I and the calibration graphs for cephalexin at various water bath temperatures is shown in Figure 2.

TABLE 1:

Effect of temperature of hydrolysis water bath on calibration graph of cephalexin

Concentration of cephalexin in presented solution /µg ml	Absorbance at				
	50°C	60°C	70°C	80°C	90°C
10	0.075	0.147	0.155	0.199	0.199
20	0.147	0.259	0.304	0.366	0.366
30	0.208	0.387	0.420	0.509	0.509
40	0.299	0.497	0.588	0.699	0.700

The calibration graphs show good rectilinearity. Problems of reproduciblity were experienced at 95°C and it was necessary to replace water lost by evaporation from the water bath. This latter problem could have been overcome by using silicone oil in the bath, but for these reasons 80°C was adopted for routine use. Samples remain in the water bath for about 30 minutes. Previously several cephalosporins were shown to give their maximum yield of sulphide after 30 minutes in a boiling water bath, but others required up to 60 min hydrolysis at this temperature.



Autoanalyser System

FIGURE 1

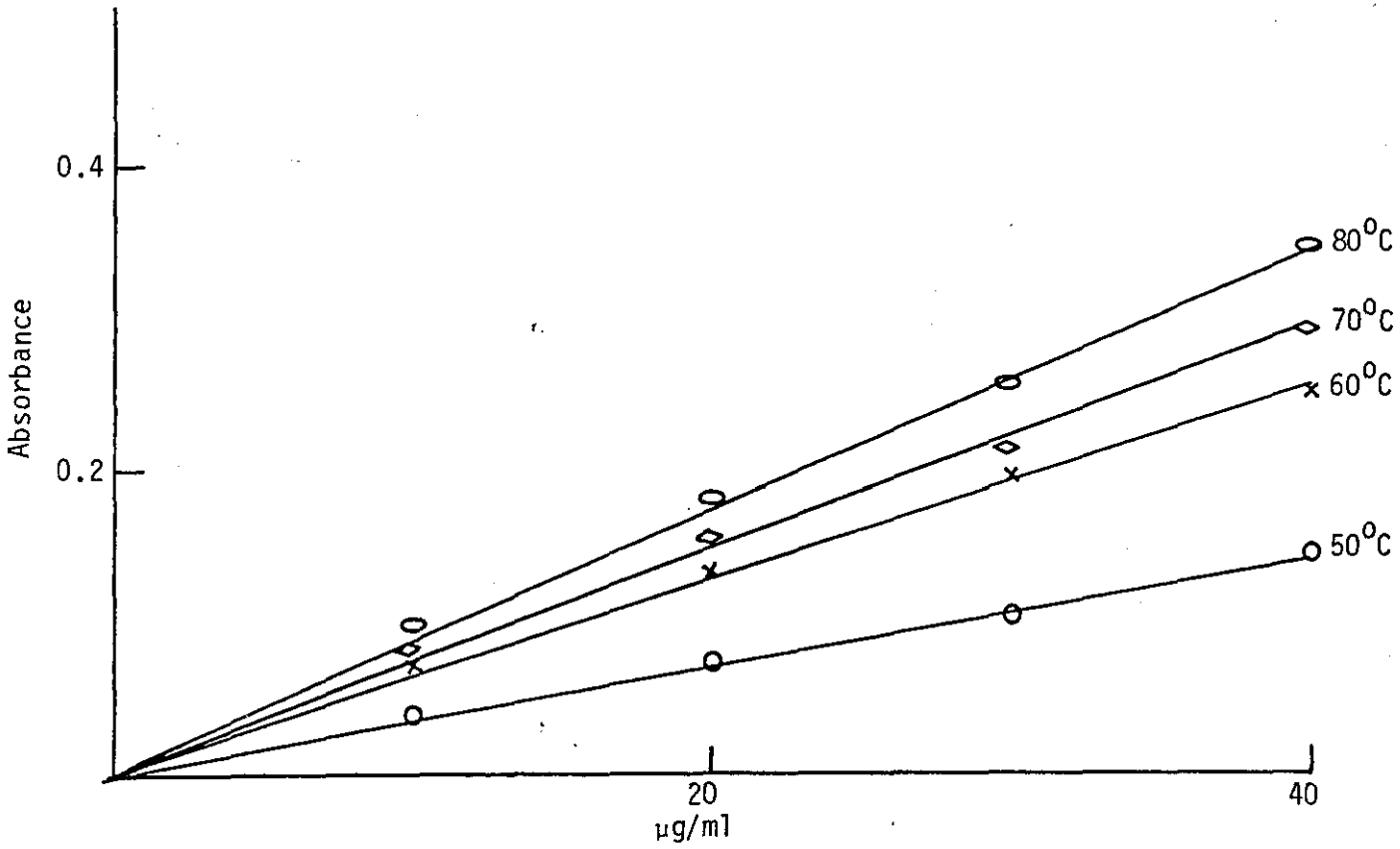


FIGURE 2: Calibration graphs obtained for cephalexin at various temperatures

Clearly maximum formation of sulphide is not achieved in the present Autoanalyser system. The minimum wash time was determined by the usual method of observing the time to reach a steady state on changing from the standard solution of lowest concentration to that of highest concentration and vice versa. From this it was clear that over sixty samples an hour could be run even without curve regeneration but all the present work was carried out at a rate of 30 samples an hour. The wash time was twice the sample time. Rectilinearity was achieved when water was used as the wash liquid but a steadier baseline was achieved by using a 0.5M sodium hydroxide solution as wash liquid without loss of rectilinearity and this was adopted. The system was cleaned after use by passing a 10% w/v aqueous solution of Triton X-100 through all the liquid channels.

The reproducibility of the recommended method was checked by running a calibration graph of a cephalexin sample starting from the lowest concentration to the highest concentration and then from the highest concentration to the lowest concentration. The results were also used to check the sample carry-over effect and the linearity of the method. The recorder trace for the reproducibility studies is shown in Figure 3. Clearly the reproducibility of the method is excellent, the base line is stable and the rectilinearity is very good. It also shows that the procedure is free from any sample carry over or interaction.

Precision and day-to-day reproducibility, however, were checked by running ten consecutive samples of the cephalosporin standards and recording its absorbances, and obtaining the absorbance of the same

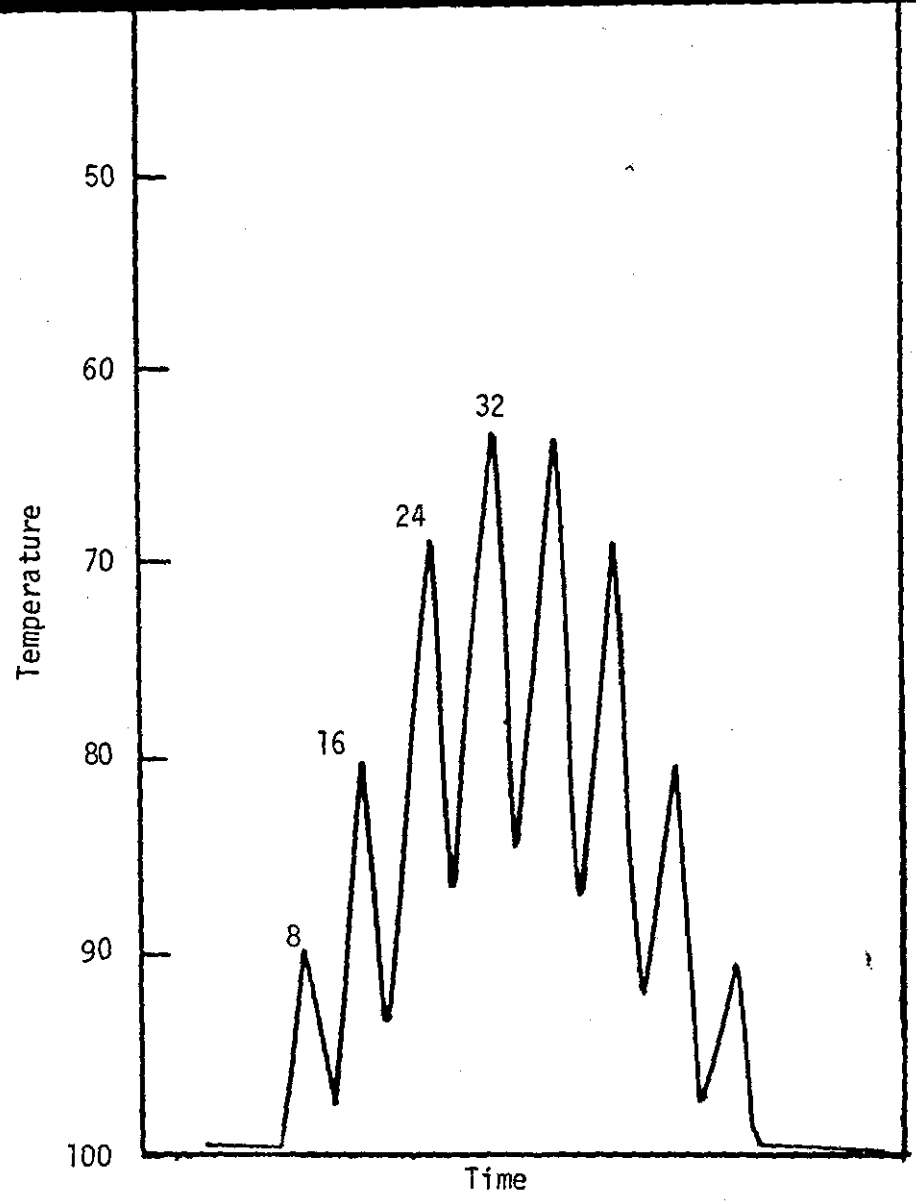


FIGURE 3: Calibration graph for cephalexin by continuous flow- monitoring of transmittance at fixed wavelength of 667 nm for the concentration range of 10-40 μg ml^{-1} at a rate of 30 samples/hr with 1:1 sample to wave ratio

standards 48 hours later. Data for the previous experiments are shown in Table 2. Clearly the reproducibility and day-to-day precisions of the method are good and the method is reliable for the routine determination of cephalosporins in solutions. The same experiment has been conducted with cephalosporins in the presence of penicillins and similar results were obtained.

TABLE 2:

Studies on the reproducibility of the method and day-to-day precision of the technique

Cephalosporins 16 μg ml^{-1}	Absorbance (mean of 10 readings)	Coefficient of variation %	Absorbance after 48 hr (mean of 10 readings)	Coefficient of variation %
Cephalexin	0.1145	0.50%	0.1146	0.56%
Cephradine	0.1073	0.60%	0.1080	0.67%
Cephaloglycin	0.0753	0.60%	0.0758	0.62%
Cefuoxime	0.0524	0.60%	0.0531	0.70%
Cephoxazole	0.0714	0.5%	0.0708	0.46%
Cephalori dine	0.0458	0.55%	0.0460	0.50%
Cephalothin	0.0431	0.62%	0.0433	0.60%
7-ACA	0.0851	0.52%	0.0853	0.50%
7-ADCA	0.1183	0.44%	0.1179	0.42%
Cephalonium	0.0457	0.66%	0.0461	0.70%
Cefaclor	0.1025	0.40%	0.1031	0.51%
Cefazolin	0.0804	0.51%	0.0809	0.50%

Data for calibration graphs for the determination of twelve cephalosporins is given in Table 3 together with results obtained on passing a standard sulphide solution through the system. The cali-

bration graphs show good rectilinearity with low coefficients of variation. Detection limits (twice the standard deviation above the blank) were in the range 0.3 - 0.9 $\mu g\ ml^{-1}$ of cephalosporin in the solutions presented to the analyser.

TABLE 3:

Calibration graphs for determination of cephalosporins in standard solutions

Cephalosporin	Concentration range presented in sample cups/$\mu g\ ml^{-1}$	Slope of calibration graph /$10^{-3} ml\ g^{-1}$	Coefficient of variation * %	Detection limit / $\mu g\ ml^{-1}$
Sodium sulphide	8-32	10.0	1.01	0.3
Cephalexin	8-32	7.0	0.41	0.3
Cephradine	8-32	6.8	0.73	0.3
7-AXA	10-40	2.1	0.62	0.5
7-ADCA	10-40	3.5	0.66	0.4
Cephaloglycin	20-80	3.6	0.66	0.5
Cefuroxime	20-80	2.0	0.72	0.8
Cephoxazole	20-80	3.8	0.71	0.8
Cephaloridine	20-80	1.1	0.63	0.9
Cephalothin	20-80	2.3	0.81	0.7
Cephalonium	20-80	0.89	0.42	0.3
Cefaclor	20-80	3.0	0.45	0.4
Cefazolin	20-80	7.0	0.51	0.6

* Based on calibration graphs of four points plus blank.

Results for the determination of three cephalosporins in penicillin G and penicillin V samples are given in Table 4, and the calibration graphs are shown in Figure 4. Several points emerged from this study. First, penicillin G and penicillin V at the high concentrations used (0.2 mg ml^{-1} in the solutions presented) give appreciable blanks (equivalent to about 0.25 µg ml^{-1}) of cephalosporin) owing to the slight colour of the degradation products. The size of this blank was readily determined by replacing the N,N-dimethyl-p-phenylenediamine sulphate solution with sulphuric acid solution of the same acidity. Maintaining the same acidity is important as the colour decreases with increasing acidity. Secondly, rectilinear calibration graphs with low coefficients of variation were obtained and detection limits for the determination of the cephalosporins in samples of penicillin were calculated to be in the range 1.25 - 2 µg g^{-1}. Comparison of the slopes of the calibration graphs for particular cephalosporins in Tables 3 and 4 clearly indicate that the high concentration of penicillin affects the yield of sulphide obtained from the cephalosporins. Results with standard sulphide solutions indicate a slight decrease in the yield of methylene blue (48% instead of 52%) from sulphide in these solutions. Appreciably increased overall yields of methylene blue are obtained from cephaloridine and cephalothin in the presence of high concentrations of penicillin G or penicillin V, whereas for cephalexin there is a decreased overall yield in the presence of penicillin V but not penicillin G.

