School of Pharmacy

Factors Influencing The Rate of Degradation of Amoxycillin Sodium and Potassium Clavulanate in the Liquid and Frozen states

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"This thesis is presented as part of the requirements for
the award of the Degree of Doctor of Philosophy
of the
Curtin University of Technology"

March 2000

"..This scientific power investigates and apprehends created objects and the laws surrounding them. It is the discoverer of the hidden and mysterious secrets of the material universe and is peculiar to man alone... "

From Bahá'í Writings

To my beloved parents

ABSTRACT

Kinetics of the reactions of amoxycillin sodium and potassium clavulanate alone and in combination were investigated in the liquid and frozen states at selected pH values of 2.0, 4.6 and 7.0. A stability indicating HPLC assay was developed to perform simultaneous quantification of these compounds validated under stressed conditions.

Amoxycillin and clavulanate degradation obeyed first-order kinetics under all conditions of this study. The effect of temperature, buffer, concentrations and complexation were investigated. Both compounds showed acceleration in rates due to general acid catalysis from buffer species. The buffer catalysis rate constants due to total phosphate and total acetate at 55°C were 5.84×10^{-1} (mol $dm^{-3})^{-1}$ h^{-1} and 1.53×10^{-1} (mol $dm^{-3})^{-1}$ h^{-1} for amoxycillin, 2.33 (mol $dm^{-3})^{-1}$ h^{-1} and 4.4×10^{-1} (mol $dm^{-3})^{-1}$ h^{-1} for clavulanate respectively. The buffer independent rate constant values were obtained and interpreted according to the available literature data. Increase in the initial concentration of amoxycillin or clavulanate did not change the first-order rate constant values of these antibiotics significantly at liquid state temperatures. However in the buffer systems, the rate of hydrolysis of amoxycillin in the combination was significantly subject to clavulanate catalysis. This novel finding was influenced by phosphate buffer concentration. A kinetic model was proposed and the second-order catalytic rate constant values at pH 7.0 and 55°C were estimated for clavulanate catalysis of amoxycillin (k_{cvc}) to be $k_{cvc} = 1.75\times10^{2}$ (mol $dm^{-3})^{-1}$ h^{-1} and for phosphate catalyzed of clavulanate catalysis of amoxycillin (k_{phccv}) as $k_{phccv} = 2.87$ (mol $dm^{-3})^{-1}$ h^{-1}.

The temperature dependence of the rate of amoxycillin sodium degradation over the pH range evaluated did not change significantly. However the E_a values of potassium clavulanate decreased slightly with increase in pH. Both the compounds showed similar E_a values at pH 4.6 in acetate system. Hence 71.2 kJ mol^{-1} for amoxycillin and 75.1 kJ mol^{-1} for clavulanate.

The investigation on complexation effects by HPβCD on the rate of hydrolysis of amoxycillin and clavulanate indicated no significant change in the rate of reaction of amoxycillin in the acetate buffer system. But the rate of clavulanate hydrolysis in combination was decreased by approximately 10%. The rate constant

within the cyclodextrin complex and the stability constant of the complex obtained for clavulanate at pH 4.6 and 55°C were $k_c = 1.54\times 10^{-1}\ h^{-1}$ and $K_c = 74.2$ (mol $dm^{-3})^{-1}$.

Extrapolation of the rate constant values to the frozen state from the liquid state data indicated marked acceleration of the rate of amoxycillin and clavulanate in all the pH values investigated. The highest acceleration in rate recorded was 15.0 fold for clavulanate in the hydrochloric acid system and the lowest value was 4.4 fold for amoxycillin at –7.3 °C. The rate constant values obtained were interpreted in terms of the concentration model (Pincock and Kiovsky 1966), phase-temperature relationship of the solutes, buffer catalysis, pH change and polymerization reactions.

In the hydrochloric acid system a kinetic model was deduced providing adequate explanation of the experimental results. The stabilizing effect of sodium chloride used for maintaining constant ionic strength (μ=0.5) was enormous in this system. The shelf-life of amoxycillin was increased from 2.2 h to 58.3 h at –7.3°C when sodium chloride was included in the system. It also stabilized the rates of the reactions significantly in the buffer systems.

The buffer systems used in this study stabilized the rates of the reaction of both the drug compounds considerably. The shelf-life of amoxycillin in phosphate buffer was 621.3 h at –13.5 °C and in acetate buffer the shelf-life of clavulanate was 71.9 h at the same temperature. These are the highest shelf-life values recorded so far in the literature for amoxycillin and clavulanate at this frozen temperature.

ACKNOWLEDGMENTS

First and foremost, I would sincerely thank Prof. Bruce Sunderland for introducing me to the subject and his continual support and encouragement in his roles both as supervisor and as Head of the school of Pharmacy, Curtin University of Technology. His patience and understanding all through my difficult times, and his excellent guidance through out various stages of my work is very much appreciated.

I, also, would like to thank Associate Professor Charles McDonald as co-supervisor for going through my manuscript and providing valuable comments. Also thanks to Associate Professor John Parkin for his comments on NMR.

I am grateful to Mr. Michael Boddy for technical support and assistance with instrument maintenance. I would, also, like to thank Mr. Michael Stack for material and equipment supplies, as well as other staff of School of Pharmacy, Curtin University of Technology for their support in many ways.

I must sincerely thank Associate Professor David Parry, Director of Postgraduate and Research Studies, Faculty of Science, Northern Territory University for providing me with equipment and facilities. I would also like to thank all the technical staff of the Faculty of Science at Northern Territory University, for their support and assistance during my stay in Darwin where I carried out my major experimental work.

I am grateful to Dr Steve Aldous, Head of the Tasmanian School of Pharmacy, and Dr Omar Hassan for providing me with equipment and facilities, as well as other staff members when we moved to Hobart.

I thankfully acknowledge the award granted by Curtin University Postgraduate Scholarship (CUPS).

Finally, appreciation to my family members. Saeid thank you for your patience, support and looking after Samir. And thank you Samir for being patient and so lovingly tolerating the situation. Also thanks to my other family members who are far away, and everyone else who showed understanding and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

GLOSSARY

ABBREVIATIONS AND SYMBOLS

Amox	amoxycillin
Amox-Comb	amoxycillin in combination with clavulanate
ACT	acetate buffer
BP	British Pharmacopoeia
Clav	clavulanate
Comb	combination
CD	cyclodextrin
c_i	concentration of ions
C_l	concentration in the liquid region of the frozen system
C_s	concentration in the thawed solution
β-CD	β-cyclodextrin
HPβCD	hydroxypropyl β-cyclodextrin
d.f	degrees of freedom
Di	dimer
D_2O	deuterium oxide
DMSO	dimethyl sulphoxide
DS	drug substance
FP	formulated product
Ea	energy of activation
Hy	hydrolysis
HPLC	high performance liquid chromatography
HAc	acetic acid
NaAc	sodium acetate
IP	ion pair
I-PrOH	iso propanol
n-PrOH	normal propanol
MeOH	methanol
MeCN	acetonitrile

PO_4	phosphoric acid
PHOS	phosphate buffer
REF	references
RP	reverse phase
SE	standard error
TEAA	tetraethyl ammonium acetate
Temp	temperature
TLC	thin layer chromatography
t cal	calculated student's t test
t tab	tabulated student's t test
h	hours
min	minutes
°C	degree celcius
kJ	kilojoule
MHz	megahertz
K_a	ionization constant
k	rate constant
k_H	specific proton catalyzed rate constant
k_{obs}	observed rate constant
a_H	hydrogen activity
k_{OH}	specific hydroxide ion catalytic rate constant
k_{hyd}	rate constant due to hydrolysis
k_w	spontaneous water catalyzed rate constant
K_w	dissociation constant of water
k_c	rate constant within the complex
K_c	stability constant of the complex
k_{cvc}	rate constant due to clavulanate catalysis of amoxycillin
k_{Phccv}	rate constant for phosphate catalyzed of clavulanate catalysis of amoxycillin
k_{Exp}	rate constant obtained by experiment
k_{pred}	predicted rate constant
r	correlation coefficient
t	temperature

$t_{½}$	half-life
t_{90}	shelf-life
A_{mx}	amoxycillin
A	frequency factor
K_f	cryoscopic constant
R	gas rate constant
T	absolute temperature
ΔT_f	freezing point depression
i	van't Hoff factor
μ	ionic strength
w/v	weight per volume
USP	United States Pharmacopoeia
UV	ultraviolet-visible
z_i	valence of species
HCl	hydrochloric acid
KCl	potassium chloride
NaCl	sodium chloride
Na^+	sodium ion
NaOH	sodium hydroxide
Cl^-	chloride ion
H^+	proton
H_3C	citric acid
H_2C^-	dihydrogen citrate ion
HC^{2-}	monohydrogen citrate ion
C^{3-}	unprotonated citrate ion
$H_2PO_4^-$	dihydrogen phosphate ion
HPO_4^{2-}	monohydrogen phosphate ion
H_3BO_3	boric acid
NH_2	amino group
NH_3^+	protonated amino group

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE SURVEY

The stability of medicinal compounds has gained in importance. Various institutions and pharmaceutical manufacturing companies have introduced essential programs necessary to study systematically the decomposition of drugs and their excipients. The standard testing procedures introduced by the regulatory authorities are constantly being reviewed and upgraded to ensure high quality and optimum storage conditions of drug compounds are achieved. Drug stability testing has become mandatory in new drug applications. It has become more evident that the stability of the pharmaceutical compounds not only applies to the chemical stability of a drug substance in a particular dosage form but also to its pharmaceutical properties such as dissolution, disintegration, hardness and also the microbiological requirements that need to be met.

In the last decade intravenous additive services have become an essential part of many hospital pharmacies and community health care centres. To make this service efficient it is often necessary for the pharmacist to prepare the intravenous solutions in advance. Recognizing this fact, many manufacturers of intravenous solutions have been working to produce readily useable intravenous drug solutions. These dosage forms are usually called "premixed" drug solutions. There are already several premixed solutions available, commercially, as either liquid or frozen aqueous solutions. One of the primary advantages of these dosage forms is time and cost savings for pharmacists and other health care workers (Chilmakurti 1992; Lynn 1982). As the premixed dosage forms are manufactured products, quality assurance standards are established to minimize the risk of medication errors and contamination which could be associated with hospital ward-based admixing processes. Many of the intravenously administered drugs, particularly antibiotics such as penicillins including ampicillin and amoxycillin and the cephalosporins, do not posses sufficient stability in aqueous solutions for long term storage. Therefore, freezing the drug solutions had been an option to improve the shelf-life. However many data in the literature indicate that freezing could reduce the shelf-life of some drug substances such as ampicillin and amoxycillin (Chilamkurti and Vieira 1994; McDonald *et al.* 1989b; Concannon *et al.* 1986; Lynn 1982; Dinel *et al.* 1977;

Ashwin and Lynn 1975; Savello and Shangraw 1971; Lynn 1970). Therefore an understanding of the nature and behaviour of these drug substances under the particular storage conditions is essential.

Various factors such as the chemical nature of the drug, diluent, pH, method of freezing and frozen storage conditions affect the stability of drug substances. Each drug may show different physico-chemical characteristics when subjected to freezing and thawing process. Therefore each drug and diluent combination must be carefully evaluated, to enable specification of the storage conditions.

Development of frozen dosage forms requires a thorough understanding of concentration and phase changes as freezing occurs, as well as the kinetics of drug degradation under these modified conditions. Several reasons have been proposed for an enhanced reaction rate in frozen systems. These include, increased concentrations of reactants in the liquid region of a frozen system when the temperature is above the eutectic temperature, enhanced proton mobility of ice, crystal imperfections, the dielectric properties of ice and the existence of catalytically active sites on the ice surface (Pincock 1969). With the exception of the concentration effect none of the others has as yet been shown experimentally to be the cause of increased reaction rates. Theoretically these accelerated effects should not affect the first-order rate constants, but may occur in such reactions, where one of the reactants is present in a large excess of concentration, or the pH of the buffers have changed under freezing conditions. It would, however, expect to influence the rate constant of higher order reactions.

Amoxycillin as its sodium salt is widely used in Australia for the treatment of uncomplicated penicillin-sensitive infection in appropriate patients. But its use is limited since it can easily be destroyed by a wide range of β-lactamase producing clinically important bacteria, such as *H. influenzae, E. coli*. Potassium clavulanate on the other hand is a potent inhibitor of the enzyme lactamase and hence broadens the antibacterial spectrum of amoxycillin by exerting a pronounced synergistic effect, when administered concomitantly, but exhibits only weak antibacterial activity itself (Todd and Benfield 1990; Yogev, Melick and Kabat 1981; Stein and Gurwith 1984; Brown 1986). The trihydrate form of amoxycillin in combination with potassium clavulanate is currently used in a tablet dosage form and as a dry powder for reconstitution as a suspension. The sodium salt of amoxycillin (more soluble form)

in combination with potassium clavulanate tends to be used for intravenous dosage formulations. Aminopenicillins such as amoxycillin are generally stable in their solid dosage forms above 0°C whereas the stability is significantly decreased when they are reconstituted into liquid preparations. In a study (Ashwin and Lynn 1987) on the stability of intravenous (IV) Augmentin® (amoxycillin sodium in combination with potassium clavulanate) recommendations were made for maximum periods permissible between reconstitution of IV Augmentin® and completion of the infusion (in the commonly used electrolyte or lactate fluid or water for injections). These were reported to range from between three to four hours. The study concluded that if refrigeration of the reconstituted IV infusions were to occur, a maximum of 8 hours at 5°C could be allowed. Hence the liquid dosage form of this combination drug has limited application in premixed formulations. Although there is a report on the stability of amoxycillin sodium in aqueous solution below 0°C (McDonald *et al.* 1989b), there are no data on stability studies of amoxycillin sodium in combination with clavulanate in the frozen state.

This project aims to investigate the stability of amoxycillin, which undergoes autocatalytic and hydrolytic degradation in its combination with potassium clavulanate in liquid and frozen states. In addition factors affecting the rate of the reaction and additives that might influence the reaction rate in the frozen state will be evaluated. The project was designed to provide further insight into the reaction rates in the frozen state, so that it may lead to the production of solutions of drugs with longer shelf-lives which would be economically beneficial especially with the health policy movement from hospital-based to community -based medical care.

1.1 Literature Survey

Amoxycillin an amphoteric penicillin, is β-amino-p-hydroxybenzyl penicillin, a semi-synthetic penicillin with a broad spectrum of antibacterial activity. It was first discovered at Beecham Laboratories in 1971 and marketed by Beecham Pharmaceuticals in 1972. A co-formulation with potassium clavulanate [amoxycillin trihydrate-potassium clavulanate (Augmentin®)], marketed by Beecham in 1981 extended the antibacterial spectrum to include β-lactamase producing organisms. Amoxycillin, which is on the World Health Organisation's list of essential drugs, is used as the trihydrate in oral products and as the sodium salt in parenteral products.

Although the chemical, microbiological and pharmacological properties of the two drugs are being constantly studied, there has been very little work done on the stability of this important combination dosage forms in the liquid and particularly the frozen states. The aim of this literature survey is to consider those aspects of the studies relevant to this research project.

1.1.1 The chemical stability of amoxycillin

1.1.1.1 Stability of amoxycillin solution in the liquid state

Several workers (Tsuji *et al.* 1978; Zia, Shalchian and Borhanian 1977; Bundgaard 1977a) have studied the kinetics of the degradation pathway of amoxycillin in aqueous solution. Studies in dilute aqueous solutions (10^{-4} – 10^{-3} mol dm^{-3}) have been carried out over the pH range 0.3 to 10.5 at 35°C (Tsuji *et al.* 1978), 1.1 to 10.8 at 35°C (Zia, Shalchian and Borhanian 1977), 8.2 to 12.6 at 35°C (Bundgaard 1977a), 2 to 7 at 30°C, 40°C,50°C and 60°C (Doadrio and Sotelo 1988) and 1.5 to 9 at 37°C (Moll and Esperester 1984). These studies show that at constant pH the degradation followed first-order kinetics, with a minimum rate at about pH 6 (Tsuji *et al.* 1978; Zia, Shalchian and Borhanian 1977; Doadrio and Sotelo 1988). The results also indicated that degradation was subject to general acid base catalysis by citrate and phosphate buffers (Tsuji *et al.* 1978; Zia, Shalchian and Borhanian 1977), with a 10 fold increase in rate being ascribed to phosphate in one study (Moll and Esperester 1984). Increasing ionic strength was reported to have a positive effect on reaction rate in alkaline and a negative effect in acidic media (Sapena *et al.* 1985).

At higher concentrations (6×10^{-2} - 3×10^{-1} mol dm^{-3}) and at pH 8.6 to 10 and 35°C the rate of degradation was found to follow a higher order of reaction than first order. This was thought to be indicative of a dimerization reaction (Bundgaard 1977a).

Therefore it can be concluded that amoxycillin has two routes of degradation (Scheme 1.1), namely dimerization and hydrolytic cleavage of the β-lactam ring. Based on the literature reports (Hou and Pool 1969a; Bundgaard 1977a; Bird *et al.* 1983; Haginaka and Wakai 1986) the mechanism of amoxycillin degradation is illustrated in Scheme 1.1. The dimerization pathway proceeds through nucleophilic attack by the free side chain amino group in one molecule upon the β-lactam carbonyl group in a second molecule. Dimerization is subjected to general base

catalysis by amino and ionized phenolic groups of the side-chain of other amoxycillin molecules.

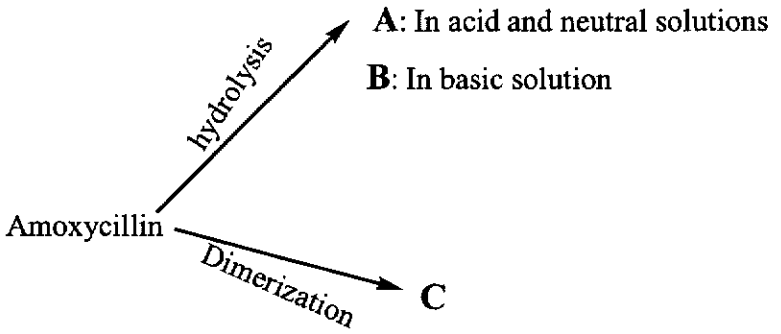
Degradation by hydrolysis includes the hydrolytic opening of the β-lactam ring to give the corresponding penicilloic acid (VI) and penicillinic acid (III) in alkaline and acidic solutions respectively (Scheme 1.1). Epimerisation of penicilloic acid in aqueous solution has been reported (Bird *et al.* 1983; Fong, Johnson and Kho 1983; Haginaka and Wakai 1986) to occur in wide pH range between 2.5-13. Studies (Bird, Jennings and Marshal 1986) on degradation products of amoxycillin have indicated compounds such as pyrazine, small amounts of penicillamine (in acidic pH) and a small amount of N-formylpenicillin (in neutral pH) were found in acidic and neutral pH systems.

The concentration dependent dimerization pathway is the predominant degradation pathway at higher concentration and alkaline pH values. Bundgaard (1977a) concludes that in low amoxycillin concentration solutions, the concentration independent hydroxide-ion-catalyzed hydrolysis becomes of greater significance, dimerization still plays an important part in the total degradation pathway, particularly at low alkaline pH values.

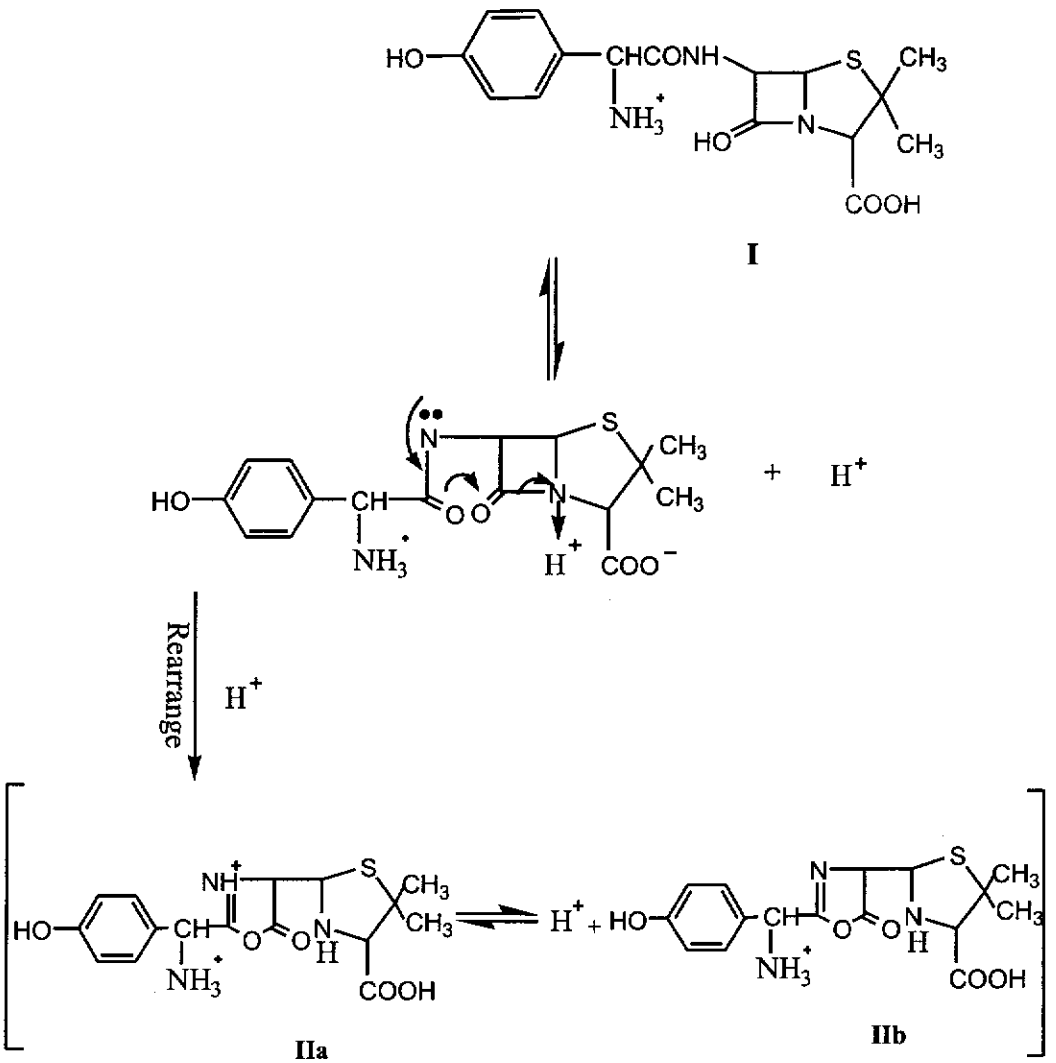
1.1.1.1.1 Kinetics of rates of reactions of amoxycillin solution

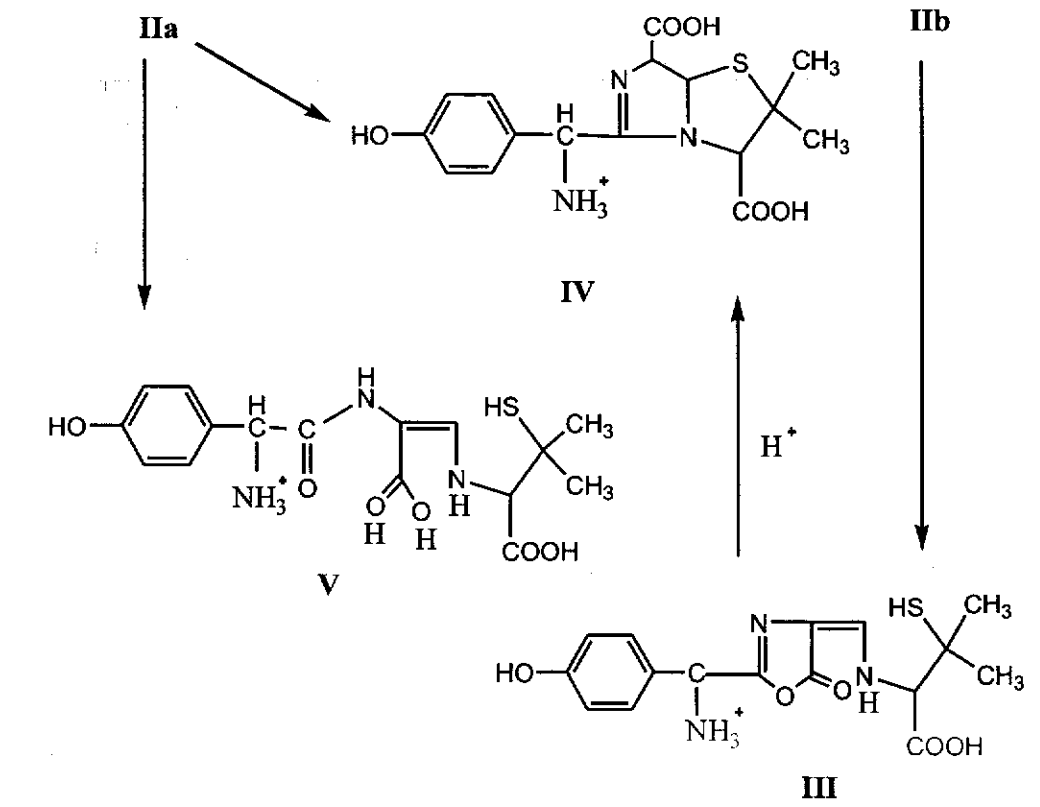
The work done thus far on the kinetics of the degradation of amoxycillin suggests that the two degradation pathways occur concomitantly as shown in Scheme 1.1. Amoxycillin in aqueous acidic solution, contains three dissociable protons, attached to the carboxyl, the aromatic hydroxyl, and the α-ammonium groups. Accordingly amoxycillin may exist in different ionized forms depending on the pH of the solution. Bundgaard (1977a) has studied various ionized forms of amoxycillin in alkaline pH (Scheme 1.2). The values of microscopic and macroscopic ionization constants for these ionic forms of amoxycillin have been reported as shown in Table 1.1 (Bundgaard 1977a; Bird 1992).

Scheme 1.1: Degradation paths of amoxycillin

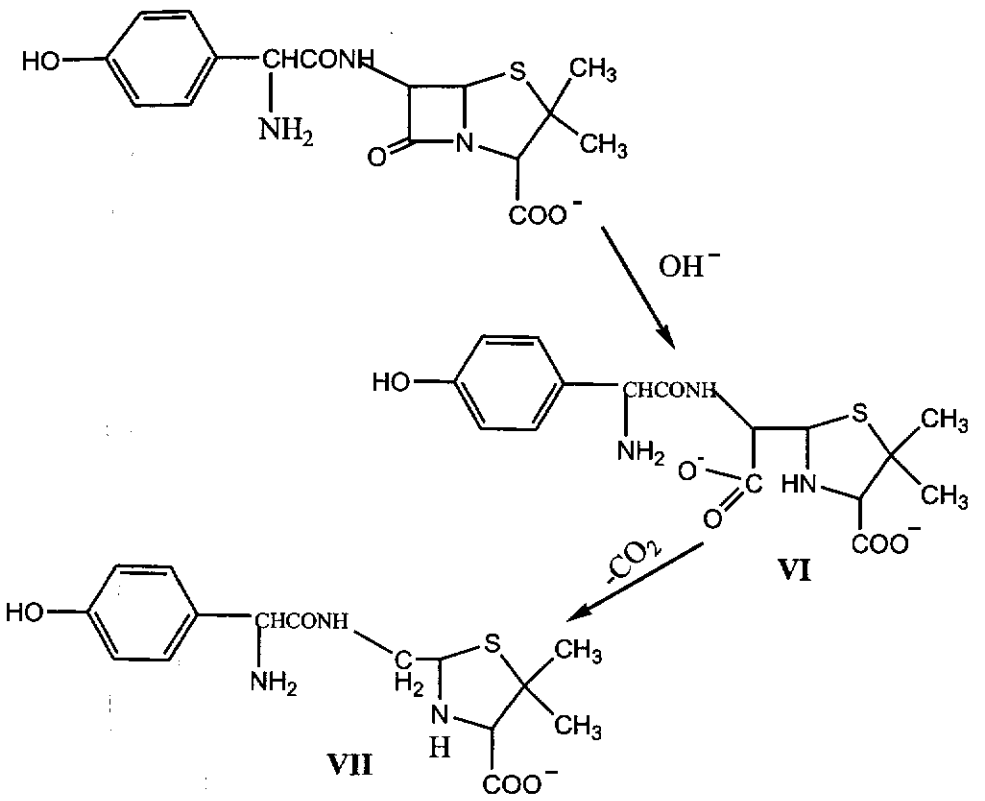


A: In acid and neutral solutions

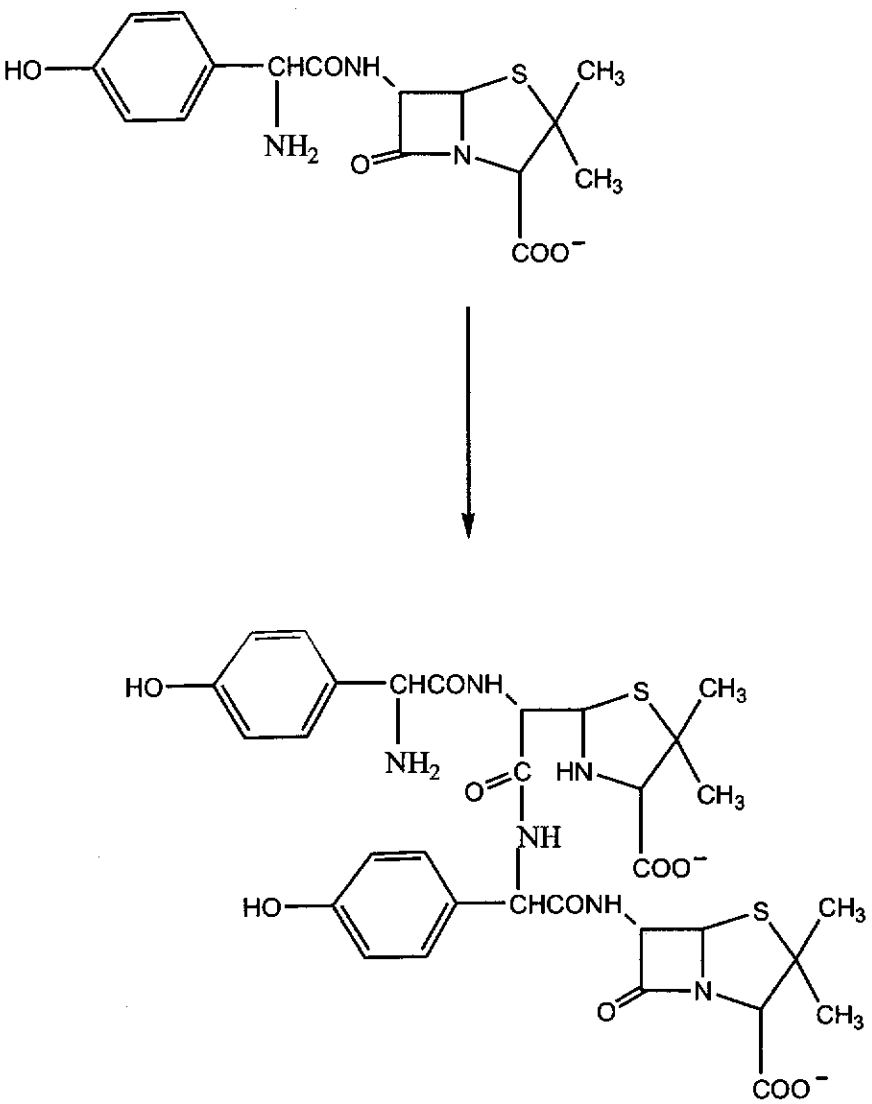




B: In basic solution



C: Dimerization reaction



VIII

Scheme 1.2: Possible ionized forms of amoxycillin in solutions of weak acidic pH to alkaline pH.

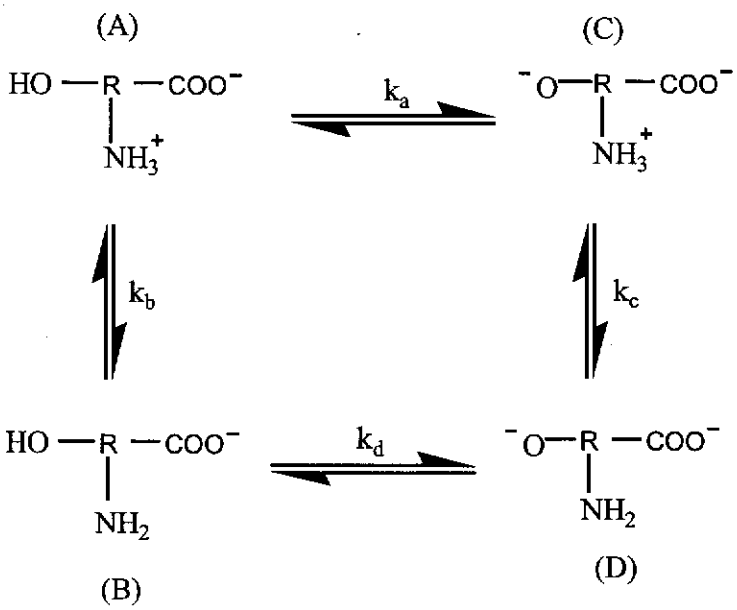


Table 1.1: The macroscopic and microscopic dissociation constants for the three ionizable groups of amoxycillin, (a) Macro dissociation constants

SOLVENT	TEMP (°C)	pK_a COOH	NH_2	OH	REF
Water	22	2.4	7.4	9.6	(Bird 1992)
Aq. KCl, μ* 0.5	37	2.67	7.11	9.55	(Tsuji *et al.* 1978)
Aq. KCl, μ 0.5	35	2.87	7.28	9.65	(Zia, Shalchian and Borhanian 1977)
Aq. KCl, μ 1.0	23	2.63	7.55	9.64	(Bundgaard 1977a)
Aq. KCl, μ 1.0	35	2.61	7.30	9.45	(Bundgaard 1977a)

* μ = ionic strength

Table 1.1: The macroscopic and microscopic dissociation constants for the three ionizable groups of amoxycillin, (b) Micro dissociation constants (Bundgaard 1977a)

SOLVENT	TEMP (°C)	pK_a			
		NH_2^* (OH)	NH_2 (O^-)	OH (NH_3^+)	OH (NH_2)
Aq. KCl, μ 1.0	23	7.58	8.49	8.70	9.61
Aq. KCl, μ 1.0	35	7.33	8.24	8.51	9.49

* The column headed NH_2 (OH) gives the dissociation constant of the NH_2 group for the form in which the carboxyl group is ionised and the phenolic group is not ionised, etc.