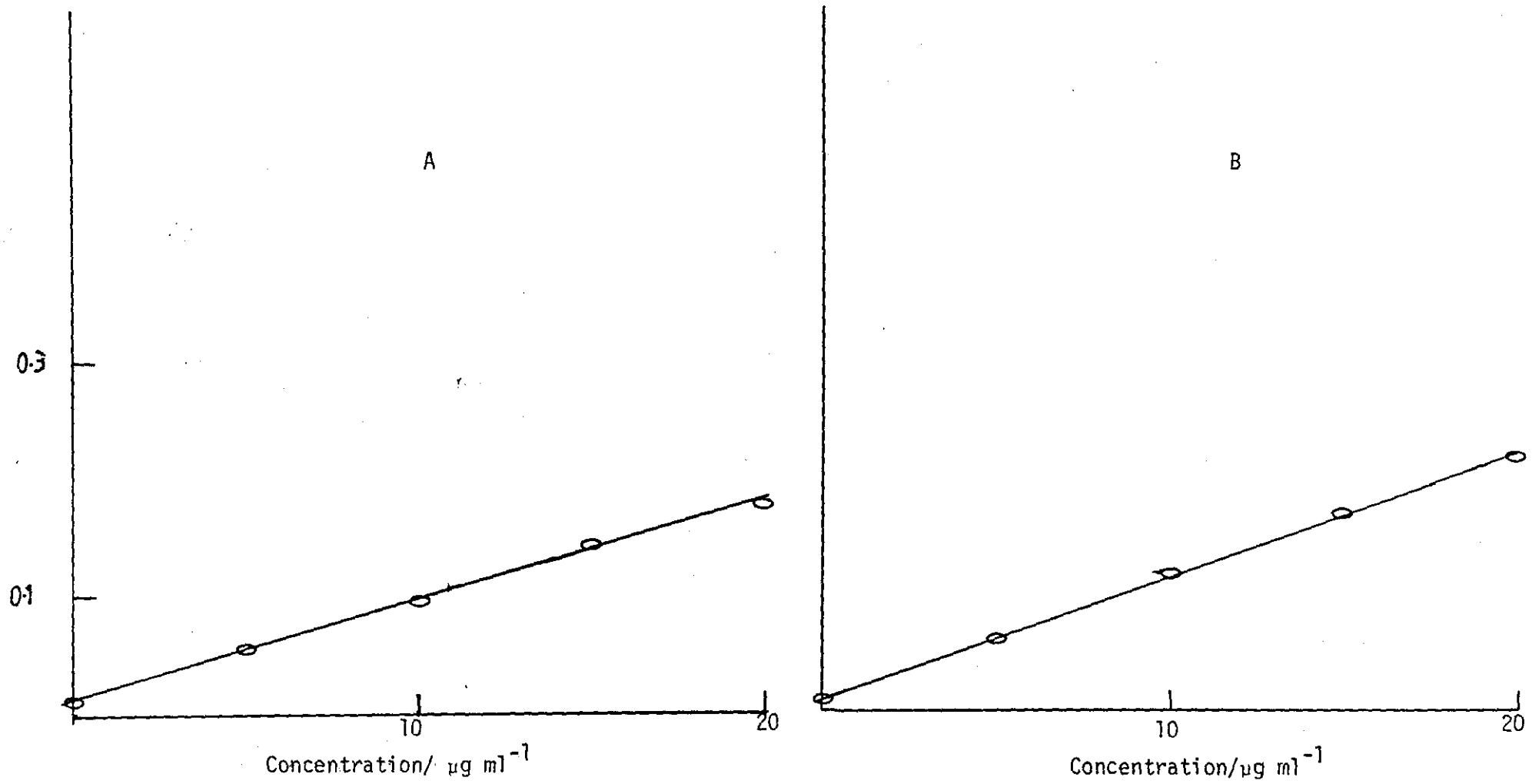


FIGURE 4: Calibration graphs obtained for cephalexin in (A) penicillin G (B) penicillin V

TABLE 4:

Calibration graphs for determination of cephalosporins in penicillin G and penicillin V samples

Cephalosporin	Equivalent concentration range in penicillin G /μg g^{-1}	Slope of calibration graph+ /10^{-3}ml μg^{-1}	Coefficient of variation* %	Detection limit /μg g^{-1}
(a) Penicillin G sample				
Sodium sulphide	0.5-10	9.2	1.02	1.25
Cephalexin	0.5-10	3.6	0.67	1.25
Cephaloridine	1-20	3.4	0.70	1.5
Cephalothin	1-20	3.1	0.68	1.5
(a) Penicillin V sample				
Sodium sulphide	0.5-10	9.2	1.03	1.25
Cephalexin	0.5-10	7.0	0.53	1.23
Cephaloridine	1-20	4.0	0.70	2.0
Cephalothin	1-20	5.1	0.68	2.0

* Based on calibration graph of four points plus blank
\+ The absorbance of the blank was 0.0130 (Penicillin G) and 0.0132 (Penicillin V)

Where a 350 μg ml^{-1} solution of ampicillin was presented no methylene blue was formed. Insufficient ampicillin was available to study the determination of cephalosporins in ampicillin but there should be no difficulty in doing this.

Discussion

The recommended automatic procedure is highly satisfactory for the determination of cephalosporins. The method is not stability indicating as several cephalosporin degration products are known to degrade further to sulphide. In this respect the method is inferior to the imidazole method for cephalosporins and penicillins which is stability indicating owing to the direct reaction of the imidazole with β-lactam ring. The present method, however, is selective for cephalosporins in the presence of penicillins, uses the visible rather than the UV spectrum and does not require the use of mercury(II).

In dilute aqueous solutions both the manual and automatic procedures have been shown to be highly reproducible despite the fact that the molar yield of sulphide from a cephalosporin will be between 14 and 65% (manual procedure) and the fact that the yield of methylene blue from sulphide goes from 52% to 48% in the automatic procedure in the presence of high concentrations of penicillins. On the other hand increased overall yields of methylene blue were observed in the presence of high concentrations of penicillins for cephaloridine and cephalothin. The yield of hydrogen sulphide from cephalexin is known to be highly dependent on pH, and more significantly, degradation routes depend markedly on other matrix effects such as buffer constituents and degredand concentration. Thus the observation of matrix effects in the recommended procedure are not unexpected. Precision and day-to-day reproducibility, however, have been shown to be excellent for determination of cephalosporins in penicillin samples. Clearly a standard addition method must be used

in developing any limit test, and the blank owing to the colour of penicillin degradation products must be determined and subtracted. The method will not distinguish between cephalosporins and other sulphide producing degradation products or impurities in the penicillin samples. It should be possible to distinguish traces of inorganic sulphide from cephalosporins and other sulphide producing impurities, if this were felt to be necessary, by flushing out weakly acidic solutions of the penicillin sample with nitrogen gas before presenting the solution to the Autoanalyser system.

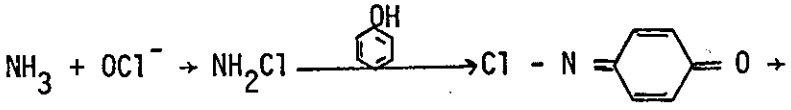
The procedure and system used here is capable of providing a limit test for 1-2 $\mu g\ g^{-1}$ of cephalosporin and other sulphide-producing impurities in penicillin samples. By using an even more concentrated solution of the penicillin this detection limit can be reduced slightly. The use of a modified system incorporating a double beam spectrophotometer would allow the automatic subtraction of the blank owing to the colour of the penicillin degradation products. The detection limit might well be reduced by the use of a more modern spectrophotometer.

CHAPTER 5

DETERMINATION OF CEPHALOSPORINS AND PENICILLINS BY DEGRADATION TO AMMONIA AND FORMATION OF INDOPHENOL COLOUR

Introduction

The Berthelot reaction[202] in which ammonia is oxidised by hypochlorite to form chloramine which reacts with phenol to form an indophenol dye is frequently used for determination of ammonia.



The indophenol dye is measured spectrophotometrically generally at 625 nm. The formation and intensity of the colour depends on the sequence of addition of the reagents[203], as well as on time, temperature, the pH of the solution and its redox potential as indophenol is both an acid-base and redox indicator[204,205].

The blue colour of indophenol formed by ammonia, hypochlorite and phenol in alkaline medium has been employed since its discovery in 1859 in numerous analytical procedures which however, still differ very much from each other[206-209]. Various species have been suggested to catalyse the reaction, amongst which manganese (II), acetone[210], and sodium nitroprusside[209] are the most effective, the latter allowing full colour development to be attained at room temperature in 20 mins[209].

The phenol-hypochlorite method for determining ammonia in water using nitroprusside as catalyst has been investigated[211]. The method is simple and does not require accurate time intervals between addition of reagents, nor expensive equipment. The method is well suited for routine use.

The indophenol reaction has been used to determine the amount of ammonia released from several cephalosporins and penicillins before and after hydrolysis in 0.5M NaOH. The reaction has also been made the basis of a colorimetric method of determining cephalosporins and penicillins.

Experimental

Reagents

All reagents were of analytical grade except where otherwise indicated.

1. Phenol solution, 3% w/v.

Dissolve 3 grams of phenol in 100 ml distilled water. (Care must be taken in handling concentrated or solid phenol to avoid contact with skin as it is corrosive and highly toxic by absorption.

2. Sodium nitroprusside solution

Dissolve 10 mg of sodium nitroprusside in 100 ml distilled water.

3. Sodium hypochlorite solution

Dilute 20 ml of commercial sodium hypochlorite solution (10-14% available chlorine) to 1 litre with distilled water.

4. Standard hydrochloric acid solution.

Stock solution of 10^{-1}M standard hydrochloric acid was used.

5. Sodium hydroxide solution.

Dissolve 20g of sodium hydroxide in water, cool and dilute to 1 litre with distilled water.

6. Alkaline phenol solution 3% w/v.

Dissolve 3 grams of phenol in 100 ml of 0.5M sodium hydroxide solution.

Samples

The following samples were used in this chapter: cephalexin, cephradine, cephaloglycin, cefaclor, cefazolin, cephaloridine, cephalothin, cephalonium, cephoxazole, 7-ACA, 7-ADCA, penicillin G, penicillin V, ampicillin, diketopiperazine derivative and pyrazine derivative.

Experimental Techniques

The following set of experiments were carried out in order to optimise the experimental conditions to produce the maximum colour intensity.

1. Effect of phenol concentration on the apparent molar absorptivity of the coloured dye formed from standard ammonia formed from cephalexin

Into six 50 ml volumetric flasks containing 5 ml of ammonia derived from cephalexin, from 0.5-5 ml of phenol solution (3% w/v) were added. To each flask, 5 ml of sodium nitroprusside (100 ppm), 2 ml of sodium

hypochlorite solution were added. The flasks were heated in a water bath at 100°C for 2-3 mins. The absorbances were measured at 625 nm against water as reference in 1 cm cell. The results are shown in Table (i).

TABLE (i):
Effect of Phenol Concentration on the apparent molar absorptivity of the coloured dye formed

Volume of Phenol used	0.5 ml	1 ml	2 ml	3 ml	4 ml	5 ml
Molar absorptivity 10^4 l ml^{-1} cm^{-1}	1.02	1.42	1.91	2.02	1.95	1.55

The optimum concentration of phenol is found to be 3 ml of (3% w/v) solution.

2. Effect of sodium hypochlorite concentration on the apparent molar absorptivity of the coloured dye formed

The experiment was repeated as before, except that the phenol solution volume was fixed to 3 ml and different volumes of sodium hypochlorite stock solution were used. The results are shown in Table 2.

TABLE (ii):

Effect of sodium hypochlorite concentration on the apparent molar absorptivity of the dye formed

Volume of Sodium Hypochlorite Used	0.4 ml	0.6 ml	0.8 ml	2 ml	3 ml	4 ml
Molar absorptivity 10^4 l mol^{-1} cm^{-1}	1.34	1.59	1.64	1.74	1.65	1.54

The optimum volume of sodium hypochlorite is found to be 2 ml.

3. Effect of sodium nitroprusside concentration on the apparent molar absorptivity of the coloured dye formed

Into seven 50 ml volumetric flasks, to each flask the following additions were made: 5 ml of standard ammonia from cephalexin, from 0-10 ml of sodium nitroprusside solution (100 ppm), 3 ml of phenol solution (3% w/v and 2 ml of sodium hypochlorite solution. The experiment carried out as before. The results are shown in Table (iii).

TABLE (iii):

Effect of sodium nitroprusside concentration on the apparent molar absorptivity of the coloured dye formed

Concentration of sodium nitroprusside	0 ppm	200 ppm	400 ppm	500 ppm	600 ppm	800 ppm	1 mg
Molar absorptivity 10^4 ℓmol^{-1}cm^{-1}	0.005	1.61	1.68	1.74	1.69	1.62	1.63

The optimum concentration is found to be 500 ppm of sodium nitroprusside.

4. Effect of heating time on the apparent molar absorptivity of the coloured dye formed

The effect of heating time on the colour intensity was studied at 100^{o}C at the optimum reagents concentration, namely, sodium hypochlorite solution 2 ml, phenol solution concentration of 3 ml of (3% w/v) and 500 ppm of sodium nitroprusside. Results are shown in Table (iv).

TABLE (iv):

Effect of heating time on the apparent molar absorptivity of the coloured dye obtained at 100°C

Heating Time min	1	2	3	4	5	6	7
Molar absorptivity $10^4 l\ mol^{-1} cm^{-1}$	1.64	1.68	1.67	1.67	1.66	1.65	1.60

From the results obtained, the optimum heating time is seen to lie between 2-4 minutes at 100°C.

i) Determination of the apparent molar absorptivities based on the indophenol reaction of cephalosporins and penicillins and the molar yields of ammonia after distillation of ammonia from the degraded solutions

The distillation apparatus was composed of the following parts: a Pyrex glass distillation flask provided with a two-holed stopper which carries a tap funnel and a glass spray trap. The purpose of the trap is to prevent any droplets of sodium hydroxide solution being driven over in the subsequent distillation. A condenser was attached to the trap and to the conical flask which served as a receiver.

Procedure:

Weigh out accurately about 0.1g of AR ammonium chloride or sample, dissolve it in water, and make up to 250 ml in a volumetric flask. Transfer 25.0 ml of the solution into a distillation flask and dilute with 200 ml of water. Add a few fragments of porous porcelain. Place 100 ml of standard 0.1M hydrochloric acid in the receiver and adjust the flask so that the end of the condenser just dips into the acid.

Make sure that all the connections are fitting tightly. Run the sodium hydroxide solution into the distillation flask by opening the tap, close the tap as soon as all the alkali has entered. Heat the flask so that the contents boil gently. Continue the distillation for 40-50 minutes, by which time all the ammonia should have passed over into the receiver. Open the tap before removing the receiver and rinse the condenser with a little water. Transfer the contents of the receiver to a 500 ml volumetric flask quantitatively and dilute to volume. Transfer, by means of a pipette, aliquots (50 ml) of the solution into six 50 ml calibrated flasks. Add by pipette, 3.0 ml of alkaline phenol solution and mix. Add 5.0 ml of sodium nitroprusside and 2.0 ml of sodium hypochlorite. The flasks were then heated in a water bath at 100°C for 3-4 minutes, cool and dilute to volume with distilled water, measure the absorbance at 625 nm against water as reference. The percentage yields of ammonia from each cephalosporin and each penicillin was determined by comparing the apparent molar absorptivity of the β-lactam compound with that of the standard ammonium compound.

ii) Study of the direct formation of the indophenol colour with heating of the alkaline solution for various cephalosporins and penicillins and some of their degradation products without distillation of the ammonia

Dissolve about 0.1g of the β-lactam compound or degradation derivative, accurately weighed in water if soluble or in a small volume of dilute hydrochloric acid, and dilute to 100 ml in a calibrated flask. Transfer, by means of a pipette, aliquots (10 ml) of the solutions into a series (7-13) stoppered flask and make up the solution to 100 ml in such a way that the final concentration of sodium hydroxide is 0.5M.

Heat the flasks in a boiling water bath. Remove individual flasks in sequence at suitable time intervals, cool them quickly after removal from the bath. Continue with each flask as follows: add, by pipette, 5 ml of the contents of each flask to 50 ml calibrated flasks and add 3 ml of phenol solution and mix. Add 5 ml of sodium nitroprusside and 2 ml of sodium hypochlorite solution. Heat each flask in a water bath at 100°C for 3-4 minutes, cool, and dilute to volume with distilled water. Measure the absorbance at 625 nm against water as reference.

iii) Recommended procedures for the colorimetric determination of the cephalosporins and penicillins

A. *Determination of cephalosporins and penicillins as indophenol by distillation of ammonia*

Weigh out accurately about 0.1g of cephalosporin or penicillin, dissolve in distilled water, and make up to 250 ml in a volumetric flask. Transfer, by means of a pipette, aliquots (0-10 ml) of the solution into 250 ml distillation flasks. Add a few fragments of porous porcelain. Place 100 ml of standard 0.1M hydrochloric acid in the receiver and adjust the flask so that the end of the condenser just dips into the acid. Run the sodium hydroxide solution into the distillation flask by opening the tap of the separating funnel, close the tap as soon as all the alkali has entered. Heat the flask so that the contents boil gently. Continue the distillation for 40 minutes, by which time all the ammonia should have passed over into the receiver. Open the tap before removing the receiver and rinse the condenser with a little water. Transfer the contents of the receiver to a 250 ml volumetric flask quantitatively and dilute to volume.