The microscopic ionization constant referred in Scheme 1.2 and Table 1.1 is defined as follows:

$$k_a = \left(\frac{a_H[C]}{[A]}\right) \qquad k_b = \left(\frac{a_H[B]}{[A]}\right) \qquad k_c = \left(\frac{a_H[D]}{[C]}\right) \qquad k_d = \left(\frac{a_H[D]}{[B]}\right) \qquad (1.1)$$

where a_H is the hydrogen ion activity. Bundgaard (1977a) has defined the relationship between the macroscopic ionization constant K_2 (for ammonium group) and K_3 (for phenol group) to microscopic ionization constants as in the following equations:

$$K_2 = k_a + k_b \qquad (1.2)$$

$$1/K_3 = (1/k_c) + (1/k_d) \qquad (1.3)$$

$$K_2 K_3 = (k_a k_c) + (k_b k_d) \qquad (1.4)$$

The macroscopic ionization constants values reported were determined by potentiometric and spectrophotometric titration methods.

Knowledge of each possible ionized species of amoxycillin and its concentration at particular pH values is fundamental, since each ionised species can have different chemical reactivities. The relative concentrations of each ionized form of amoxycillin present as at pH range 8-12, has been provided by Bundgaard (1977a) as a function of pH (Figure 1.1).

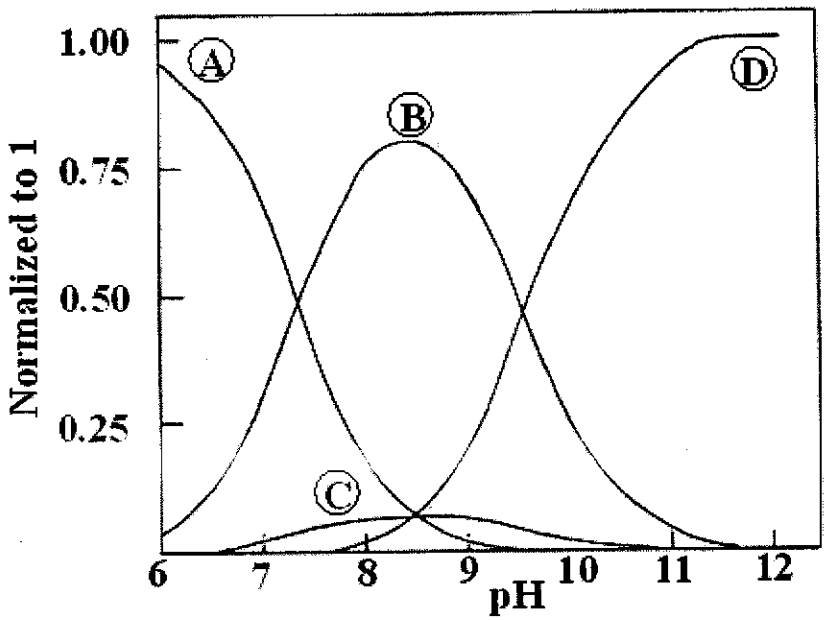


Figure 1.1: Relative concentrations of the various ionic forms of amoxycillin at different pH at 35°C and μ =1.0 ionic strength (Bundgaard 1977a). A, B, C and D are the ionic species shown in Scheme 1.2.

i. Kinetics of dimerization: The work of Bundgaard (1977a) has shown that at constant temperature and pH, the rate of dimerization of amoxycillin shows both second-order and third-order dependencies on amoxycillin concentration. If A_{mx} represent amoxycillin and *Di* the dimer product, then the important reactions contributing to the dimerization are as follows:

$$A_{mx} + B \xrightarrow{k_1} Di \qquad (1.5)$$

$$A_{mx} + D \xrightarrow{k_2} Di \qquad (1.6)$$

$$A_{mx} + B \xrightarrow[k_3]{+B} Di \qquad (1.7)$$

$$A_{mx} + B \xrightarrow[k_4]{+D} Di \qquad (1.8)$$

$$A_{mx} + D \xrightarrow[k_5]{+D} Di \qquad (1.9)$$

where B and D are the ionic species shown in Scheme 1.2 and $k_1 \ldots k_5$ represent specific rate constants for uncatalysed or water-catalyzed aminolysis (k_1 and k_2) and general base catalysis by amino and phenolate groups (k_3 to k_5). The overall rate equation for loss of amoxycillin by dimerization therefore can be written as,

$$\text{Rate} = k_1[B][A_{mx}] + k_2[D][A_{mx}] + k_3[B]^2[A_{mx}] + k_4[B][D][A_{mx}] + k_5[D]^2[A_{mx}] \qquad (1.10)$$

where *[B]* and *[D]* represent the molar concentration of the relevant amoxycillin species.

In Equation 1.10, Bundgaard (1977a) has excluded general-acid catalyzed dimerization reactions due to the limited solubility of the compound at pH values where the concentration of amino-protonated amoxycillin would be significant which makes it experimentally difficult to investigate.

Also excluded from the Equation 1.10 are the reactions involving the zwitterion C species (Scheme 1.2). It has been shown (Bundgaard 1977; Connors, Amidon and Stella 1986) that inclusion of these reactions in Equation 1.10 would not result in different kinetic behaviour, it would only influence the values of k_3 and k_4. The reported rate constants for the loss of amoxycillin via dimerization is shown in Table 1.2.

Table 1.2 : Rate constants values for dimerization of amoxycillin at 35°C

(Bundgaard 1977a)

k_1 $*M^{-1}h^{-1}$	k_2 $M^{-1}h^{-1}$	K_3 $M^{-2}h^{-1}$	k_4 $M^{-2}h^{-1}$	k_5 $M^{-2}h^{-1}$
0.09	0.5	0.4	1.6	2.0

*M = mol dm^{-3}

ii. Kinetics of hydrolytic degradation: As illustrated in Scheme 1.1, concomitantly with the intermolecular self-aminolysis reaction, amoxycillin also undergoes degradation by hydrolysis via opening of the ß-lactam ring giving the corresponding penicilloic acid. Data provided by Bundgaard (1977a) indicate that, at low amoxycillin concentrations such as 10^{-4} mol dm^{-3} to 10^{-3} mol dm^{-3}, the rate of hydrolysis is first-order in amoxycillin concentration and first–order in hydroxide ion activity in the pH range 8.2-12.6, see Equation 1.10 and Figure 1.2.

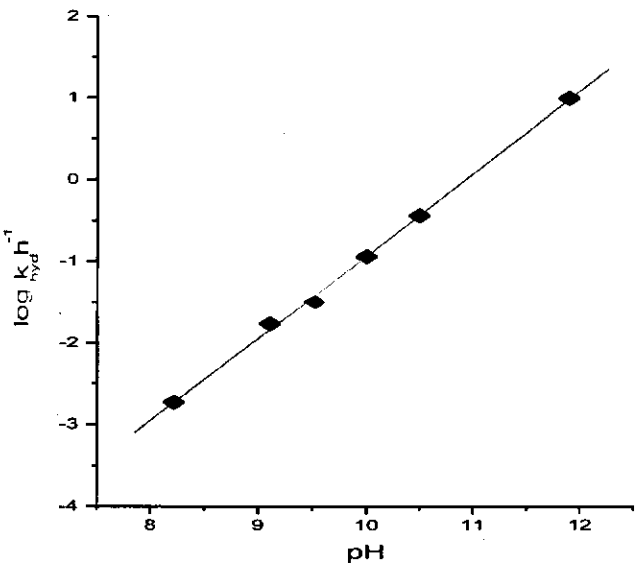


Figure 1 .2 : Plot of the logarithm of observed pseudo-first-order rate constants (k_{hyd}) for hydrolysis of amoxycillin at 35° C and μ = 1.0 versus pH. (Bundgaard 1997a)

$$-\frac{d[A_{mx}]_T}{dt} = k_{hyd}\,[A_{mx}]_T = k_{OH}\,a_{OH}\,[A_{mx}]_T \qquad (1.11)$$

In Equation 1.11, k_{OH} is the specific hydroxide ion catalytic rate constant. It is evident from Figure 1.2 that there exists a linear relationship between the first-order rate constant, k_{hyd} and pH, implying that the different ionic forms of amoxycillin, do not differ in regard to their susceptibility to react with hydroxide ions in the pH range 8-12.5. This fact also explains why the different ionic forms of amoxycillin

possess identical ß-lactam reactivities in aminolytic reactions. Bundgaard (1977a) has obtained the value of k_{OH} to be 1.15×10^3 (mol $dm^{-3})^{-1}$ h^{-1} at 35°C. This value is similar to that of ampicillin [1.33×10^3 (mol $dm^{-3})^{-1}$ h^{-1}] reported previously by Bundgaard (1976) under the same experimental conditions. Therefore, amoxycillin and ampicillin possess similar ß-lactam reactivity in aminolytic reactions.

The rate of hydrolysis of amoxycillin at higher amoxycillin concentrations (0.06 - 0.3 mol dm^{-3}) has shown greater than a first-order dependence on the amoxycillin concentration. Bundgaard (1977a) found that amoxycillin also undergoes self-catalyzed hydrolysis by the phenoxide ion of one amoxycillin molecule which hydrolyses the ß -lactam ring in a second molecule. As it is seen in Scheme 1.2, of the two ionic species (C and D) of amoxycillin containing the ionized phenol group, species D is said to be predominantly involved as a catalyst of hydrolysis. The self-catalyzed hydrolysis is unique to amoxycillin, because ampicillin, which differs from amoxycillin by lacking the hydroxyl group, undergoes no self-catalyzed hydrolysis under identical conditions.

Taking into account the various protonated and deprotonated species of amoxycillin, the important reactions due to the hydrolysis pathway of amoxycillin degradation can be written as follows (Connors, Amidon and Stella 1986).

$$A_{mx}H_3^+ + H^+ \xrightarrow[H_2O]{k_6} PDS \qquad (1.12)$$

$$A_{mx}H_2^{\pm} + H^+ \xrightarrow[H_2O]{k_7} PDS \qquad (1.13)$$

$$A_{mx}H_2^{\pm} \xrightarrow[H_2O]{k_8} PDS \qquad (1.14)$$

$$A_{mx}H^- \xrightarrow[H_2O]{k_9} PDS \qquad (1.15)$$

$$A_{mx}H^- + OH^- \xrightarrow[H_2O]{k_{10}} PDS \qquad (1.16)$$

$$A_{mx}^{2-} + OH^- \xrightarrow[H_2O]{k_{11}} PDS \qquad (1.17)$$

$$A_{mx}^{2-} + A_{mx}^{2-} \xrightarrow[H_2O]{k_{12}} PDS \qquad (1.18)$$

where A_{mx} indicates amoxycillin presented in various protonated and deprotonated states and *PDS* represents products. Combining the acid-base and self-catalyzed hydrolysis reactions, the following rate equation describes the total rate of hydrolysis reactions.

$$\text{Rate} = k_6 [A_{mx}H_3^+] [H^+] + k_7 [A_{mx}H_2^\pm] [H^+] + k_8 [A_{mx}H_2^\pm] + k_9 [A_{mx}H^-] + k_{10} [A_{mx}H^-] [OH^-] + k_{11} [A_{mx}^{2-}] [OH^-] + k_{12} [A_{mx}^{2-}]^2 \quad (1.19)$$

In Equation 1.19, k_6 and k_7 are the second order rate constants for specific acid catalysis of $A_{mx}H_3^+$ and $A_{mx}H_2^\pm$ respectively, k_8 and k_9 are the first order rate constants for reaction of $A_{mx}H_2^\pm$ and $A_{mx}H^-$ with water, and k_{10} and k_{11} are the second-order rate constants for specific base catalysis of $A_{mx}H^-$ and A_{mx}^{2-}. The second–order rate constant for the self-catalyzed hydrolysis of amoxycillin is k_{12}.

Zia and co-workers (1977) and Tsuji *et al.* (1978) investigated the hydrolysis kinetics of amoxycillin over a wide range of pH values in order to abstract the rate constants shown in Equation 1.19. These workers monitored the concentration of amoxycillin during the time course of the degradation. However, their analytical method was not able to differentiate between the monomer and dimer species. Moreover the hydrolysis and dimerization reactions have different pH dependencies. Since these workers used a low concentration of amoxycillin such as 10^{-3} mol dm^{-3} where hydrolysis plays the major role in the degradation pathway, their data are to be considered useful whenever low concentrations of amoxycillin are required. It can be argued that even at low concentrations and at the lower pH (8.6) values, the work of Bundgaard (1977a) indicates that dimerization is a significant degradation pathway. However the concentration of amoxycillin used by these workers is about 10 times lower than that used by Bundgaard to calculate the relative contribution of dimerization and hydrolysis pathways to the overall degradation of amoxycillin. The lowest pH value used by Bundgaard (1977a) was 8.6.

iii. Equation for the overall degradation of amoxycillin: The rate of the overall degradation of amoxycillin can be expressed as the following sum of the rates of hydrolysis and dimerization (Bundgaard 1977a; Connors, Amidon and Stella 1986).

$$(\text{rate})_{\text{total}} = (\text{rate})_{\text{hydrolysis}} + (\text{rate})_{\text{dimerization}}$$

or

$$-\frac{d[A_{mx}]_T}{dt} = \frac{d[A_{mxOH}]}{dt} + 2\frac{d[Di]}{dt} \qquad (1.20)$$

The relative contribution of dimerization (Di) and hydrolysis (Hy) to overall degradation of amoxycillin is shown in Table 1.3 as a function of pH and initial concentration.

Table 1.3: Calculated relative contributions (in percentage) of hydrolysis and dimerization reactions to the overall initial rate of degradation of amoxycillin in aqueous solution at 35°C and μ = 1.0 (Bundgaard 1977a; Connors, Amidon and Stella 1986)

AMOXYCILLIN CONCENTRATION		pH 8.6		pH 9.2		pH 10.0	
mol dm^{-3}	(% w/v)	Hy	Di	Hy	Di	Hy	Di
0.477	20	7	93	10	90	9	91
0.238	10	13	87	21	79	24	76
0.119	5	24	76	38	62	51	49
0.048	2	46	54	63	37	79	21
0.024	1	64	36	78	22	91	9

The results in Table 1.3, show that dimerization becomes the predominant degradation reaction with increased amoxycillin concentration. Even at lower amoxycillin concentration dimerization still plays a significant role especially at the lowest pH evaluated.

1.1.1.1.2 Buffer effects on stability of amoxycillin

Several workers (Zia, Shalchian and Borhanian 1977; Tsuji *et al.*1978; Girona *et al.* 1984) have investigated the effect of various buffers on amoxycillin degradation. In these studies Zia and coworkers (1977) investigated the catalytic effect of citrate buffer pH 3.03-6.55 and phosphate buffer pH 6.73-8.00. The study indicates that various buffer species have different catalytic effects on the amoxycillin

moiety (Table 1.4a). The buffer catalytic rate constants determined by these workers are listed in Table 1.4b. In another report (Girona *et al.* 1984) the catalytic effect of acetic acid-acetate mixture on amoxycillin was studied. Acetic acid-acetate mixture was found to have an advantage over citrate buffer owing to a lower catalytic rate constant value.

Table 1.4: (a) Kinetic data on degradation of amoxycillin at 35°C and μ = 0.5 (Zia, Shalchian and Borhanian 1977)

Buffer Ingredients, mol $dm^{-3} \times 10^{-2}$											
pH	KCl	HCl	H_3C	H_2C^-	$HC^{=}$	C^{3-}	$*P_1$	$*P_2$	$*B_1$	$*B_2$	$*k_{obs}$
1.10	40.0	10.0	-	-	-	-	-	-	-	-	13.82
1.50	45.0	5.0	-	-	-	-	-	-	-	-	7.57
2.10	49.0	1.0	-	-	-	-	-	-	-	-	3.07
3.03	45.3	-	5.41	4.50	0.09	-	-	-	-	-	7.37
3.72	40.4	-	1.83	7.47	0.70	0.001	-	-	-	-	3.84
4.45	33.4	-	0.29	6.44	3.23	0.034	-	-	-	-	1.68
5.50	20.0	-	0.01	1.37	7.70	0.926	-	-	-	-	0.55
6.55	03.0	-	-	0.08	4.23	5.702	-	-	-	-	0.29
6.73	29.7	-	-	-	-	-	4.83	5.17	-	-	1.96
7.00	26.7	-	-	-	-	-	3.34	6.66	-	-	2.42
7.48	22.9	-	-	-	-	-	1.42	8.58	-	-	2.85
8.00	21.0	-	-	-	-	-	0.48	9.52	-	-	3.05
8.26	48.3	-	-	-	-	-	-	-	8.30	1.70	2.88
9.22	43.5	-	-	-	-	-	-	-	3.49	6.51	11.52
10.8	40.1	-	-	-	-	-	-	-	0.09	9.91	63.33

where, $* k_{obs} \times 10^{-2}$ (h^{-1})

$*P_1 = H_2PO_4^-$ $*P_2 = HPO_4^{=}$ $*B_1 = H_3BO_3$ $*B_2 = H_4BO_4^-$

Table 1.4: (b) Buffer catalytic rate constants of amoxycillin at 35 °C and μ = 0.5 (Zia, Shalchian and Borhanian 1977)

BUFFER	RATE CONSTANTS (mol $dm^{-3})^{-1}$ h^{-1}					
	*k_1	k_2	k_3	k_4	k_5	k_6
Citrate	5.27×10^{-1}	3.12×10^{-1}	3.0×10^{-2}	1.0×10^{-4}	-	-
Phosphate	-	-	-	-	7.15×10^{-2}	2.87×10^{-1}

*Rate constants corresponding to the following buffer species:

$k_1 = H_3C$; $k_2 = H_2C^-$; $k_3 = HC^=$; $k_4 = C^{3-}$; $k_5 = H_2PO_4^-$ and $k_6 = HPO_4^=$

1.1.1.1.3 Effect of temperature on the stability of amoxycillin

The effect of temperature on the stability of amoxycillin was investigated by Zia, Shalchian and Borhanian (1977), in 0.1 mol dm^{-3} citrate buffer at pH 4.45 and μ = 0.5 and in non-buffered solution of pH 10.47 and μ = 0.5. The apparent activation energies (E_a) reported by these workers were 75.7 and 104.6 kJ mol^{-1} respectively. In another report (Doadrio and Sotelo 1988) the temperature dependence of amoxycillin was studied at various pH values (Table 1.5). The results provided by these authors at pH around 4.0 is consistent with that of reported by Zia and co-workers (1977).

Table 1.5: Activation energy data for amoxycillin at various pH range and μ = 0.5 (Doadrio and Sotelo 1988)

pH	E_a (kJ mol^{-1})
2.0	102.93
3.0	78.66
4.0	77.40
5.0	61.09
6.0	39.96
7.0	46.86

1.1.1.1.4 Effect of pH on the degradation rates of amoxycillin

There are two reports of pH – rate profiles for amoxycillin in the literature. One is the work of Bundgaard (1977) which is over a limited pH range 8.2-12.6 and considered by others (Connors, Amidon and Stella 1986) to be of greater value due to the analytical method used, which differentiates between the dimeric and monomeric forms. The pH–rate profile reported in that study shows a linear proportionality between pseudo-first-order rate constant k_{hyd} and hydroxyl ion concentration (Figure 1.2).

The second report is the work of Zia and co-workers (1977) and Tsuji *et al.* (1978). Both have demonstrated similar pH-rate profiles for amoxycillin over a much wider pH range (0.3 - 10.8). However their analytical method may be flawed. These workers used a low concentration of amoxycillin 10^{-3} mol dm^{-3} and the iodometric titration method, which does not differentiate between the monomeric and dimeric species of amoxycillin. Tsuji *et al.* (1978) reported at constant pH, with excess buffer and a low amoxycillin concentration (5×10^{-3} mol dm^{-3}), the degradation of amoxycillin followed first-order kinetics and the β-lactam cleavage of amoxycillin molecule was subjected to general acid-base catalyses (this may explain the differences in catalysis rate in buffers). This is in agreement with other workers (Zia, Shalchian and Borhanian 1977 and Bundgaard 1977a). The pH dependence curve of amoxycillin reported by these workers is U shaped and similar to that obtained by Zia and co-workers (1977), see Figure 1.3. The pH-rate profile was also found to be similar to that of ampicillin (Hou and Pool 1969). According to Zia, Shalchian and Borhanian (1977) at the region of pH = pK_{a2} (second dissociation constant due to α- ammonium group) in the pH-rate profile of amoxycillin (Figure 1.3) the shoulder type break in the ascending part of the curve is more evident than that of ampicillin. Tsuji and co-workers (1978) have concluded that since there is no break near pK_{a3} (third dissociation constant due to aromatic hydroxyl group) of the pH-rate profile of amoxycillin, the dissociation of the phenolic moiety, apparently has no effect on the rate of β-lactam cleavage. This claim can only be true for low concentrations of amoxycillin whereas at higher concentration where self-catalysed hydrolysis plays an important role, the ionized phenolic moiety in one molecule

catalyses the hydrolysis of the β-lactam ring of another molecule (Bundgaard 1977a) as it was described in Section 1.1.1.1.1 ii. The pH for the minimum rate at zero buffer concentration was reported to be 5.77 by Zia and co-workers (1977). Where as in citrate buffer the pH for maximum stability was found to be 6.5 by the same workers.

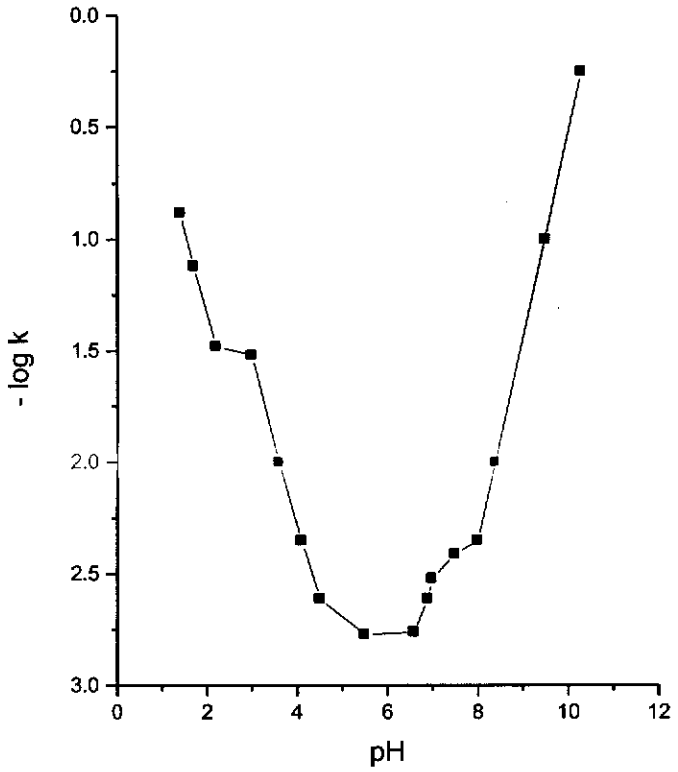


Figure 1.3: pH-rate profile of amoxycillin degradation at 35°C and 0.5 ionic strength. (Data taken from: Zia, Shalchian and Borhanian 1977)

1.1.1.1.5 Stability of amoxycillin in intravenous fluids

There are several reports on the stability of amoxycillin sodium in various intravenous infusion fluids (Cook, Hill and Lynn 1982; Wildfeuer and Rader 1996). Degradation was reported to be faster at higher amoxycillin concentrations (Cook, Hill and Lynn 1982) and particularly in fluids containing dextrose, dextran or sorbitol (Cook, Hill and Lynn 1982; Wildfeuer and Rader 1996). Other studies also have shown that carbohydrates and alcohols have a deleterious effect on the stability of amoxycillin in solution (McDonald *et al* 1989b; Pujol *et al.* 1986). These reports

have shown that compounds containing poly-hydroxyl groups, such as carbohydrates and polyhydric alcohols, are highly reactive with penicillins in infusion solutions. These compounds in alkaline solution undergo nucleophilic reactions with penicillins to form penicilloyl esters, which subsequently hydrolyse to give penicilloic acids. Also in the case of α-aminopenicillins such as amoxycillin, aminolysis products analogous to those formed in the absence of such adjuvants (Bundgaard and Larsen 1978; Bundgaard and Larsen 1979) have been reported. Amoxycillin was found to be most stable in water and in solutions of sodium chloride or sodium chloride with potassium chloride and far less stable in glucose or dextran fluids. Little difference in stability was found between 1% and 2% amoxycillin solutions in various infusion fluids, but the antibiotic was significantly less stable in a 5% solution. Solutions in lactate or bicarbonate had intermediate stability. The antibiotic was, for practical purposes, unstable in 30% sorbitol solution (Cook, Hill and Lynn 1982). Table 1.6 lists the t_{90} values (the time necessary for potency to fall to 90% of its initial value) for various concentrations of amoxycillin in selected intravenous fluids. These workers also compared their data with the published data on ampicillin (Cook, Hill and Lynn 1982; Stjernstrom *et al.* 1978; Jacobes *et al.* 1970) and concluded that amoxycillin was less stable than ampicillin in saline and that they had similar stability in 5% glucose solutions.

A more recent study on the stability of ampicillin infusions (Allwood and Brown 1993), in unbuffered and buffered saline revealed that ampicillin is too unstable to recommend storage after reconstitution or dilution in infusions, including 0.9% sodium chloride. The study recommends that such infusions must be used within 12-24 h after preparation unless buffered. A 13.6% w/v potassium acid phosphate solution was used by these workers as a buffering agent, which improved the shelf-life to the extent of 6-12 days, depending on the ampicillin concentration. Cook and co-workers (1982) ruled out the possibility of buffering amoxycillin with sodium bicarbonate, which was reported by some workers (Stjernstrom *et al.* 1978) to be valuable in stabilizing ampicillin.

There are further reports on the stability of amoxycillin in liquid and frozen states, which will be discussed under the stability in the frozen state (Section 1.1.1.3).

Table 1.6: Stability periods in terms of t_{90} (time for the antibiotic concentration to decrease to 90% of its initial concentration) of amoxycillin at different concentration in intravenous fluids at 25°C (Cook, Hill and Lynn 1982)

INTRAVENOUS FLUID	INITIAL AMOXYCILLIN CONCENTRATION (W/V)		
	1%	2%	5%
Water	8 h	8 h	3 h
Sodium chloride 0.9%	8 h	8 h	3 h
[a]Sodium chloride 0.9%	8 h	8 h	3 h
[b]Compound sodium lactate	6 h	6 h	3 h
Sodium lactate M/6	6 h	6 h	3 h
Sodium bicarbonate 2.74%	6 h	4 h	2 h
Sodium bicarbonate 8.4%	4 h	3 h	1 h
Glucose 5%	2 h	1.5 h	1 h
Dextran 40, 10% in sodium chloride 0.9%	2 h	1.5 h	≤ 1.5 h
Dextran 40, 10% in glucose 5%	1 h	≤ 1 h	≤ 1 h

[a] = with potassium chloride 0.3% ; [b] = Hartmann's solution

In summary the review in the liquid state suggests that though there are reports on the kinetics of degradation of amoxycillin, these have various limitations such as the methods of analysis, concentration effect and temperature effect. No thorough investigation of this area with adequate stability indicating analytical methods to cover a full range of pH values has yet been reported.

1.1.1.2 Stability of amoxycillin in the solid state

Penicillins such as amoxycillin are generally stable in their powdered forms for an extended period of time, however when they are reconstituted into liquid preparations, stability is significantly decreased (Tu *et al.* 1988). There are two reports (Mehta *et al.* 1994; Tu *et al.* 1988) available on the stability of reconstituted amoxycillin trihydrate in combination with clavulanate in oral suspension. In one report (Mehta *et al.* 1994) the stability was evaluated at 20°C and 8°C by determining the shelf-life of the reconstituted suspension over the period stated on

the label. The authors concluded their investigation by indicating that the suspension should be refrigerated at all times during the period of use (7 days). Because the clavulanate component which is less stable than amoxycillin lost 10% of its labelled content after 2 days at 20°C. The study however reports no further investigation on kinetics and mechanism of degradation of the drug compounds.

In the other report (Tu *et al.* 1988), the stability of the reconstituted oral suspension antibiotics were studied in original containers and in prepackaged commercially available oral syringes stored at temperatures 25, 5 and -10°C. According to this study the degradation of amoxycillin trihydrate followed zero-order kinetics and that of potassium clavulanate, pseudo-first-order kinetics. The study reports no significant degradation of either amoxycillin or clavulanate when stored at –10°C for 14 days. However the report indicates that prepakaging the product in the oral syringes caused substantial fall in the drug's activity especially for clavulanate when the storage temperature was increased from –10°C to 25 °C. These authors concluded that amoxycillin was far more stable than the clavulanate in all the conditions investigated and that loss of clavulanate was the overall stability limiting factor in these oral suspensions.

Kinetic studies on the rate of decomposition of amoxycillin trihydrate and sodium salt in the solid state at 37°C-110°C has been reported (Mendez *et al.* 1989). Results for the sodium salt were reported as indicating a sequential two step degradation. The trihydrate showed first order kinetics at 37°C and 50°C but at the higher temperature its degradation rate was consistent with formation of a solid and a gas. Rate constants were derived which were extrapolated to 20°C and used to calculate the time for 10% degradation as 1.2 and 3.2 years for the sodium salt and trihydrate respectively. These authors also calculated the Arrhenius parameters for both forms of amoxycillin. However no mention was made of the possible effects of water content which is well known to be important for the solid state stability of all penicillins.

Results consistent with a sequential two step degradation were found for both amoxycillin trihydrate and the sodium salt in open containers at 80°C to 140°C (Plotkowiak 1987). The same author found that under controlled humidity conditions degradation was first order at 23 to 90% relative humidity (RH) (at 64°C

- 90°C) for the trihydrate and at 50 to 90% RH (at 40°C - 70°C) for the sodium salt, although at 23% RH sequential reactions occurred with the sodium salt (Plotkowiak and Nogowska 1989).

The logarithm of the first order rate constants at a fixed temperature increased linearly with RH (Plotkowiak and Nogowska 1989) or with the logarithm of the vapour pressure (Plotkowiak 1989), confirming the importance of water in the degradation of these compounds.

1.1.1.3 Stability of amoxycillin solution in the frozen state

The literature indicates that several drugs in solution such as ampicillin sodium and amoxycillin sodium degrade at frozen state temperatures at faster rates than in the liquid state at higher temperatures (Dinel *et al.* 1977; Savello and Shangraw 1971; Schwartz and Hayton 1972; Hiranka, Frazier and Gallelli 1972; Lynn 1970; Ashwin and Lynn 1975; Concannon *et al.* 1986; Ashwin, Lynn and Taskins 1987; McDonald *et al.* 1989a; McDonald *et al.*1989b). This has been explained due to the concentration effect of solutions in the frozen state (Savello and Shangraw 1971; McDonald *et al.*1989b). Savello and Shangraw (1971) reported that a 1% ampicillin sodium solution in water degraded 2% after 24 h at 50°C, but when frozen at -20°C the same solution showed 5.2% degradation in same period of time.

There are a few reports (Concannon *et al.* 1986; McDonald *et al.* 1989a; McDonald *et al.*1989b) in the literature on the rate of degradation of amoxycillin in the frozen state. McDonald and coworkers (1989b) reported that the t_{90} for a 1% amoxycillin sodium solution in normal saline decreased from 252 h at 0°C (thawed state) to 8 and 14 h at -6.5°C and -19.2°C respectively, when stored in the frozen state. Another report (Concannon *et al.* 1986) on the rate of decomposition of amoxycillin sodium at temperatures of 19.5°C to -30°C indicated that amoxycillin sodium was unstable in aqueous solutions when stored between 0°C and -20°C. It was recommended therefore to improve the stability of the admixture, the drug solution should be kept at storage temperature below -30°C. These workers did not present any mechanism on kinetics of degradation of amoxycillin in the apparently frozen state. McDonald *et al.* (1989b), have studied the stability of amoxycillin in normal saline and glucose (5%) solutions in the liquid and the frozen states over the temperature range –26°C to 60°C. Based on this study they recommended that

amoxycillin should not to be diluted in glucose (5%) and, where solutions are to be stored in normal saline, that this be done in the liquid state in a refrigerator preferably just above but close to 0°C. Table 1.7 illustrates the stability data in terms of t_{90} and $t_{1/2}$ of amoxycillin in normal saline and in glucose (5%) solutions. These authors also calculated the initial concentration of amoxycillin sodium in the frozen state at several temperatures of investigation. The data indicate that as the temperature decreases to sub zero degrees the relative concentration of amoxycillin species increases, Table 1.8.