Transfer, by means of a pipette, aliquots (5 ml) of each solution to 50 ml calibrated flasks and add 3 ml of alkaline phenol and mix. Add 5 ml of sodium nitroprusside and 2 ml of sodium hypochlorite solution. Heat the flasks in a water bath at 100°C for 3-4 minutes, cool, and dilute to volume with distilled water, measure the absorbance at 625 nm against water as reference.

Sample should be treated in a similar manner to the standard and the amount of cephalosporin or penicillin present can be calculated by comparison with the appropriate calibration graph.

B. *Determination of penicillins and cephalosporins as ammonia without distillation after hydrolysis in 0.5M NaOH at 100°C in a water bath and determination of the apparent molar absorptivities of ammonia without distillation*

Dissolve about 0.1g of the β-lactam compound accurately weighed in water or in small volumes of hydrochloric acid, and dilute to 100 ml in a calibrated flask. Transfer, by means of a pipette, aliquots (0-10 ml) of the solution into stoppered flasks and make up the solution to 100 ml in such a way that the final concentration of sodium hydroxide is 0.5M. Heat the flask in a boiling water bath for a length of time appropriate for the particular compound to produce maximum formation of ammonia and then cool.

Add by pipette, 5 ml of each solution to 50 ml calibrated flasks and add 3 ml of phenol solution and mix. Add 5 ml of sodium nitroprusside and 2 ml of sodium hypochlorite solution. Heat the flasks in a water bath at 100°C for 3-4 minutes, cool, and dilute to volume with distilled water, measure the absorbance at 625 nm against water as reference.

C. *Determination of penicillins and cephalosporins as ammonium compound and the determination of the apparent molar absorptivities of the β-lactam compound without hydrolysis*

Dissolve about 0.1g of the β-lactam compound accurately weighed in water or in small volumes of hydrochloric acid, and dilute to 500 ml in a calibrated flask. Transfer, by means of a pipette, aliquots (0-10 ml) of the solutions into 50 ml calibrated flasks and add 3 ml of alkaline phenol solution and mix. Add 5 ml of sodium nitroprusside and 2 ml of sodium hypochlorite solution. Heat the flasks in a water bath at 100°C for 3-4 minutes, cool, and dilute to volume with distilled water, measure the absorbance at 625 nm against water as reference.

Results and Discussion

All the cephalosporins and penicillins studied have been shown to give ammonia. During degradation the yield of ammonia varies widely for different cephalosporins and penicillins, but is highly reproducible for individual compounds. The ammonia formed can be distilled into standard hydrochloric acid and reacted with the indophenol reagent, and this procedure has been made the basis of a colorimetric method of determining cephalosporins and penicillins. The apparent molar absorptivities at 625 nm for the indophenol dye formed from ammonium chloride and from cephalosporins, penicillins and some of their degradation products are given in Table 1. As in the case of sulphide different cephalosporins were shown to give different but reproducible yields of ammonia. The yield (based on one nitrogen atom per molecule) varied from 18.0% for cephoxazole to 136.8% for cephalonium. In the case of penicillins the yield varied from 28.1% for penicillin G to 45.1 for ampicillin. The effect of hydrolysis prior to distillation was

investigated using cephalexin. The same experiment was carried out, as in the distillation experiment, except that the flask containing the sample in 0.5M sodium hydroxide was first heated in a boiling water bath to ensure complete hydrolysis, the water bath was then removed and the distillation was performed as before. No difference in the yield of ammonia from cephalexin was observed and hence the hydrolysis step before distillation was excluded as being unnecessary. Experience with other cephalosporins and penicillins indicates that this is always the case.

The rate of formation of ammonia with time of heating of the alkaline solution for various cephalosporins and penicillins and some of their degradation products are given in Table 2, and Figures 1, 2 and 3. For cephaloridine in particular, the reaction between the reagent and the neutral, freshly prepared solution of cephaloridine, produces a higher absorbance than the same solution after complete hydrolysis. With increasing heating time the molar yield of the indophenol dye produced started decreasing to a point where absorbance value near that of the blank reading and then they increased again until a plateau is eventually reached, see Figures 2 and 3. With all compounds ammonia production eventually ceases and an optimum time for heating can be selected for each, see Table 4. For cephalolothin the same behaviour was observed as for cephaloridine except that the initial reading of the indophenol is ∿50% that of the final value: the value decreases almost to zero before increasing again to the maximum value. Most of the other cephalosponins and penicillins give significant initial readings which then increase to the final maximum value without any decrease when a neutral solution is reacted with

the indophenol reagent. The exceptions are α-aminocephalosporins such as cephalexin, cephradine, cephaloglycin and cefaclor which give very small initial readings, see Table 5.

Calibration graphs obtained to determine individual cephalosporins and penicillins using optimum heating times were found to be rectilinear and coefficients of variation were typically less than 2%, see Table 4. Method A, that of distillation, was found in six cases to be more sensitive than method B. In this case the yield of ammonia varies from 12.1% for cephoxazole to 136.7% for cephalonium, see Table 3. For cephalothin, cephaloridine, cefazolin, cephoxazole 7-ACA, 7-ADCA and cephalonium the yields of ammonia using method B, were found to be less than that obtained by method A. It was not clear whether the decrease of the molar yield of ammonia is due to the loss by reaction of ammonia as it formed with some of the degradation products in solution or the percentage in the case of cephalexin, cephaloglycin, cefaclor, cephradine, penicillin G, penicillin V and ampicillin was high because of interference from other degradation products as the reagent is not selective and reacts with many functional groups such as $-NH_2$.

The method of the determination of cephalosporins and penicillin as ammonia with or without distillation is very simple and the reaction of ammonia with alkaline phenol and hypochlorite is a well known reaction and can be easily automated. The method is as sensitive as the methylene blue method reported previously but has the disadvantages of not differentiating between cephalosporins and penicillins. Together with the information from the sulphide work the ammonia percentages from different cephalosporins and penicillin can provide considerable

information of help in studying the reaction mechanism of the degradation of cephalosporins and penicillins. The identification of these small molecular weight degradation products can provide useful information on the allergic reaction to penicillin and cephalosporins because considerable evidence now exists that the induction of allergy to penicillins may be mainly or at least partly brought about by contaminants of higher molecular weight which become chemically associated by covalent linking with the degradation products of penicillin.

TABLE 1:

Apparent molar absorptivities of cephalosporins and penicillins and molar yields of ammonia after distillation

	Apparent Molar Absorptivity $10^4 l mol^{-1} cm^{-1}$	Molar Yield of Ammonia	Coefficient of Variation %*
Ammonium chloride	2.35	100	1.2
Cephalexin	1.90	83.0	1.4
Cephradine	1.76	75.1	1.3
Cephaloglycin	2.20	93.7	1.2
Cephalothin	1.04	45.3	1.4
Cephaloridine	1.01	43.0	1.3
Cephalonium	3.21	136.8	1.5
Cefoxazole	0.44	18.0	1.2
Cefaclor	1.08	45.9	1.1
Cefazolin	2.38	10.13	1.3
7-ACA	1.50	64.2	1.4
7-ADCA	2.49	105.0	1.3
Diketopiperazine-derivative	2.01	85.5	1.2
Pyrazine derivative	1.40	59.7	1.3
Penicillin V	1.04	45.1	1.1
Penicillin G	0.66	28.1	1.3
Ampicillin	1.06	45.1	1.2

* Three distillations

TABLE 2:

Effect of time of heating in 0.5M sodium hydroxide solution on the formation of ammonia from: cephalexin (9.8 ppm), cephaloglycin (7.4 ppm), cefaclor (11 ppm), cephradine (8.2 ppm) and ampicillin (21 ppm) as indicated by the indophenol absorbance at 625 nm

Time/min	Cephalexin	Cephaloglycin	Cefaclor	Cephradine	Ampicillin
	Absorbance at 625 nm				
0	0.028	0.092	0.022	0.021	0.210
10	0.124	0.104	0.095	0.101	0.301
20	0.185	0.255	0.142	0.202	0.422
30	0.307	0.300	0.195	0.315	0.539
40	0.413	0.322	0.252	0.421	0.547
50	0.530	0.350	0.320	0.430	0.548
60	0.540	0.389	0.319	0.429	0.549
70	0.545	0.397	0.321	-	-
80	0.540	0.399	-	-	-

TABLE 2... continued

Effect of time of heating in 0.5M sodium hydroxide solution on the formation of ammonia from: Cephalothin (30 ppm), cephaloridine (60 ppm), cephalonium (43 ppm), penicillin G (18.8 ppm) and penicillin V (28 ppm) as indicated by the indophenol absorbance at 625 nm

Time/min	Cephalothin	Cephaloridine	Cephalonium	Penicillin G	Penicillin V
	Absorbance at 625 nm				
0	0.240	0.523	0.526	0.124	0.203
5	0.079	0.184	0.742	-	-
10	0.061	0.053	-	0.201	0.297
15	0.120	0.047	0.943	-	-
20	-	-	-	0.254	0.317
25	0.201	0.062	0.997	-	-
30	-	-	-	0.299	0.359
35	0.264	0.084	1.099	-	-
40	-	-	-	0.315	0.399
45	0.355	0.097	1.099	-	-
50	-	-	-	0.331	0.404
55	0.428	0.174	1.112	-	-
60	-	0.250	-	0.330	0.405
65	0.473	-	1.116	-	-
70	-	0.295	-	-	-
75	0.474	-	1.115	-	-
80	-	0.345	-	-	-
85	0.473	-	-	-	-
90	-	0.483	-	-	-
100	-	0.484	-	-	-
110	-	0.485	-	-	-

TABLE 2 ... continued

Effect of time of heating in 0.5M sodium hydroxide solution on the formation of ammonia from: Cefazolin (46 ppm), cephoxazole (14 ppm), 7-ACA (13 ppm) and 7-ADCA (8.2 ppm) and diketopiperazine derivative (30 ppm) as indicated by the indophenol absorbance at 625 nm

Time/min	Cefazolin	Cephoxazole	7-ACA	7-ADCA	Diketopiperazine derivative
	Absorbance at 625 nm				
0	0.412	0.145	0.024	0.031	0.221
10	0.490	0.187	0.123	0.204	0.403
20	0.519	0.201	0.292	0.397	0.511
30	0.602	0.292	0.450	0.502	0.791
40	0.720	0.373	0.603	0.699	0.850
50	0.797	0.401	0.779	0.841	0.869
60	0.892	0.409	0.899	0.842	0.870
70	0.942	0.410	0.901	0.843	-
	0.941	-	-	-	-

TABLE 3:

Molar yield of ammonia as determined spectrophotometrically with and without distillation

	Molar Yield %	
	With distillation	Without distillation
Ammonium chloride	100.0	100
Cephalexin	83.0	82.5
Cephradine	75.1	75.0
Cephaloglycin	93.7	92.9
Cephalothin	45.3	24.2
Cephaloridine	43.0	21.0
Cephalonium	136.8	136.7
Cephoxazole	18.0	12.1
Cefaclor	45.9	45.5
Cefazolin	101.3	56.1
7-ACA	64.2	41.2
7-ADCA	105.0	72.1
Penicillin V	45.1	45.0
Penicillin G	28.1	29.0
Ampicillin	45.1	44.9
Diketopiperazine derivative	85.5	86.0

TABLE 4:

Calibration graphs for determination of cephalosporins as ammonia, without distillation, in standard solutions

	Optimum Hydrolysis Time/min	Concentration range µg/ml	Coefficient of Variation %
Ammonium chloride	-	10-60	0.75
Cephalexin	60	10-60	0.90
Cephradine	50	10-60	0.95
Cephaloglycin	60	10-60	0.78
Cephalothin	65	10-60	0.92
Cephaloridine	90	10-60	0.82
Cephalonium	55	10-60	0.87
Cephoxazole	50	10-60	0.80
Cefaclor	50	10-50	0.91
Cefazolin	70	10-60	0.82
7-ACA	70	10-60	0.91
7-ADCA	50	10-60	0.95
Penicillin V	50	10-60	0.85
Penicillin G	50	10-60	0.95
Ampicillin	40	10-60	0.92

TABLE 5:

Apparent molar absorptivities of cephalosporins and penicillins before and after hydrolysis

	Apparent molar absorptivities before hydrolysis x $10^4/l^4 mol^{-1} cm^{-1}$	Apparent molar absorptivities after hydrolysis x $10^4/l$ $mol^{-1} cm^{-1}$
Ammonium chloride	2.35	2.35
Cephalexin	0.09	1.90
Cephaloglycin	0.50	2.18
Cefaclor	0.06	1.06
Cephradine	0.08	1.76
Ampicillin	0.40	1.05
Cephaloridine	0.56	0.33
Cephalothin	0.35	0.49
Cephalonium	0.56	3.21
Penicillin G	0.24	0.68
Penicillin V	0.24	1.05
Cefazolin	0.41	1.31
Cephoxazole	0.41	0.28
7-ACA	0.49	0.96
7-ADCA	0.08	1.69

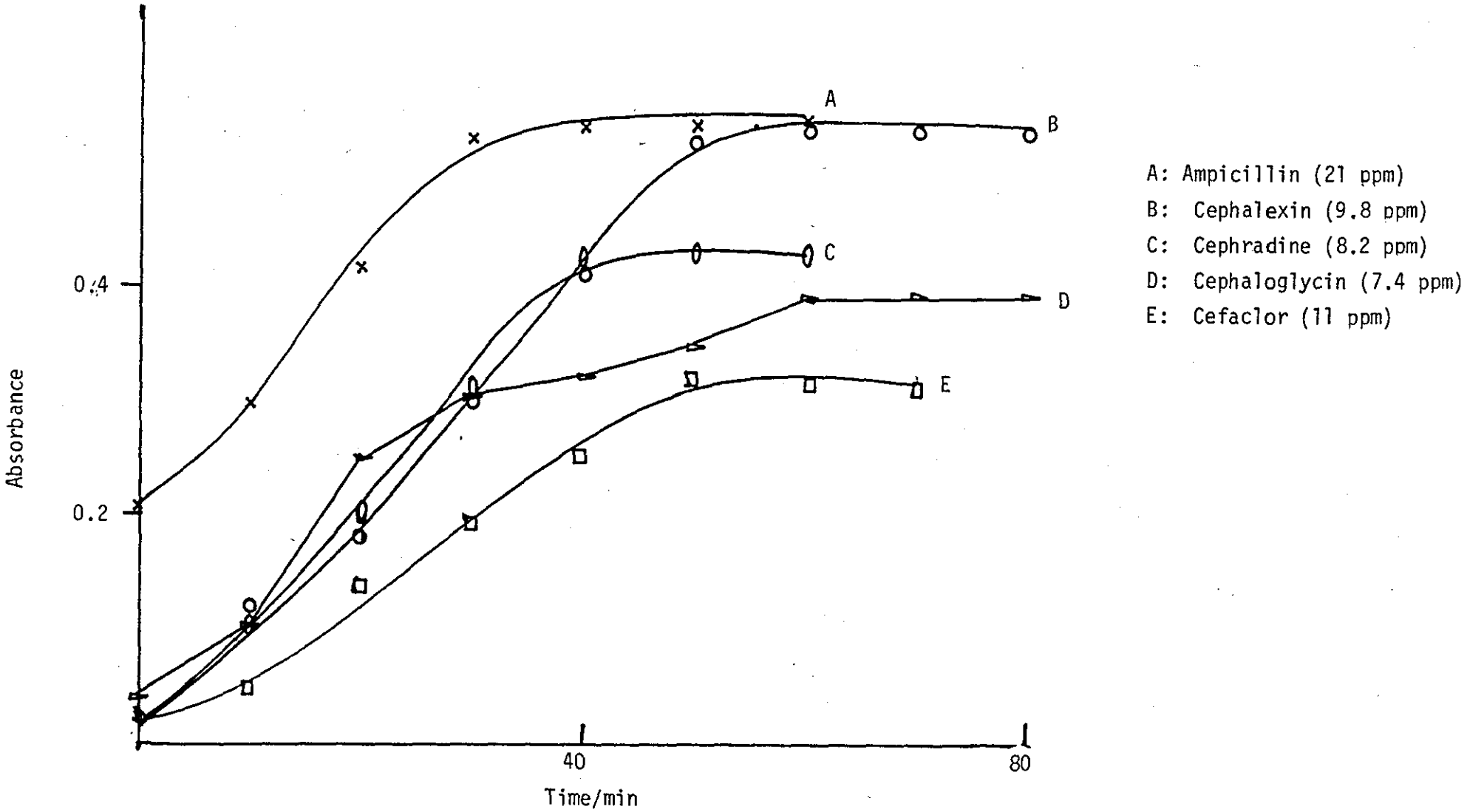


FIGURE 1: Effect of time of heating in 0.5M sodium hydroxide solution on the formation of ammonia from A, B, C, D, E as indicated by the indophenol absorbance at 625 nm

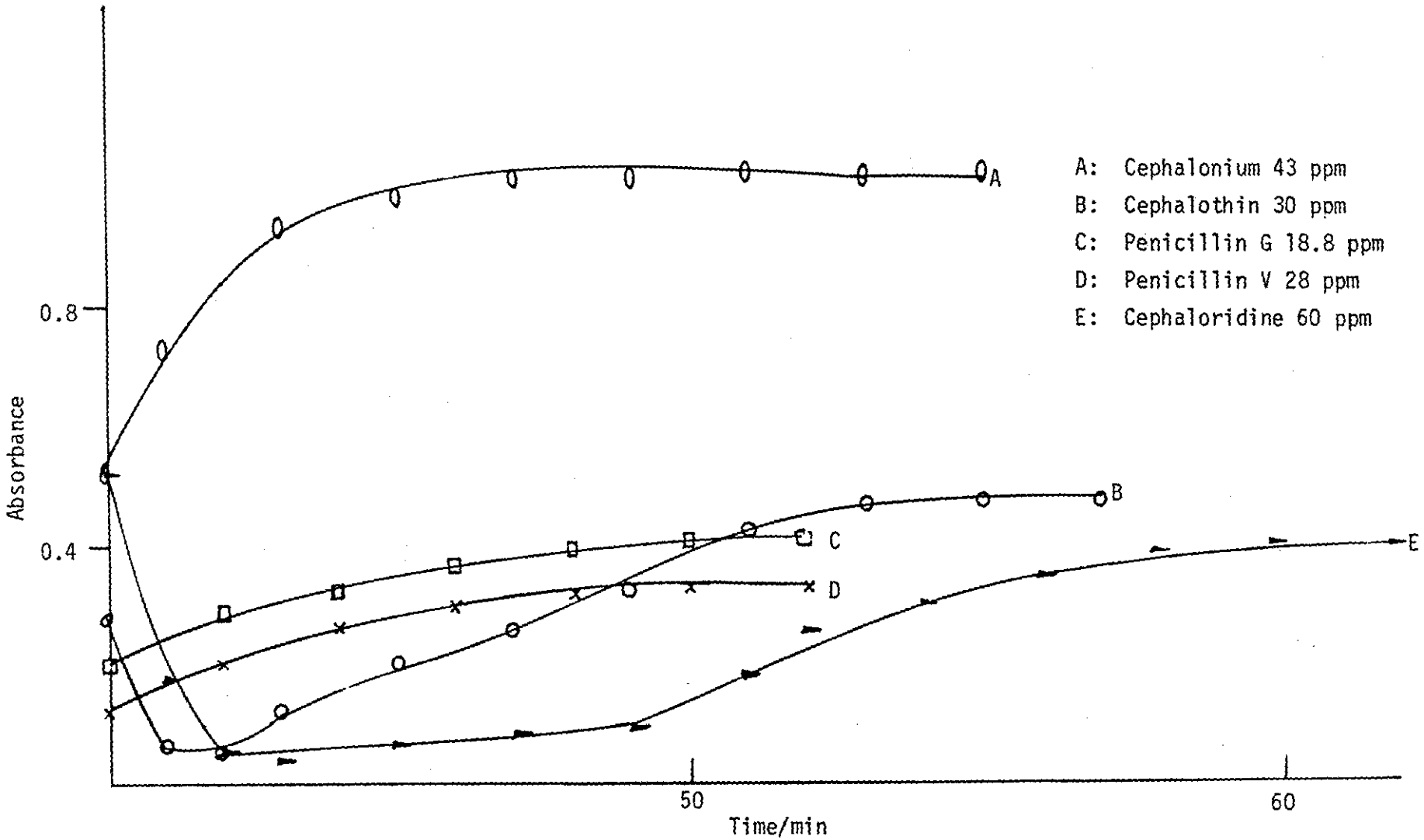
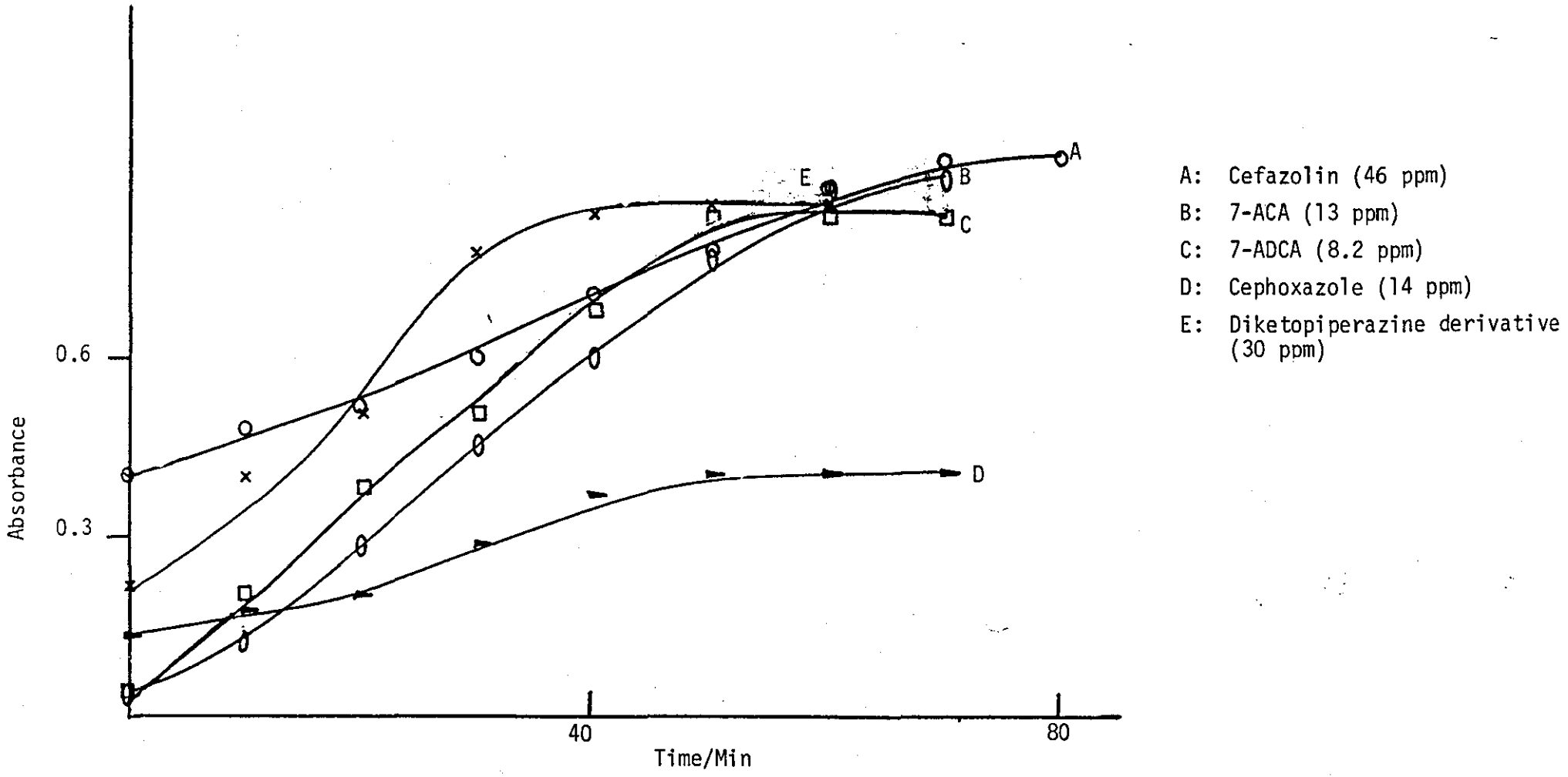


FIGURE 2: Effect of time of heating in 0.5M sodium hydroxide solution on the formation of ammonia from A, B, C, D, E as indicated by the indophenol absorbance at 625 nm



A: Cefazolin (46 ppm)
B: 7-ACA (13 ppm)
C: 7-ADCA (8.2 ppm)
D: Cephoxazole (14 ppm)
E: Diketopiperazine derivative (30 ppm)

FIGURE 3: Effect of time of heating in 0.5M sodium hydroxide solution on the formation of ammonia from A, B, C, D, E as indicated by the indophenol absorbance at 625 nm

CHAPTER 6

FLOW INJECTION VOLTAMMETRIC DETERMINATION OF NITRITE BY REDUCTION AT A GLASSY CARBON ELECTRODE IN ACIDIC BROMIDE OR CHLORIDE MEDIA

Introduction

Flow injection analysis is a technique that allows for the rapid and automated determination of a wide range of species with excellent reproducibility using simple and inexpensive instrumentation[212]. Electrochemical detection has been used extensively in liquid chromatography for the determination of electroactive species in biological fluids, industrial effluents, and in pollution monitoring[213,214]. Ion-selective and voltammetric electrodes are the main electroanalytical detectors used for carrying out analysis in flowing solutions. Voltammetry is reported to have a wide application and some specificity. Very low detection limits are feasible ($\sim 10^{-9}$M). Detection in flowing systems has a number of advantages compared with static electrode systems, these are:

a) The current is increased because of increased mass transport to the electrode, the diffusion layer being thinner in flowing streams.

b) The background current is decreased because at constant potential no current is needed to charge the double layer and the oxidation states of the functional group in the carbon electrode surface are in equilibrium.

c) Hydrodynamic voltammetry is time independent, which permits steady state measurements. The need for high speed electronics is diminished.

d) The residual charging current is independent of flow rate. Therefore the signal-to-noise ratio is increased by increasing the flow rate of the analyte, as long as mass transfer rates do not exceed charge transfer rates. This is rarely met in practice. This advantage is gained, however, only if a high purity electrolyte is used.

Chan and Fogg[215] used a Metrohm EA 1069/2 electrochemical detector cell together with the Metrohm injection unit to determine the phenolic analgesic meptazinol. Calibration graphs were rectilinear over the range 0.01 - 10 $\mu g\ ml^{-1}$. In order to improve the reliability of the technique it was necessary to modify some of the manufacturer's instructions, namely the reference electrode was placed after the detector electrode in the flowing stream. Further, a pre-saturation of internal reference electrode with silver chloride prevented large drifts in potential.[216]

Fogg and Bhanot[216] have observed the blocking of the frit of the reference electrode (EA 442), provided with the flow cell, causing some difficulties in carrying out flow injection work. Therefore they used the flow cell in a similar manner to the PAR 310 mercury electrode detector cell. The working electrode only was inserted into the detector cell, which was then partly immersed in electrolyte (0.01M sulphuric acid) contained in a beaker. Contact from the electrolyte to the counter and reference electrodes was made by means of salt bridges.

This system has been used successfully by Fogg and Bsebsu[217,218] for the voltammetric determination of phosphate and silicate by direct injection of neutral solution of phosphate or silicate in a carrier stream of acidic molybdate eluent or injection of preformed heteropolyacids in a carrier stream which had the same composition as the blank.

In developing an automated amperometric microtitration technique for the determination of low concentrations of pharmaceutically-important sulphonamides by diazotisation with nitrite Coenegracht et al[219] investigated the use of the biamperometric end-point detection method that is commonly used for determination at higher levels of sulphonamides[220]. Extrapolation of current versus volume plots is necessary at low concentration in order to obtain satisfactory precision and unfortunately the biamperometric plots after the end point were curved. They then studied the system monoamperometrically using a rotating platinum working electrode and showed that for a 5×10^{-6}M solution that is 1M in hydrochloric acid and 20% m/v in potassium bromide there is no potential range over which zero current is obtained. Instead a composite oxidation-reduction wave is obtained with zero current at 0.6V versus SCE. The anodic part of this wave is due to the oxidation of bromide and the cathodic part to the reduction of nitrosyl bromide formed by reaction of nitrite and bromide. At these high levels of bromide the current on the anodic side is not limited, but a well-formed cathodic voltammetric wave is given by nitrosyl bromide.

Coenegracht et al also showed that in a 5×10^{-6}M solution of nitrite that is 1M in hydrochloric acid and 20% m/v sodium chloride zero current was obtained over the potential range +0.7 - +0.9V.

Oxidation of chloride occurred above +0.9V and reduction on nitrite below +0.7V, but the reduction wave due to nitrosyl chloride was not as well-formed as that due to nitrosyl bromide.

Although the end-point detection method in previous work was not adapted to the direct determination of nitrite it clearly can be used for this purpose. In this chapter the solution conditions have been optimised for the determination of nitrite using voltammetric flow-injection analysis at a glassy carbon electrode.

Experimental

Apparatus and Reagents

Flow of eluent was produced with an Ismatec Mini-S peristaltic pump, and injections (25 µl) were made with a Rheodyne injection valve (5020). The injection valve was connected to a metrohm detector cell (EA 1096), fitted with a glassy carbon electrode (EA 286), by means of suitable lengths of 0.58 mm bore tubing.

Difficulties were encountered with the blocking of the frit of the reference electrode provided with the flow cell and for this reason the flow cell was dismantled and used in a configuration similar to that of the PAR 310 mercury electrode detector cell. Thus of the three electrodes only the working electrode was inserted in the detector cell which was then partly immersed vertically in the electrolyte (0.01M sulphuric acid) contained in a beaker. This beaker was kept in a larger container to prevent spillage of acid on the bench. Electrical contact from a platinum counter electrode and a potentiometric calomel reference electrode to the sulphuric acid solution was made by means of a salt

bridge. It was found to be unnecessary to de-gas the eluent. The potential of the glassy carbon electrode was held at 0.03V versus SCE, except as indicated otherwise, using a PAR 174 polarographic analyser (Princeton Applied Research). Current signals were monitored on a Tarkan 600 y-t recorder.