1.1.1.4 Methods of analysis for amoxycillin in solution

There are several assay methods reported in the literature for amoxycillin. The US Pharmacopoeia (United States Pharmacopoeia 1990d) gives an HPLC method of assay for amoxycillin content in various dosage forms. A different HPLC method is specified for amoxycillin content in co-formulation products with potassium clavulanate (United States Pharmacopoeia 1990b). The British and European Pharmacopoeias (British Pharmacopoeia 1993a; European Pharmacopoeia 1988) use the mercurimetric titration assay for both the trihydrate and sodium salt. This method is also specified for the determination of degradation products in the sodium salt monograph. The BP (British Pharmacopoeia 1993c) and the Veterinary BP (British Pharmacopoeia (Veterinary) 1993) use the spectrophotometric method involving reaction with imidazole for the content of amoxycillin in formulated products.

Table 1.7: Stability of amoxycillin sodium (1% w/v) in normal saline and in glucose (5%) solutions. (McDonald *et al.* 1989b)

STORAGE TEMP (°C)	NORMAL SALINE		GLUCOSE 5%	
	t_{90} (h)	$t_{1/2}$(h)	t_{90} (h)	$t_{1/2}$(h)
-26.0	55.0	390.0	25.5	220.0
-20.0	16.0	-	-	-
-19.2	14.0	130.0	8.4	50.0
-15.0	11.0	95.0	4.5	30.0
-13.7	10.0	90.0	4.0	-
-10.0	8.5	78.0	-	-
-8.9	8.5	-	2.1	16.0
-7.5	8.0	72.0	-	-
-6.5	8.0	-	2.5	19.5
-5.1	13.0	58.0	-	-
-5.0	14.0	-	-	-
-4.1	-	-	3.2	24.5
0.0	252.0	-	12.5	-
10.0	91.2	-	5.2	-
25.0	24.0	-	1.8	-
35.0	-	-	1.0	-
45.0	2.6	-	-	-
60.0	0.9	-	-	-

Table 1.8: Effect of temperature on the estimated initial concentration of amoxycillin sodium in normal saline and glucose (5%) solutions. (Based on the initial amoxycillin concentration of 1% w/v in liquid state) . (McDonald *et al.* 1989b)

TEMPERATURE (°C)	CONCENTRATION Mol dm^{-3}	% W/V
0	2.58×10^{-2}	1.0
-5	1.92×10^{-1}	7.5
-10	3.86×10^{-1}	14.9
-15	5.78×10^{-1}	22.4
-20	7.71×10^{-1}	29.9
-25	9.63×10^{-1}	37.3

The following is a brief account of various methods of analysis reported in the literature.

i. Titrimetric methods (Iodometric): Most penicillins including amoxycillin have been assayed (Bird 1992) by the classical iodometric method. This method was the required procedure in the US Pharmacopoeia (United States Pharmacopoeia 1990a) prior to the introduction of an HPLC method in 1991. The method is based on the fact that iodine does not react with the intact penicillin nucleus, but reaction does occur after hydrolysis of penicillin to penicilloic acid. A blank titration is performed to correct for any penicilloic acid or other impurities present in the sample that are reactive to iodine. The standard procedure is slightly altered in the case of ampicillin and amoxycillin by the addition of a small amount of hydrochloric acid to the blank to release bound iodine, which would otherwise cause a false result (Bird 1992). The reaction between iodine and penicilloic acid does not have an exact stoichiometry. Therefore the results calculated are relative to the purity of a reference sample which is assayed simultaneously with the sample.

ii. Potentiometric: Non aqueous potentiometric titrations in dimethyl-sulphoxide/methanol and glacial acetic acid, with lithium methoxide and perchloric

acid titrants, has been included with the iodometric assay in the US Pharmacopoeia prior to the introduction of HPLC (United States Pharmacopoeia 1990a). The method is not specific for amoxycillin, degradation products and other potential impurities also respond.

The mercurimetric titration method included in British and European Pharmacopoeias (British Pharmacopoeia 1993, pp42-43; European Pharmacopoeia 1988, mono No 577) is based on the fact that the thiazolidine ring of penicilloic acid reacts with mercuric ion (Bird and Redrup 1977) with a 1 to 1 stoichiometry. Therefore the sample is hydrolysed to penicilloic acid with alkali and titrated at pH 4.6 with mercuric nitrate. A blank titration of the unhydrolysed penicillin estimates any penicilloic acid and other impurities, which might react with mercuric ion. Preliminary acetylation of the amino group of amoxycillin is required to prevent its interference in the titration (Bird and Redrup 1977).

iii. Spectrophotometric methods

- **Ultraviolet:** The measurement of a second derivative peak at 280.7 nm in pH 5.8 buffer (Bird 1992) and the fourth derivative peak at 308.5 nm in 0.1 mol dm^{-3} NaOH (El-Walily *et al.* 1992) have been reported to assay amoxycillin in formulated products.
- **Ultraviolet spectrophotometry of a derivative:** At pH 5.2 degradation of amoxycillin in the presence of cupric ion gives a compound absorbing at 320 nm. In the British Pharmacopoeia (1973) measurement of this compound was used as the assay method. The compound was thought to be the penicilloic acid of amoxycillin (Bhattacharyya and Cort 1978), but this is unlikely because it is expected that the compound is unstable due to a simple reaction of the amino group with the oxazolone ring (Bird 1992). This assumption is supported by a failure to get the penicilloic acid from the reaction with imidazole and mercuric chloride under conditions which produced a stable penicillenic acid appropriate for assay purposes from penicillins without an α- amino group (Bird 1992).

  A stable product with strong absorbance at 325 nm has been reported (Bundgaard 1977b) to be formed following acetylation of the amino group followed by reaction with imidazole and mercuric chloride, which gave the mercaptide of the penicilloic acid of amoxycillin. This provided a sensitive (limit of quantitation = 0.5 μg/ml) and specific assay method for amoxycillin in the

presence of its acid and alkali degradation products. The method is not specific for the dimer and higher polymers of amoxycillin containing an intact β-lactam ring. However specificity with respect to these polymers can be achieved by treating the sample in 0.1 mol dm^{-3} hydrochloric acid at 60°C (Bundgaard 1977b). This method without preliminary acid treatment, was introduced into the British Pharmacopoeia (1980) for assay of amoxycillin and its degradation products which is still utilized for these products in the current edition.

Penicillins when reacting with 1,2,4-triazole and mercuric chloride are said to produce penicillic acid in a faster and more sensitive procedure than the imidazole (Haginaka *et al.* 1984). However, amoxycillin, without acetylation, is reported (Bird 1992) to produce an unstable product with the interfered penicilloic acid, giving a response equivalent to 30% of that of amoxycillin itself. In another report (Csiba and Czeh 1979), reaction with acetylacetone and formaldehyde followed by measuring the product at 339 nm, offered a method of analysis for amoxycillin in the 5 to 60 μg/ml range.

- **Colourimetric:** This method is based on the fact that a reaction with formaldehyde and sulphuric acid or with chromotropic acid and sulphuric acid (British Pharmacopoeia 1993b; Singh, Roy and Mandal 1985) produce a colour which has been used as an identity test to differentiate various penicillins and cephalosporins. None of these methods differentiate amoxycillin from ampicillin, yet the formaldehyde reaction is one of the identity tests used in the British and European Pharmacopoeias (British Pharmacopoeia 1993a; European Pharmacopoeia 1988) under amoxycillin monograph.

Several other colourimetric methods have been reported for the assay of amoxycillin, particularly in formulated products. However few of these reports (Dubois *et al.* 1981; Bird 1992) include any specificity of the method relative to amoxycillin degradation products, although in some cases such as for the reaction with ninhydrin, it is obvious from the functional group that they will respond.

iv. Chromatographic methods: The most widely used method in recent years has been high performance liquid chromatography (HPLC).

- **High Performance Liquid Chromatography (HPLC):** In order to obtain an accurate, sensitive and rapid method of analysis many workers have used HPLC

to assay, identify and isolate the degradation product of amoxycillin. Table 1.9 summarizes most HPLC methods, which have been published for analysis of amoxycillin and its impurities and degradation products in drug substances and formulated products. Most of these methods use reverse phase C18 columns, with UV detection and the mobile phase containing a small amount of methanol or acetonitrile in phosphate buffer at pH 4 to 6. In more complex conditions, ion pairing and post column derivatisation, have been used. But the simpler conditions used in the first few entries of Table 1.9 have been shown (Bird 1992) to be adequate for most normal assay purposes.

- **Thin Layer Chromatography (TLC):** TLC also has been used in the analysis of amoxycillin but its use is mainly restricted to an identity test (United States Pharmacopoeia 1990d; United States Pharmacopoeia 1990b; British Pharmacopoeia 1993a; European Pharmacopoeia 1988). TLC systems that separate amoxycillin from its major degradation products were used to monitor the purity of the amoxycillin sodium salt during process development (Tico *et al.* 1988).

Table 1.9: HPLC methods of analysis for amoxycillin content, its impurities and degradation products

TYPE OF SAMPLE	TYPE OF METHODS AND MOBILE PHASE	COMMENTS
FP	β-CyD/UV MeOH/TEAA-buffer pH 4.5	Simultaneous determination of amoxycillin and clavulanate (Tsou *et al.* 1997)
DS,FP	RP/C18/UV MeOH/Borax buffer pH 4.0	Determination of amoxycillin sodium with clavulanate for injection (Zhefeng *et al.* 1996)
DS,FP	RP/C18/UV MeCN/pH5.0 PO_4	USP method (United States Pharmacopoeia 1990d)
FP	RP/C18/UV MeOH/pH4.4 PO_4	With clavulanate (United States Pharmacopoeia 1990d)
DS,FP	RP/C18/UV MeOH/pH6.0 PO_4	Simultaneous assay with clavulanate (Abounassif *et al.* 1991)
DS	RP/C18/UV MeOH/pH2.5 PO_4	To measure the rate of hydrolysis, pH2 to 7 (Doadrio and Stelo 1988)
DS	RP/C18/UV MeOH/pH6.5 PO_4	Stability studies in simulated gastric juice (Moll and Esperester 1984)
DS	RP/C18/IP/UV MeCN/Bu_4NOH/PO_4	Stability studies in intravenous solutions (Wildfeuer and Rader 1996)
DS	RP/C18/UV MeOH/PO_4	Penicilloic measured, other degradation products detected (Tico *et al.* 1988)
DS	RP/C18/Gradient/UV MeOH/PO4	Solid state stability, qualitative method for degradation products (Mendez *et al* 1989)

TYPE OF SAMPLE	TYPE OF METHODS AND MOBILE PHASE	COMMENTS
FP	RP/C18/UV MeOH/ pH4.0 PO_4	With clavulanate (Ashwin, Lynn and Taskins 1987)
FP	RP/C18/DualUV MeOH/ pH4.0 PO_4	With clavulanate which is assayed simultaneously but detection is done separately (Tu *et al.* 1988)
DS	RP/C18/UV MeOH 6%/ pH5.5 PO_4 phenoxyacetic acid as internal standard	Stability in aqueous and frozen state (Concannon *et al.* 1986)
DS	RP/C18/IP/UV And RP/Ph/UV Various mobile phases	Investigation of retention mechanism (Huang, Wu and Chen 1991)
DS	RP/C18/UV i-PrOH/pH7.25 PO_4	Effect of temperature on separation of 6 penicillins (Martin, Mebendez and Negro 1988)
DS,FP	RP/C18/UV MeCN/MeOH/pH4.7PO_4	Identity test, separation of 9 penicillins (Briguglio and Lau-Cam 1984)

where, DS = drug substance; FP = formulated product; β-CyD = β-cyclodextrin stationary phase; RP = reverse phase; IP = ion pair; UV = ultraviolet; MeOH = methanol; TEAA = tetraethylammonium acetate; MeCN = acetonitrile; I-PrOH and n-PrOH = iso-and normal propanol; PO_4 = Na or K phosphate or phosphoric acid. Where mobile phase pH is not stated the original reference gives a defined composition without specifying pH.

Apart from the said methods other methods such as capillary electrophoresis, polarography (Bird 1992), flow injection analysis (Garcia *et al.* 1994) also have been used for determination of amoxycillin.

1.1.2 The chemical stability of clavulanate

1.1.2.1 Stability of clavulanate solution in the liquid state

The hydrolytic degradation of clavulanic acid in aqueous solution has been documented (Haginaka *et al.* 1985; Haginaka *et al.* 1983; Finn *et al.* 1984).

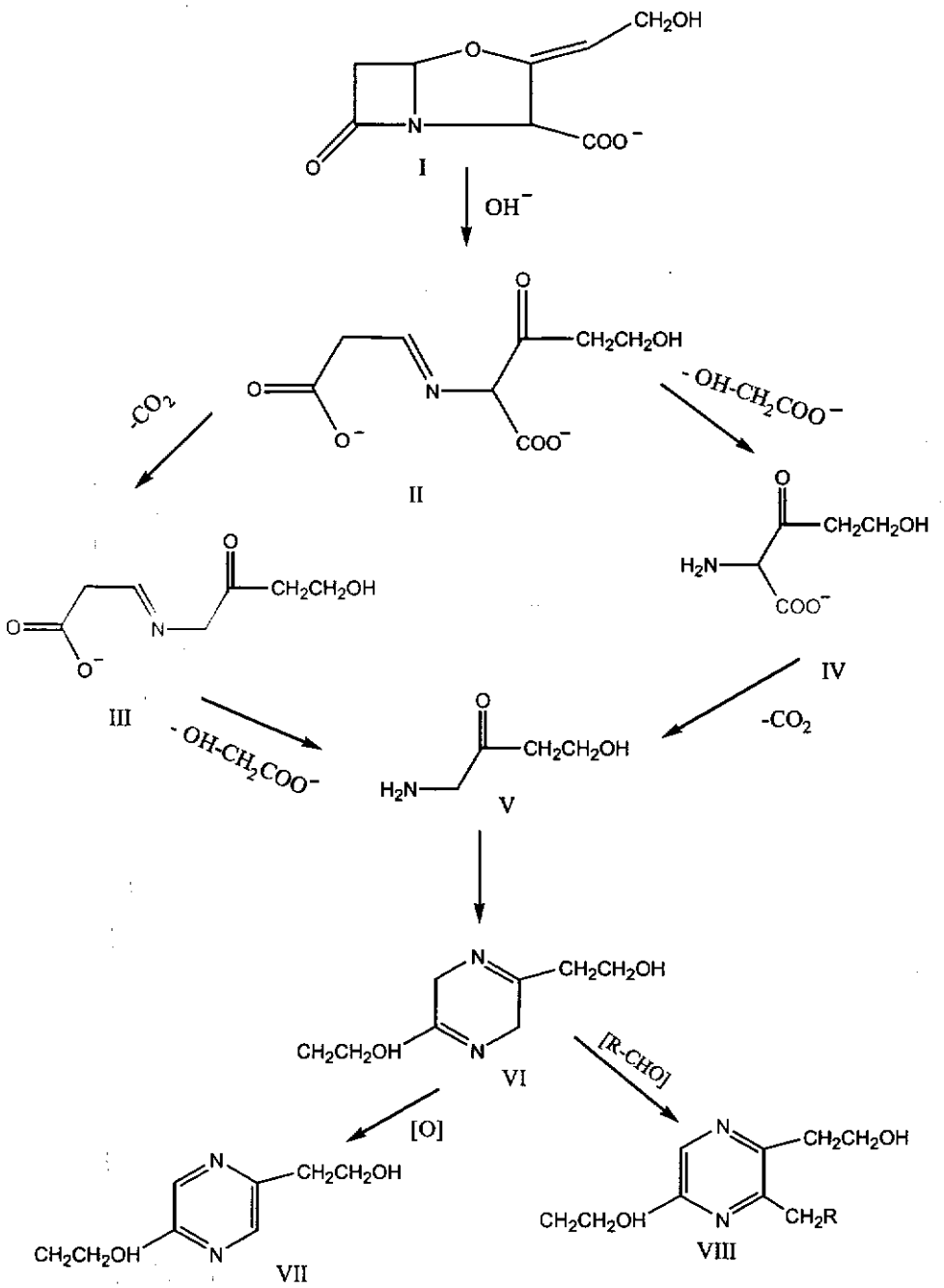
It is well known that penicillins are hydrolyzed by alkali and β-lactamase enzymes to give penicilloic acids. A common feature of these reactions is that the product retains the five membered ring of the original 4,5-fused bicyclic system. However, this generalization is not true for the 4,5-fused ring system of clavulanic acid. Finn *et al.* (1984) investigated the hydrolysis of clavulanic acid in acidic, neutral and alkaline conditions. These workers concluded that in all three conditions an amino ketone (V) was one of the major products of the hydrolysis of clavulanic acid. However, in alkali or neutral solutions as the hydrolysis proceded, this compound (V) gave rise to other products including the pyrazines (VII) and (VIII), see Scheme 1.3.

A possible mechanism of degradation of clavulanic acid has been explained by Haginaka *et al.* (1985). Scheme 1.3 represents the various possible steps in the degradation of clavulanic acid as illustrated by Haginaka *et al.* (1985).

1.1.2.1.1 Kinetics of rate of reactions of clavulanate

The stability of clavulanic acid was investigated (Haginaka, Nakagawa and Uno 1981) in aqueous solution over a pH range of 3.15 to 10.10 at 35 °C and at ionic strength of 0.5. The reaction was reported to follow pseudo-first-order kinetics with respect to clavulanic acid. The data are summarized in Table 1.10. These workers (Haginaka, Nakagawa and Uno 1981) also estimated the catalytic rate constants for the buffer species used in their study (Table 1.11). In the case of phosphate buffer it was reported that the catalytic effect of HPO_4^{2-}could be dominant over that of $H_2PO_4^-$ in accelerating the degradation of clavulanic acid within the mid pH region of their study.

Scheme 1.3: Degradation mechanism of potassium clavulanate to pyrazine derivatives (Haginaka *et al.* 1985)



VII: R=H
IX: R=CH_2COOH
X: R=CH_3

Table 1.10: Rate constants data for degradation of clavulanic acid at 35°C and $\mu = 0.5$ (Haginaka, Nakagawa and Uno 1981)

BUFFER	pH	k_{obs} (h^{-1})			
		0.30M*	0.20M	0.10M	k_{PH} (h^{-1})
CITRATE	3.15	1.04	0.849	0.655	0.462
ACETATE	3.58	0.308	0.289	0.265	0.244
	3.94	0.163	0.158	0.151	0.145
	4.41	0.059	0.054	0.044	0.038
	4.79	0.036	0.033	0.028	0.025
	4.99	0.029	0.025	0.020	0.016
PHOSPHATE	5.63	0.055	0.042	0.026	0.012
	6.28	0.112	0.080	0.045	0.012
	6.67	0.149[a]	0.080[b]	0.057[c]	0.011
	7.12	0.138[d]	0.100[e]	0.053[f]	0.012
	7.74	0.143[g]	0.104[h]	0.058[i]	0.016
	7.96	0.130[j]	0.100[k]	0.057[l]	0.023
BORATE	8.30	0.117	0.111	0.097	0.088
	8.52	0.180	0.165	0.144	0.127
	8.74	0.248	0.212	0.172	0.134
	9.09	0.400	0.350	0.268	0.208
CARBONATE	9.45	0.472	0.424	0.397	0.356
	10.10	1.43[a]	1.34[m]	1.24[c]	1.12

where, * 0.30M = buffer concentration (0.30 mol dm^{-3}, etc)

k_{pH} = pseudo-first-order rate constant, corresponding to the non-buffer-catalyzed degradation.

buffer concentration (mol dm^{-3}) : a = 0.25, b = 0.13, c = 0.08, d = 0.21, e = 0.14, f = 0.07, g = 0.18, h = 0.12, i = 0.06, j = 0.15, k = 0.10, l = 0.05, m = 0.17

Table 1.11: Catalytic rate constants of buffers species at 35°C and μ = 0.5

(Haginaka, Nakagawa and Uno 1981)

BUFFER	k_1 $(mol\ dm^{-3})^{-1}\ h^{-1}$	k_2 $(mol\ dm^{-3})^{-1}\ h^{-1}$
Phosphate[a]	0.112	0.770
Acetate[b]	0.182	0.010
Borate[c]	0.025	1.12

where, a = k_1 ; $H_2PO_4^-$, k_2 ; HPO_4^{2-}

b = k_1 ; CH_3COOH , k_2 ; CH_3COO?

c = k_1 ; H_3BO_3, k_2 ; H_4BO_4?

1.1.2.1.2 Effect of pH on the degradation rates of clavulnate in the liquid state

The work of Haginaka, Nakagawa and Uno (1981) on the pH-rate profile of clavulanic acid is presented in Figure 1.4. According to them, the curve obtained from the pH-rate profile demonstrated the best fit to the following equation,

$$k_{pH} = k_w + k_H a_H + k_{OH}(K_W/a_H) \qquad (1.21)$$

where

k_{pH} = non-buffer-catalyzed first-order rate constant

k_H and k_{OH} = represent second-order rate constants for proton and hydroxide ion-catalysed degradation reactions, respectively

k_w = the rate constant of spontaneous or water-catalysed degradation

a_H = the proton activity

$K_W = 2.09 \times 10^{-14}$ at 35°C, μ= 0.5 (Haginaka, Nakagawa and Uno 1981)

It is of value to compare the pH-rate profile of clavulanic acid with that of amoxycillin (Figure 1.4), because they are formulated in combination. As it is seen in Figure 1.4, unlike the pH-rate curve of amoxycillin, there is no break in pH-rate curve of clavulanate. This is because clavulanate has only one ionization state with its pK_a reported (Haginaka, Nakagawa and Uno 1981) to be 2.4, otherwise the two compounds seem to exhibit similar pH-rate profiles. For instance it is interesting to note that the pH of maximum stability for clavulanate reported by these authors as 6.4, is close to that of amoxycillin (5.8 to 6.5) reported by Zia and co-workers (1977).

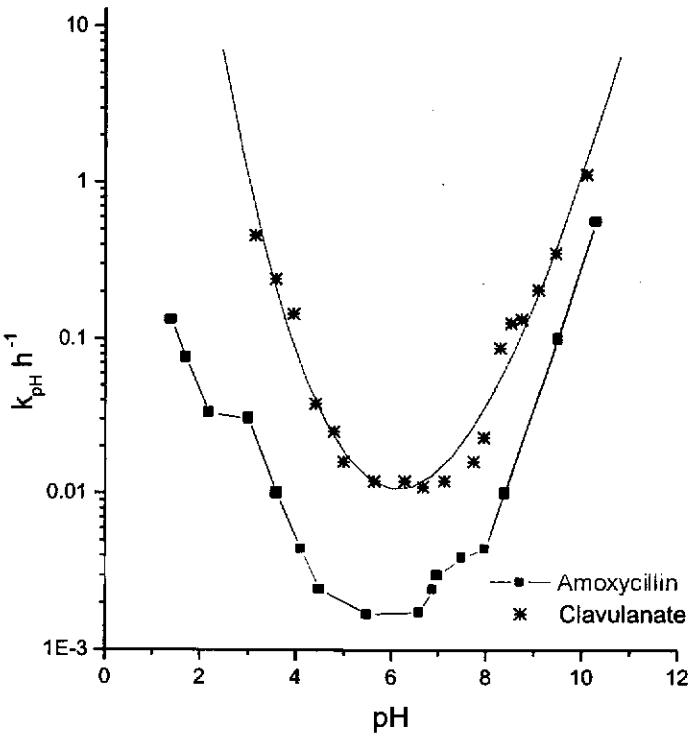


Figure 1.4: Comparison of the pH-rate profile of amoxycillin with clavulanate at 35° C and μ = 0.5. (Haginaka, Nakagawa and Uno 1981; Zia, Shalchian and Borhanian 1977)

The experimental values are represented by ¦ and * for amoxycillin and clavulanate respectively. The solid curve in the case of clavulanate indicates the theoretical curve expressed in Equation 1.21.

Thus this agreement in pH is beneficial for formulation purposes of the combination antibiotic. However, it is important to note, as evident from Figure 1.4, the degradation rate of clavulanate is markedly greater than amoxycillin at all the pH values. For instance around the pH of maximum stability, the rate of degradation of clavulanate is about 8 times greater than amoxycillin.

When formulating the combination antibiotic in buffer systems, the effect of buffers on the degradation rates of the individual antibiotic needs to be considered. The catalytic effect of buffers on amoxycillin, reported in the literature is already discussed in Section 1.1.1.1.2 and the data are presented in Table1.4b. Also the catalytic effect of buffer on the rate of clavulanate hydrolysis is presented in Table 1.11. Comparing these data, it is evident that in the case of clavulanate in phosphate buffer, the catalytic effect due to HPO_4^{2-} species is almost 2.5 times greater than amoxycillin.

Thus these factors need to be carefully considered as they can control the shelf–life of the combination antibiotic.

1.1.2.1.3 Effect of temperature on the rate of degradation of clavulanate

The temperature effect on the degradation of clavulanic acid has been documented (Haginaka, Nakagawa and Uno 1981) at different pH values in acidic, neutral and alkaline media at constant ionic strength μ= 0.5. The observed rate constants at these temperatures along with the apparent activation energies (E_a) are listed in Table 1.12. The data in Table 1.12 indicate that the E_a of clavulanic acid at pH 3.94 and 8.74 are almost similar, while that of pH 6.67 is a slightly lower value.

As described under Section 1.1.1.1.3, the activation energies reported for amoxycillin (Zia, Shalchian and Borhanian 1977; Doadrio and Sotelo 1988) indicate that at pH around 4.0, amoxycillin and clavulanate posses a similar E_a values. This may suggest that both these antibiotics posses a similar mechanism of β-lactam ring hydrolysis at about pH 4. However, the literature indicate a wide difference between E_a values of amoxycillin (Table 1.5) and clavulanate (Table 1.12) in neutral media.

Table 1.12: Rate constants and Arrhenius activation parameters for the degradation of clavulanic acid at $\mu = 0.5$ (Haginaka, Nakagawa and Uno 1981)

pH	TEMPERATURE (°C)	k_{obs} (h^{-1})	E_a (kJ mol^{-1})	log$A(h^{-1})$
3.94	35	0.151	79.5	12.7
	50	0.733		
	65	2.550		
6.67	35	0.057	61.5	9.17
	50	0.181		
	65	0.479		
8.74	35	0.172	76.6	12.3
	50	0.728		
	65	2.45		

1.1.2.1.4 Stability of clavulanate in intravenous fluids

There are reports (Swenson *et al.* 1990; Wildfeuer and Radar 1996; Ashwin, Lynn and Taskins 1987) available on the stability of potassium clavulanate in combination dosage forms in intravenous vehicles. Wildfeuer and Radar (1996), performed a comparative study using four different infusion solutions at 4°C, 25°C and 37°C and reported the following descending sequence of stability: sulbactam, ampicillin, amoxycillin and clavulanic acid. These workers extended their investigation into fluids at 37°C and concluded that clavulanic acid was the least stable compound in all the fluids. Although these authors reported the stability of clavulanic acid in combination with amoxycillin and various infusion solutions their results are inconsistent with the other data (Ashwin *et al.* 1987) under similar conditions. Wildfeuer and Radar (1996) found t_{90} values for clavulanic acid in water for injection to be 1 hour at 4°C against 15 hours at 5°C reported by Ashwin, Lynn and Taskins (1987). Also at 25°C the t_{90} value for clavulanic acid reported by Wildfeuer and Radar (1996), was 30-45 minutes compared with 4-5 hours claimed by Ashwin and coworkers.

The stability of potassium clavulanate in combination with ticarcillin has been studied (Swenson *et al.* 1990) in various intravenous solutions in different types of plastic containers. These authors concluded that the combination dosage form was most stable at -15°C , however the report indicated minimum degradation at 35°C for 24 hours and at 4°C for 7 days.

The stability of potassium clavulanate has been reported (Ashwin, Lynn and Taskins 1987) to be highly concentration dependent. The authors also investigated the stability of clavulanate in the presence of amoxycillin at refrigeration (5°C) and freezing temperatures (-20°C) in water and sodium chloride 0.9% vehicles. The report indicates that a satisfactory degree of activity was maintained at 5°C in water and sodium chloride 0.9% for 15 hours and 12.5 hours, respectively, compared to 4 to 5 hours at 25°C . However the stability was found to be inadequate at -20°C. After 4 hours of storage at -20°C only 65% of the initial clavulanate content remained.

1.1.2.2 Stability of clavulanate in the solid state

There are no literature data on the stability of clavulanate alone in the solid state. However, there are reports on the stability of its combination dosage form in oral suspension (see amoxycillin and clavulanate in combination Section 1.1.3).

1.1.2.3 Methods of analysis of clavulanate solution

There are number of methods by which clavulanic acid has been assayed. These include spectrophotometric (Bird, Bellis and Gasson 1982; Izquierdo, Gomez-Hens and Perez-Bendito 1993), microbiological (Kanazawa, Kuramata and Matsumoto 1988; Ball *et al.* 1980), enzymatic (Cullmann and Dick 1986), polarographic (Perez, Martin and De Aldana 1991) and chromatographic (Haginaka, Wakai and Yasuda1987; Haginaka, Wakai and Yasuda 1986; Haginaka, Yasuda and Nakagawa 1986; Foulstone and Reading 1982; Haginaka, Nakagawa and Uno 1981) methods. Recent editions of the British (1994) and United States (1990c) pharmacopoeias have used HPLC methods.

The spectrophotometric methods of analysis are based upon the reaction of clavulanic acid with imidazole in water. The acylation of imidazole by the ß-lactam carbonyl results in the formation of 4-(4-aza-8-hydroxy-6-oxo)oct-2-en-1-oylimidazole which has an intense absorbance with a maximum at 312nm (Kenig 1988). This method has been reported to be sensitive and useful for stability studies of clavulanate and its derivatives in biological fluids (Kenig 1988).

Table 1.13: HPLC methods of analysis for clavulanate, its impurities and degradation products

SAMPLE	TYPE OF METHODS AND MOBILE PHASE	COMMENTS
DS,FP	RP/C18/UV MeOH/Borax buffer pH 4.0	With amoxycillin sodium (Zhefeng *et al.* 1996)
FP	RP/C18/UV MeOH/pH4.4 PO_4	USP, with amoxycillin. (United States Pharmacopoeia 1990c)
DS	RP/C18/UV MeOH/pH6.0 PO_4	With amoxycillin (Abounassif *et al.* 1991)
DS,FP	RP/C18/UV TBAB+NaH_2PO_4 + Na_2HPO_4/MeOH/pH7.25	Variable UV detector. (Haginaka, Nakagawa and Uno 1981)
DS	RP/C18/UV MeOH/ PO_4/ pH7.0	Pre-column derivatisation by 1,2,4-triazole reagent In serum and urine samples (Martin and Mendez 1988)
FP	RP/C18/UV MeOH/ pH4.0 PO_4	With amoxycillin (Ashwin, Lynn and Taskins 1987)
FP	RP/C18/DualUV MeOH/ pH4.0 PO_4	With amoxycillin which is assayed simultaneously but detection is done separately. (Tu *et al.* 1988)

where, DS = drug substance; FP = formulated product RP = reverse phase; IP = ion pair, UV = ultraviolet; MeOH = methanol; TBAB = Tetra-n-butylammonium bromide; PO_4 = sodium or potassium phosphate or phosphoric acid. Where mobile phase pH is not stated the original reference gives a defined composition without specifying pH.

The HPLC assay method used by Foulstone and Reading (1982) was based on pretreatment of amoxycillin and clavulanic acid in an imidazole reaction. Subsequently others (Martin and Mendez 1988; Shah, Adlard and Stride 1990) used a pre-column 1,2,4-triazole reaction method for assay of clavulanic acid.

Other methods developed have involved post-column techniques (Haginaka, Yasuda and Uno 1985; Haginaka, Wakai and Yasuda 1987; Haginaka, Wakai and Yasuda 1986), ion-interaction chromatography (Salto and Alemany 1984) and reverse-phase chromatography (Abounassif *et al.* 1991). Since an objective of this project was to develop a suitable stability indicating HPLC method, selected HPLC methods used for the determination of clavulanic acid are listed in Table 1.13.

1.1.3 The stability of amoxycillin in combination with clavulanate

Several workers (Wildfeuer and Radar 1996; Ashwin, Lynn and Taskins 1987; *Tu et al.* 1988; Mehta *et al.* 1994; Moore *et al.* 1996) have reported on the stability of these drugs in combination. These studies conclude that clavulanate is less stable than the amoxycillin, hence clavulanate is the stability limiting component. In one report (Ashwin, Lynn and Taskins 1987), the increase in ratio of amoxycillin to clavulanate in aqueous solution was reported to decrease the shelf-life of clavulanate. These authors tried to explain the result by suggesting that, the presence of a free side-chain amino group in amoxycillin could participate in the nucleophilic opening of the beta-lactam ring of clavulanate. *Tu et al.* (1988) and Mehta *et al.* (1994), have studied the stability of the combination in oral suspension over a limited temperature range (see 1.1.1.2). Wildfeuer and Radar (1996) provided a comparative study of two ß-lactam antibiotics (amoxycillin and ampicillin) in parenteral combination dosage forms with their respective ß-lactamase inhibitors (clavulanate and sulbactam) over a limited temperature range. Ashwin and coworkers (1987) have studied the stability of amoxycillin sodium in combination with potassium clavulanate in various intravenous fluids (Talbe 1.14). The results reported by Ashwin and coworkers (1987) however are in conflict with those of Wildfeuer and Radar (1996) as stated in Section 1.1.2.1.4.