Reagents

Standard sodium nitrite solution, approximately 1×10^{-2}M

Dissolve approximately 0.172g of analytical reagent grade sodium nitrite accurately weighed in water and dilute to 250 ml in a calibrated flask. This solution is 1×10^{-2}M in nitrite. Prepare more dilute standard solutions from this solution.

Hydrochloric acid solution, 0.3M

Dilute 13.8 ml of concentrated hydrochloric acid to 500 ml with water.

Acidic bromide solution, 20% m/v in KBr and 3.2M in hydrochloric acid

Dissolve 100g of potassium bromide in 350 ml of water, add 138 ml of concentrated hydrochloric acid, cool the solution, dilute to 500 ml and mix.

Sodium chloride solution, 30% m/v

Dissolve 150g of sodium chloride in water and dilute to 500 ml with water.

Procedures:

1. Direct injection into acidic bromide eluent

Nitrite sample or standard solution (25 µl) was injected into acidic bromide solution carrier stream. The response was observed over three injections at each level of concentration.

2. Injection of nitrite pre-reacted with acidic bromide solution

Transfer by pipette an aliquot (⩽ 45 ml) of nitrite sample or standard solution (1×10^{-7}M - 1×10^{-4}M) into a 50 ml calibrated flask. Add 4 ml of acidic bromide solution, mix the solution and allow it to stand for 20 minutes. Dilute to 50 ml, mix and inject 25 µl into 0.3M hydrochloric acid solution. Note the reduction current signal. Use a 3m delay coil.

3. Direct injection into acidic chloride eluent

Inject 25 µl of nitrite sample or standard solution (1×10^{-5}M - 5×10^{-4}M) into acidic chloride solution and note the reduction current signal. A 3m delay coil was used.

4. Injection of nitrite pre-reacted with acidic chloride solution

Place 30 ml of 30% sodium chloride solution and 8.6 ml of concentrated hydrochloric acid in a 50 ml calibrated flask. Add by pipette an aliquot (⩽ 10 ml) of nitrite sample or standard solution (1×10^{-5} - 5×10^{-4}M, dilute to 50 ml with water and mix. Inject 25 µl into 0.3M hydrochloric acid solution and note the reduction current signal. A 3m delay coil was used for this experiment.

Preliminary Studies

Initial studies were made at 2 x 10^{-4}M level of nitrite using the solution conditions of Coenegracht et al[219].

The chloride system was studied first. Injections of nitrite with added sodium chloride into hydrochloric acid and of a pre-reacted nitrite-sodium chloride-hydrochloric acid solution into acid chloride eluent were made. For the injection of neutral nitrite containing chloride into hydrochloric acid of different concentrations a double peak indicating incomplete reaction was observed at sodium chloride concentration less than 15%, but only a single peak was observed at 15%. The peak height increased with increasing acidity from 0.01M to 2M hydrochloric acid. At 5M hydrochloric acid salt precipitated and a double peak was observed above 3.8M.

The study of injecting nitrite pre-reacted with acidic chloride indicated that complete reaction was reached rapidly, no difference in peak height being observed between solutions kept for up to 40 minutes before injection and those injected immediately after mixing. Comparison of peak heights with a 4m delay coil for neutral nitrite and pre-reacted nitrite injection indicated that the yield in the former case was 74% that in the latter case (see Figure 1).

Whereas solutions in which nitrite reacted with acidic chloride remained colourless, those in which nitrite reacted with acidic bromide became increasingly coloured more yellow with time. Furthermore the injection of these pre-reacted solutions gave a voltammetric signal which was some ten times larger than that obtained with the chloride system and which increased continuously and markedly for pre-reaction times of

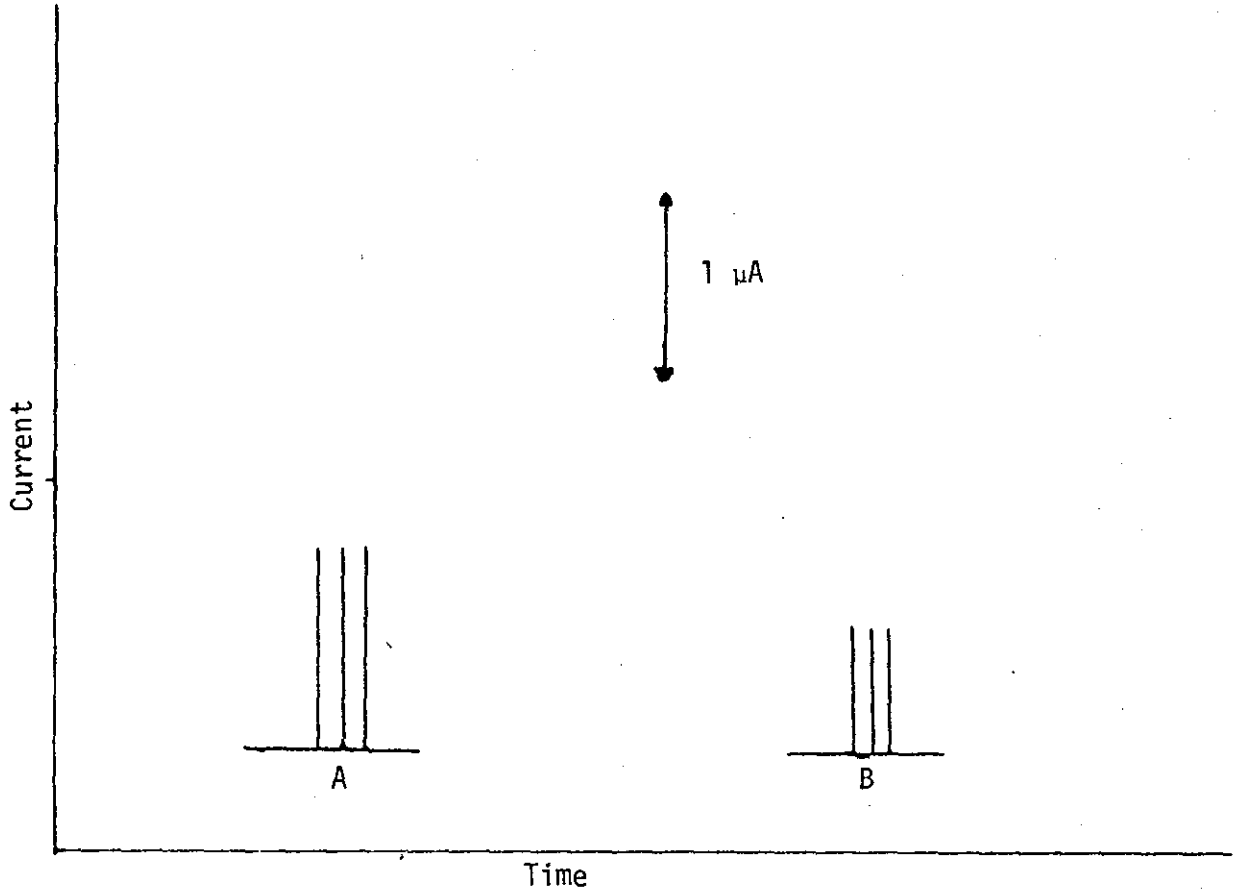


FIGURE1: Injection of (A) Pre-reacted nitrite, 2×10^{-5}M, (B) Direct injection of nitrite 2×10^{-5}M

up to more than 40 minutes. The injection of neutral nitrite containing potassium bromide into acid, however, gave voltammetric signal of the same order of magnitude as for the pre-reacted chloride system. For the pre-reacted system increased acidity gave more rapid formation of colour and signal. At 20% KBr and 3.2M hydrochloric acid the voltammetric peak obtained was 18.5 μA after 5 minutes and 23 μA after 20 minutes.

Measurable peaks were obtained for 1×10^{-5}M nitrite for direct injection of nitrite into the chloride system and for 1×10^{-6}M for injection of pre-reacted acidic chloride solution. For bromide system 1×10^{-6}M of nitrite was detectable by direct injection. A much lower limit was expected from the pre-reacted system in view of the large increase in yield of nitrosyl bromide on standing the pre-reacted 10^{-4}M nitrite solution for 20 minutes, but in practice the signal surprisingly disappeared rather abruptly at nitrite concentrations of 10^{-6}M and less. This seemed to indicate that, although rapid formation of nitrosyl bromide occurs at high acidity, low concentrations of nitrosyl bromide are unstable in this medium.

Results

The manifold used for all experiments is shown in Figure 2.

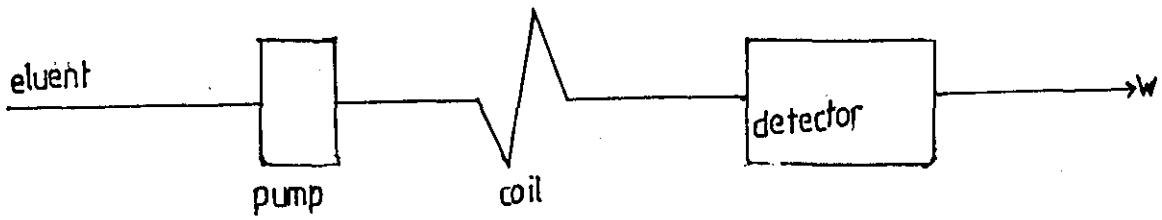


FIG. 2

Results in Table 1 show the effect on the signal obtained of the amount of acidic bromide reagent added to various levels of nitrite before injection into 0.3M hydrochloride acid.

TABLE 1:

Pre-reacted acidic bromide system: effect of volume of acidic bromide reagent added to give 50 ml of pre-reacted solution on the reduction current signal (μA) at various levels of nitrite concentration. Delay coil length = 3m

Volume of acidic bromide reagent added/ml	Equivalent concentration of nitrite in pre-reacted solution			
	2×10^{-4}M	2×10^{-5}M	2×10^{-6}M	2×10^{-7}M
2	2.50	0.30	0.03	0.009
4	12.0	1.18	0.118	0.011
6	13.0	1.40	0.070	0
8	12.4	1.50	0	0

The instability of the reductand at lower concentrations in higher acidity solutions is clearly seen. The use of 4 ml of reagent was adopted as this gave a rectilinear signal over three orders of magnitude from 2×10^{-7}M to 2×10^{-4}M nitrite. This is illustrated by the signals shown in Figure 3. The delay coil length between 1 and 4m has a relatively small effect on peak height although the peaks become broader. The peak heights at different delay coil lengths are given in Table 2 which also includes the flow rates obtained. These flow rates apply to all the results given in this chapter.

The effect of acid concentration of eluent containing 20% m/v potassium bromide, and that of potassium bromide concentration of eluent

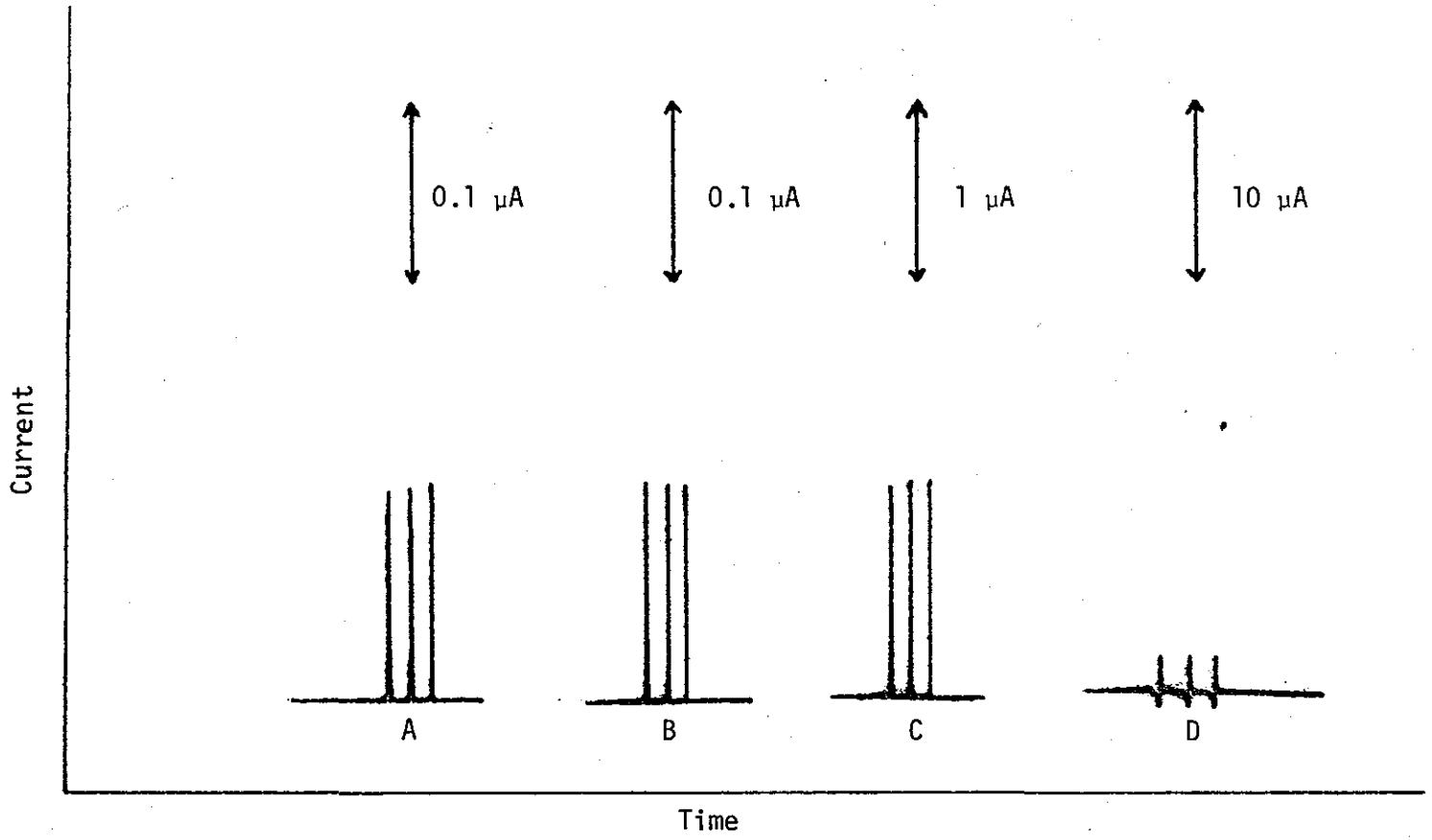


FIGURE 3: Injection of pre-reacted nitrite into acidic bromide medium using recommended procedure
Equivalence nitrite concentration in pre-reacted solution injected:
A. 2×10^{-7}M; B. 2×10^{-6}M; C. 2×10^{-5}M; D. 2×10^{-4}M.

3.2M hydrochloric acid, on the signal obtained on direct injection of nitrite are shown in Tables 3 and 4.

TABLE 2:

Pre-reacted bromide system: effect of delay coil length on the signals obtained and the flow rate using the recommended procedure. Equivalent nitrite concentration = 2×10^{-5}M

Delay coil length/m	0.5	1.0	2.0	3.0	4.0	5.0
Peak current/μA	1.70*	1.40	1.36	1.26	1.04	0.85
Flow rate/ml min^{-1}	9.6	8.0	5.2	4.2	3.3	2.9

* Double peak

TABLE 3:

Direct injection into acidic bromide: effect of acid concentration of eluent on signal obtained. Potassium bromide concentration = 20% m/v. Nitrite concentration = 2×10^{-4}M

Hydrochloric acid Concentration/M	1	2	3	3.2	3.4
Peak Current/μA	1.90*	3.90*	5.30	6.90	6.60

*Double peaks

TABLE 4:

Direct injection into acidic bromide: effect of potassium bromide concentration on signal obtained.Hydrochloric acid concentration = 3.2M. Nitrite concentration = 2×10^{-5}M.