Table 1.14: Stability of amoxycillin sodium in combination with potassium clavulanate in infusion solutions at 25°C (presented in terms of clavulanate stability) (Ashwin, Lynn and Taskins 1987)

INTRAVENOUS VEHICLE	t_{90} (h)	MEAN t_{90} (h)
Water for injection BP	*4.8-5.2	4.9
Sodium chloride 0.9%	*5.3-5.8	5.6
Compound Sodium chloride	4.1-4.7	4.4
Ringer's solution for injection	4.1-4.2	4.1
[(a)]Sodium chloride 0.9%	3.8-4.0	3.9
Compound sodium lactate	*4.0-4.2	4.1
Ringer-lactate; Hartmann's solution	*3.9-4.1	4.0
Sodium lactate M/6	4.2-4.3	4.3
Stability (presented in terms of clavulanate) at 5°C		
Water for injection BP	15.0	-
Sodium chloride 0.9%	12.5	-
Glucose 5%	1.2	-

* Values obtained using materials stored at 20°C for 36 months; [(a)] with potassium chloride 0.3%

t_{90} is defined as the time for which 90% of the initial activity is maintained

1.1.3.1 Methods of analysis for amoxycillin in combination with clavulanate

This has been already discussed under amoxycillin and clavulanate (Sections 1.1.1.4 and 1.1.2.3).

1.1.4 Stability studies in the frozen state

Before exploring the kinetics of the reactions in frozen systems a general prelude relevant to reactions in the frozen systems is discussed first.

i. General aspects: The earliest report in the literature concerning reactions in the frozen state is devoted primarily to an important practical problem, the preservation of food- stuff stored at frozen temperatures. It was concluded in 1930's that enzyme action in food stored at sub-zero temperatures was very important in food

preservation. As a comparison frozen and supercooled systems were reported (Lineweaver 1939), it indicated that when an enzyme system freezes a sharp discontinuity occurred in the velocity-temperature curve.

Most drug systems studied show a rate decrease in the frozen system, but there are other reports (Grant, Clark and Alburn 1966; Tappel 1966) showing faster enzymatic reactions in frozen solutions relative to an identical supercooled system. The use of freezing is growing rapidly for large numbers of foodstuffs, pharmaceuticals, biomedical, organic and inorganic compounds. The unusual experimental data and theoretical models from stability studies of frozen systems reinforce the need for detailed and systematic investigations for each individual frozen system.

In recent years the practice of freezing drug solutions has become more prevalent. Antibiotics such as penicillins, which do not have sufficient stability in aqueous solution, have been evaluated in frozen systems with the objective of improved stability.

A common understanding regarding storage at lower temperature is that the rate of reaction is slower than at higher temperatures. However the decrease in temperature causing a system to freeze, may result in faster reactions (Grant, Clark and Alburn 1962; Grant *et al.* 1961; Pincock and Kiovsky 1965a; Pincock and Kiovsky 1965b; Larsen and Jenseng 1969; Larsen 1971a; Larsen 1971b; McDonald *et al.* 1989b; Concannon *et al* 1986) than in unfrozen samples of the same solution. This is partly because the apparently frozen state may not be a one-component system (solid) below its freezing point. The frequent existence of liquid in equilibrium with a solid at temperatures below the freezing point is often neglected. It is therefore important to note that only below the eutectic temperature is a system completely solid. Therefore, an apparently frozen state exists at a range of temperatures below the freezing point and above the eutectic point where solid is in equilibrium with a liquid phase.

ii. Some theoretical aspects of reactions in frozen systems: The concept of the rate of reaction takes a special meaning when dealing with frozen systems. The usual definition of "rate" can be defined as rate = d(concentration) / d(time). However when the volume of a liquid phase changes due to freezing or thawing, accordingly there would be changes in the number of moles of reactant. Hence for many frozen

reactions, in order to obtain meaningful rate constants, the rate definition has to be modified (Pincock 1969) to, rate = (1/V)(dM/dt). Where V is the volume of the reactive phase and M is the moles of the reactants in that phase. Further explanation to this definition is given by deducing Equation 1.28, discussed under kinetics of frozen solution reactions (Section 1.1.4 iv.).

Other possible theories have been suggested by Grant and co-workers (Grant, Clark and Album 1961; Grant and Album 1967) describing the base-catalysed hydrolysis of penicillin in frozen systems at –5°C to -30°C. These workers found that the reaction was not influenced by the manner in which samples were frozen, rather the presence of some solutes such as glycerol or ethanol, stopped the reaction in the frozen samples. The authors suggested an explanation involving concentration of reactants on freezing, a favourable substrate-catalyst positional constraint, and the possibility of exceptionally high proton mobility in ice. They also suggested that reactant and product diffusion, crystal imperfections, as well as dielectric properties of ice may play some role in certain reactions of frozen systems. Also in the study of hydroxylaminolysis of some amino acid esters in frozen solutions the same authors showed that the reactions were often inhibited by addition of compounds which are structurally analogous to the reactants; the kinetic relationship which resulted from this investigation was a Lineweaver-Burk plot for competitive inhibition. This can suggest the presence of catalytically active sites on the ice surface (Pincock 1969; Grant and Album 1965; Grant, Clark and Album 1961).

Another theory was the possibility of ice structure taking part in a proton transfer reaction. This has been suggested by Butler and Bruice (1964) while investigating the reaction of morpholine with two thiolactones. They found that the overall observed reaction rate order changed from three in liquid state to two in frozen solution. This result was contrary to the anticipated concentration phenomenon. The authors explained the result by saying that the ice itself had taken part in a proton transfer reaction in place of one of the morpholine molecules.

iii. Physico- chemical aspects of the frozen state: When an aqueous drug solution containing excipients such as buffer is frozen, the solutes concentrate in solution as pure water crystallizes to form ice. Thus the concentrated solutes exist in a network. When the solution is further cooled, these fluids become more concentrated eventually the solute(s) precipitate out partially or completely. Therefore an

understanding of phase changes in the frozen state is essential and can be studied by number of techniques such as differential thermal analysis (DTA), differential scanning calorimetry (DSC) and resistivity measurements (Chilamkurti 1992; Van Gorp*et al.* 1987; Jennings 1980).

A phase change can have an impact on degradation kinetics in the frozen state and thus affect drug stability. For instance, if a drug and excipients exist as concentrated solutions, the degradation rate may be increased due to increased molecular mobility, increased buffer concentration (buffer catalysis), increased sugar concentration (carbohydrate catalysis), increased ionic strength, and changes in pH. However if solute(s) precipitate partially or completely, the composition of the frozen solution will change and possibly affect in the rate of degradation. The precipitation of a drug will generally enhance the stability due to the drug's presence as a solid phase. Therefore, based on these factors, it could be possible to try to alter the composition of the formulation in order to obtain favourable phase changes to enhance drug stability.

Another aspect is the difficulty of prediction of degradation rates in the frozen state based on degradation rates at higher thawed temperatures, using Arrhenius type extrapolations. This can arise due to the various effects stated before. However, according to Chilamkurti (1992), it is better to perform accelerated kinetic studies on concentrated solutions, if the degradation in the frozen state is largely due to concentration effects. This approach was demonstrated by Marsh and co-workers (Marsh *et al.* 1987) in an investigation on degradation rates of 2% aztreonam in 5% dextrose solution at thawed and frozen states. The study reported that in order to establish an Arrhenius relationship between the rates obtained in the liquid and frozen states, concentrated solutions representative of the frozen conditions were evaluated. The estimated frozen rates obtained through the Arrehenius extrapolation of the liquid state data were then in agreement with the actual data from the frozen states.

1.1.4.1 Kinetics of frozen solution reactions

The kinetics of reactions in the frozen state have been explained in detail by Pincock and Kiovsky (1966) based on a solute concentration model.

Considering a bimolecular reaction,

$$A + B \rightarrow \text{Product} \qquad (1.22)$$

The rate of the reaction in the liquid phase of the frozen solution is given by

$$\frac{d[A_l]}{dt} = -k_2[A_l][B_l] \quad (1.23)$$

where k_2 is the second order rate constant which is dependent only on temperature and the subscript "l" refers to the liquid region of the frozen system.

The rate of reaction in the frozen solution is experimentally obtained in terms of concentration changes in thawed solutions. Therefore, the experimentally determined rate may be related to the rate in the reaction regions of a frozen solution as follows:

Rate in reaction regions in terms of total volume

$$V_l = \frac{d[A_l]}{dt} = -k_2[A_l][B_l] \quad (1.24)$$

where moles converted per unit time is,

$$-k_2[A_l][B_l]V_l \qquad (1.25)$$

Rate in thawed solution of volume V_s is,

$$V_s = \frac{d[A_s]}{dt} = -k_2[A_l][B_l]\frac{V_l}{V_s} \qquad (1.26)$$

Considering the concentration $[A_l], [B_l]$ as well as the volume V_l in Equation 1.26 is related to the measurable concentration in thawed solutions and assuming all the solutes present in the thawed solution are present in the liquid reaction regions of the frozen solution then,

$$[A_l]V_l = \text{moles of } A = [A_s]V_s \qquad (1.27)$$

$$[B_l]V_l = \text{moles of } B = [A_s]V_s \qquad (1.28)$$

then

$$\frac{d[A_s]}{dt} = -k_2[A_s][B_{sl}]\frac{V_s}{V_l} \qquad (1.29)$$

The volume V_l can similarly be related to the concentration of solutes in a thawed solution; C_lV_l = total number of moles of solute = C_sV_s where C_s is the total

concentration of all solutes in a thawed solution. Therefore Equation 1.28 can be written as

$$\frac{d[A_s]}{dt} = -k_2 C_l \frac{[A_s][B_s]}{C_s} \qquad (1.30)$$

Equation 1.30 relates the rate of reaction of a frozen solution as measured in thawed solution, to the concentrations of solutes in the thawed solution. A number of general features of reactions in the frozen state have been illustrated by Pincock and Kivosky (1966) on the basis of Equation 1.30, which are discussed below:

i. The rate constant for a frozen reaction will involve the product of the normal second order rate constant, k_2 and the total concentration of the reaction regions, C_l, both of these are dependent on temperature only.

ii. If the activation energy and entropy are known, the value of k_2 can be calculated; the value of C_l at various temperatures is obtained from the phase temperature relationship of the system.

iii. At lower temperatures, the second order rate constant decreases and the concentration factor increases, thus k_2C_l will have a maximum at some temperature below the freezing point of the solution.

iv. Comparing the rate of reaction in the frozen state with that of ordinary non-frozen reaction (ie., $k_2\ [A_s][B_s]$)at the same temperature, the "frozen" rate is greater than the "unfrozen" rate by the ratio C_l/C_s.

v. Since C_s includes reactant and product concentrations, as well as the concentrations of all other soluble, but otherwise reactively inert solutes, $C_s = A_s + B_s + P_s + I_s$, hence it is apparent that relative reaction rates are sensitive to many variations in conditions.

vi. Because of the inverse relation of C_s to rate constant, if C_s is increased for any reason , the rate of reaction will decrease.

vii. The numbers of moles of products and their solubilities in the reaction volumes can affect the rate of reaction. For instance, production of greater or smaller numbers of moles of product than reactant results in changes in C_s during a run. Thus, it will result in changes in the volume V_l and will lead to rate depressing or accelerating effects.

viii. Compounds, which are not involved in the reaction and have no effect on the rate of non-frozen reactions can have striking effects on the rate of reaction when frozen. For instance a solute like benzene has no effect on the rate of reaction of t-butylproxy formate with 2,6-lutidine in p-xylene, but if the solution is frozen, the measured rate is one half as great as in the same frozen solution without benzene (Pincock and Kiovsky 1965b). This is because benzene increases the value of C_s and decreases the rate in frozen state. It acts only to dilute the reactants concentration by increasing the reaction volume, and hence decrease the rate of a second order reaction with in the liquid regions of the frozen solution.

ix. In Equation 1.30 the presence of reactant concentrations in both the denominator and numerator, indicates that the experimentally observed order of a frozen reaction could be different from that of non-frozen. A bimolecular reaction would turn from a second order to a pseudo-first order when one of the reactants is in great excess in a non-frozen solution. In the case of a frozen solution if one reactant is in excess concentration the rate of the reaction would become pure first- order because the observed rate constant is independent of the concentration of the reactant.

Although these general features of reactions in the frozen state are based on an ideal behaviour of solutes, it appears to correlate with many qualitative as well as some quantitative experimental results. Equation 1.30 can be integrated to obtain expressions giving reactant concentrations in thawed solutions as a function of time. Many different concentration versus time relationships are possible and further variations may arise when the frozen system has unusual phase properties such as when the solid phase has catalytic effects, or a true solid phase reaction might occur. This will lead to deviation from the general treatment given above and result in other interesting features of reactions in the frozen state.

1.1.4.1.1 First-order reactions

First order reactions can be derived in similar way from Equation 1.30. Hence the rate of reaction of a frozen solution as measured in thawed solution can be denoted as follows

$$\frac{d[A_s]}{dt} = -k_2 C_l \frac{[A_s]}{C_s} \qquad (1.31)$$

where the term k_2 (second order rate constant) and C_l (concentration in the frozen state) are dependent on temperature.

Integrating Equation 1.31,

$$\log[A_s]_t = \log[A_s]_0 - \frac{k_{obs}t}{2.303} \qquad (1.32)$$

where $k_{obs} = k_2 \frac{C_l}{C_s}$

The subscript 't' and '0' refer to concentration at time t and zero (initial concentration) respectively. The term k_2 is dependent upon the temperature of the frozen state. Hence from the Arrhenius equation (Martin 1993a):

$$k_2 = Ae^{-E_a/RT} \qquad (1.33)$$

then

$$\log k_2 = \log A - \frac{E_a}{2.303RT} \qquad (1.34)$$

The k_2 values can be calculated by extrapolation of the reaction rates obtained in the liquid state to those of frozen temperatures using the Arrhenius equation.

If the value of the concentration factor $\frac{C_l}{C_s}$ is one or close to unity then $k_{obs} = k_2$ and the reaction is similar to a first- order reaction in the liquid state where the rate constant is independent of the initial concentration of the reactants. However if the observed rate constant is different ie. $k_{obs} \neq k_2$ then several factors (*eg.* concentration, buffer effect, pH change etc.) as will be discussed in the following sections can effect the rate of a first order reaction in a frozen solution.

1.1.4.2 Factors effecting the stability of frozen formulations

A brief review of some of the factors influencing the stability of drugs in solution, in the frozen state is discussed below.

i. Solute precipitation: When the concentration of a solute in a frozen solution is beyond its solubility, the solute may precipitate either as an amorphous or crystalline form. There are a number of reports (Mishra *et al.*1988; Chilamkurti *et al.* 1989) in the literature indicating that drug solutions stored below the crystallization temperature of the drug should demonstrate significant improvement in stability when compared to solutions stored at temperatures above the drug crystallization

temperature. It is also interesting to note that crystallization of a drug during lyophilization is also a favourable factor improving post lyophilization stability (Chilamkurti 1992). The dissolution of a crystalline solute in thawed solution is another factor to be considered. In some case drugs such as mitomycin, the crystallized drug has a limited solubility, the dissolution of the precipitated drug could then cause problems (Chilamkurti 1992). In such cases the dissolution of the crystallized drug could be enhanced by optimization of the formulation using factors like drug concentration, pH, diluent etc.

ii. Concentration: The concentration of solutes in a frozen system may influence the rate of degradation of certain drugs. It has been documented that drugs such as ampicillin sodium (Savello and Shangraw 1971; Ashwin and Lynn 1975), amoxycillin sodium (Concannon *et al.* 1986; McDonald *et al* 1989b), imipenem (Bigley, Forsyth and Henley 1986), and ceftriaxone disodium (Kedzierewicz *et al.* 1989) degrade at a faster rate when in concentrated solution. If a solution of any of these drugs were frozen the rate of degradation in the frozen state would be much higher than that of the expected rate in the liquid state. This has been explained to be due to the concentration effect. Another type of concentration effect, arises from micellization. Micellization of penicillin G has been reported (Thakkar and Wilham 1971; Ong and Kostenbauder 1975) and the acid catalyzed degradation of penicillin G is increased by two-fold in micellar solution. However the base catalyzed degradation was decreased by two to three-fold. Therefore, when a dilute solution of penicillin G is frozen , it could form micelles due to the concentration effect in the frozen state. Also depending on the extent of the concentration and the pH of the solution the rate or the kinetics of the reaction could change accordingly.

iii. Diluent: Diluent effects on the stability of drugs in intravenous fluids are well documented (Allwood and Brown 1993; Wildfeuer and Radar 1991; Cook, Hill and Lynn 1982; Ashwin, Lynn and Taskins 1987; McDonald *et al* 1989b).

Penicillins are known to degrade rapidly in the presence of glucose at alkaline pH. This effect can be observed in the liquid state, but may not be obvious in frozen systems due to some changes such as precipitation of the drugs or change in pH. Savello and Shangraw (1971) have reported the acceleration in rate of degradation of ampicillin sodium in the presence of glucose both in frozen and thawed states. According to this report loss of ampicillin in 5% dextrose at -20°C after 24 h was

about 13 times higher than the amount observed in normal saline. However, at 27°C the increase was only about 6 fold. Other factors which could affect the stability of drug formulations in the frozen state include, increased ionic strength due to the presence of sodium chloride or increased buffer concentration due to the presence of a buffer such as in lactated Ringer's solution which is used as a diluent.

iv. Buffer: Buffers as stated before can influence the stability of the frozen formulations in several ways, which, could be different from their influence in the liquid state. If the drug compounds precipitate in the frozen storage state, then the interaction between the buffer and the drug would be very little with no significant effect of buffer on drug stability. But if the buffer precipitates and not the drug, then changes in pH and the buffer capacity could occur leading to changes in drug stability. These arguments are supported by reports (Murase, Echlin and Franks 1991; Murase and Franks 1989; Van den Berg and Rose 1959; Larsen 1973) on phosphate buffer. The reports indicate that disodium hydrogen phosphate can selectively precipitate from a sodium phosphate buffer solution at –0.5°C leaving monosodium dihydrogen phosphate in solution (Larsen 1973). According to Murase and Franks (1989), mono sodium dihydrogen phosphate did not precipitate at its recorded eutectic temperature (-9.9°C) or lower temperatures used in their study. The salt rather became concentrated, subsequently supersaturated and then turned into an amorphous solid. These authors also reported that of the two potassium phosphate salts, potassium dihydrogen phosphate precipitated readily at –2.7°C while dipotassium phosphate did not precipitate easily. These changes in buffer composition resulted in significant changes in pH. In another report Hill and Buckley (1991) found that the rate of decomposition of NADPH (nicotinamide adenine dinucleotide phosphate) and hydrolysis of 4-nitrophenyl acetate were effected on freezing in phosphate buffer due to pH shifts. Other factors which could effect the rate include increased ionic strength or buffer catalysis effects.

v. pH : As stated above freezing an aqueous solution containing buffer(s) can result in pH changes. It was reported by Larsen (1973) that significant pH changes occur when buffers such as neutral sodium phosphate, neutral borax-monopotassium phosphate, and neutral potassium phosphate were frozen. Orii and Morita (1977) examined more than 30 buffer solutions to study the pH changes and reported that

almost half exhibited pH change. It is also reported that addition of some excipients such as sodium chloride or glycerol to buffer solutions often prevented pH shifts (Larsen 1973; Orii and Morita 1977). Therefore this can be investigated in formulations whenever a pH shift is not desirable.

A report (Chilamkurti 1992) suggests that micellization of some drugs in solution could result in pH change in the frozen state. Ong and Kostenbauder (1975) have reported a significant shift in the pH-rate profile of penicillin G when the drug was present in concentrated micellar solution. Hence if a dilute solution of penicillin G is frozen a similar effect could result, due to the concentration effect and the micellization of penicillin G. Another factor which can result in pH change is the degradation of the drug in the frozen state itself which may result in the formation of acidic or basic degradation products that can affect the pH of the system. Therefore, the pH change could have either an enhancing or reducing effect on the stability of the frozen drug, when in solution.

1.1.4.3 Some considerations of analytical methods

In developing an analytical method for frozen systems there are some issues, which need to be considered. The primary consideration should be to develop a method which ensures minimum or prevents drug degradation during the assay period. This can be achieved by establishing controlled time and temperature limits for thawing, sample preparation and analysis. Another issue is the possibility of other degradation pathways arising in frozen systems. Hence the assay methods need to be validated under these conditions.

i. pH measurements : It is often difficult to measure the pH of a frozen solution. Measuring the pH of the thawed solution may not provide a true indication of pH of the frozen solution. There are several reports in the literature on pH measurement of frozen systems. Each method has its limitations. Van Den Berg (1968) has suggested a modified calomel and glass electrodes for low temperature pH measurements. Commercial calomel electrodes contain solutions saturated with potassium chloride. Addition of compounds such as ethylene glycol or glycerol can provide superior results and can be used for temperatures down to –30°C. However the author indicated that one of the limitations of this method is that the pH values obtained in frozen materials should be compared with similar kinds of frozen materials and

within narrow temperature ranges. It is evident there will be changes in activity of H^+ in different solvent systems.

Another method is the use of pH indicators (Orii and Morita 1977). These workers examined about 30 buffer solutions and almost half of the buffers showed a pH change. Some disadvantages of this method are time consuming, poor reproducibility, possible extrinsic colour changes due to a temperature-dependent shift in pH indicator constant, salt errors and different solubilities of different forms of indicators.

ii. Effect of rapid thawing: There are reports of rapid thawing of frozen admixtures using a microwave oven (Tabor and Norton 1985; Tredree 1986; Tidy, Sewell and Jeffries 1988; Holmes *et al.* 1982). In all cases the results indicate that these antibiotics were unaffected by microwave energy. It is important that when dealing with microwave thawing of any frozen admixture the method is standardized and documented (Tabor and Norton 1985). There are certain variables which can effect the thawing process (Tabor and Norton 1985), these include, the dose of radiation and the resultant temperature, the location of the container in the oven, and the most importantly the number and the size of the containers loaded in the oven. The pH and ionic concentration of the antibiotic solution and the chemical structure of the particular antibiotic also can influence the rate of the degradation during thawing.

1.1.5 Influence of hydroxypropyl β-cyclodextrin on the stability of drug formulation

The use of complexing agents such as cyclodextrin in aqueous solution has been extensively investigated to improve the stability of various drugs and formulation products. For instance as early as 1955, benzocaine was first (Higuchi and Lachman 1955) stabilized by complex formation with caffeine and later by cyclodextrin complexation (Lach and Chin 1964).

Cyclodextrins are formed by the enzymatic cyclization of starch by cycloglycosyltransferase. The important structural (Figure 1.5) characteristics of the cyclodextrin molecules are their fairly cylindrical shape, with a somewhat hydrophobic central cavity and the hydrophilic hydroxyl groups on their outer surface. These structures are said to be cone shaped to some extent, because of the

lack of free rotation about the bonds connecting the glucopyranose units (Loftsson 1995).

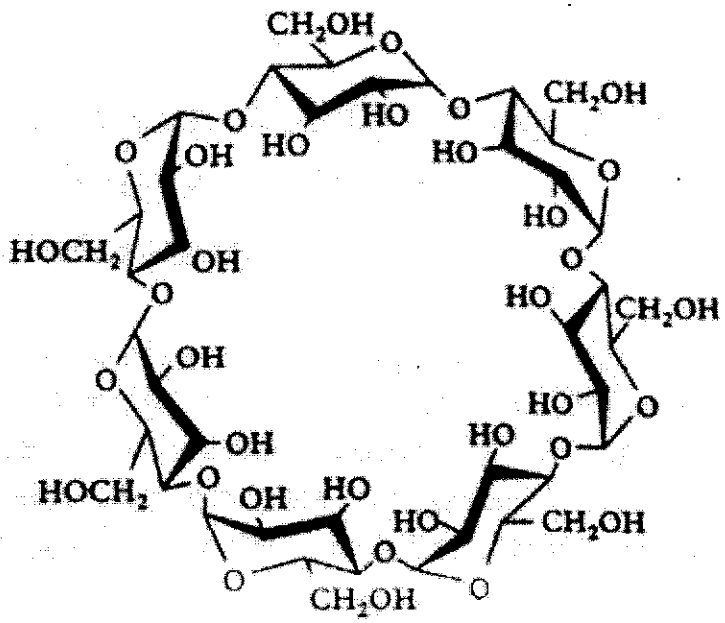


Figure 1.5 : Structure of β-cyclodextrin.

(Loftsson 1995, inserted with permission from Nature publishing groups UK)

Cyclodextrins act as complexing agents by forming inclusion complexes (Szente 1993; Szejtli 1991) with guest molecules or parts of them, into their cavity. Covalent bond formation does not occur during the complexation. The free guest molecules are in equilibrium with the molecules bound, the driving force of the complex formation is said to be the release of enthalpy rich water from the cyclodextrin cavity (Loftsson 1995). These water molecules are freely replaced by the appropriate guest molecules, which are less polar than water.

The most common cyclodextrins are α-cyclodextrin (or cyclohexaamylose), β-cyclodextin (or cycloheptaamylose), and γ-cyclodextrin (or cyclooctaamylose), consisting of six, seven or eight α-1,4- linked glucopyranose units, respectively. β-cyclodextin is the one that is most useful for complexing average size molecules such as many drugs.

To improve the physicochemical and biological properties of cyclodextrins, branched substituted cyclodextrins have been developed. One such derivative is hydroxypropyl β- cyclodextrin , in which some of the hydroxyl groups of the β-cyclodextrin molecule are substituted by hydroxypropyl groups. These derivatives have much greater solubility in aqueous solutions and extended surface area for complexation than the parent cyclodextrin (Brewster *et al.* 1991; Irie *et al.* 1988; Yoshida *et al.* 1988; Uekama and Irie 1990: Bekers *et al.* 1991). Thus hydroxypropyl β-cyclodextrin has a solubility of 60g/100ml and no reported toxic effects on parenteral administration (Loftsson *et al.* 1991; Brewster *et al.* 1989; Pitha *et al.* 1986).

Hence, cyclodextrins can be useful in many ways. In the solid state, cyclodextrin complex formation has been used to increase the rate of dissolution of the guest molecule (Islam and Nurukar 1991), increase its chemical stability, reduce its volatility and sublimation. In aqueous solutions cyclodextrin complexes also have been used to improve the stability and solubility of guest molecules and reduce volatility and absorption into or on surfaces. They have also been used to modify liquid drugs into microcrystalline powders, and decrease or abolish unpleasant tastes or odours (Loftsson 1995).

Although there are several reports on the stability of cyclodextrin complexes with β-lactam antibiotics (Ong, Sunderland and McDonald 1997; Loftsson and Olafsdottir 1991; Hsyu *et al.* 1984), there is no report for amoxycillin and clavulanate alone or in combination dosage form. Hsyu *et al.* (1984) investigated, ampicillin-β-cyclodextrin complexes and reported a reduction in the incidence of gastro-intestinal side effects in comparison with the uncomplexed drug. In another report the inhibitory effect of β-cyclodextrin on polymerization of ampicillin has been documented (Aki *et al.* 1990).

In this project hydroxypropyl β-cyclodextrin was chosen to investigate its effects on the stability of amoxycillin and clavulanate combination dosage form.

1.1.5.1 Kinetics of the reactions in presence of complexing agents

The effects of cyclodextrins on chemical stability of drug compounds in aqueous solutions has been widely studied. A review of various stability constants based on complexing effects of cyclodextrins has been presented by Loftsson (1995).

In aqueous solutions the molecules forming cyclodextrin complexes are in equilibrium with other free molecules in the solution, as indicated in the following equation:

$$mD + nCD \underset{}{\overset{K_{m:n}}{\rightleftharpoons}} D\text{-}CD(m+n) \qquad (1.35)$$

where

mD = m drug guest molecules

nCD = n cyclodextrin molecules

$K_{m:n}$ = the stability constant of the complex

In most cases one guest molecule forms a complex with one molecule of the cyclodextrin and the stability constant ($K_{1:1}$) is denoted by K_c. In kinetic studies K_c can be determined from the stabilizing or destabilizing effects of the cyclodextrin. When the drug concentration is kept constant and the cyclodextrin concentration is increased, the observed first-order rate constant (k_{obs}) for the rate of disappearance of the drug will asymptotically approach some minimum or maximum value. The observed rate constant within the cyclodextrin complex (k_c) and K_c can be determined by the Lineweaver-Burk plot (Lineweaver and Burk 1934). Hence in dilute aqueous solution assuming that 1:1 complex is formed:

$$\begin{array}{ccc} D + CD & \overset{K_c}{\rightleftharpoons} & D\text{-}CD \\ \downarrow k_0 & & \downarrow k_c \end{array} \qquad (1.36)$$

Degradation products

where

k_0 = observed first order rate constant for the degradation of free drug 'D'

The observed first-order rate constant for the total degradation of the drug (k_{obs}) is then the weighted average of k_0 and k_c (Loftsson 1995):

$$-\frac{d[D]_T}{dt} = k_{obs}[D]_T \qquad (1.37)$$

$$k_{obs} = \frac{k_0 + k_c K_c}{1 + K_c[CD]} \qquad (1.38)$$

$$\frac{1}{k_0 - k_{obs}} = \frac{1}{K_c(k_0 - k_c)[CD]} + \frac{1}{(k_0 - k_c)} \qquad (1.39)$$

In the above Equations 1.38 and 1.39, $[CD]$ is considered to be the total concentration of cyclodextrin (that is the sum of free cyclodextrin and the bound cyclodextrin in the complex). In order for this to be justified, the concentration of cyclodextrin should be at least 10 times greater than the concentration of the drug. The rate constants k_{obs} are determined at three or four concentrations of cyclodextrin, and k_c and K_c are calculated. The system without cyclodextrin gives the value of k_0.

1.1.5.2 Stabilization effects of cyclodextrin

There are several reports (Ong, Sunderland and McDonald 1997; Hsyu *et al.* 1984; Loftsson *et al.* 1993; Aki *et al.* 1990; Bekers *et al.* 1989), indicating that drug-cyclodextrin complex formation has improved the stability of β-lactam antibiotics and other drug formulations significantly.

Loftsson has investigated (1995) the various factors influencing the stabilizing abilities of different cyclodextrins. Some of these factors include:

i. The degree of complex formation is dependent on the value of K_c, the larger this value, indicates greater fraction of drug resides within the complex.

ii. The stabilizing effect is greater when the rate of the degradation of the drug within the complex (ie. the value of k_c) is smaller.

iii. The formation of a complex is not only influenced by the size of the central cavity but also by the chemical structure and the number of substitutes on the cyclodextrin molecule.

iv. the decrease in enthalpy of the system during complex formation, causes an increase in the K_c value when the temperature is lowered. Hence, increased complexation is obtained at lower temperatures than higher temperatures.

1.1.5.3 Destabilization effects of cyclodextrins

Since cyclodextrins are oligosaccharides, like many other carbohydrates they are capable of having a destabilizing effect on β-lactam antibiotics. There are reports (Loftsson and Olafsdottir 1991; Loftsson 1995; Fujiwara, Kawashima and Yamada

1985) which support this hypothesis. The destabilization effect of cyclodextrin is probably due to the interaction of the alcohol groups, located on the outer surface of the cyclodextrin molecules with the β-lactam ring of the antibiotics. This kind of base-catalysed degradation by hydroxypropyl β-cyclodextrin has been observed in cephalothin at pH values about 9.7 and aztreonam at pH above 6 (Loftsson 1995; Loftsson and Olafsdottir 1991).

Cyclodextrins have also been reported to destabilize other drugs such as prostaglandin E_1 (Adachi, Hirayama and Uekama 1992), acetylsalicylic acid (Choudhury and Mitra 1993).

1.2 Objectives of This Study

The research work was designed to achieve the following objectives.

- To further develop knowledge of the rates of degradation of amoxycillin sodium and potassium clavulanate individually and in combination dosage forms in the liquid state.
- To study the stability of amoxycillin sodium and potassium clavulanate individually and in combination dosage forms in the frozen state.
- To study the effect of concentration, buffer and complexing agents such as hydroxypropyl ß-cyclodextrin on the stability of amoxycillin sodium and potassium clavulanate in combination dosage forms.

1.3 Organisation of the Thesis

This thesis is divided into 6 chapters. Chapter 1 is the general introduction to the field of study and includes a survey of the literature. Chapter 2 presents the overall methodology on the experimental work and the interpretation of data. Chapter 3, Chapter 4 and Chapter 5 present the results obtained from the experimental data and discuss the relevant issues. Chapter 6 concludes the work by general discussion and conclusions. It also includes suggestions for future studies.

CHAPTER 2

EXPERIMENTAL

2.1 Materials

- Acetic acid glacial, Univar AR, Ajax Chemicals N.S.W., Australia
- Acetone, Univar AR, Ajax Chemicals N.S.W., Australia.
- Amoxycillin sodium reference standard, CRS, European Pharmacopoeia, batch / lot number 1, B.P. 431 R6, F-67006.
- Amoxycillin sodium, SmithKline Beecham Pharmaceuticals, Australia, batch number B0004-08904, B0004-09201 and B0004-10701.
- Disodium hydrogen phosphate, Univar AR, Ajax chemicals N.S.W., Australia, batch number 621.
- Dry ice, provided as a gift by Kleen gas, Darwin, Australia.
- 1,2-Ethanediol, Univar AR, Ajax Chemicals N.S.W., Australia.
- Hydrochloric acid, Univar AR, Ajax Chemicals N.S.W., Australia.
- Hydroxypropyl ß-cyclodextrin, Amaizo American Maize Products, USA, lot RR13 (degree of substitution = 7.0) and lot P-104-29-1 (degree of substitution = 6.5).
- Lithium clavulanate, BRL14151, provided as a gift by SmithKline Pharmaceuticals, UK, batch number BN65 and BN77.
- Lithium clavulanate, USP reference standard, cat No 13442
- Methanol, HPLC grade, Unichrom Ajax chemicals, Australia.
- Potassium clavulanate, BRL14151, provided as a gift by SmithKline Pharmaceuticals, UK, batch number BN61.
- Potassium dihydrogen phosphate, Univar AR, Ajax chemicals N.S.W., Australia, batch number 391.
- Primary buffer standard, buffer solution 4, BDH chemicals Australia Pty. Ltd., pH 4.001 ± 0.005 at 20°C.
- Primary buffer standard, buffer solution 7, BDH chemicals Australia Pty. Ltd., pH 7.00 ± 0.005 at 20°C.
- Primary buffer standard, buffer solution 9, BDH chemicals Australia Pty. Ltd., pH 9.00 ± 0.005 at 20°C.

- Sodium acetate anhydrous, Univar AR, Ajax Chemicals N.S.W., Australia, batch number 471 and BDH Analar, BDH chemicals, Australia, batch number 30104.
- Sodium chloride, Univar AR, Ajax Chemicals N.S.W., Australia, batch number 465.
- Sodium dihydrogen phosphate, Univar AR, Ajax Chemicals N.S.W., Australia batch number 471 and BDH Analar, BDH chemicals, Australia, batch number 10245.

All solutions were prepared using high purity water from either Permutit-water apparatus (Permutit, Australia), Milli-Q water (Millipore, Australia) or double distilled water from an all glass still, depending on availability.

2.2 Equipment and Instrumentation

- **High performance liquid chromatography (HPLC)**

The chromatographic system consisted of a Varian 5500 HPLC pump (Varian, USA) connected to a 20µl loop (Rheodyne, USA) injector, a Varian 5050 UV detector (Varian, USA) with a variable-wavelength and tuneable UV-Vis absorbance detection mode, an IBM computer data station (Star 10 with Delta software) and a printer (Epson LQ-570). An Alltima (Alltech, USA) reverse phase HPLC column 5µ C_{18}, packed in 25 cm × 4.5 mm was used in conjunction with a reverse phase guard-column C_{18} (Alltech, USA) as the stationary phase.

The following HPLC systems were also used at various stages of the project:

i. Waters 501 HPLC pump (Waters, USA), Waters 991 Photo diode array UV spectrophotometer (Waters, USA) connected to a 3396 Hewlett Packard integrator (Hewlett Packard, USA).

ii. Varian 9010 HPLC pump (Varian, USA), Varian 9050 UV detector connected to a Varian GC Star workstation (Varian, USA).

- **pH-meter**

Digital pH-meter, model 1852 (Australia)

Hanna pH-meter, model 8417 (Singapore)

Metrohm Herisau pH-meter, model E 520 (Switzerland)

- **Water bath**

Julabo circulating water bath, model F20-C (Germany), with variable temperature selection range of –20°C to100°C and a digital temperature display.

Grant circulating water bath, TypeZA (England)

Techne water bath, model SB-4 (England)

- **Nuclear magnetic resonance (NMR) spectroscopy**

Varian nuclear magnetic resonance spectrometer, model Gemini 200 (USA), 200 MHz.

- **Thermometers**

Dobbie thermometer, model 526.10952 (Australia) and Emil thermometer, model emil-11105 (UK) were used as the reference thermometers.

2.3 Preparation of Kinetic Runs

2.3.1 The liquid state

Kinetic studies in the liquid aqueous state were carried out at three pH values of, 7.00 ± 0.05 (1.0×10^{-1} mol dm^{-3} phosphate buffer), pH 4.60 ± 0.05 (2.2×10^{-1} mol dm^{-3} acetate buffer) and pH 2.00 ± 0.05 (1.2×10^{-2} mol dm^{-3} hydrochloric acid). Each buffer system was further studied at four different temperatures (35, 42, 49 and 55°C ± 0.2) and the experiments in hydrochloric acid were studied at temperatures of 14, 20, 27, and 35°C ± 0.2. All the runs were performed at a constant ionic strength (μ= 0.5) using sodium chloride.

2.3.1.1 Stability of amoxycillin sodium solutions: Each experimental run was prepared by adding a double strength solution of the buffer or the hydrochloric acid system, to a volumetric flask. The flask was then placed in a thermostat water bath at the required temperature for 5 minutes for equilibration. Simultaneously a double strength solution of amoxycillin sodium was prepared in water and placed in the water bath for the same period. Equilibrated solutions of amoxycillin sodium and the buffer media were mixed together and shaken well. Immediately about 2ml aliquots of the mixed solutions were removed from the flask brought to the room temperature and injected on to the HPLC column. The time when the first sample removed was denoted as time zero. Subsequently more samples were drawn at specified time intervals for analysis until at least three half-lives of the reactions were complete. Usually 10-18 samples were used for each run. Standard solutions of amoxycillin sodium prepared in water were injected onto the column between the sample runs to ascertain column reproducibility. Maximum time allowed between sampling times from the flask, until injection on to the HPLC column was 3 minutes.

The temperature of the thermostat bath was monitored with a reference thermometer with a least temperature specification of 0.2°C.

2.3.1.2 Stability of potassium clavulanate solutions: The same method of sample preparation stated for amoxycillin sodium (Section 2.3.1.1) was used. The theoretical concentrations of potassium clavulanate were 1.05×10^{-3} mol dm^{-3} in the buffers and 7.38×10^{-4} mol dm^{-3} in hydrochloric acid systems. Standard solutions of potassium clavulanate were used between the sample runs to determine reproducibility. Usually 8-12 samples were used for each individual run.

2.3.1.3 Stability of amoxycillin sodium and potassium clavulanate in combined solutions: The specified amounts of the drugs were placed in a volumetric flask, mixed and dissolved in water and treated as for the individual drugs stated in Sections 2.3.1.1 and 2.3.1.2.

2.3.1.4 The catalytic effect of potassium clavulanate on stability of amoxycillin sodium solutions: These runs were prepared using four different concentrations of potassium clavulanate (5.3×10^{-4} mol dm^{-3}, 1.05×10^{-3} mol dm^{-3}, 2.10×10^{-3} mol $dm^{-3}$and 3.15×10^{-3} mol dm^{-3}) and a constant concentration of amoxycillin sodium (1.29×10^{-3} mol dm^{-3}). Experimental runs were executed with various concentrations of potassium clavulanate alone and in combination with amoxycillin. Each of the runs was prepared in two buffer systems, phosphate buffer (pH 7.00 ± 0.05) and acetate buffer (pH 4.60 ± 0.05). All solutions were adjusted to constant ionic strength $\mu = 0.5$ and a temperature of 55°C ± 0.2. For sample preparation refer to Section 2.3.1.1.

2.3.1.5 The catalytic effect of amoxycillin sodium on stability of potassium clavulanate solutions: A similar procedure was adopted as in Section 2.3.1.4 except that four different concentrations of amoxycillin sodium were used (1.29×10^{-3} mol dm^{-3}, 6.45×10^{-3} mol dm^{-3}, 12.90×10^{-3} mol $dm^{-3}$and 25.80×10^{-3} mol dm^{-3}). The concentration of potassium clavulanate in all the experiments was kept constant at 1.05×10^{-3} mol dm^{-3}. Experimental runs were executed with various concentrations of amoxycillin sodium alone and in combination with potassium clavulanate. For the runs with higher concentrations of amoxycillin sodium, samples were diluted with water (1 into 2ml, 1 into 5ml and 1into 10 ml respectively) prior to injecting onto the HPLC column.

2.3.1.6 The catalytic effect of the buffers used on the stability of amoxycillin sodium and potassium clavulanate solutions: These experiments were performed at constant temperature (55°C ± 0.2), in acetate buffer pH 4.60 ± 0.05 and phosphate buffer pH 7.00 ± 0.05.

The effect of acetate buffer on the rate of reactions of amoxycillin sodium and potassium clavulanate was studied by executing experimental runs in four different total acetate concentrations of 1.1×10^{-1} mol dm^{-3}, 2.2×10^{-1} mol dm^{-3}, 4.0×10^{-1} mol $dm^{-3}$and 5.5×10^{-1} mol dm^{-3} at constant pH (4.6). While the effect of phosphate buffer on the stability of amoxycillin sodium and potassium clavulanate was studied similarly using four different total phosphate concentrations of 5.0×10^{-2} mol dm^{-3}, 1.0×10^{-1} mol dm^{-3}, 1.5×10^{-1} mol dm^{-3} and 2.0×10^{-1} mol dm^{-3} at constant pH (7.0).

All the solutions were adjusted to constant ionic strength (0.5μ) using sodium chloride. Samples were prepared according to the method specified in Section 2.3.1.1.

2.3.2 The frozen state

Experiments in the frozen state were carried out at three sub zero temperatures, -7.3 ± 0.2, -9.8 ± 0.2, -13.5 ± 0.1° C and at pH values, pH 7.00 ± 0.05 (1.0×10^{-1} mol dm^{-3} phosphate buffer); pH 4.60 ± 0.05 (2.2×10^{-1} mol dm^{-3} acetate buffer) and pH 2.00 ± 0.05 (1.2×10^{-2} mol dm^{-3} hydrochloric acid). Solutions in hydrochloric acid were adjusted to an ionic strength of 0.5μ using sodium chloride.

2.3.2.1 Stability of amoxycillin sodium solutions: Solutions of amoxycillin sodium containing 1.29×10^{-3} mol dm^{-3}(for experiments with buffers) and 9.03×10^{-4} mol dm^{-3}(for experiments with hydrochloric acid) were prepared at double the required concentration in water. Also double strength solutions of phosphate buffer, acetate buffer and hydrochloric acid media were prepared.

Equal volumes of the double strength buffers and sample solutions were mixed together in volumetric flasks. For each set of runs 2ml samples of this mixture were added by an auto pipette into each of 16 glass stoppered test tubes. The tubes were immediately frozen in a dry ice acetone - bath mixture and kept at -75° C in a freezer for one hour. Then the tubes were transferred into a glycol- water bath mixture (~ 50% ethanediol w/v, to keep the density at 1.067) at the relevant temperature and left for about 45 minutes to equilibrate. After the set time the first

tube was removed and thawed at room temperature by placing it in a bath of lukewarm water with shaking the tube occasionally (usually about 5 minutes was required to reach room temperature) and an aliquot was immediately injected onto the HPLC column via a Rheodyne 20 µl injector loop. The time for the first sample was taken as time zero. Subsequently the remaining samples were injected by the same procedure at specified times until about 2-3 half-lives of the reaction was complete or until a minimum of 8 or maximum of 10 days of reaction was reached.
In hydrochloric acid media because of the fast rate of the reaction at room temperature extra care was taken to minimise the risk of degradation of the drug compound at that temperature during the sample preparation and after thawing. Therefore 1ml of the double strength drug solution was added to 1ml of double strength hydrochloric acid media by an auto pipette in to a glass stoppered test tube, mixed and immediately the tube was frozen to -70° C in an acetone dry ice bath-mixture. Subsequently the remaining tubes were treated in the same manner and the reminder of the procedure was the same as stated above. All the samples were injected onto the HPLC column within 2 minutes of attaining room temperature.

Standard solutions of amoxycillin sodium in water were used between the sample runs to ascertain column reproducibility. The standard solution was stored in a refrigerator at 4°C. For every standard run about 2ml of standard solution was removed, brought to room temperature and an aliquot was injected onto the HPLC column.

The temperature of the thermostat bath was monitored with a reference thermometer with a least temperature specification of 0.2°C.

2.3.2.2 Stability of potassium clavulanate solutions: Aqueous solutions of potassium clavulanate in water were prepared at the same theoretical concentrations stated under the liquid state Section 2.3.1.2. All other procedures and experimental conditions were carried out as for amoxycillin sodium Section 2.3.2.1.

2.3.2.3 Stability of amoxycillin sodium and potassium clavulanate in combined solutions: Aqueous solutions containing both the antibiotics were prepared by mixing the specified amounts of amoxycillin sodium (refer to Section 2.3.2.1) and potassium clavulanate (refer to Section 2.3.1.2) in water. Sample runs were executed using the same procedure in Section 2.3.2.1.

2.3.2.4 Effect of sodium chloride on the rates of reactions: Sodium chloride (used to maintain constant ionic strength of 0.5μ) was added to solutions of amoxycillin sodium and potassium clavulanate in various systems under the experimental conditions. These runs were compared with those containing no sodium chloride. All the experiments were conducted at constant temperature of -7.3 ± 0.2°C. The procedure for the execution of the runs remained the same as under Section 2.3.2.1.

2.3.3 Effect of hydroxypropyl β-cyclodextrin (HPβCD) on the stability of amoxycillin sodium and potassium/lithium clavulanate solutions

Kinetic runs were performed in both liquid and frozen states in 2.2×10^{-1} mol dm^{-3} acetate buffer pH 4.60 ± 0.05. In the liquid state two sets of runs were carried out at constant ionic strength 0.5 μ and a temperature of 55° C ± 0.2 as described below.

A. These runs were carried out on the basis of molar concentration ratios of the antibiotic to HPßCD from 1:2 to 1:10. Hence three different concentrations (2.1×10^{-3} mol dm^{-3}, 5.25×10^{-3} mol dm^{-3}, 1.05×10^{-2} mol dm^{-3}) of HPßCD (lot RR13) were used for a constant concentration (1.05×10^{-3} mol dm^{-3}) of lithium clavulanate solution corresponding to the molar ratios of 1:2, 1:5 and 1:10 respectively. Also three different concentrations (2.58×10^{-3} mol dm^{-3}, 6.45×10^{-3} mol dm^{-3}, 1.29×10^{-2} mol dm^{-3}) of HPßCD (lot RR13) were used for a constant concentration (1.29×10^{-3} mol dm^{-3}) of amoxycillin sodium solution corresponding to the molar ratios of 1:2, 1:5 and 1:10 respectively. For the combination runs, the amount of HPßCD added was equal to the sum of the amounts in two individual runs. Thus for the 1:2 combination run, 4.68×10^{-3} mol $dm^{-3}$of HPßCD was added to a solution containing 1.05×10^{-3} mol dm^{-3} of clavulanate and 1.29×10^{-3} mol dm^{-3} of amoxycillin. Similarly 1.70×10^{-2} mol dm^{-3} and 2.34×10^{-2} mol dm^{-3} of HPßCD were used for the 1:5 and 1:10 combination runs respectively. In all the cases the quantities of HPßCD were added to the double strength buffer solutions before mixing with the antibiotic solutions. The procedure for the execution of the runs remained the same as stated in Section 2.3.1.1.

B. This set of experiments was performed at higher concentrations of antibiotics i.e. 1.29×10^{-2} mol dm^{-3} of amoxycillin sodium and 1.05×10^{-2} mol dm^{-3} of potassium clavulanate individually and in combination. The quantities of HPßCD (lot P-104-29-1) added were 2.5%, 5% and 10% w/v. The same quantities (2.5%, 5% and 10%)

of HPßCD were used for the combination runs. The samples were prepared as stated above.

2.3.3.1 In the frozen state: Only one concentration (2.5% w/v) of HPßCD (lot P-104-29-1) was used at constant temperature of -7.3°C ± 0.2°C. The concentrations of the antibiotics used in this run were 1.29×10^{-2} mol dm^{-3} of sodium amoxycillin and 1.05×10^{-2} mol dm^{-3} of potassium clavulanate. The weighed amounts of HPßCD were dissolved in double strength acetate buffer solution, then 1ml aliquots of buffer/ HPßCD mixture were placed by an auto pipette into 14 stoppered test tubes. In to each test tube 1ml aliquots of the double strength combined solutions of sodium amoxycillin and potassium clavulanate were added and mixed well. The tubes were frozen immediately in the dry ice-acetone bath mixture and the remaining procedure was the same as described under Section 2.3.21.

All sample solutions were diluted with water (1 ml to 10 ml) prior to injecting onto the HPLC column.

2.4 Assay Method

An HPLC assay method was developed to determine the concentrations of amoxycillin and clavulanate simultaneously. The materials and equipment used were outlined in Section 2.2. The experimental conditions are given below:

- Mobile phase: The mobile phase consisted of 5.0×10^{-2} mol dm^{-3} of sodium dihydrogen phosphate adjusted to pH 4.4 ± 0.1 using dilute sodium hydroxide or phosphoric acid and mixed with methanol (95: 5).
- Detection wave-length: 228 nm
- Flow rate: 1.5 ml/min
- Injection volume: 20µl

All operations were carried out under ambient conditions.

2.5 Treatment of Kinetic Runs

Methods for calculation of rate constants are discussed in detail by Martin (1993b). The order of the reaction was confirmed by the graphic method using several half-lives of reaction. All the rate constant values were obtained from the slopes of the log concentration against time plots. Regression analysis of data were performed by a least squares method using Origin computer software. To summarize the equations relevant to those found in the result sections, consider a reaction of the type:

$$A + B \xrightarrow{k_2} \text{products} \qquad (2.1)$$

where A and B are the reacting species and the forward reaction is assumed to go to completion.

Then the rate of loss of the antibiotic A is given by

$$-\frac{d[A]}{dt} = k_2[A][B] \qquad (2.2)$$

This is a second order reaction. If [B] is in large excess then pseudo first-order kinetics are observed and equation (2.2) becomes

$$-\frac{d[A]}{dt} = k_{obs}[A] \qquad (2.3)$$

$$\text{where } k_{obs} = k_2[B] \qquad (2.4)$$

Integrating equation (2.3) gives,

$$\ln[A] = \ln[A]_0 - k_{obs}t \qquad (2.5)$$

or

$$\log[A] = \log[A]_0 - \frac{k_{obs}\,t}{2.303} \qquad (2.6)$$

or

$$k_{obs} = \frac{2.303}{t} \log\frac{[A]_0}{[A]} \qquad (2.7)$$

where

$[A]$ = Concentration of the antibiotic at time t

$[A]_0$ = Concentration of the antibiotic at time $t = 0$

The linear expression in Equation (2.6) indicates that the plot of log concentration against time is linear with the slope of $-\frac{k_{obs}}{2.303}$ from which the value of k_{obs} is obtained. A straight line obtained from these plots over 3 or more half-lives of reaction indicates that the reaction is first-order in nature. The rate constant value obtained from this plot is therefore an average k value obtained as the first-order rate constant.

The treatment of the data also involves the applicability of the Beer-Lambert Law in calculation of rate constants as the concentration of *A* varies linearly with the absorption of UV light. This was demonstrated from the concentrations used in constructing the standard curves over the concentrations relevant to this study.

The data in the frozen state were treated similarly by determining the first-order rate constants and comparing them with those at the liquid sate temperatures. For theory of the frozen state refer to Section 1.1.4, Chapter 1. Further treatment of data and the evaluation of the rate equations will be discussed under individual drugs in Chapter 4.

2.5.1 Activation parameters

Activation energy data were obtained using the Arrhenius temperature dependence theory as described by Martin (1993a). The effect of temperature on the rate of the reaction is shown in Equation (2.8)

$$k = Ae^{-E_a/RT} \qquad (2.8)$$

or

$$\log k = \log A - \frac{E_a}{2.303\,RT} \qquad (2.9)$$

where k = Specific reaction rate constant

A = A constant known as Arrhenius frequency factor

E_a = Energy of activation

R = Gas constant

T = Temperature (Kelvin)

The values of these constants (E_a and A) are obtain by plotting log k against $1/T$. As seen in Equation 2.9 the slope of the line so obtained is $-E_a / 2.303\,R$ and the intercept is $log\,A$, from which E_a and A can be obtained.

The data in the liquid state were extrapolated by linear regression to the frozen temperature to compare the rate constant values with respect to the frozen state.

2.6 pH Measurements

Routine pH measurements of sample and buffer solutions were carried out at room temperature using a digital pH meter. Prior to pH measurement the instrument was standardised using standard buffer solutions, one of lower and the other of higher pH than the solution under study.

Two pH measurements for each kinetic run were carried out, one at the beginning of the experiment and the other towards the end of the runs. In the frozen state with every run, 5ml of the sample solutions were placed in two separate tubes. Following the same procedure for sample preparation discussed under individual runs, the frozen tubes were thawed to room temperature and the pH values of the solutions were measured accordingly.

2.7 Preparation of Buffers

Preparation of the buffers used in the study was based on the Henderson-Hasselbalch equation for a weak acid and its salt (Martin 1993c):

$$pH = pK_a + \log \frac{[salt]}{[acid]} \qquad (2.10)$$

where the pK_a was the value listed or calculated at the required ionic strength.

Whenever constant ionic strength 0.5μ was required the concentration of sodium chloride required was calculated by the following equation (Martin 1993d):

$$\mu = \frac{1}{2} \sum_{1}^{j} c_i z_i^2 \qquad (2.11)$$

where $\sum_{1}^{j}$ = Summation of the products of cz^2 terms for all the ionic species in the solution from the first one to the j^{th} species

c_i = Concentration in moles per litre of any of the ions.

z_i = Valence of the species

The buffers constituents used in this study are given below.

- Phosphate buffer (1.0×10^{-1} mol dm^{-3}), pH 7.00 ± 0.05, containing 6.3×10^{-2} mol dm^{-3} of disodiun phosphate and 3.6×10^{-2} mol dm^{-3} of potassium dihydrogen phosphate.
- Acetate buffer (2.2×10^{-1}mol dm^{-3}), pH 4.60 ± 0.05, containing 1.3×10^{-1} mol $dm^{-3}$of sodium acetate and 9.3×10^{-2} mol $dm^{-3}$of acetic acid.

Other buffer concentrations indicated in Section 2.3.1.6 were calculated based on the above concentrations.

2.8 General Discussion

In the frozen state some preliminary work performed before the main experimental work was necessary to establish the experimental conditions. Studies at lower sub- zero temperatures showed that the drug compounds were highly stable under these experimental conditions. Therefore rate studies at lower sub-zero temperatures were avoided due to the lengthy experimental time. The presence of sodium chloride used as a compound for constant ionic strength was found to have significant rate stabilising effect on the rate of the reaction of the antibiotics at the temperatures of this study. Therefore sodium chloride was deleted from the sample preparation in both the buffer media but included in hydrochloric acid system since the rate of the reaction would have been very fast and difficult to monitor especially in the case of potassium clavulanate. Also preliminary studies at higher concentrations such as 10 times the usual concentration of the drug combination in acetate buffer resulted in precipitation, which appeared to be not completely soluble upon shaking. This problem made it difficult to carry out experiments at higher concentrations under the conditions of this study.

2.9 Errors

To eliminate determinate and indeterminate errors, the instruments used were constantly checked and calibrated. The HPLC column was checked for plate counts and whenever there was a significant change in the column efficiency it was replaced by a new column. In each experimental run every day standard solutions of the drug samples were used from time to time in between the sample runs to check the reproducibility. The thermostat bath was calibrated from time to time using a reference thermometer.

The maximum temperature variation observed was ± 0.2°C resulting in a maximum error of ± 4% in the rate constant. Other errors involved in the analysis of the runs could be due to column reproducibility where a maximum of ± 2% was allowed when comparing with the day to day standard solutions. Where volumetric methods were involved for sample preparation, a further ± 1% error could have occurred. This brings the maximum theoretical errors to about ± 7%. Where

repeated runs were performed the rate constants were reproducible within this figure.

Linear relationships were fitted by the method of least squares. The errors in slope and intercept were expressed at the 95% confidence interval. Typical standard errors were within 5% of the rate constant values however wherever nonlinear curve fitting program or the stripping technique were used, some standard error values were up to about 25% of the rate constants. The correlation coefficient (r) on each sample size in the liquid state was equal or above 0.99 and in the frozen state the lowest correlation coefficient observed was 0.93.

Measurement of each pH value involved an error of ± 0.05 of pH units. Because standardisation could also induce an error of ± 0.05 unit, the total error involved in a pH measurement could result in a maximum of ± 0.1 of a pH unit.

CHAPTER 3

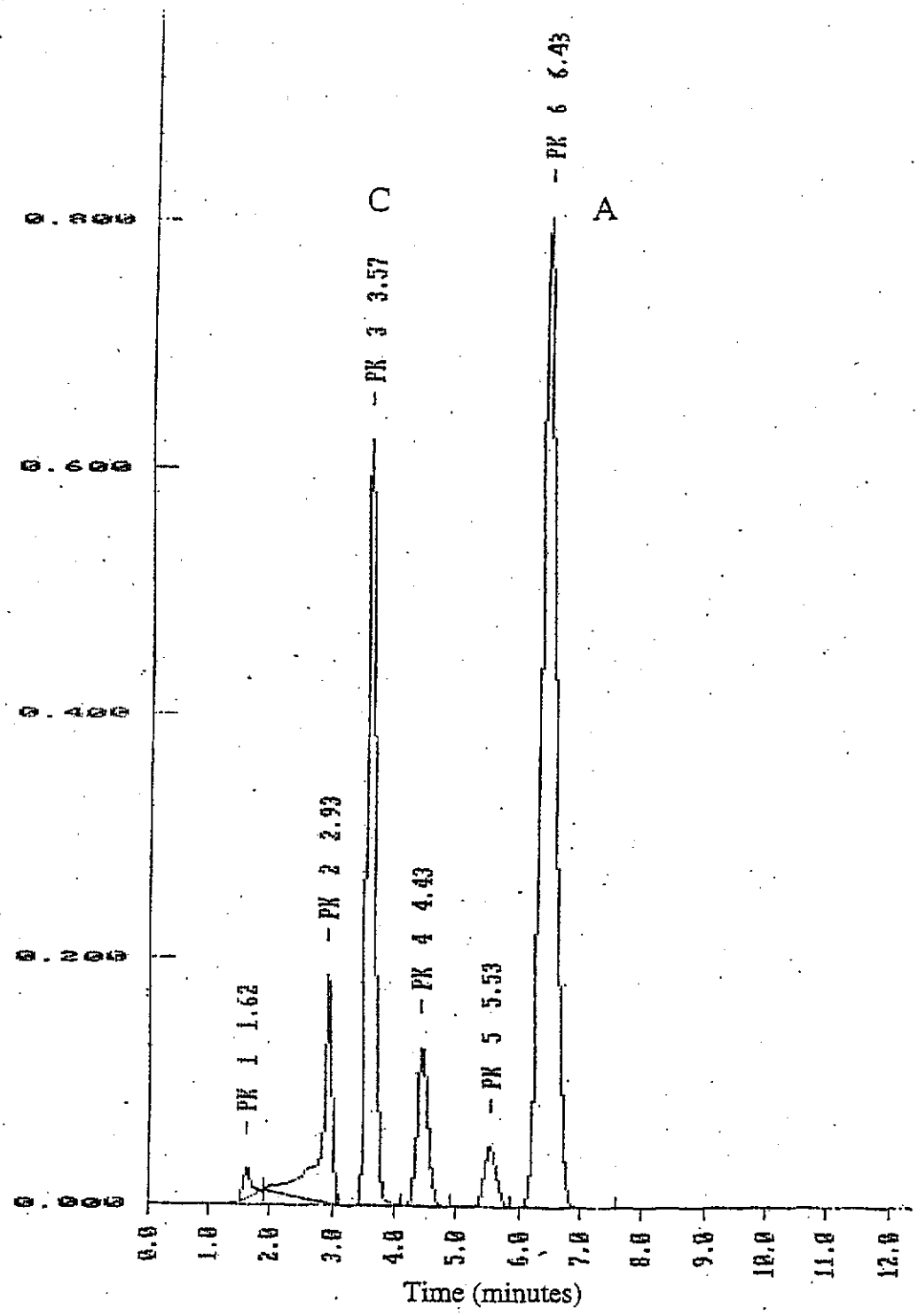
STABILITY IN THE LIQUID STATE

Kinetics of the hydrolysis of amoxycillin sodium and potassium clavulanate individually and in combination were studied in the liquid state and under the conditions of this study as specified in Chapter 2, Section 2.3. The amounts of the reactants remaining with time were monitored by the HPLC assay method described previously, and data were obtained by dividing the height of each individual peak obtained at each sampling time into the one obtained at time zero which was designated as 100%. This quotient was expressed as a percentage. The responses due to standard samples of both the reactants were used to monitor the reproducibility of the assay method. Typical HPLC chromatograms of the partially degraded antibiotics are illustrated in Figure 3.1. In this figure it is evident that amoxycillin and clavulanate show full baseline resolution.

3.1 Justification of the Assay Method

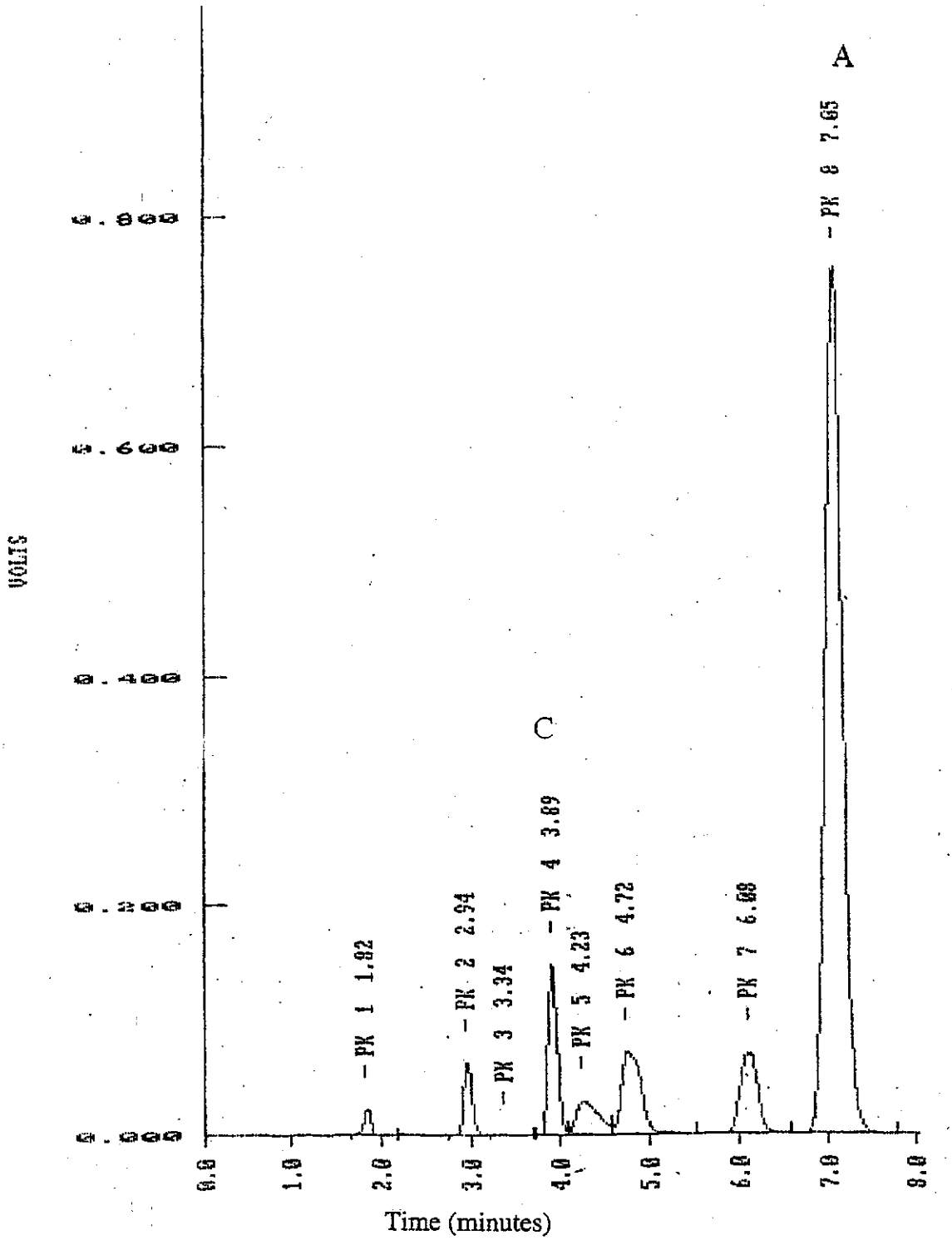
Most existing methods of analysis (Ashwin, Lynn and Taskis 1987; Abounassif *et al.* 1991; United States Pharmacopoeia 1990) were investigated in order to establish a stability indicating method to enable measurements of the concentrations of both compounds simultaneously. The USP method (1990) was found to be the most appropriate one. However this method was modified to give improved resolution and a better specificity for the desired peak. Refer to Chapter 2 Section 2.4 for the assay methodology.