Potassium bromide Concentration/% m/v	5.0	10.0	15.0	20.0	25.0
Peak Current/μA	0.30	0.41	0.49	0.69	ppt

The effect of delay coil length on the signal for direct injection into the optimised eluent is shown in Table 5. A 4m coil is required to remove the double peak but otherwise there is little change in peak height between 0.5 and 4m. Signals obtained for direct injection of 2×10^{-6}M - 2×10^{-4}M nitrite solutions are shown in Figure 4.

TABLE 5:

Direct injection into acidic bromide: effect of delay coil length on signal obtained using recommended eluent. Nitrite concentration = 2×10^{-4}M.

Mixing coil length/m	0.5	1.0	2.0	3.0	4.0	5.0
Peak Current/μA	6.50*	6.40*	6.40*	6.50*	6.90	5.60

*Double peak

Signals obtained for the injection of nitrite pre-reacted in acidic chloride solution are shown in Figure 5. The effect of acid concentration of eluent containing 18% m/v of sodium chloride, and that of the sodium chloride concentration of eluent 2M in hydrochloric acid, on the signal obtained on direct injection of nitrite are shown in Tables 6 and 7. The effect of delay coil length on the signal for direct injection into the optimised eluent is shown in Table 8.

The effect of the potential of the glassy carbon electrode on the signal obtained for nitrite injected after pre-reaction with acidic bromide or chloride into 0.3M hydrochloric acid solution is shown in Figure 6.

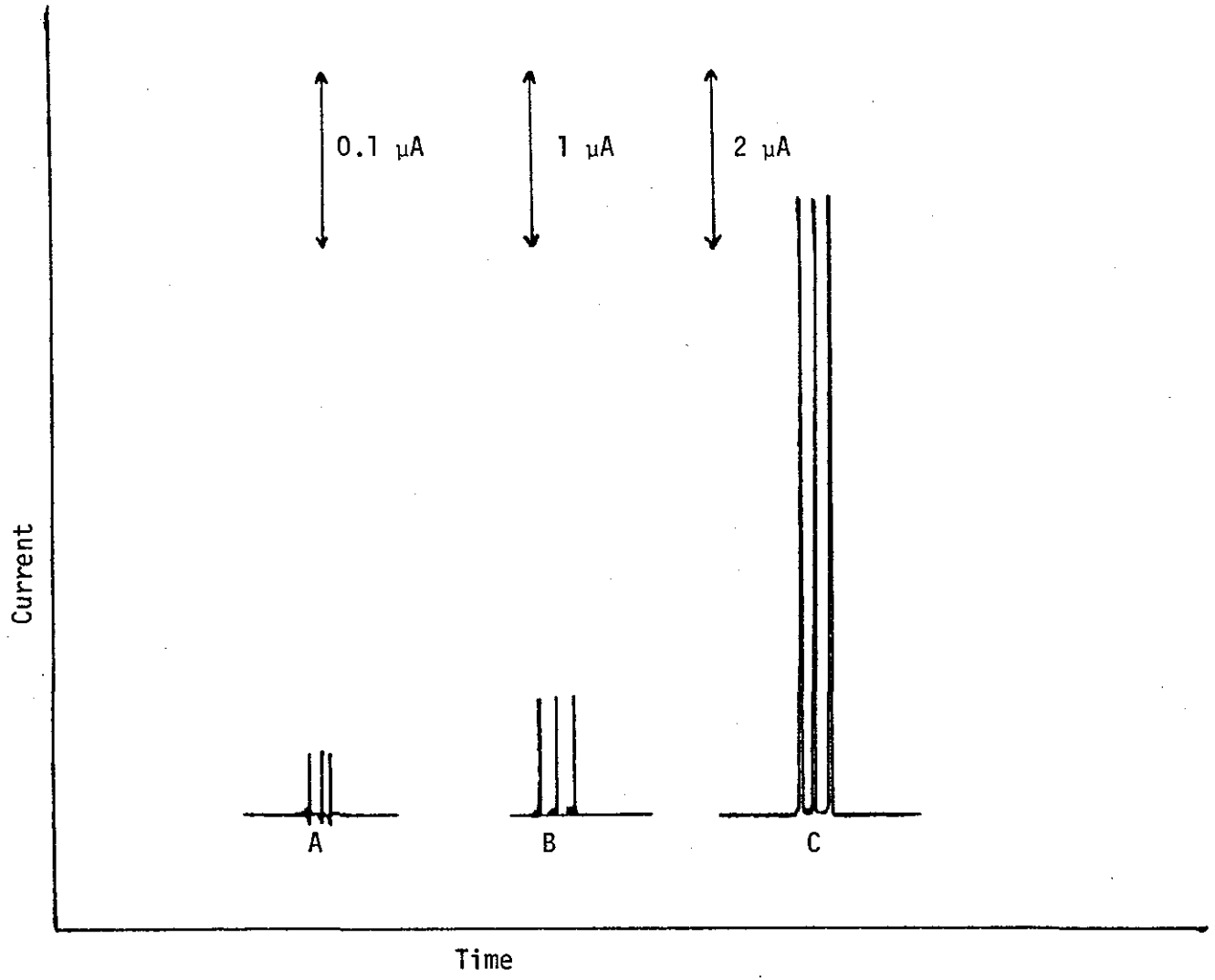


FIGURE 4: Direct injection into acidic bromide solution using recommended procedure. Equivalent nitrite concentration in injection solution: A. 2 a 10^{-6}M; B. 2 x 10^{-5}M; C. 2 x 10^{-4}M.

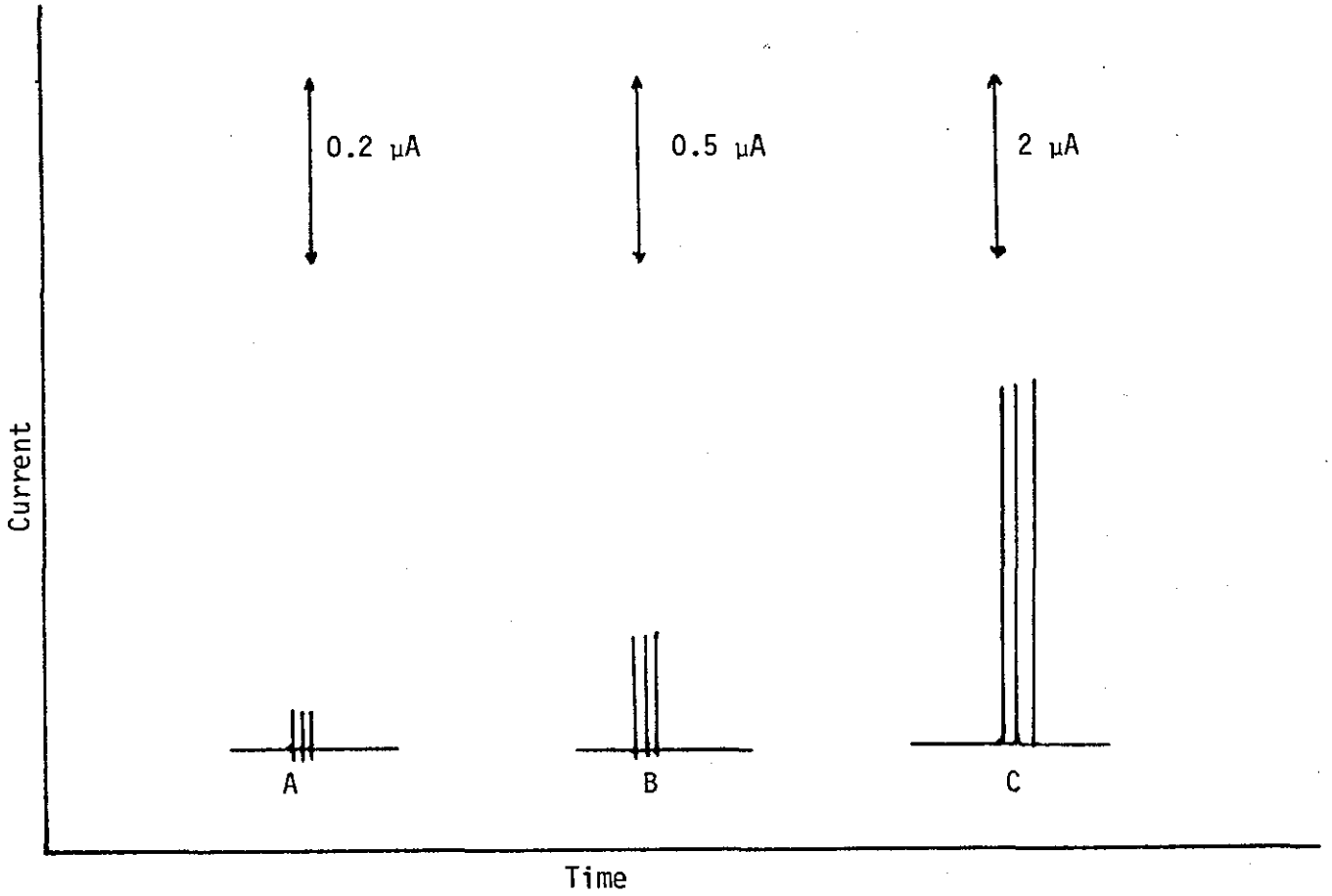


FIGURE 5: Injection of nitrite pre-reacted with acidic chloride solution. Equivalent nitrite concentration in pre-reacted solution injected: A. 2×10^{-6}M; B. 2×10^{-5}M; C. 2×10^{-4}M.

FIGURE 6: Effect of the potential of the glassy carbon electrode on the signal obtained for injection of nitrite pre-reacted with (A) acidic bromide; and (B) acidic chloride into 0.3M hydrochloric acid.
Equivalent nitrite concentration: 2×10^{-5}M.

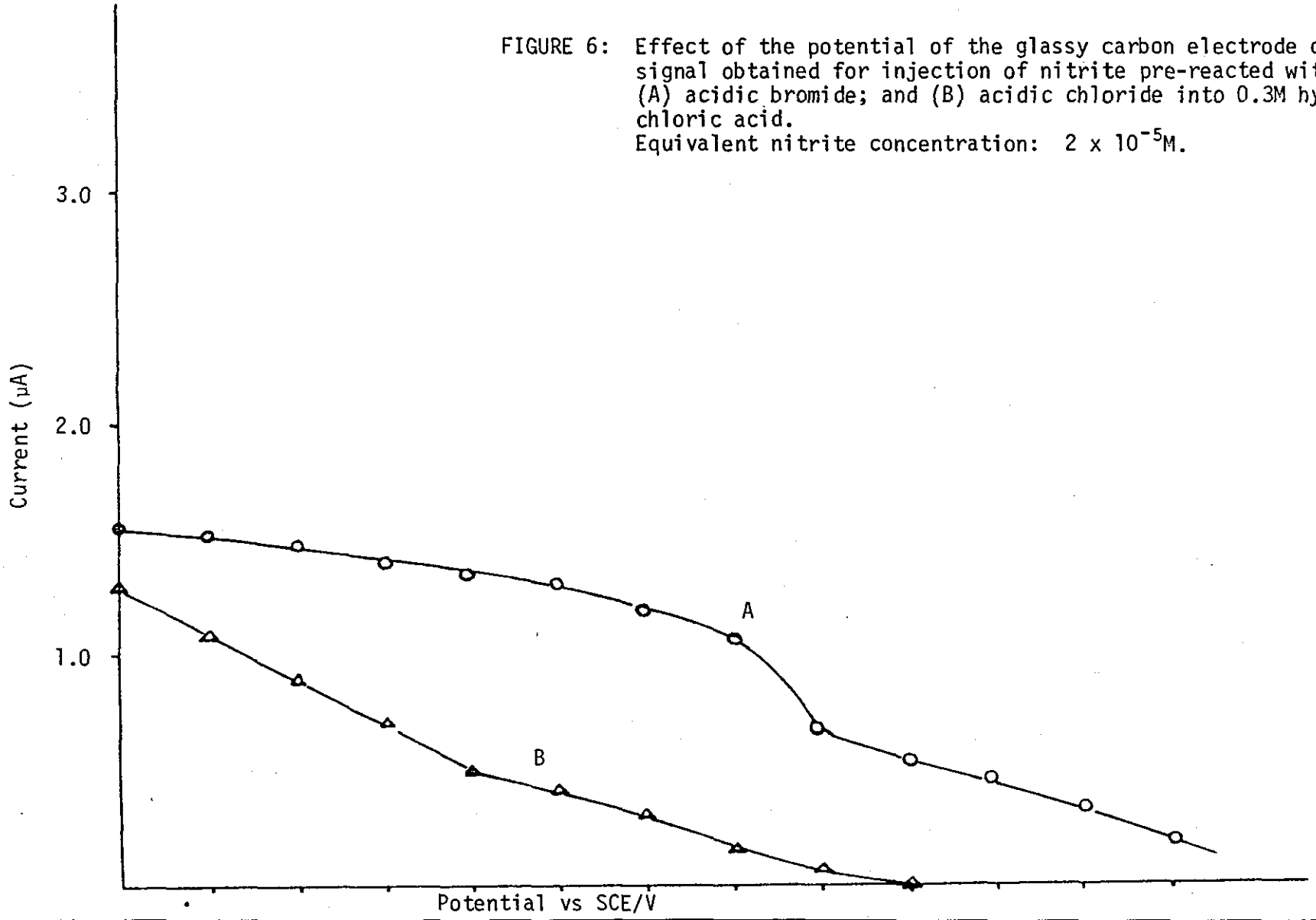


TABLE 6:

Direct injection into acidic chloride: effect of acid concentration of eluent on signal obtained. Sodium chloride concentration = 18% m/v. Nitrite concentration = 2×10^{-4}M

Hydrochloric Acid Concentration/M	0.5	1.0	1.5	2.0
Peak Current/μA	0.20	0.26	0.50	1.60

TABLE 7:

Direct injection into acid in chloride: effect of sodium chloride concentration of eluent on signal obtained. Hydrochloric acid concentration = 2M. Nitrite concentration = 2×10^{-4}M

Sodium chloride Concentration/% m/v	3.0	6.0	9.0	12.0	15.0	18.0	20.0
Peak Current/μA	0.38	0.50	0.70	0.85	1.12	1.30	ppt

TABLE 8:

Direct injection into acidic chloride: effect of delay coil length on signal obtained using recommended eluent. Nitrite concentration = 2×10^{-4}M

Delay coil length/m	0.5	1.0	2.0	3.0	4.0
Peak Current/μA	3.50*	3.00*	2.50*	1.50	0.70

* Double peak

Discussion

Nitrite down to the 10^{-6}M level can be determined very conveniently by flow-injection voltammetry at a glassy carbon electrode by direct injection into an acidic bromide solution. By injecting nitrite pre-reacted with acidic bromide into 0.3M hydrochloric acid solution the determination can be extended to the 10^{-7}M level. A high potassium bromide concentration (20% m/v) is used in the eluent for the direct injection procedure, and therefore the use of the pre-reacted system which uses a lower concentration of bromide would allow a considerable saving of reagent. Clearly, pre-reaction could be carried out in a smaller volume than that used in the recommended procedure (50 ml).