The method was validated by taking standard solutions of the drug combination in high purity water over the concentration range of 6.45×10^{-5} to 2.58×10^{-3} mol dm^{-3} (amoxycillin sodium) and 4.2×10^{-5} to 1.68×10^{-3} mol dm^{-3} (potassium clavulanate) where linearity $r > 0.999$ was established for both the compounds. The precision of the method was found by calculating the coefficient of variation ($n = 6$) to be 0.66% for amoxycillin (1.29×10^{-3} mol dm^{-3}) and 0.3 % clavulanate (1.05×10^{-3} mol dm^{-3}). Representative standard curves are shown in Figure 3.2.



(a) In acetate buffer degraded at 42°C, pH 4.60 and μ = 0.5. A and C represent amoxycillin and clavulanate respectively.

Figure 3.1: Typical HPLC chromatograms of the partially degraded drug compounds in combination.



(b) In hydrochloric acid system degraded at 20°C, pH 2.00 and μ = 0.5. A and C represent amoxycillin and clavulanate respectively.

Figure 3.1: Typical HPLC chromatograms of the partially degraded drug compounds in combination.

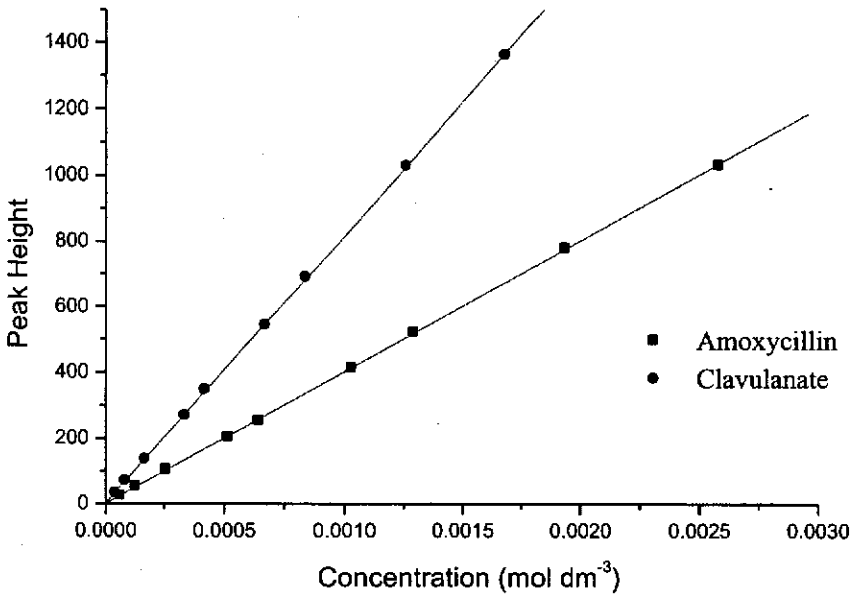


Fig 3.2: Standard curves for sodium amoxycillin and potassium clavulanate.

The stability indicating nature of the assay method was determined by inducing degradation of the drug compounds in water, acid and alkali at elevated (60°C) and room temperatures and restoring the analytical response by addition of the drugs to the completely degraded samples. The stability indicating nature of the method was also verified with a photo diode array detector to ascertain peak purity. The stability indicating experiments were conducted for each drug solution individually and in combination, as there was no significant difference of results between the two, only the runs in combination are discussed here. Stability indicating tests in acid media were performed using a solution of amoxycillin sodium and potassium clavulanate in 2.0×10^{-2} mol dm^{-3} solution in hydrochloric acid, at room temperature for a maximum of ten days where both antibiotics were almost completely degraded. Then to this degraded solution a fresh solution of amoxycillin and clavulanate was added so that the final solution showed then 75% of clavulanate and 66% of amoxycillin with respect to the initial drug concentration. Figure 3.3(a)-3.3(c) illustrates the chromatograms where recovery was calculated to be 96.08% and 99.7 % for clavulanate and amoxycillin respectively. The reason for a lower recovery value for clavulanate was due to partial degradation of clavulanate in hydrochloric acid solution because clavulanate was highly unstable in this system. Figure 3.3(b) shows the chromatogram of the almost completely degraded test solution,

magnified to show a small trace of amoxycillin remaining and the complete degradation of clavulanate. The stability test in alkali solution was conducted in 1.0×10^{-2} mol dm^{-3} sodium hydroxide by following a similar procedure to that for the acid solution with the exception that three hours was sufficient for complete degradation of the drug compounds. The recovery solution contained 110% of clavulanate and 62% of amoxycillin with respect to the initial concentrations of the degraded solution. Figure 3.4(a) - 3.4(c) illustrate the chromatograms where the assayed recovery was 99.7% and 98.4% for clavulanate and amoxycillin respectively. The stability indicating assay in water was carried out by heating the combined drug solution at 60°C for two days leading to complete degradation of the drug compounds. Then to the degraded solution equal quantities (with respect to the initial concentration) of clavulanate and amoxycillin was added. Figure 3.5(a) – 3.5(c) illustrates the chromatograms obtained. The recovery was 101.04% and 98.67% for clavulanate and amoxycillin respectively.

Thus the over all recovery values obtained from these experimental results fall within the range 100% ± 4% which is considered to be within the experimental error limits.

The stability indicating nature of the assay method was further investigated by use of a diode array detector for spectrum analysis (200-350 nm) and peak purity tests. Standard samples of amoxycillin sodium RS and potassium clavulanate RS were dissolved in water and evaluated by the spectrum analysis and the peak purity tests. These data were used as standard data for further evaluation of the assay method. Subsequently kinetic studies on the reactants in hydrochloric acid, acetate buffer and phosphate buffer media were carried out at 60°C. The absorption ratios at certain retention times were calculated by taking the ratio of two absorption readings of each spectrum. This ratio was then compared with other ratios obtained arbitrary from a different retention time within the particular peak. Generally three ratios obtained across three regions (around peak starting point, mid point and ending point) of each peak were compared. As there was no significant change in ratios it was concluded that the area under the curve was pure. These data were further confirmed with the peak purity test, which is a program (991 Photo diode array version 6.22a) by which the instrument shades the area of the peak under analysis assessing it to be pure. A combination of these evaluations has confirmed the assay method as being specific and stability indicative in nature. A wave- length of 228 nm

was found to be most suitable, providing a high response for both the antibiotics when used in combination under all the experimental conditions. The peak height rather than the peak area was concluded to give a more specific result, on combination with the degraded compounds. Therefore the entire assay calculations are based on the height. Traces of the peak purity runs are shown in Figure 3.6(a)-3.6(d).

The slight variation in retention times evident over the entire stability indicating experiments are the result of a consistent small variation with time and day to day analysis and also using different HPLC instruments (Chapter 2, Section 2.2). These were evident from the series of standard solutions tested each time between the runs.

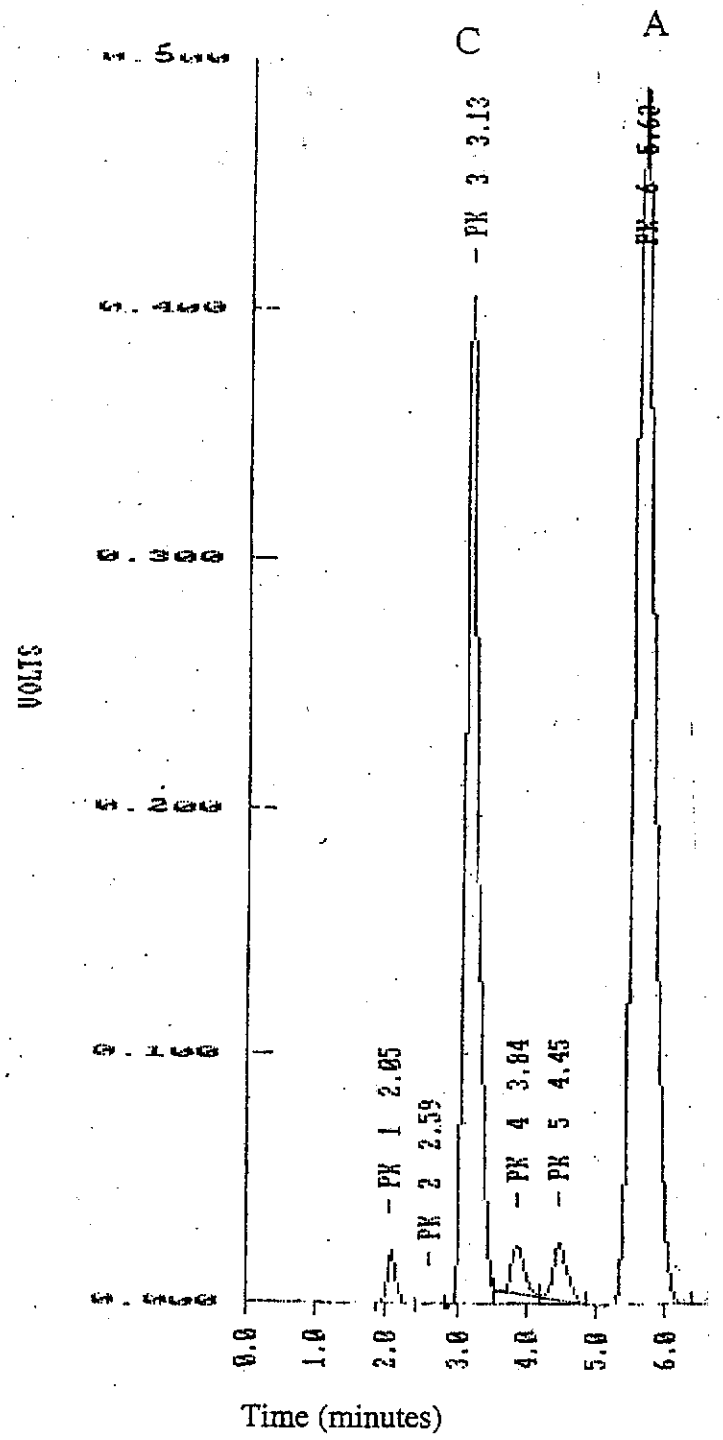
Thus the results indicate that there was no significant interference from degradation products to the amoxycillin and clavulanate peaks over a wide range of pH and under the conditions of total degradation. The peak purity test demonstrated that occasionally during the course of degradation of the drug compounds a small impurity was observed (Figure 3.6(d)) in some peaks if tailing occurred, this issue was largely overcome by taking the height response instead of area. Thus the assay method developed is a stability indicating method and suitable for the purpose of this project.

3.2 Kinetics of the Reactions

Experimental runs for amoxycillin and clavulanate showed a linear relationship when log percentage concentration was plotted against time over 2-4 half-life times of reaction (Figure 3.7), indicating that simple first-order kinetics was obeyed. However in the case of amoxycillin in combination with clavulanate in the buffer systems, the rate plots (Figure 3.8) were nonlinear. These data were treated by application of a first-order biexponential model.

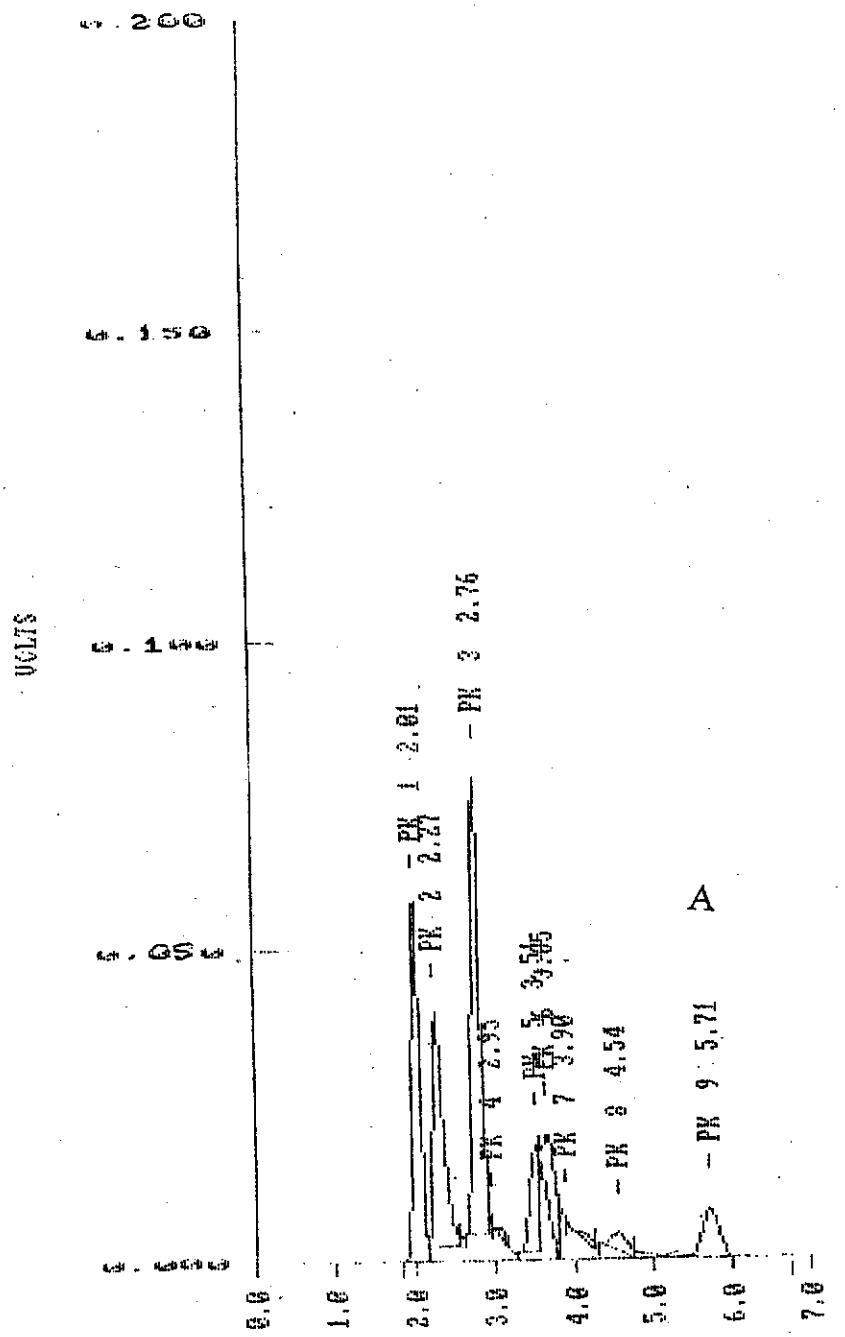
3.2.1 First-order reaction

General features of the mechanism of the reaction were provided in Chapter 2 Section 2.5.



(a) Zero time solution. A and C represent amoxycillin and clavulanate respectively.

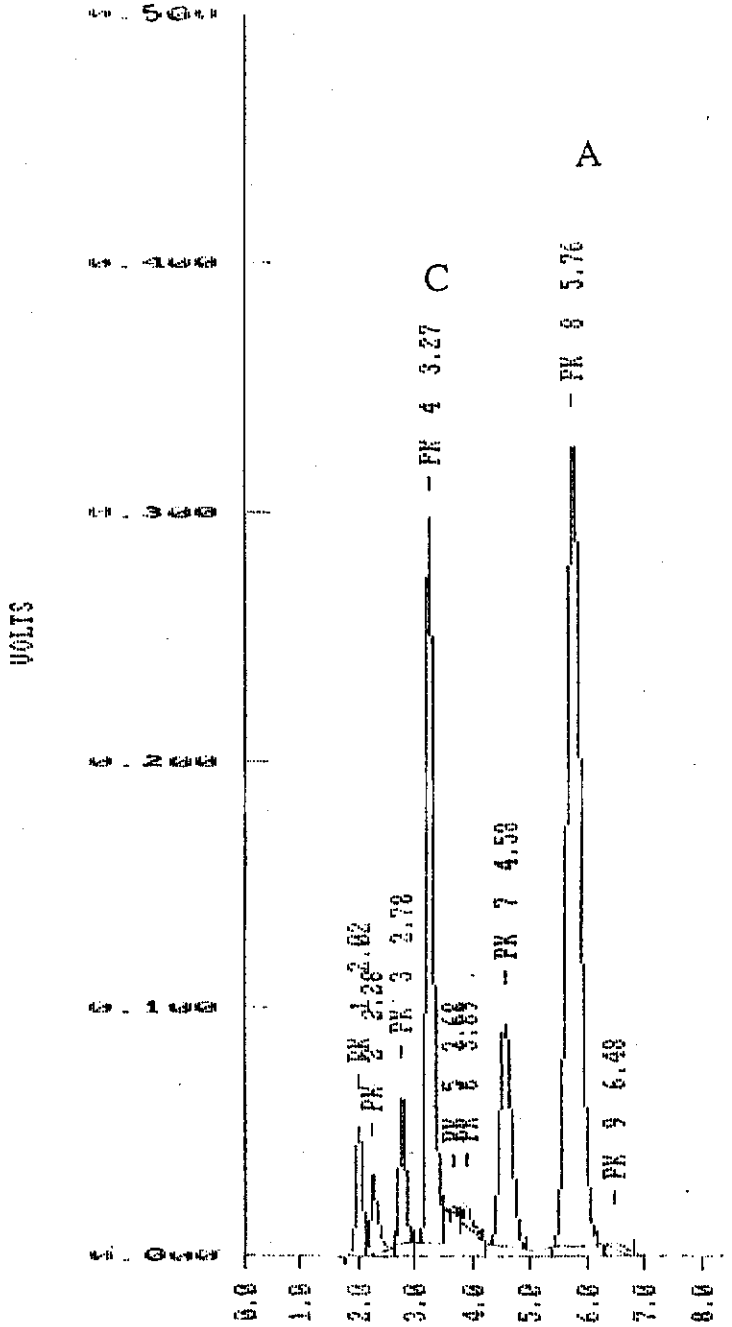
Figure 3.3: Stability indicating test in acid solution (2.0×10^{-2} mol dm^{-3} HCl) at 24°C.



Time (minutes)

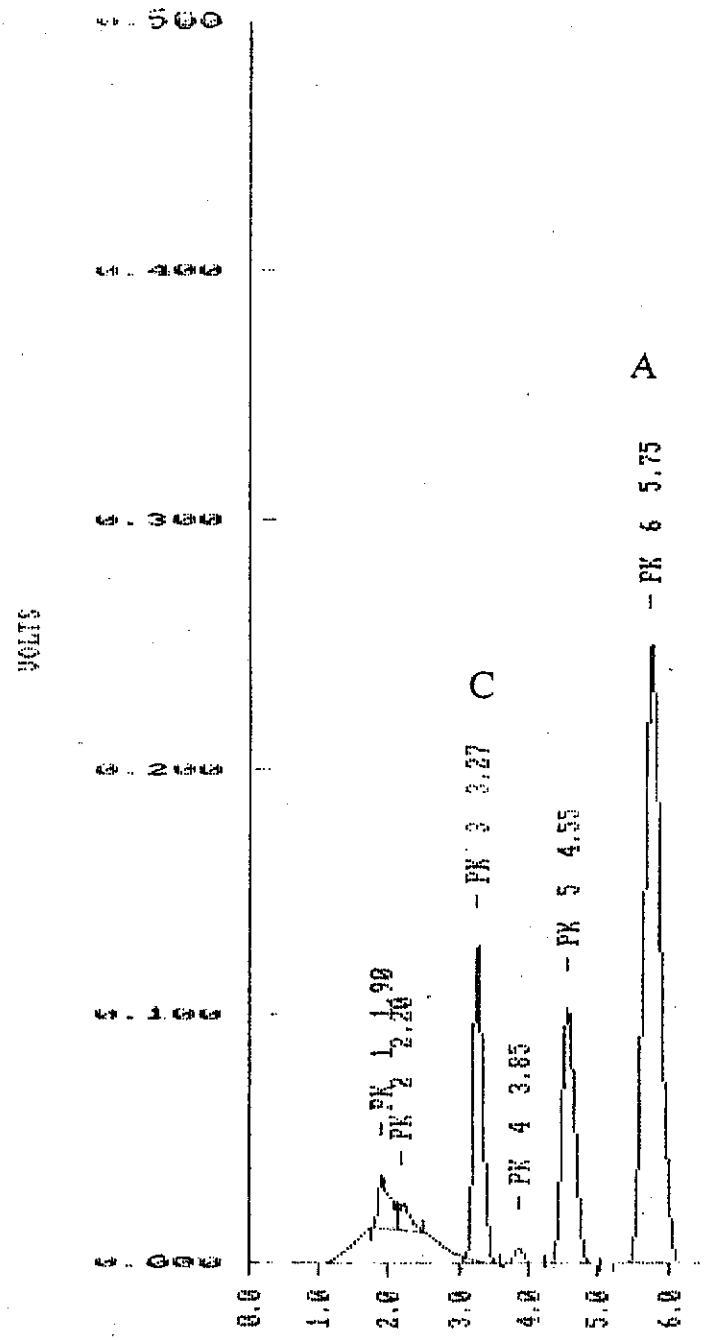
(b) Almost completely degraded solution. A represents amoxycillin.

Figure 3.3: Stability indicating test in acid solution (2.0×10^{-2} mol dm^{-3} HCl) at 24°C.



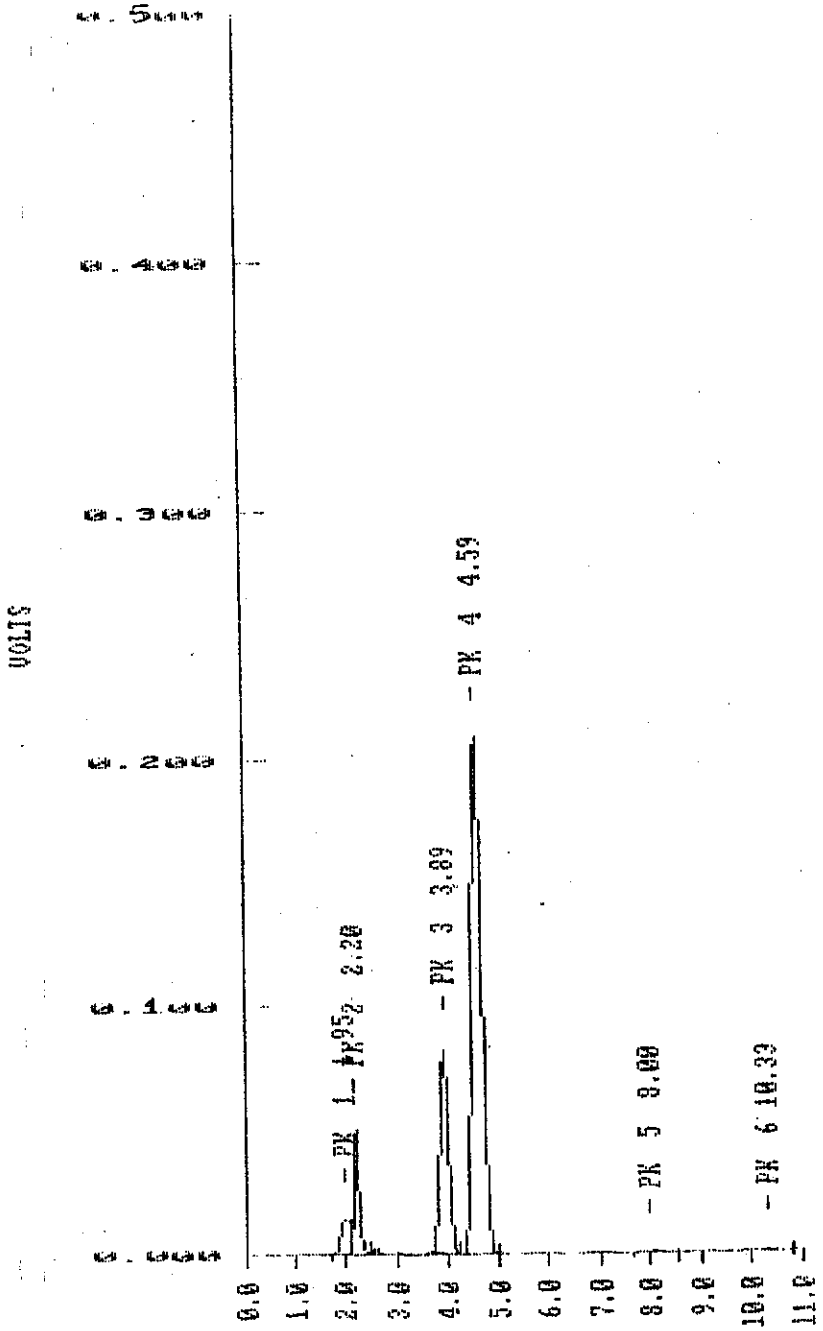
(c) Spiked solution. A and C represent amoxycillin and clavulanate respectively.

Figure 3.3: Stability indicating test in acid solution (2.0×10^{-2} mol dm^{-3} HCl) at 24°C.



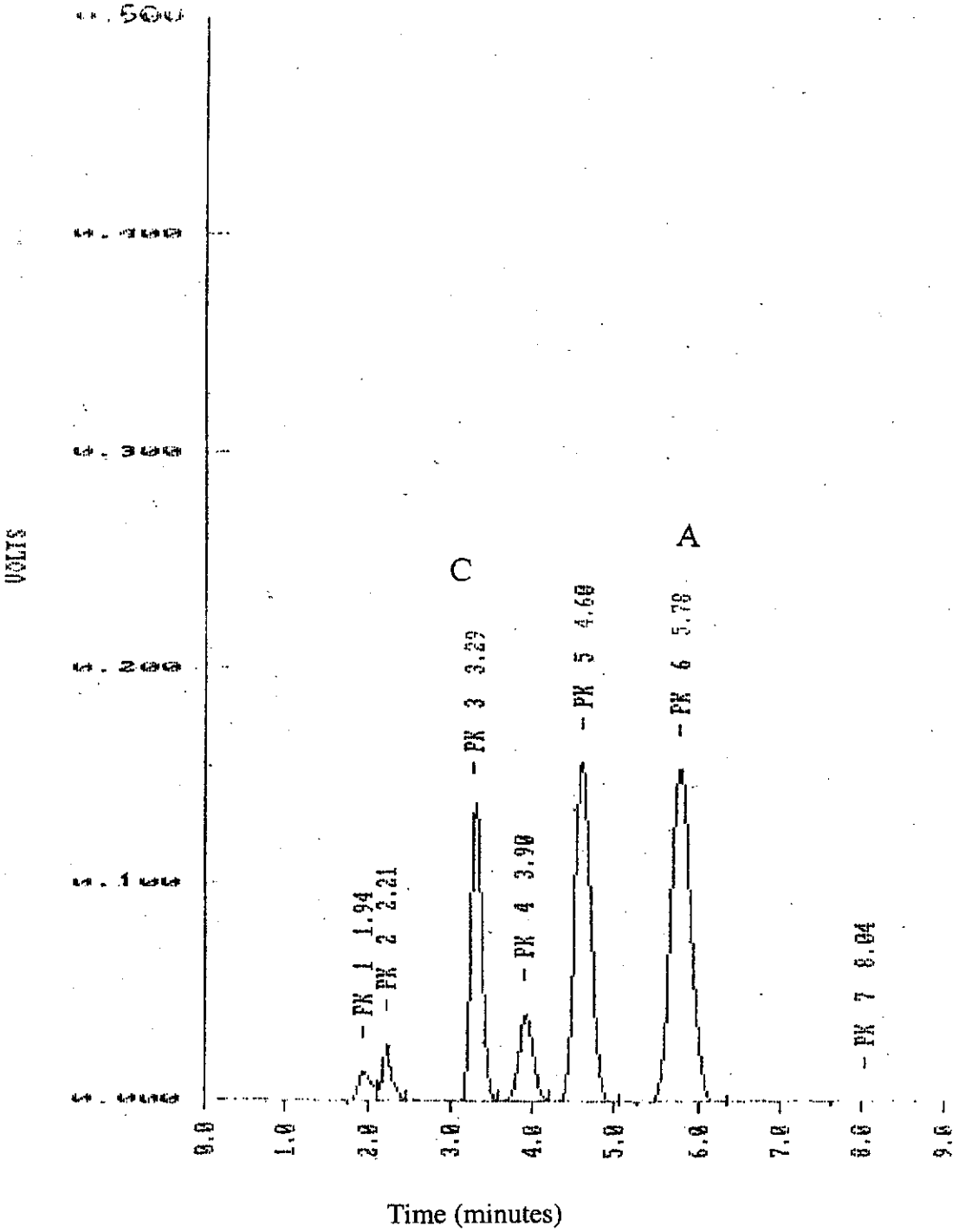
(a) **Zero time solution. A and C represent amoxycillin and clavulanate respectively.**

Figure 3.4: Stability indicating test in alkali solution (1.0×10^{-2} mol dm^{-3} NaOH) at 24°C.



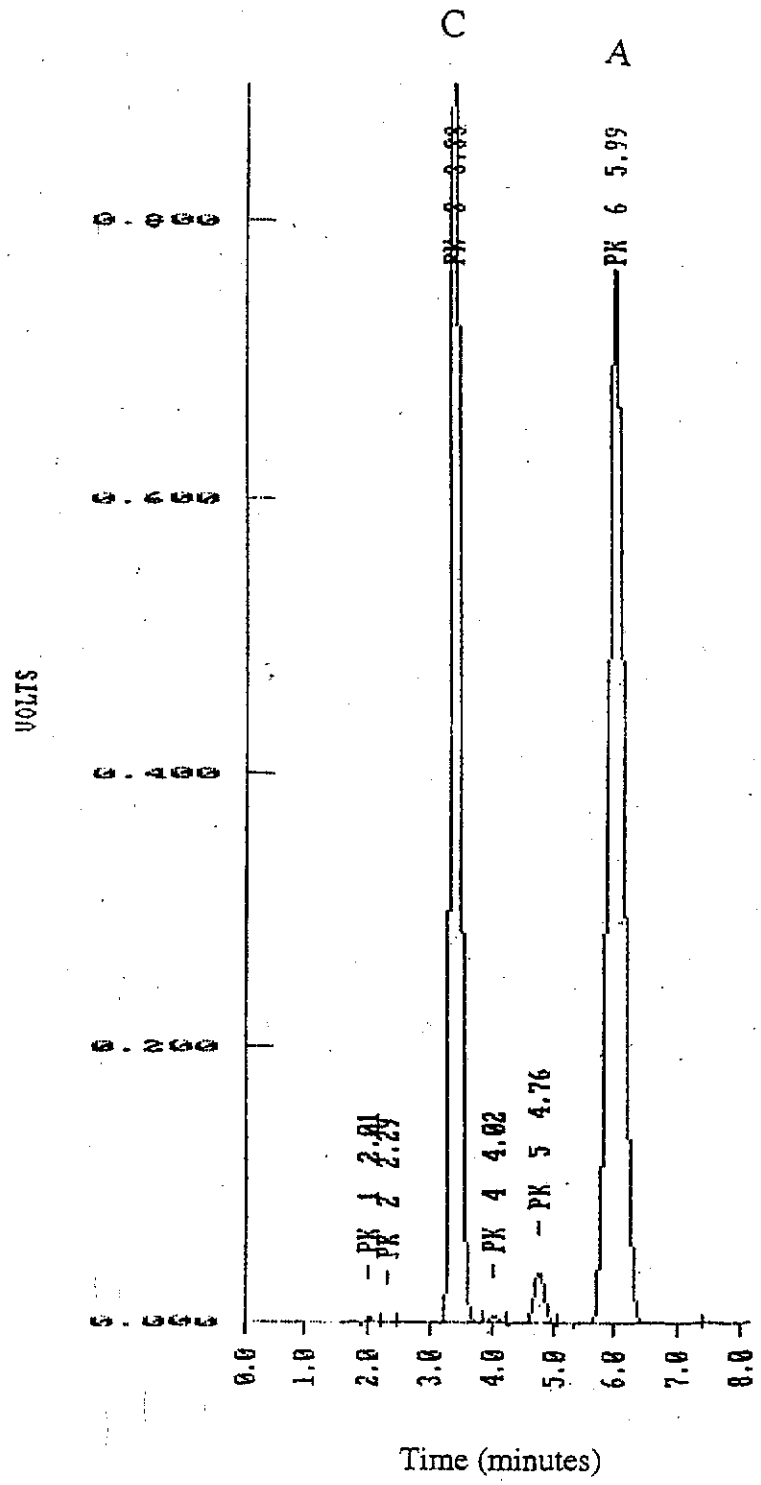
(b) Completely degraded solution.

Figure 3.4: Stability indicating test in alkali solution (1.0×10^{-2} mol dm^{-3} NaOH) at 24°C.



(c) Spiked solution. A and C represent amoxycillin and clavulanate respectively.

Figure 3.4: Stability indicating test in alkali solution (1.0×10^{-2} mol dm^{-3} NaOH) at 24°C.



(a) Zero time solution. A and C represent amoxycillin and clavulanate respectively.

Figure 3.5: Stability indicating test in water at 60°C.

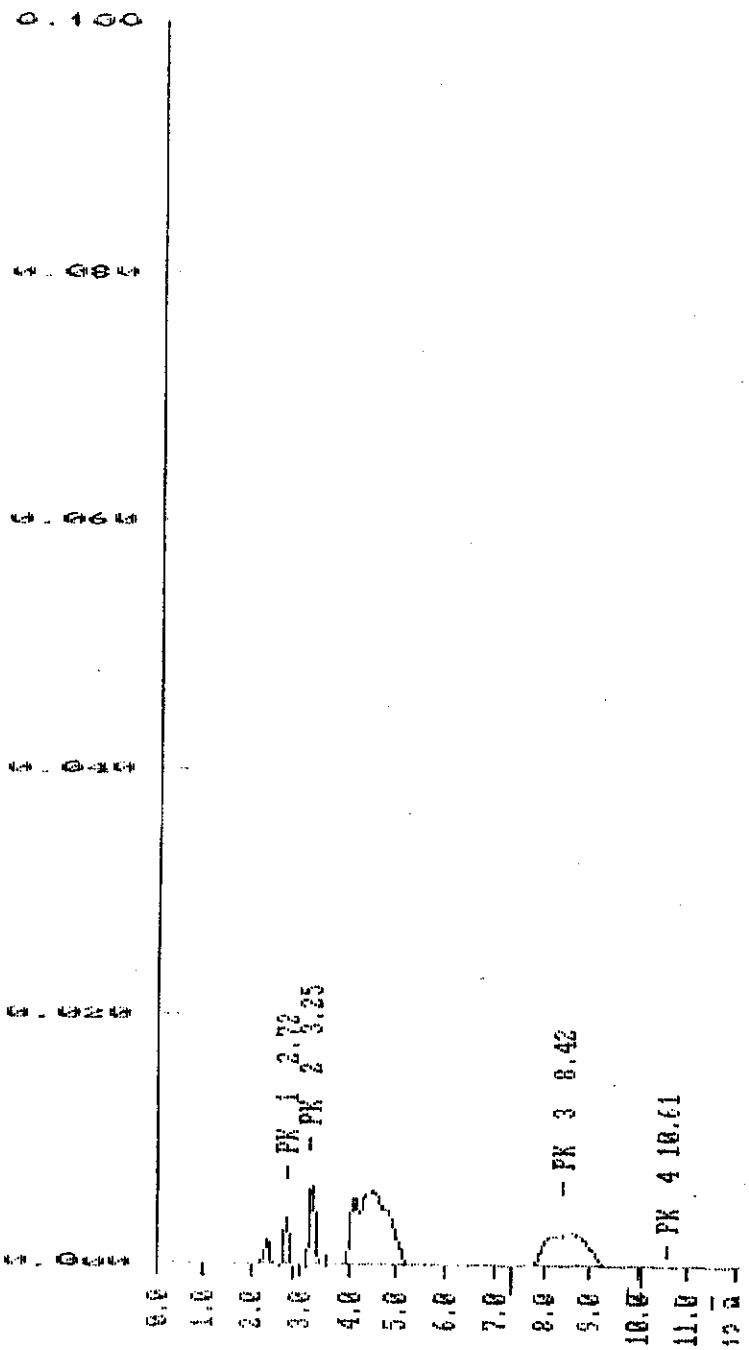
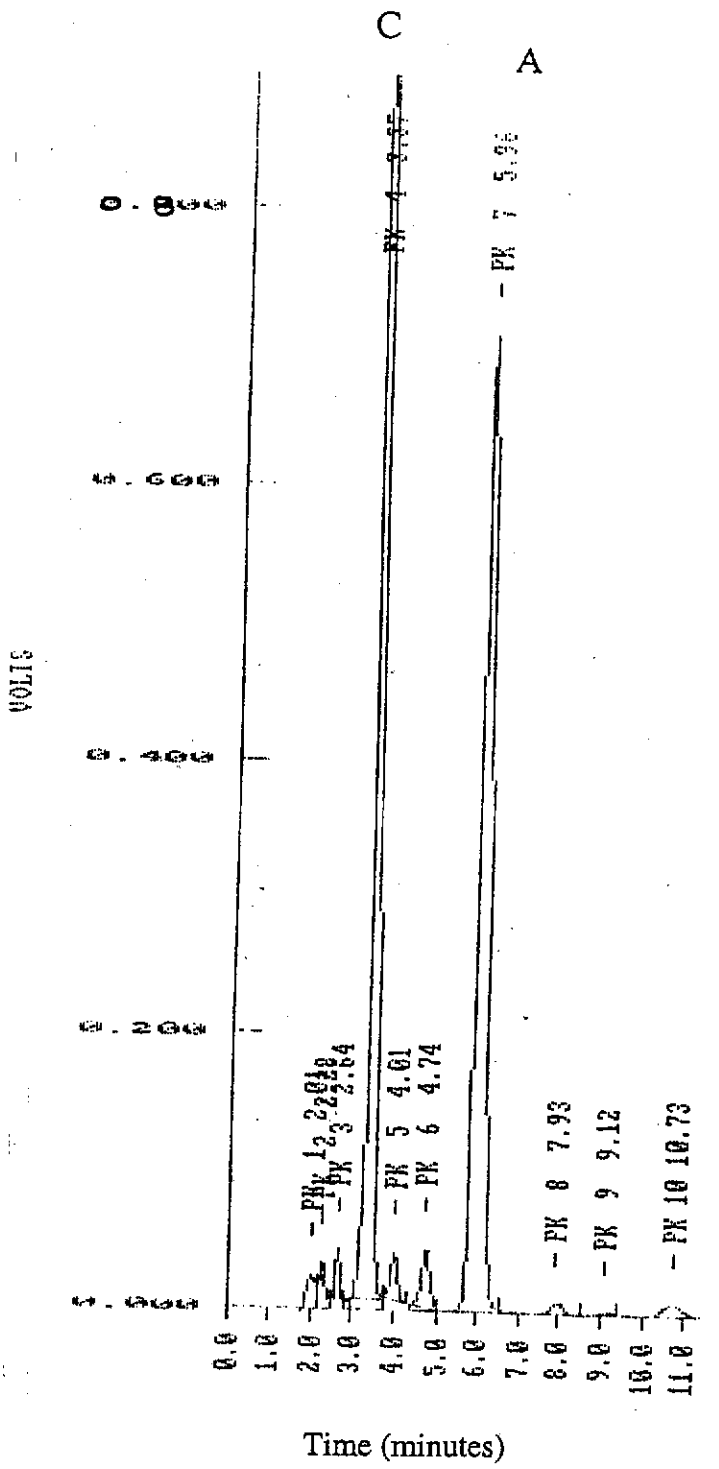


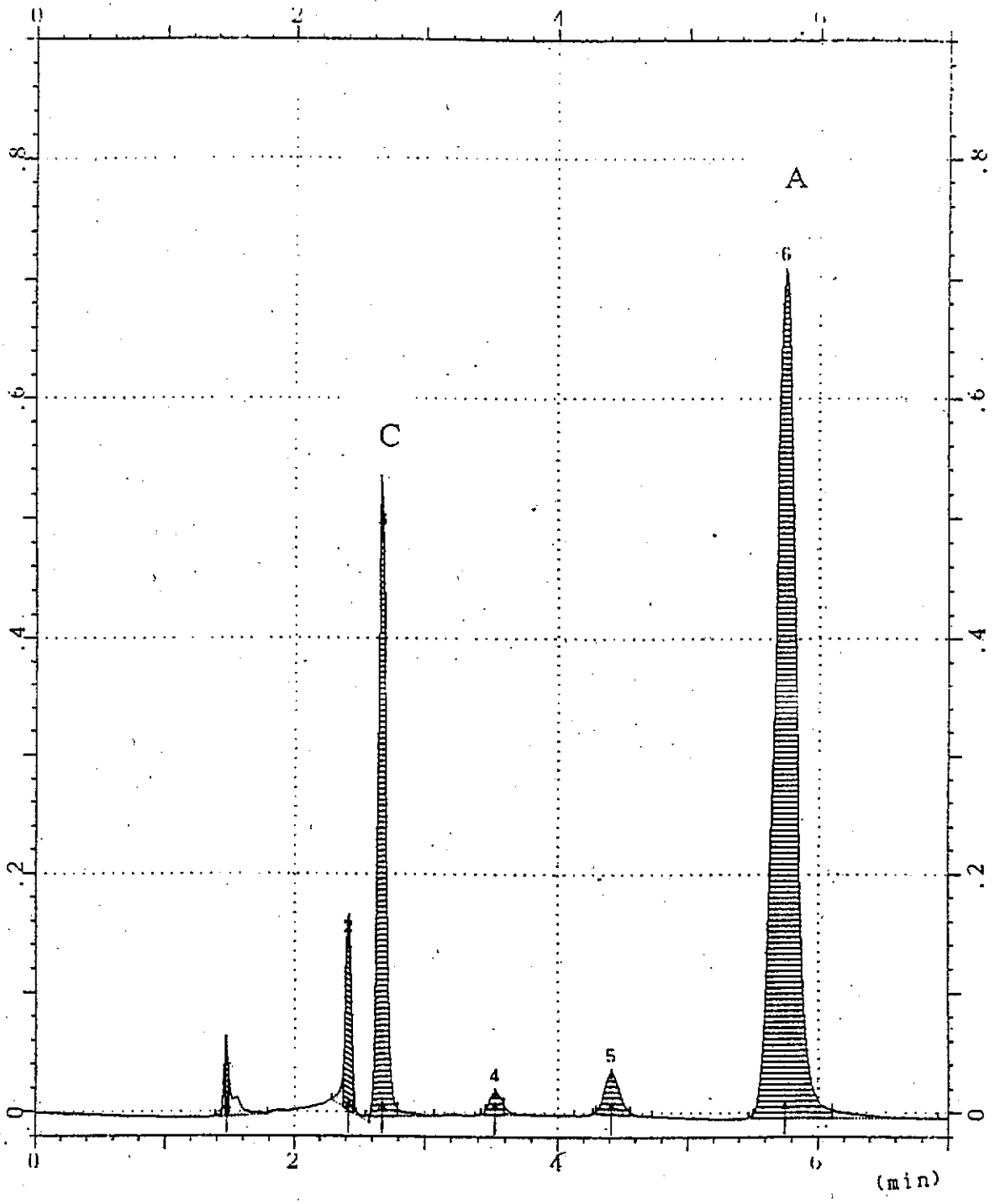
Figure 3.5: (b) completely degraded solution

Stability indicating test in water at 60°C



(c) Spiked solution. A and C represent amoxycillin and clavulanate respectively.

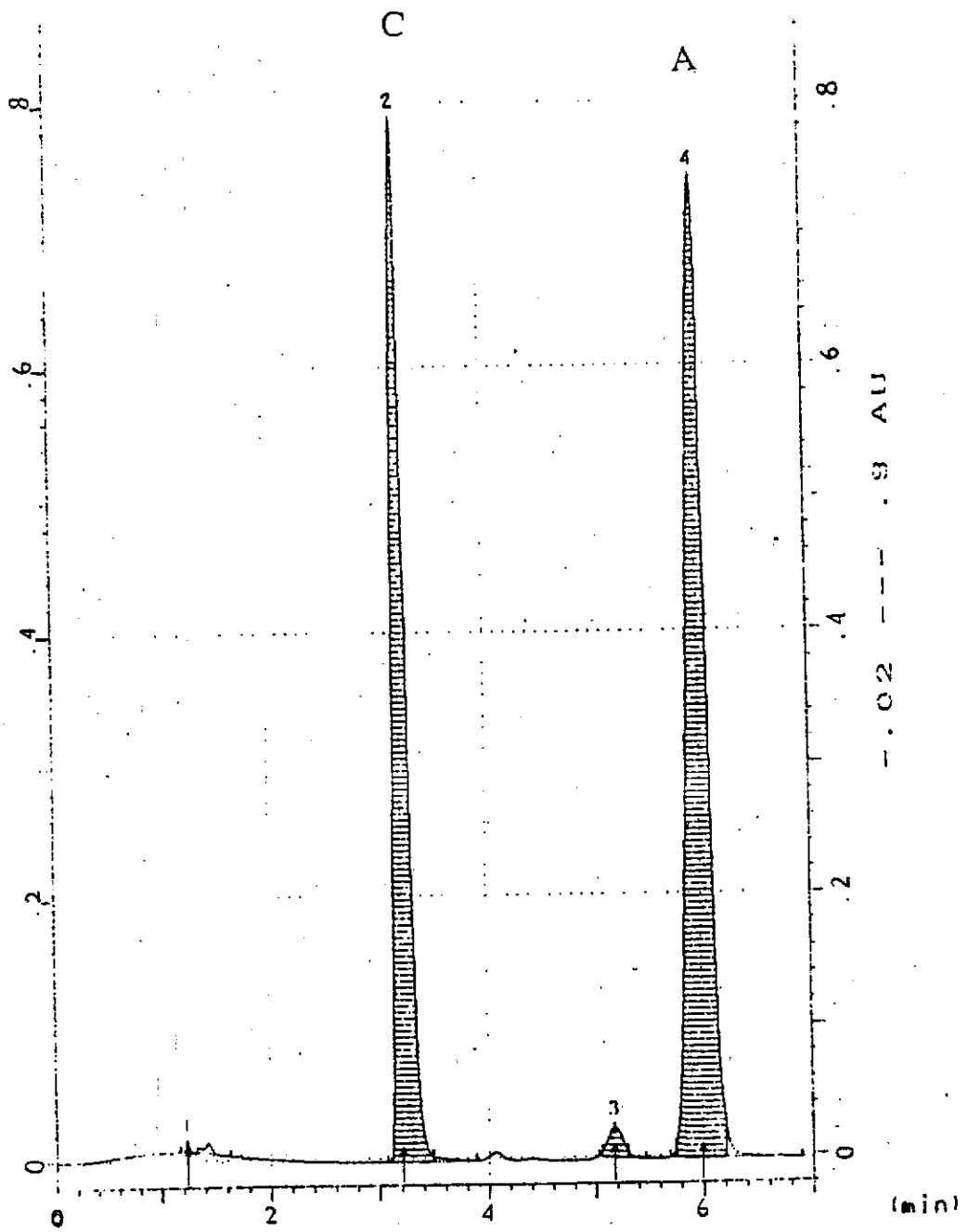
Figure 3.5: Stability indicating test in water at 60°C.



(a) Zero time solution, in acetate buffer at 24°C, pH 4.6 and μ = 0.5. A and C represent amoxycillin and clavulanate respectively.

Figure 3.6: Representative traces of Purity Test* using photo diode array detector.

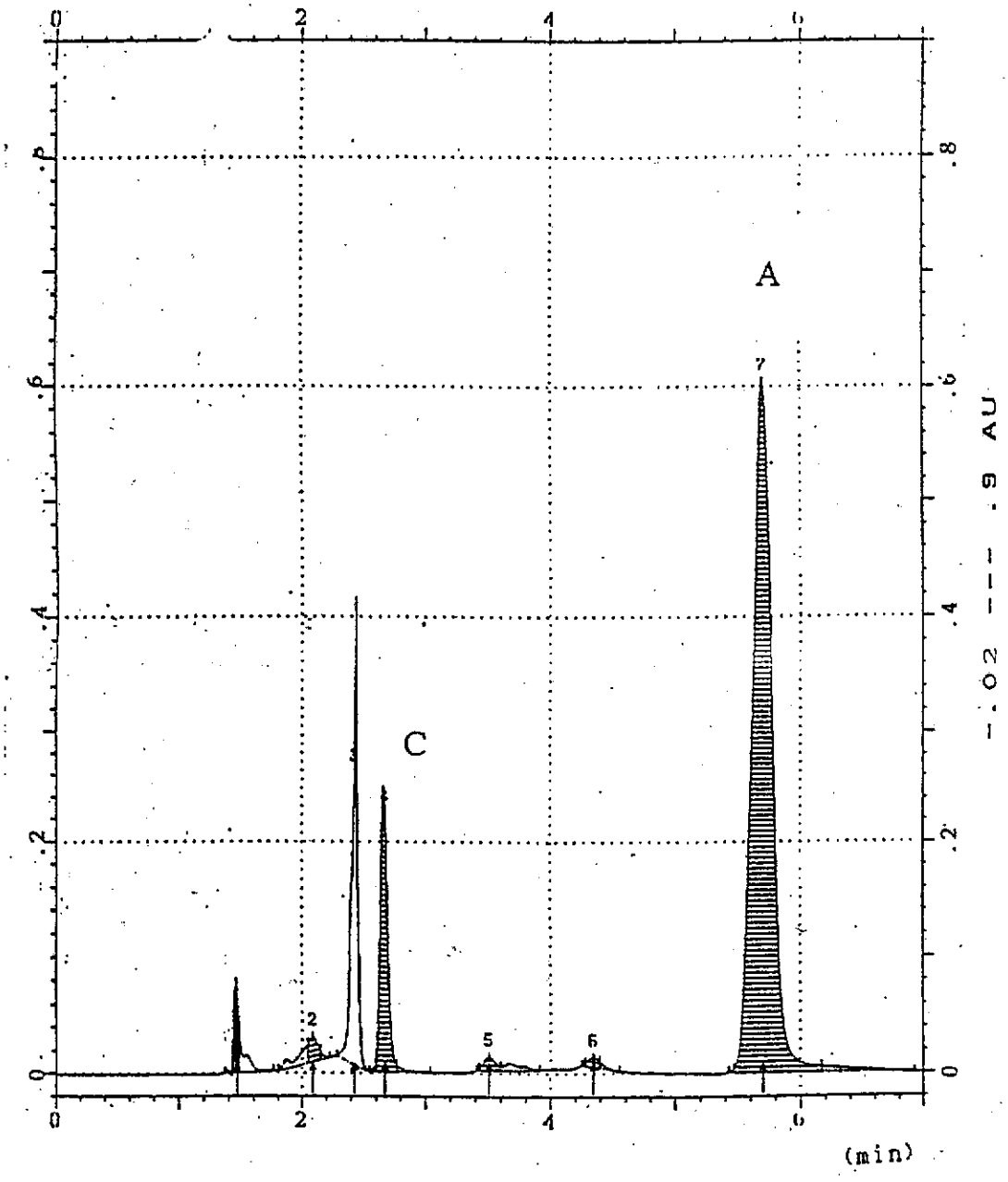
* shading indicates the area under the peak is pure.



(b) Zero time solution, in phosphate buffer at 24°C, pH 7.0 and μ = 0.5. A and C represent amoxycillin and clavulanate respectively.

Figure 3.6: Representative traces of Purity Test* using photo diode array detector.

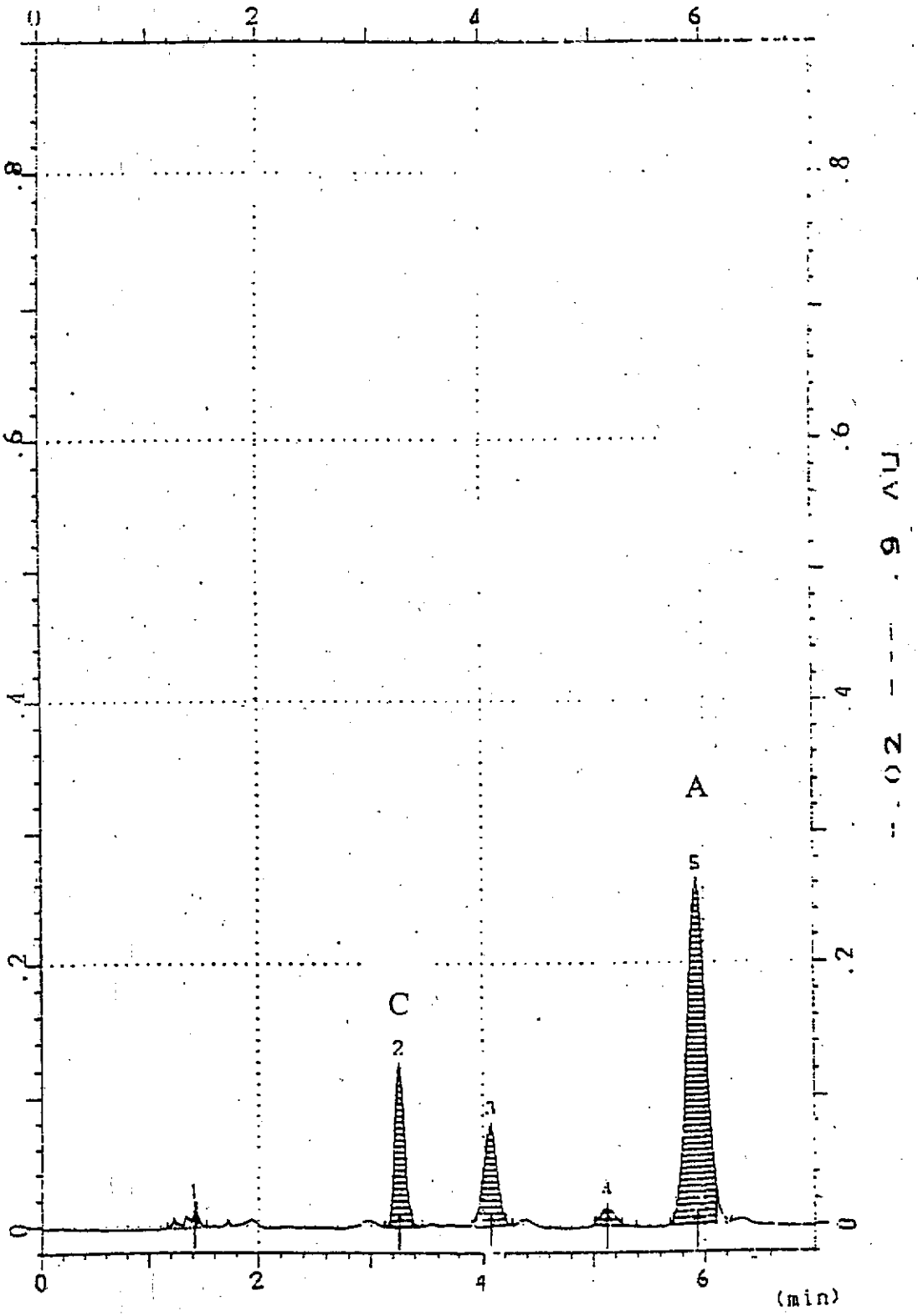
* shading indicates the area under the peak is pure.



(c) Partially degraded, in acetate buffer at 60°C, pH 4.6 and μ = 0.5. A and C represent amoxycillin and clavulanate respectively.

Figure 3.6: Representative traces of Purity Test* using photo diode array detector.

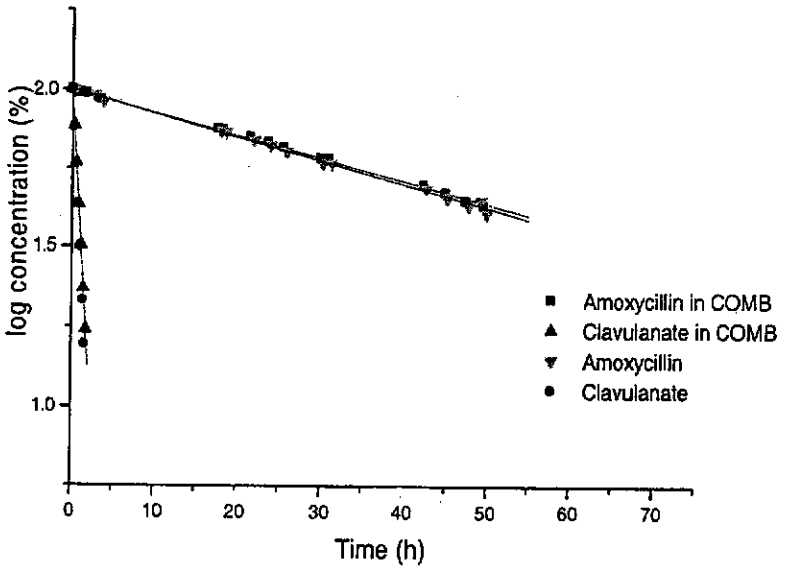
* shading indicates the area under the peak is pure.



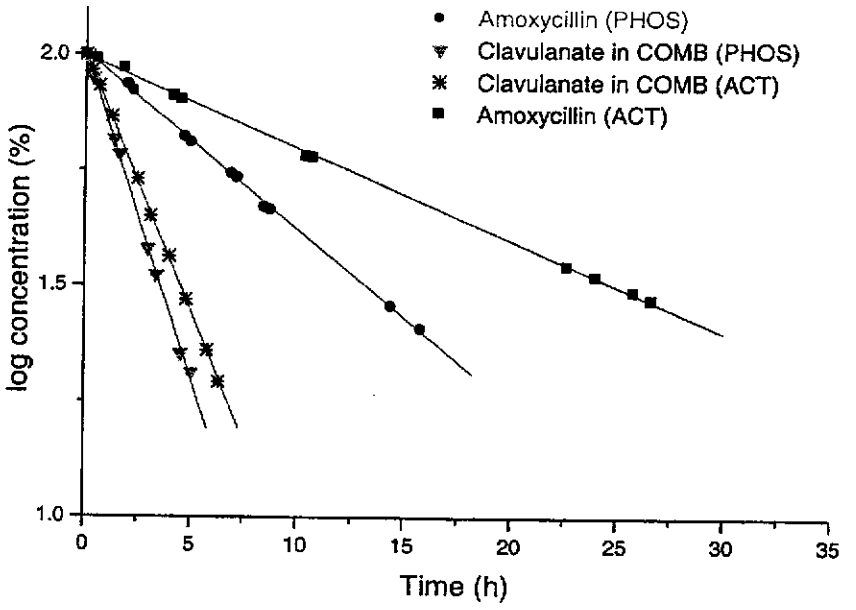
(d) Partially degraded, in phosphate buffer at 60°C, pH 7.0 and μ = 0.5. A and C represent amoxycillin and clavulanate respectively.

Figure 3.6: Representative traces of Purity Test* using photo diode array detector.

* shading indicates the area under the peak is pure.



(a)



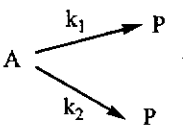
(b)

Figure 3.7: Typical simple first order plots for amoxycillin and clavulanate.
(a) In 1.0×10^{-2} mol dm^{-3} HCl, pH 2.0 at 27°C and constant ionic strength μ= 0.5.
(b) In 2.2×10^{-1} mol dm^{-3} acetate buffer pH 4.6 and 1.0×10^{-1} mol dm^{-3} phosphate buffer pH 7.0 at 55°C and constant ionic strength μ= 0.5.

(PHOS)= phosphate buffer; (ACT) =acetate buffer; COMB =combination form

3.2.2 First-order biexponential decay

As mentioned previously the degradation of amoxycillin in combination with clavulanate in buffers and under the experimental conditions of this study followed a first order biexponential decay. The mechanism of this type of reactions is given below. Amoxycillin degraded in two parallel steps:



(3.1)

where k_1 and k_2 are the first-order rate constants and A and P are amoxycillin and the products formed during the degradation period.

The initial rate constant (k_1) is affected by the catalytic influence of clavulanate and k_2 is the final rate constant where the clavulanate effect is no longer significant. Hence the curves obtained under these conditions (Equation 3.1) can be expressed in the following biexponential equation:

$$C = A\,e^{-k_1 t} + B\,e^{-k_2 t} \qquad (3.2)$$

The data obtained from the experimental runs were treated by a nonlinear curve stripping technique which consisted of stripping the linear part of the end data of a run and plotting the log concentration verses time by a least square method on a computer. The slope and the intercept of these plots were used to obtain k_2 and B in Equation 3.2 respectively. These data were incorporated in Equation 3.2 to obtain the values of k_1 and A by the same procedure. See data in Table 3.1 and Figure 3.8 for typical plots. In all the cases the linearity obtained was $r > 0.98$. Table 3.1 also provides a typical statistical analysis of data showing the calculated standard error values corresponding to each rate constant value. The calculated student's t values (t cal) for k_1 and k_2 are greater than the tabulated student's t [t tab (0.01)] values which indicates that there is a significant difference between the initial rate constant (k_1) and the final rate constant (k_2) values.

The thermal degradation of amoxycillin sodium in the solid state has been reported in the literature (Mendez *et al.* 1989) as being in accordance with Equation 3.2.

3.3 pH Effect

The effect of pH on the rate of degradation of amoxycillin and clavulanate individually and in combination was studied under the experimental conditions stated

Table 3.1: (a) First-order k_{obs} values of amoxycillin and clavulanate individually and in combination at constant ionic strength (μ = 0.5)

*pH	t (°C)	AMOX h^{-1}	SE	CLAV h^{-1}	SE	CLAV-COMB h^{-1}	SE	AMOX-COMB h^{-1}	SE		
2.0	14	4.40×10^{-3}	5.09×10^{-5}	2.22×10^{-1}	2.53×10^{-5}	2.07×10^{-1}	2.22×10^{-5}	4.28×10^{-3}	4.42×10^{-5}		
	20	8.18×10^{-3}	4.96×10^{-5}	5.03×10^{-1}	1.32×10^{-5}	4.44×10^{-1}	2.93×10^{-5}	7.99×10^{-3}	2.67×10^{-5}		
	27	1.72×10^{-2}	1.18×10^{-4}	1.12×10^{0}	1.19×10^{-4}	1.02×10^{0}	2.45×10^{-5}	1.66×10^{-2}	7.44×10^{-5}		
	35	3.44×10^{-2}	1.44×10^{-4}	2.80×10^{0}	1.02×10^{-4}	2.67×10^{0}	1.95×10^{-4}	3.46×10^{-2}	2.51×10^{-4}		
								k_1 h^{-1}	SE	k_2 h^{-1}	SE
4.6	35	8.06×10^{-3}	5.62×10^{-5}	3.59×10^{-2}	2.5×10^{-6}	3.59×10^{-2}	3.33×10^{-6}	1.91×10^{-2}	1.2×10^{-3}	7.99×10^{-3}	2.48×10^{-4}
	42	1.46×10^{-2}	9.16×10^{-5}	7.24×10^{-2}	4.85×10^{-4}	7.29×10^{-2}	8.41×10^{-5}	5.85×10^{-2}	1.36×10^{-3}	1.27×10^{-2}	1.55×10^{-4}
	49	2.37×10^{-2}	2.1×10^{-4}	1.44×10^{-1}	2.51×10^{-4}	1.36×10^{-1}	4.35×10^{-4}	1.02×10^{-1}	9.6×10^{-3}	2.45×10^{-2}	3.59×10^{-4}
	55	4.59×10^{-2}	1.65×10^{-4}	2.08×10^{-1}	8.31×10^{-4}	2.00×10^{-1}	6.12×10^{-4}	1.20×10^{-1}	6.1×10^{-3}	4.75×10^{-2}	8.02×10^{-4}
7.0	35	1.54×10^{-2}	7.5×10^{-5}	6.50×10^{-2}	216×10^{-4}	6.03×10^{-2}	9.5×10^{-4}	1.83×10^{-1}	5.0×10^{-2}	1.56×10^{-2}	1.87×10^{-3}
	42	2.91×10^{-2}	1.66×10^{-4}	1.14×10^{-1}	1.76×10^{-4}	1.17×10^{-1}	2.25×10^{-4}	2.28×10^{-1}	4.13×10^{-2}	3.02×10^{-2}	2.04×10^{-3}
	49	5.51×10^{-2}	2.09×10^{-4}	2.17×10^{-1}	1.39×10^{-3}	2.05×10^{-1}	7.98×10^{-4}	4.06×10^{-1}	8.39×10^{-2}	5.70×10^{-2}	2.88×10^{-3}
	55	9.06×10^{-2}	1.6×10^{-3}	3.36×10^{-1}	1.52×10^{-5}	3.29×10^{-1}	1.63×10^{-3}	5.83×10^{-1}	1.66×10^{-2}	9.40×10^{-2}	1.06×10^{-3}

(b) Student's t-test for k_1 & k_2

pH	t (°C)	t cal	d.f	t tab (0.01)
4.6	35	13.8	16	2.921
	42	51.9	16	2.921
	49	10.5	12	3.055
	55	13.5	10	3.169
7.0	35	4.36	16	2.921
	42	5.5	12	3.055
	49	4.42	10	3.169
	55	25.5	6	3.707

*pH 2.0 is in 1.24×10^{-2} mol dm^{-3} hydrochloric acid; pH 4.6 is in 2.2×10^{-1} mol dm^{-3} acetate buffer; pH 7.0 is in 1.0×10^{-1} mol dm^{-3} phosphate buffer.
Amoxycillin sodium initial concentration was 1.29×10^{-3} mol dm^{-3} in buffers and 9.03×10^{-4} mol dm^{-3} in hydrochloric acid system.
Initial concentration of potassium clavulanate was1.05×10^{-3} mol dm^{-3} in buffers and 7.38×10^{-4} mol dm^{-3} in hydrochloric acid system.