As well as giving a lower detection limit the acidic bromide medium has the additional advantage that adsorption is not a problem with this system. The glassy carbon electrode can be used extensively with this system without the need to clean or polish it. A glassy carbon electrode used with the acidic chloride system requires more frequent cleaning, for example after approximately 5 or 10 injections at the 10^{-4} and 10^{-5}M respectively.

In the reaction of nitrite with bromide and chloride in acidic media an equilibrium appears to be set up, and clearly in the procedures recommended here conversion of nitrite into nitrosyl bromide or nitrosyl chloride is incomplete. More extensive formation of nitrosyl bromide was shown to be obtained at the 10^{-4}M level, for example, by increasing the acidity and bromide concentration even higher.

CHAPTER 7

INDIRECT FLOW INJECTION VOLTAMMETRIC DETERMINATION OF AROMATIC AND ALIPHATIC AMINES

Introduction

Aromatic amines are determined routinely by diazotisation methods. The mechanism and kinetics of nitrosation and diazotisation of aromatic amines have been investigated by Hughes et al[220], among others, and the results have been summarised in two reviews[221-222]. The course of the reaction can be depicted by the following series:

$$\underset{}{Ar.NHR} \rightarrow \underset{I}{ArN(R).NO} \rightarrow \underset{II}{Ar\ N.NOH} \longrightarrow \underset{III}{Ar\ N_2^+} \qquad (1)$$

(R = H, alkyl or aryl)

For primary amines, the final stage is represented by (III), whereas in secondary amines, the ultimate product is I. The introductory step is the rate-determining one.

The reaction can follow different mechanisms, depending on the conditions. In acidic environments $C_{HCl} > 0.1M$ and in the presence of a sufficient concentration of halogenides, nitrosation by means of nitrosyl halides is predominant:

$$HNO_2 + X^- + H^+ \xrightarrow[fast]{} NOX + H_2O \qquad (X = Cl \text{ or } Br) \quad (2)$$

$$NOX + Ar\ NH_2 \longrightarrow Ar.NH_2.NO^+ \xrightarrow[fast]{} Ar.N_2^+ \qquad (3)$$

At higher concentrations aromatic amines may be determined by titration with standard nitrite solution and the end-point determined

biamperometrically[223] preferably in the presence of bromide[224]. Lower concentrations (< 10^{-3}M) of amines are determined frequently by visible spectrophotometry by coupling the diazotised amine with a coupling agent such as N-1-naphthylethylenediamine hydrochloride. In this chapter the flow injection voltammetric procedure given in the previous chapter has been extended to the determination of aromatic amines and aliphatic amines by monitoring the excess of nitrite remaining after diazotization of the amine. Aniline, sulphanilic acid and sulphanilamide were chosen for study as being representative of amines that are diazotised readily. Cephalexin, cephalonium, and penicillin G were chosen to represent the cephalosporins.

Experimental

Flow of eluent was produced with an Ismatec Mini-S peristaltic pump, and injections (25 µl) were made with a Rheodyne injection valve (5020). The injection valve was connected to a Metrohm detector cell (EA 1096), fitted with a glassy carbon electrode (EA 286), by means of suitable lengths of 0.58 mm bore tubing. The detector cell was used as previously partially immersed in 0.01M sulphuric acid solution with the counter and reference electrodes removed. Electrical contact from a platinum counter electrode and a potentiometric calomel reference electrode to the sulphuric acid solution was made by means of salt bridges. It was found to be unnecessary to de-gas the eluent. The potential of the glassy carbon electrode was held at +0.30V versus S.C.E, except as indicated otherwise, using a PAR 174 polarographic analyser (Princeton Applied Research). Current signals were monitored on a Tarkan 600 y-t recorder.

Determination of Amines

Reagents

Standard sodium nitrite solution, approximately 1×10^{-2}M

Dissolve approximately 0.172g of analytical reagent grade sodium nitrite accurately weighed in water and dilute to 250 ml in a calibrated flask. This solution is 1×10^{-2}M in nitrite. Prepare more dilute solutions from this solution.

Hydrochloric acid solution, 0.3M (approx).

Dilute 13 ml of concentrated hydrochloric acid to 500 ml with distilled water.

Hydrochloric acid solution, 3.2M

Dilute 138 ml of concentrated hydrochloric acid to 500 ml with distilled water.

Potassium bromide, 40% m/V

Dissolve 40g of potassium bromide in water and dilute to 100 ml.

Neutral nitrite/bromide eluent, 1×10^{-4}M in nitrite

Dissolve 50g of potassium bromide in water, add by pipette 25 ml of 1×10^{-3} sodium nitrite solution and dilute to 250 ml in a calibrated flask.

Neutral nitrite/bromide eluent, 1×10^{-5}M in nitrite

Prepare as above but use 25 ml of 1×10^{-4}M sodium nitrite solution.

Standard amine solution for pre-reaction with nitrite 1 x 10^{-2}M. Dissolve 0.233g of aniline, 0.433g of sulphanilic acid, 0.431g of sulphanilamide, 0.868g of cephalexin, 1.148g of cephalonium or 0.931g of penicillin G in small volume of dilute hydrochloric acid in case of aromatic amines and, directly in distilled water in case of the β-lactam compounds, dilute to 250 ml with distilled water in a calibrated flask. Prepare less concentrated standard solutions by dilution of these stock solutions with distilled water.

Standard amine solutions for direct injection into neutral nitrite/bromide eluent, 1 x 10^{-2}M

Prepare in the same way as in the standard amines solutions for pre-reaction with nitrite except that solutions should be prepared in 3.2M hydrochloric acid solution. Prepare less concentrated solutions by dilution of these stock solutions with 3.2M hydrochloric acid solution.

Procedures for obtaining calibration graphs:

a) By injection of pre-reacted solutions

Add by pipette 5 ml of 1 x 10^{-3}M sodium nitrite solution and 1-4 ml of 1 x 10^{-3}M amine solution into a 50 ml calibrated flask. Dilute to volume, mix and allow to stand for 20 minutes. Inject 25 µl of this solution into an eluent consisting of 0.3M hydrochloric acid solution, using a 3m delay coil.

For determinations at the 10^{-5}M and 10^{-6}M levels use the same procedure but with standard sodium nitrite and standard amine solutions of 10^{-4}M and 10^{-5}M concentrations.

b) By direct injection into neutral nitrite/bromide eluent

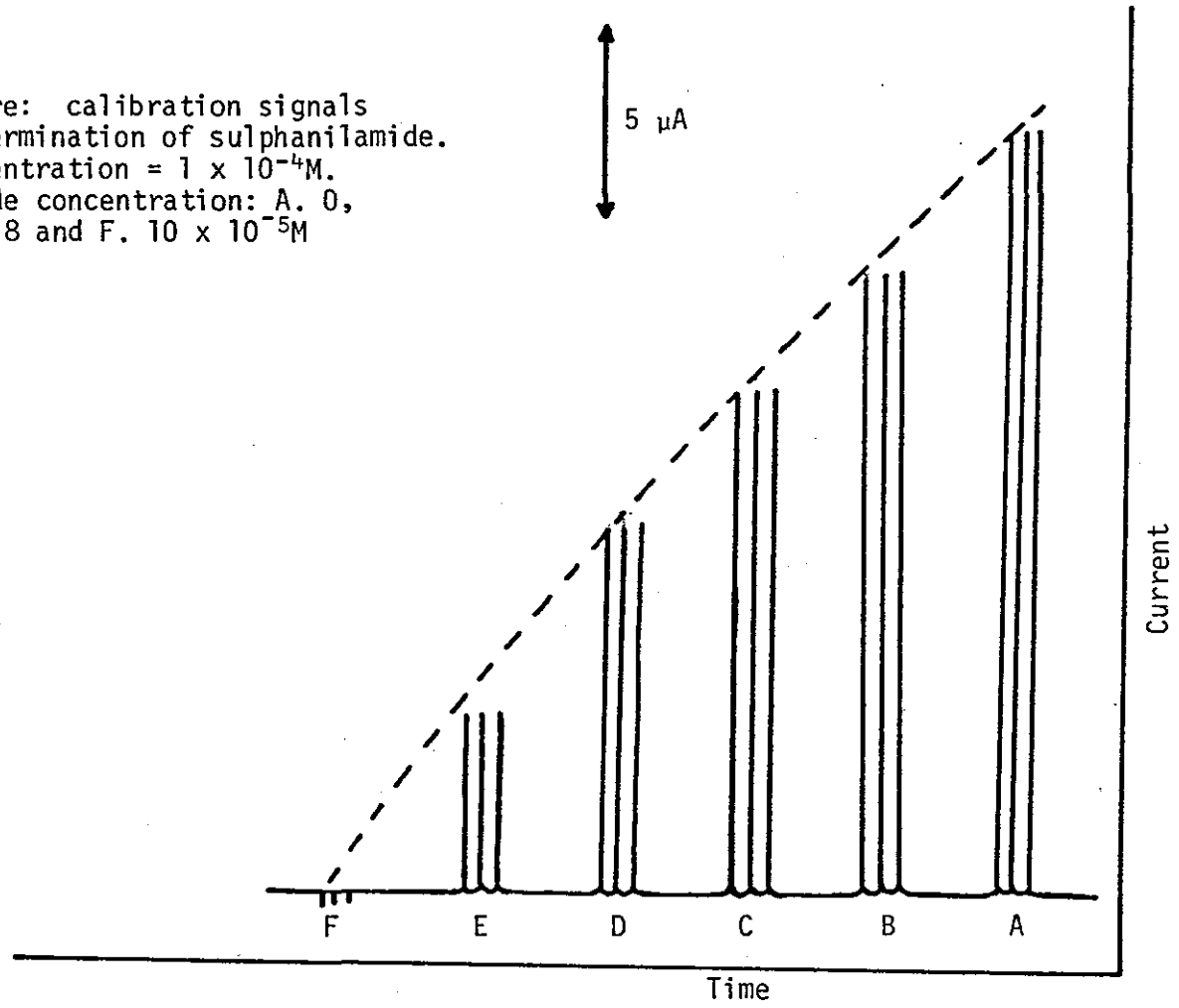
Inject 25 µl of a standard amine (0.2 - 1 x 10^{-4}M) in 3.2M hydrochloric acid solution directly into neutral nitrite/bromide eluent, 1 x 10^{-4} in nitrite. Use a 4m delay coil.

Results

Calibration signals obtained for the determination of sulphanilamide and cephalexin (1-9 x 10^{-5}M) by injection of pre-reacted solutions of amine and nitrite at the 10^{-4}M initial level of nitrite are shown in Figures 1 and 2. The signals have been spaced uniformly in Figure 1 so that the stoichiometry and near reactilinearity of the response are apparent. The small negative blank observed when stoichiometric amounts of nitrite and amine are present remains constant for excess of amine. The calibration graphs for the determination of 1-9 x 10^{-6}M of amine solutions after reaction with 1 x 10^{-5}M nitrite solutions are similar to that shown in Figure 1. In the previous chapter nitrite was determined by using a lower level of acidity in the pre-reacted solutions owing to the apparent instability of low levels of nitrosyl bromide in solutions of higher acidity. This allowed the nitrite to be determined at the 10^{-7}M level. Unfortunately this acidity was too low for diazotisation to take place effectively and the higher acidity had to be used. The use of this higher acidity causes the detection limit obtained using the present pre-reacted method to be higher than might have been expected.

Calibration signals obtained for the determination of sulphanilamide (0.2 - 1.6 x 10^{-4}M) by direct injection of an acidic solution of

FIGURE 1: Pre-reaction procedure: calibration signals obtained for the determination of sulphanilamide. Initial nitrite concentration = 1×10^{-4}M. Initial sulphanilamide concentration: A. 0, B. 2, C. 4, D. 6, E. 8 and F. 10×10^{-5}M



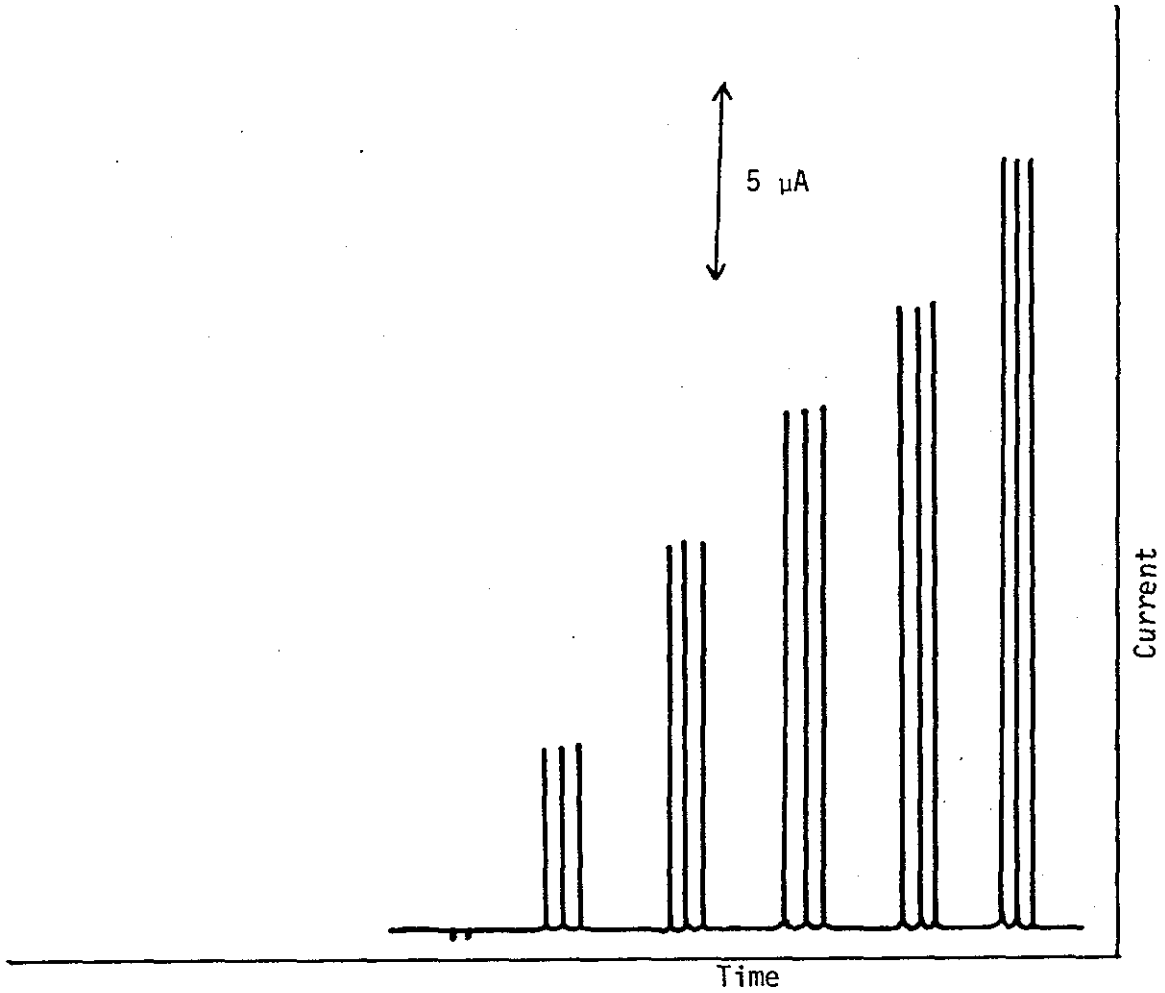


FIGURE 2: Pre-reacted procedure: calibration signals obtained for the determination of cephalexin. Initial nitrite concentration = 1×10^{-4}M. Initial cephalexin concentration A. 0, B. 2, C. 4, D. 6, E. 8 and F. 10 x 10^{-5}M.

sulphanilamide into eluent containing nitrite (1×10^{-4}M) and bromide are shown in Figure 3 which can be compared directly with the signals in Figure 1. The calibration graph obtained for cephalexin by direct injection is shown in Figure 4. The blank signal, which is the full signal from nitrite present, in the eluent, is obtained by injection of 3.2M hydrochloric acid solution. Clearly reaction between nitrite and amines as seen from the sulphanilamide results in Figure 3, is incomplete but although the calibration graph is not rectilinear the decrease in nitrite signal is appreciable between 0 and 1×10^{-4}M (about 42%). Amines above the 1×10^{-4}M level have a decreased effect on the nitrite signal. Above about 1.6×10^{-4}M amine a double peaked signal is obtained indicating reduced reaction at the centre of the sample slug.

Similar non-rectilinear calibration graphs were obtained at lower levels of amines using eluent containing 1×10^{-5}M and 1×10^{-6}M nitrite. The loss of 10^{-5}M and 10^{-6}M nitrite signals between 0 and 1×10^{-5}M and 0 and 1×10^{-6}M amine are both about 29%. The limits of detection for both sulphilamide and cephalexin was calculated to be 1×10^{-10}M. Using an eluent 2×10^{-6}M in nitrite the decrease in signal owing to the injection of sample solution 5×10^{-8}M amine was clearly observed.

Discussion

Two simple methods of determining aromatic and aliphatic amines indirectly by flow injection voltammetry are recommended here. In these the amines are determined by monitoring the excess of nitrite remaining after diazotization. In the first method the nitrite and

FIGURE 3: Direct injection procedure: calibration signals obtained for the determination of sulphanilamide. Nitrite concentration in eluent = 1×10^{-4}M. Sulphanilamide concentration of injected sample: A. 0, B. 2, C. 4, D. 6, E. 8, F. 10, G. 12 and H. 14×10^{-4}M.

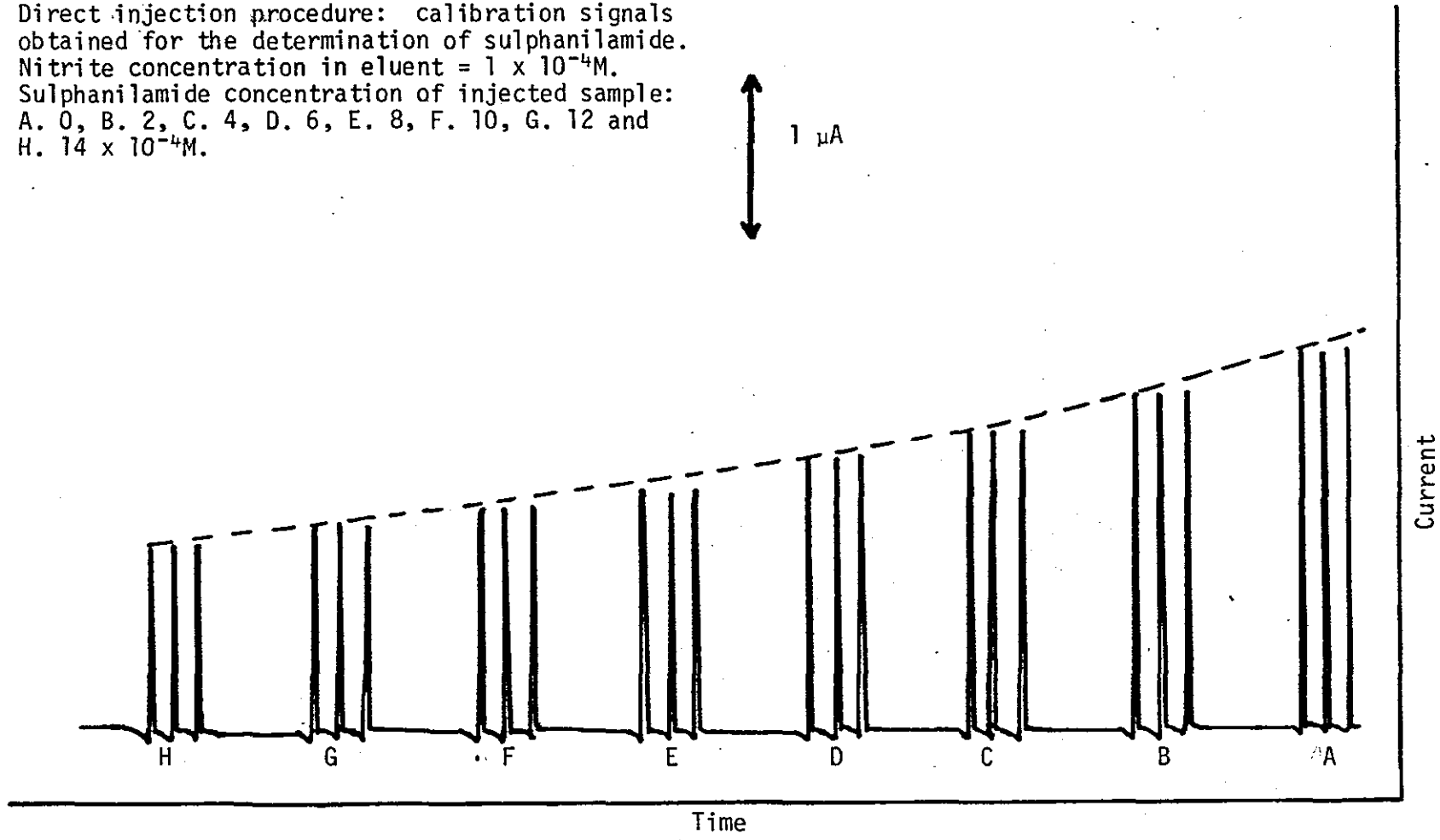
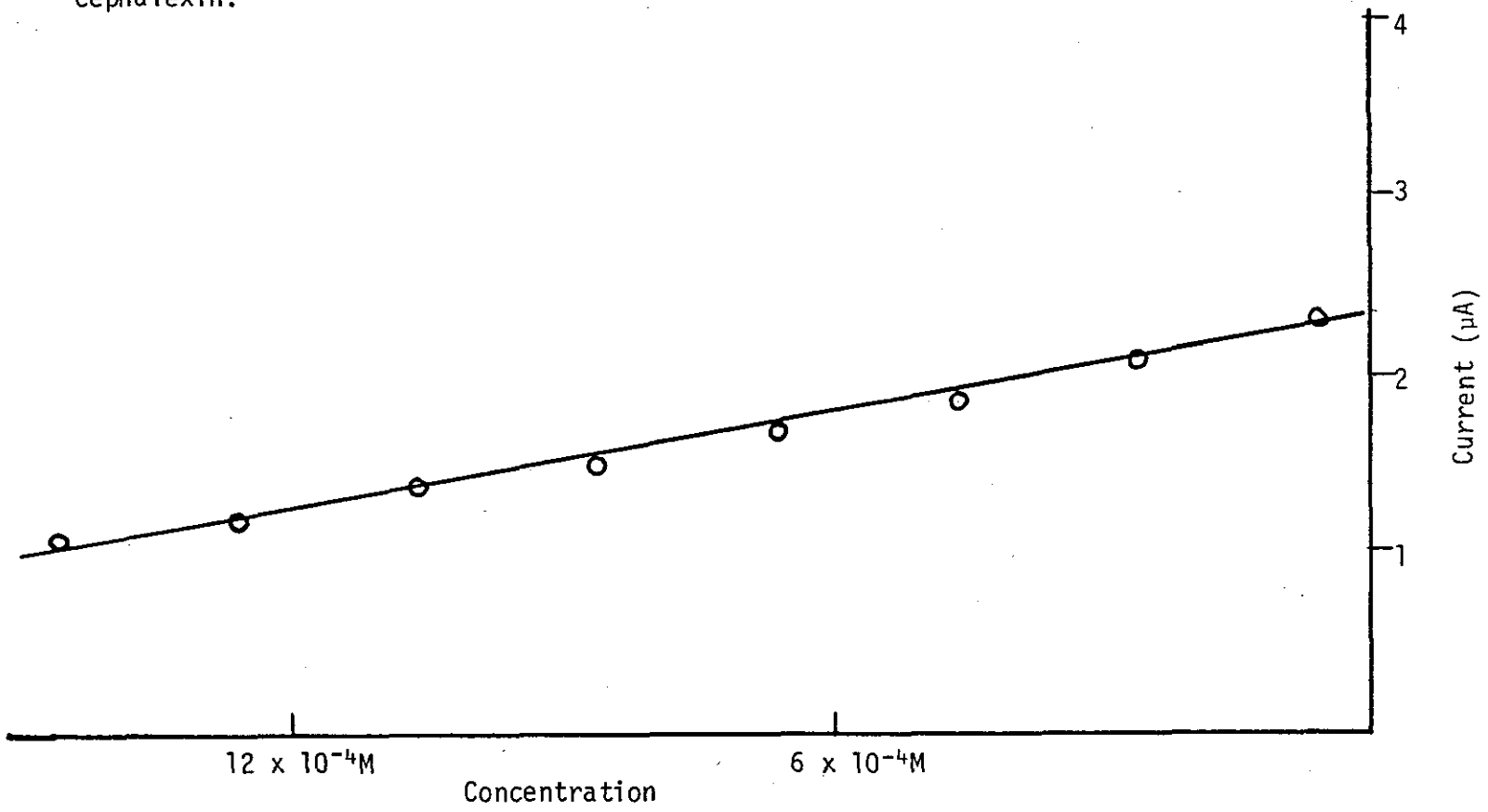


FIGURE 4: Direct injection procedure. Calibration graph obtained for the determination of cephalexin.



amine are pre-reacted before injection. Complete reaction occurs in this case and in many applications this will be preferred. Because of the relatively low apparent stability of the nitrosyl bromide at these acidities, the detection limit for amines is quite high at about 0.5×10^{-6}M. The calibration graphs deviate slightly from rectilinearity. In the second method an acidic solution of the amine is injected directly into a neutral eluent containing nitrite and bromide. Reaction here is incomplete but the method may be more convenient for some applications. Further, because the excess of nitrosyl bromide is determined immediately after it is formed in the delay coil the detection limit (calculated as 1×10^{-10}M) is considerably lower than in the pre-reaction method. For flow injection voltammetric methods in which complete reaction is attained in both pre-reacted and direct injection procedures, the pre-reaction method would normally be expected to give a lower detection limit. The method suffers from the non-rectilinearity of the calibration graphs and the smaller reduction in nitrite signal that occurs relative to the pre-reaction method.

The effect of the degradation products on the signals and the calibration graphs of the β-lactams has not been investigated, however the method proved to be simple, quick and gave reasonable results. The proposed voltammetric method is simpler than related visible spectrophotometric methods in which destruction of the excess of the nitrite with sulphamic acid and coupling of the diazonium salt with a suitable reagent are also required. Many amines, of course, can be determined directly by oxidation at a glassy carbon electrode and for many applications this may be simpler and preferred method. Adsorption

of oxidation products at the glassy carbon electrode with resulting loss of signal can be a problem, however, especially in purely aqueous systems, whereas adsorption in the present method is minimal. Further the present method is more selective for aromatic amines than is direct electrochemical oxidation in which interference may be experienced, for example, from phenol.

CONCLUSION

Previous work had shown that hydrogen sulphide is produced by cephalexin and cephradine in neutral solution. The hydrogen sulphide evolved had been determined indirectly, after blowing it off and absorbing it in cadmium nitrate solution, by means of the decrease in the height of cadmium d.p. peak. The present work has shown that hydrogen sulphide is produced from all cephalosporins in alkaline solution. The hydrogen sulphide produced can be determined in the degraded solution by means of the methylene blue visible spectrophotometric method. Different cephalosporins were shown to give different but reproducible yields of sulphide; the yields varied from 140% for cefuroxime to 64.4% for cephalexin. The yields were calculated assuming that the yield of methylene blue in the reaction of sulphide with N,N-dimethyl-p-phenylenediamine and iron(III) as oxidising agent, which had been determined as 52.2% using standard sulphide solutions, was unchanged in the presence of the cephalosporin degradation products and that the degradation products did not react with the cyclization reagents. The yields of sulphide for all cephalosporins has been confirmed by determining the sulphide formed on degradation by potentiometric titration with standard lead nitrate solution using an Orion sulphide ion-selective indicator electrode.

The alkaline degradation and conversion of the resulting sulphide to methylene blue has been adapted as a sensitive visible spectrophotometric method for the determination of cephalosporins. The manual

visible spectrophotometric method for the determination of cephalosporins by alkaline degradation to sulphide and formation of methylene blue has been adapted for use with an air segmented Autoanalyser I system. The system has been tested for the determination of twelve cephalosporins: rectilinear calibration graphs were obtained with good precision in the general range 8-80 $\mu g\ ml^{-1}$ of cephalosporins. The automated procedure was tested as a method of determining trace amounts of cephalosporins and other sulphide-producing impurities in penicillin G and penicillin V samples. The detection limit was calculated to be 1-2 $\mu g\ g^{-1}$ of cephalosporin in penicillin.

Present work has shown that all cephalosporins and penicillins studied produce ammonia on alkaline degradation. Ammonia reacts with alkaline phenol and sodium hypochlorite to produce indophenol blue. Certain cephalosporins themselves have been shown to give indophenol type reactions. These reactions have been made the basis of three types of sensitive methods of determining cephalosporins and penicillins as follows: (1) ammonia is distilled off from an alkaline solution of the cephalosporin or penicillin into standard hydrochloric acid and then reacted with the indophenol reagent: (2) ammonia is formed by degrading the cephalosporins and penicillins in 0.5 M sodium hydroxide solution for a sufficient period which gives maximum yield of ammonia and the ammonia is then reacted directly without distillation with the indophenol reagent; and (3) neutral freshly prepared β-lactam solutions are reacted directly without hydrolysis or distillation with the indophenol reagent. The yields of ammonia on distillation has been shown to vary from 12.1% for cephoxazole to 136.7% for cephalonium.

The yield of ammonia without distillation for half the cephalosporins is the same but for the other half a lower yield is obtained. For cephaloridine the yield of method (3) is the highest.

The nitrite work originally done in conjunction with Mr N K Bsebsu as a separate work outside this project was shown to have applications in the β-lactam work. Cephalosporins and penicillins can be determined indirectly by flow injection analysis using the nitrite procedure. In this method cephalosporins and penicillins were determined by direct injection at the 10^{-7} - 10^{-4}M level in 3.2M hydrochloric acid solution eluent 10^{-4} or 10^{-5}M in nitrite and 20% m/v in potassium bromide, or by pre-reacting them with the acid-bromide reagent that is 3.2M in hydrochloric acid and 20% m/v sodium bromide.

Finally the sulphide and ammonia results can be used together with the d.p.p. work carried out by others in this laboratory on the degrädation reactions of cephalosporins and penicillins in solution to answer some questions regarding the mechanism of reaction of these compounds in solution. The indophenol methods have not been automated using an air segmented Autoanalyser in the present work, but this would readily be done as the method has proved to be simple and precise. Combination of any two of the methods can be used as a confirmation of identity of the β-lactam. Alternatively two methods could be used for the determination of two compounds.

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