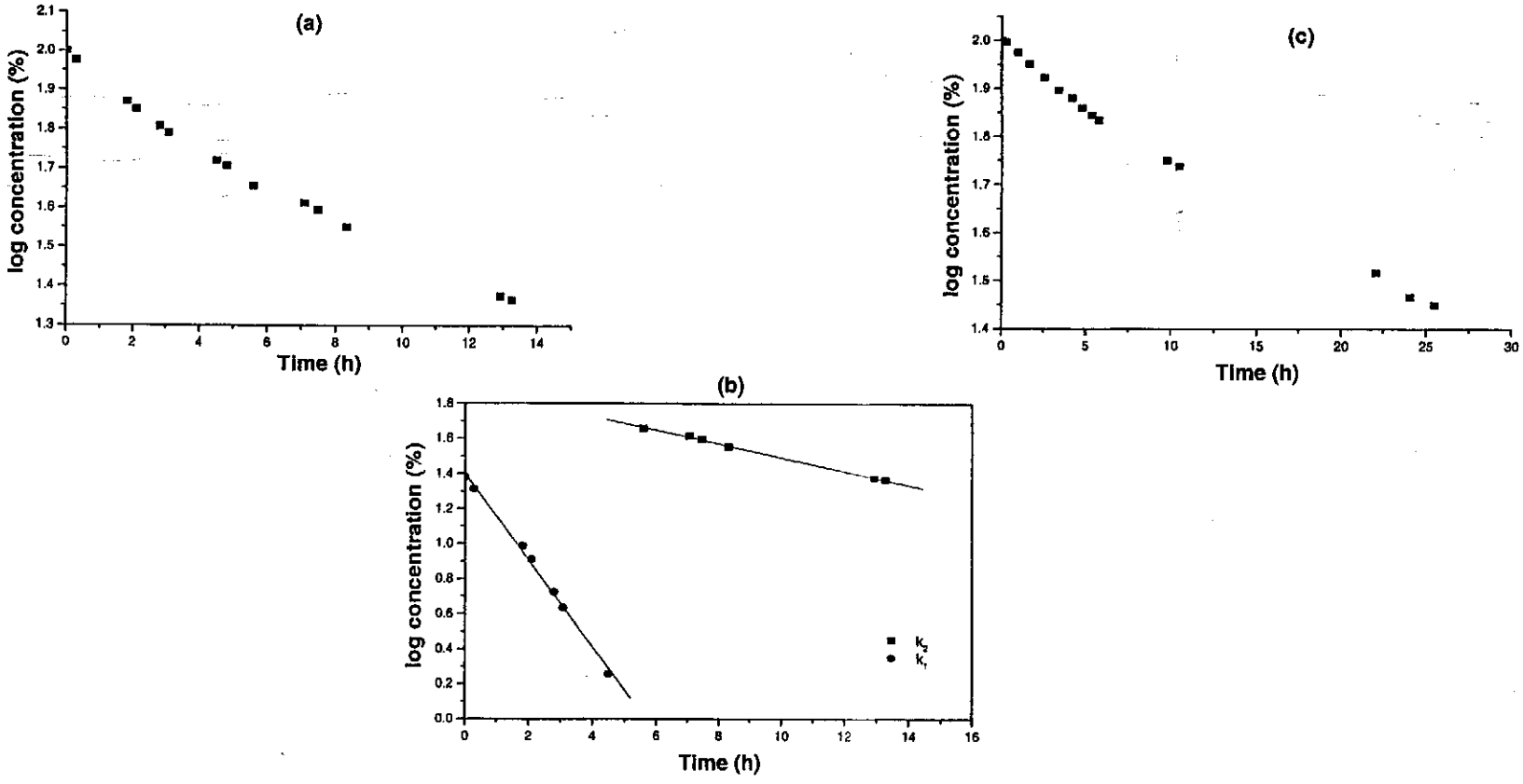


Figure 3.8: First order plots of amoxycillin in combination with clavulanate at constant ionic strength (0.5) and 55^{0}C
(a) In phosphate buffer pH 7.0
(b) First-order-biexponential plot of plot (a)
(c) In acetate buffer pH 4.6 where the concentration of clavulanate (3.15×10^{-3} mol dm^{-3}) is three times higher than (a)

in Section 2.3.1. The observed rate constant values corresponding to each pH value are listed in Table 3.1.

3.3.1 Hydrochloric acid system pH 2.0: The data in Table 3.1 indicate that there were no significant changes between the first-order rate constant values of amoxycillin in individual and combination runs, demonstrating that at this pH and solvent system any effect of clavulanate on the rate of degradation of amoxycillin in combination dosage was undetectable. The overall rate constant for clavulanate is much greater than amoxycillin, about 60 fold, indicating that clavulanate is much more susceptible to acid hydrolysis than amoxycillin. Data in Table 3.1 also show that the observed rate for the degradation of clavulanate in individual runs is generally slightly faster than that of the combination. Although this effect is not significant, it may be related to the slight pH change (0.1 unit) observed between the two runs. This was due to the basic nature of amoxycillin solution, thus when added to the hydrochloric system tended to increase the pH of the system slightly.

There was no significant change in pH observed during the course of each experimental run. The maximum pH change observed was 0.07 pH unit.

3.3.2 Acetate buffer pH 4.6: In this system as noted in Table 3.1 the degradation of amoxycillin in combination followed a first order biexponential decay with initial rate constant k_1 being greater than the rate constant for amoxycillin at later times. This final rate constant k_2 was similar to the rate constant for amoxycillin alone. This suggests the possibility of a catalytic effect of clavulanate on amoxycillin leading to larger rate constant values than amoxycillin alone. It was observed that as the clavulanate component degraded more rapidly than amoxycillin the rate of the reaction slowed and became similar to the rate of reaction of amoxycillin without clavulanate. However amoxycillin did not appear to have any effect on the rate of clavulanate decomposition in the combination runs, since there did not appear any significant change between the rate constant of clavulante in combination and alone. There was no significant change in pH during the course of each experiment. The maximum pH change recorded was 0.06 pH unit. This could not account for the increased rate.

3.3.3 Phosphate buffer pH 7.0: The data in Table 3.1 also show that the rate of degradation of clavulanate did not change significantly when used in combination with amoxycillin or alone at all the temperatures studied. However as in the pH 4.6 system, the initial rate of degradation of amoxycillin (k_1) in combination was

enhanced considerably in this buffer system. The extent of this increase in rate was greater than the pH 4.6 system. This observation has lead to the assumption that other factors apart from clavulanate could cause the increase in rate of amoxycillin in combination, which will be discussed in detail under catalytic effects in Section 3.6. No significant change in pH was observed during the course of each run.

The pK_a values of clavulanate and amoxycillin are 2.4 and 2.6 respectively related to deprotonation of the carboxyl groups. The pK_a for the α-amino group of amoxycillin is 7.6. At pH 4.6 the deprotonation of both clavulanate and amoxycillin would be ≥ 99%; hence little difference in the carboxyl group ionization would be evident.

Amoxycillin would also be fully protonated with respect to the amino group. Hence this antibiotic would be zwitterionic at this pH. Small changes in pH at pH 4.6 are likely to have only minor modifications to the ionic characteristics of these species. Therefore the increase in rates of amoxycillin in the combination runs can not be due to a pK_a influence.

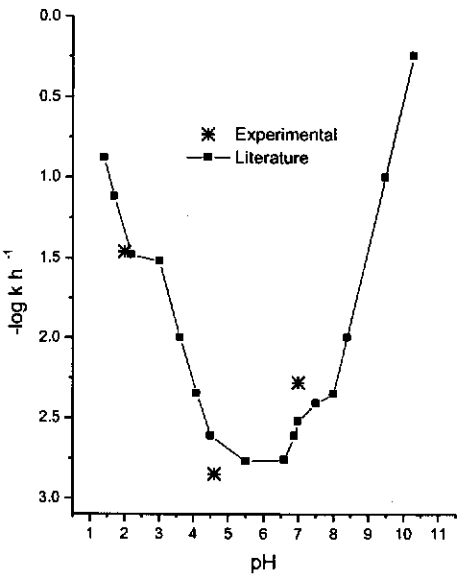
The data in Table 3.1 indicate that the overall rate of degradation of clavulanate is faster than amoxycillin (except for the combination runs in phosphate system). This is in agreement with the literature reports (Wildfeuer and Radar 1996; Ashwin, Lynn and Taskins 1987) that clavulanate is the stability limiting factor of the combination dosage form.

3.3.4 pH-rate data: The pH-rate data of amoxycillin and clavulanate under the conditions of this study are compared with the available literature data in Figure 3.9. To compare the literature data with the experimental results of this study, first-order rate constant values of amoxycillin and clavulanate at various buffer concentration and constant pH were estimated at 35°C using the slope of Arrhenius plot. Therefore from Arrhenius equation (Section 2.5.1) we can write,

$$\frac{E_a}{2.303R} = \frac{\log k_1 - \log k_2}{(1/T_1 - 1/T_2)} \qquad (3.3)$$

where k_1 and k_2 are the first-order rate constants at temperatures T_1 (corresponding to 55°C) and T_2 (corresponding to 35°C) respectively. Subsequently the rate constant for another buffer concentration was calculated for 35°C from Equation 3.3. The estimated first-order rate constants were then plotted against several buffer

(a)



(b)

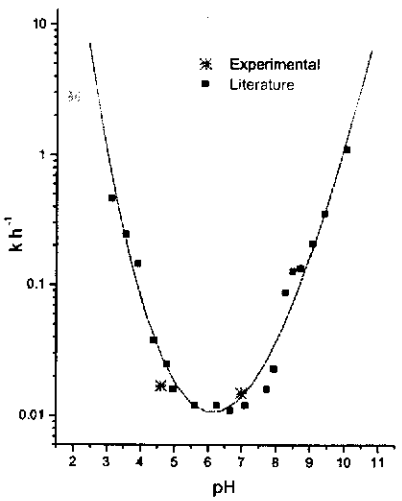


Figure 3.9: Comparison of pH-rate data of amoxycillin and clavulanate with that of the literature at 35°C and constant ionic strength μ=0.5.

(a) Amoxycillin; (b) Clavulanate

(Zia, Shalchian and Borhania 1977; Haginaka, Nakagawa and Uno 1981)

concentrations and the respective zero-buffer concentration rate constants (k_{pH}) values were obtained from the intercept values of these plots. Thus the non-buffer-catalyzed rate constants values obtained in this way for amoxycillin and clavulanate are provided in Figure 3.9.

Zia, Shalchian and Borhanian (1977) have reported the pH of maximum stability for amoxycillin in buffer free conditions to be over the range of 5.5-6.5 (Figure 3.9a). Hence below pH 5.5 the rate of reaction increases with decrease in pH whereas above pH 6.5 the rate again increases with increased pH. This is supported by the results obtained from this study. The data indicate similar pH-rate patterns with the literature data. At pH 2.1 in the hydrochloric acid system the rate constant reported by Zia and co-workers (1977) for amoxycillin is 3.07×10^{-2} h^{-1}at 35°C. This is very close to the result obtained from the present study i.e. $k_{obs} = 3.44 \times 10^{-2}$ h^{-1} at pH 2.0 in hydrochloric acid system and 35°C. At pH 4.6 and 7.0 however there is a significant difference between the rate constant values estimated from the present study and that of the literature. The maximum difference is observed at pH 7.0 where the rate constant value of amoxycillin obtained from this study is approximately 36% lower than the literature data (figure 3.9a). As will be discussed in Section 3.5 this difference in result could relate to different methods of analysis. However, taking into account the approximations and the extrapolation used to adjust the experimental data to literature data, it can be said that the results are in acceptable agreement with the literature.

The pH-rate profile of clavulanic acid documented (Haginaka, Nakagawa and Uno 1977) indicates a similar pattern of pH dependency on rate as for amoxycillin. The pH of maximum stability for clavulanate is reported as 6.4. The data in Figure 3.9b indicate close agreement between the experimental and literature results. The rate constant obtained at pH 2.0 is not reported in the literature.

Thus the results obtained from the limited pH values studied, show a similar pattern in pH-rate profile with the literature for amoxycillin (Zia, Shalchian and Borhanian 1977; Tsuji *et al.*1978) and clavulanate (Haginaka, Nakagawa and Uno 1981).

3.4 Temperature Effects

The temperature dependence of amoxycillin sodium and potassium clavulanate alone and in combination was studied under the experimental conditions

as described in Section 2.3.1. The Arrhenius plots were obtained by the normal procedure of plotting log k versus 1/T and the apparent energies of activation were calculated (Table 3.2 and Figure 3.10).

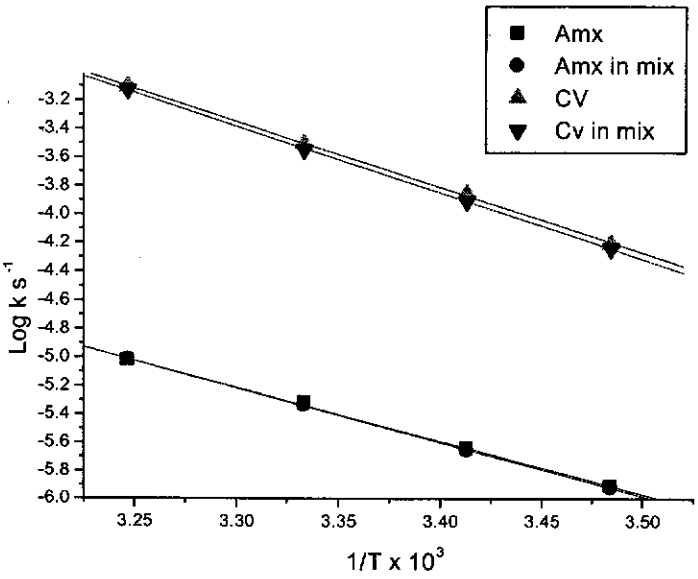
There are a few reports in the literature on activation energy values of amoxycillin and clavulanate. Zia and co-workers (1977) have reported the E_a for amoxycillin at pH 4.45 in 1.0×10^{-1} mol dm^{-3} citrate buffer solution as 75.7 kJ mol^{-1}. Doadrio and Sotelo (1988) reported the E_a values as 102.9 kJ mol^{-1}, 77.4 kJ mol^{-1} and 46.9 kJ mol^{-1} corresponding to pH 2.0, 4.0 and 7.0 respectively. These data indicate that the documented data at pH about 4.0 are consistent with the result obtained in this study (see Table 3.2). Hou and Poole (1969a) have reported the activation energies of ampicillin in acid, neutral and basic solution as 68.6 kJ mol^{-1} , 76.6 kJ mol^{-1} and 93.3 kJ mol^{-1} corresponding to pH 1.35, 4.93 and 9.78 respectively. The results obtained by these workers for ampicillin are in close agreement with the results obtained in this study (Table 3.2), thus suggesting that in amoxycillin the presence of the hydroxyl group in the side chain does not have a significant affect in the mechanism of β-lactam ring cleavage.

Table 3.2: Activation energy data of amoxycillin and clavulanate in various pH values at $\mu = 0.5$

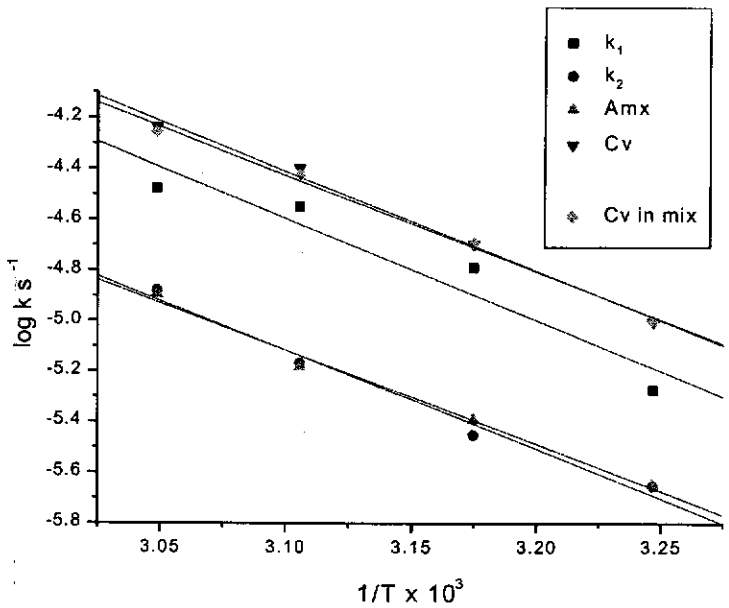
*pH	ACTIVATION ENERGY (E_a) kJ mol^{-1}			
	Amox	Clav	Amox-comb	Clav-comb
2.0	72.49	88.12	73.45	88.19
4.6	71.21	75.12	77.17(k_1) 74.83(k_2)	72.66
7.0	74.80	69.82	50.67(k_1) 75.51(k_2)	71.01

pH 2.0: 1.24×10^{-2} mol dm^{-3} hydrochloric acid; pH 4.6: 2.2×10^{-2} mol dm^{-3} acetate buffer; pH 7.0: 1.0×10^{-1} mol dm^{-3} phosphate buffer

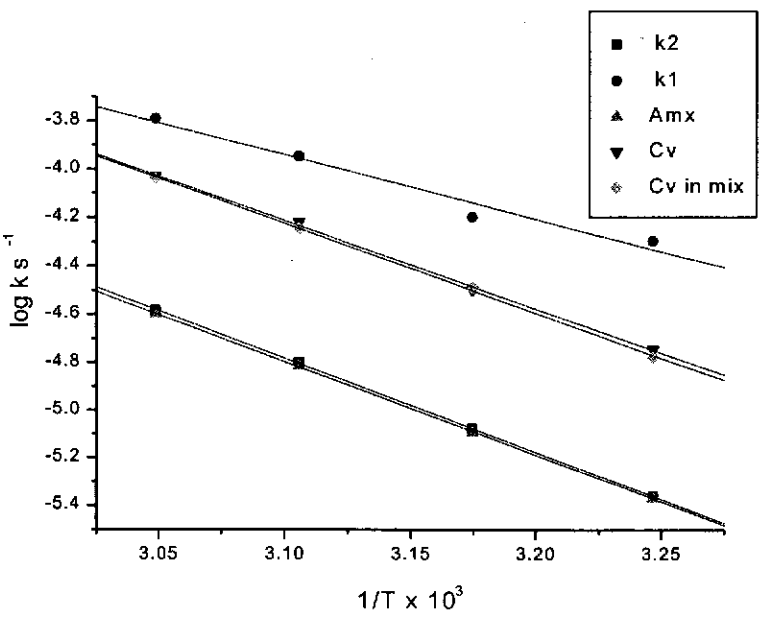
Haginaka and co-workers (1981) have reported the activation energies for clavulanic acid which are 79.5 kJ mol^{-1}, 61.5 kJ mol^{-1} and 76.6 kJ mol^{-1} at pH 3.94, 6.67 and 8.74 respectively. Although the pH values studied by these workers are not the same as the pH used in the present study, yet the reported data are in acceptable agreement with those obtained from this study (Table 3.2).


Amx
Amx in mix
CV
Cv in mix
Log k s^{-1}
-3.2
-3.4
-3.6
-3.8
-4.0
-4.2
-4.4
-4.6
-4.8
-5.0
-5.2
-5.4
-5.6
-5.8
-6.0
3.25
3.30
3.35
3.40
3.45
3.50
1/T x 10^3

(a)


k$_1$
k$_2$
Amx
Cv
Cv in mix
log k s^{-1}
-4.2
-4.4
-4.6
-4.8
-5.0
-5.2
-5.4
-5.6
-5.8
3.05
3.10
3.15
3.20
3.25
1/T x 10^3

(b)



(c)

Figure3.10: Arrhenius plots of amoxycillin sodium and potassium clavulanate in separate solution and in combination at constant ionic strength μ= 0.5

(a) In 1.0 ×10^{-2} mol dm^{-3} hydrochloric acid system pH 2.0

(b) In 2.2 ×10^{-1} mol dm^{-3} acetate buffer pH 4.6

(c) In 1.0 ×10^{-1} mol dm^{-3} phosphate buffer pH 7.0

Amx = amoxycillin sodium; Cv =potassium clavulanate, Cv in mix = potassium clavulanate in combination; k_1 and k_2 =corresponding to initial and final rate constants of amoxycillin in combination,; Amx in mix =amoxycillin in combination.

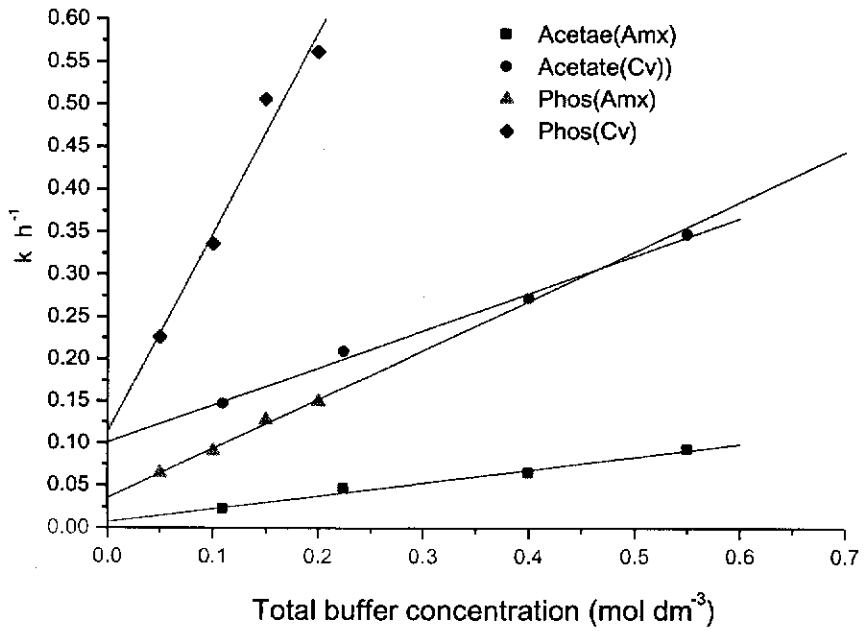


Fig 3.11: Effect of buffer concentration on rate of degradation of amoxycillin and clavulanate at 55°C and μ = 0.5.

Acetate (Amx) = amoxycillin in acetate buffer pH 4.6; Acetate (Cv) = clavulanate in acetate buffer pH 4.6; Phos(Amx) = amoxycillin in phosphate buffer pH 7.0; Phos (Cv) = clavulanate in phosphate buffer pH 7.0.

3.5 Buffer Effects

The study on buffer effects was carried out according to the procedure outlined in Chapter 2, Section 2.3.1.6. The results are consistent with the reported (Zia, Shalchian and Borhanian 1977; Tsuji *et al.* 1978; Haginaka, Nakagawa and Uno 1981) catalytic effects of acetate and phosphate buffer on amoxycillin and clavulanate. Figure 3.11 illustrates the effect of buffer concentration on the rates of degradation. Extrapolation of these plots to zero-buffer concentration provided at the intercepts, the values of non-buffer-catalysed degradation rate constants k_{pH} corresponding to each drug at the respective buffer solution. The k_{pH} values at 55°C are listed in Table 3.3. The k_{pH} of clavulanic acid at 35°C has been reported by Haginaka and co-workers (1981) at several pH values. These data are indicated in Figure 3.9b which demonstrate the proximity of literature data with these experimental results when estimated at 35°C using Equation 3.3.

The literature provides several rate constant values for amoxycillin and clavulanate in various buffer systems. For instance the rate constant reported by Zia and co-workers (1977) for amoxycillin at pH 7.0 in phosphate buffer (k_{obs} =2.42 × 10^{-2}) is ~36% higher than the one obtained from this study (k_{obs} = 1.54 × 10^{-2} h^{-1}) at 35°C. This difference is also evident in Figure 3.9a under buffer free conditions. However, in another study (Doadrio and Sotelo 1988) and under similar conditions of pH, buffer and temperature the rate of degradation of amoxycillin at 35°C is reported to be 1.2 × 10^{-2} h^{-1}. This latter reported rate constant value is in closer agreement with the data obtained from this study. The variation in results could have occurred from the methods of analysis used by the various workers. Because unlike the method of analysis of this study and the one by Doadrio and Sotelo (1988) where the estimation of amoxycillin was carried out by an HPLC method, Zia and co-workers used an iodometric titration method. Hence the results obtained from this study is supported by a more reliable assay technique.

Similarly Haginaka and coworkers have reported several rate constant values for clavulnate in acetate and phosphate buffers at various pH values. For example the first-order rate constant reported by these workers in 2.0 × 10^{-1} mol dm^{-3} acetate buffer at pH 4.41 and 4.79 and 35 °C are 5.4 ×10^{-2} h^{-1} and 3.3 ×10^{-2} h^{-1} respectively. The rate constant at pH 4.6 and 35°C was estimated from the results reported by these workers, to be 4.4 × 10^{-2} h^{-1}. As shown in Table 3.1 the value obtained from

this study in 2.2×10^{-1} mol dm^{-3} acetate buffer is 3.6×10^{-2} h^{-1}. Similarly these authors have reported three rate constant values (1.38×10^{-1} h^{-1}, 1.00×10^{-1} h^{-1} and 5.3×10^{-2} h^{-1}) corresponding to three phosphate buffer concentrations (2.1 mol dm^{-3}, 1.4×10^{-1} mol dm^{-3} and 7.0×10^{-2} mol dm^{-3}) at pH of 7.1. When these data were interpolated to 1.0×10^{-1} mol dm^{-3} buffer concentration, which is the concentration used in this study, the rate constant was estimated to be 7.1×10^{-2} h^{-1} and is thus agrees well with the result obtained from this study i.e. 6.5×10^{-2} h^{-1} at pH 7.0.

Tsuji *et al.* (1978) and Zia and co-workers (1977) have estimated the catalytic rate constants for amoxycillin in various buffer species, which is discussed in Chapter 1, Section 1.1.1.1.2. Similarly Haginaka and co-workers (1981) have reported the catalytic rate constants of phosphate and acetate buffers for clavulanate (refer Chapter 1, Table 1.11). In this study the buffer catalytic rate constant was estimated as total buffer effect from the slope of the plot of rate constants versus buffer concentration at 55°C. The results obtained for amoxycillin are 5.84×10^{-1} h^{-1} (mol dm^{-3})$^{-1}$and 1.53×10^{-1} h^{-1} (mol dm^{-3})$^{-1}$ in phosphate buffer pH 7.0 and acetate buffer pH 4.6 respectively. Similarly for clavulanate under the same procedure, these second–order rate constants are 2.33 h^{-1} (mol dm^{-3})$^{-1}$ and 4.43×10^{-1} h^{-1} (mol dm^{-3})$^{-1}$ at 55°C due to phosphate and acetate respectively. To compare these data with the literature, the procedure described in Section 3.3.4 was followed to obtain the relevant rate constant values for 35°C using Equation 3.3. Then the slope of plots of these rate constants versus buffer concentration provided the second order rate constants for total buffer catalysis at 35°C. Hence values of 1.06×10^{-1} h^{-1} (mol dm^{-3})$^{-1}$ for amoxycillin and 5.3×10^{-1} h^{-1} (mol dm^{-3})$^{-1}$ for clavulanate were obtained due to phosphate buffer. And the rate constant due to total acetate buffer estimated in this way was 7.3×10^{-2} h^{-1} (mol dm^{-3})$^{-1}$ for clavulanate. The rate constant data reported at 35°C in the literature were used to calculate the total buffer catalytic rate constants following the same procedure. The results obtained are 2.1×10^{-1} h^{-1} (mol dm^{-3})$^{-1}$ (Zia, Shalchian and Borhanian 1977) for amoxycillin in phosphate buffer and 6.07×10^{-1} h^{-1} (mol dm^{-3})$^{-1}$ and 4.0×10^{-2} h^{-1} (mol dm^{-3})$^{-1}$ (Haginaka, Nakagawa and Uno 1981) for clavulanate in phosphate and acetate buffers respectively. A comparison between the two sets of data indicates the differences between the results are over the range of 49%-12%. Considering the

differences in experimental systems and errors involved in adjusting data obtained at different temperatures, this variantion in results can be considered to be acceptable.

As these liquid state data were obtained for extrapolation to frozen state temperatures a full study of buffer effects was unnecessary since such data are already available. However when different assay methods and experimental systems are used a comparison of results is important.

Table 3.3: Effect of buffer concentration: Rate constant values of amoxycillin and clavulanate at 55°C and $\mu = 0.5$

BUFFER	k_{obs} h^{-1}		k_{pH} h^{-1}	
concentration	Amox	Clav	Amox	Clav
Acetate (pH 4.6)			7.0×10^{-3}	1.0×10^{-1}
1.1×10^{-1}*	2.17×10^{-2}	1.46×10^{-1}		
2.2×10^{-1}*	4.59×10^{-2}	2.08×10^{-1}		
4.0×10^{-1}*	6.44×10^{-2}	2.71×10^{-1}		
5.5×10^{-1}*	9.24×10^{-2}	3.47×10^{-1}		
Phosphate(pH 7.0)			3.5×10^{-2}	1.1×10^{-1}
5.0×10^{-2}*	6.45×10^{-2}	2.26×10^{-1}		
1.0×10^{-1}*	9.06×10^{-2}	3.36×10^{-1}		
1.5×10^{-1}*	1.27×10^{-1}	5.06×10^{-1}		
2.0×10^{-1}*	1.50×10^{-1}	5.62×10^{-1}		

*mol dm^{-3}

3.6 Catalytic Effects

The catalytic effects of the buffers used in this study have already been discussed under buffer effects Section (3.4). This section deals with the experiments that were designed with the objective to determine the effect of possible catalytic reaction rates, which might exist in the combination of amoxycillin and clavulanate.

i. Catalytic effect of clavulanate on amoxycillin: The procedure for these experimental runs is described under Section 2.3.1.4. As has already been stated the decomposition of amoxycillin in the presence of clavulanate in the buffer solution showed first-order-biexponential decay, with the rate plots exhibiting curvature (Figure 3.8). The extent of curvature was more distinct when the ratio of clavulanate concentration to amoxycillin was increased under constant experimental conditions. The catalytic effect of clavulanate was more prominent in phosphate buffer than acetate buffer leading to higher initial rate constant (k_1) values. Table 3.4(a)

provides the rate constant values for amoxycillin at various clavulanate concentrations.

From data in Table 3.4 (b) it is evident that there is no significant effect on the rate of the degradation of clavulanate due to change in initial concentration of clavulanate in the buffer systems. This reinforces the fact that the first-order rate constants for clavulanate are independent of initial concentration of clavulanate.

There is no reported catalytic effect of clavulanate on amoxycillin. Since this effect was more prominent in phosphate buffer, it was investigated whether phosphate catalyzed the catalysis effect of clavulanate. This hypothesis was verified by carrying out experimental runs at an additional phosphate buffer concentration i.e. 2.0×10^{-1} mol dm^{-3}. Table 3.5 compares the results of the two phosphate buffer investigations.

Comparison of the data provided in Table 3.5 indicate that the total phosphate concentration has a direct effect on the initial rate of amoxycillin k_1 which is in turn effected by the clavulanate catalysis directly. The mechanism of this behaviour is not well understood. One possible explanation may be the possibility of polymer formation between amoxycillin and clavulanate, which could have a catalytic effect on the rate of reaction of amoxycillin.

A kinetic model was developed to show the overall behaviour of the several components responsible for the catalysis of amoxycillin in presence of clavulanate in phosphate buffer.

$$k_1 = k_{Amx} + k_{Clav} \qquad (3.4)$$

$$\text{where} \quad k_{Clav} = \left[k_{cvc} + k_{phccv} \frac{[phos]}{[clav]} \right] [clav] \qquad (3.5)$$

$$\textit{and:} \quad k_{Amx} = k_0 + k_{hyd}[OH^-] + k_{ph}[phos] \qquad (3.6)$$

where

k_1 = first-order rate constant for amoxycillin (in combination) degradation at the initial stage

k_{Amx} = rate constant of amoxycillin due to hydrolysis of amoxycillin

k_{Clav} = rate constant of amoxycillin due to presence of clavulanate

k_0 = uncatalysed reaction rate constant

k_{hyd} = second-order rate constant for base catalysis of amoxycillin degradation.

k_{ph} = second-order rate constant for phosphate catalysis of amoxycillin degradation.

k_{cvc} = second-order rate constant for clavulanate catalyzed degradation of amoxycillin degradation.

k_{phccv} = second-order rate constant for phosphate catalyzed of clavulanate catalysis of amoxycillin degradation

[phos] = concentration of phosphate buffer

[clav] = concentration of clavulanate

k_0 was found from k_{pH} of the phosphate buffer (Table 3.3) and k_{hyd} was taken as 1.15 $\times 10^{-3}$ h^{-1} (mol dm^{-3})$^{-1}$ (Bundgaard 1977). The value of k_{ph} was obtained from the slope of the plot of phosphate buffer effect (Figure 3.11) and k_{phccv} was estimated to be 2.87 h^{-1} (mol dm^{-3})$^{-1}$ obtained from the slope of the plots of phosphate buffer concentration versus k_1 values. The value of k_{cvc} was found to be 1.75×10^{2} h^{-1} (mol dm^{-3})$^{-1}$ obtained from the slope of the plot of concentration of clavulanate versus k_1 (calculated for zero phosphate buffer effect) values (Figure 3.12a).

Table 3.4: (a) Catalytic effect of clavulanate on the rate of degradation of amoxycillin: First-order rate constants of amoxycillin at constant amoxycillin initial concentration of 1.29×10^{-3} mol dm^{-3}, $\mu = 0.5$ and 55°C

*BUFFER	CLAV (mol dm^{-3})	k_1 (h^{-1})	k_2 (h^{-1})
Acetate	5.3×10^{-4}	1.44×10^{-1}	3.9×10^{-2}
	1.05×10^{-3}	1.20×10^{-1}	4.8×10^{-2}
	2.10×10^{-3}	2.87×10^{-1}	4.9×10^{-2}
	3.15×10^{-3}	4.21×10^{-1}	4.6×10^{-2}
Phosphate	5.3×10^{-4}	5.02×10^{-1}	9.4×10^{-2}
	1.05×10^{-3}	5.83×10^{-1}	9.4×10^{-2}
	2.10×10^{-3}	7.99×10^{-1}	9.2×10^{-2}
	3.15×10^{-3}	8.88×10^{-1}	1.03×10^{-1}

*Acetate: 2.2×10^{-1} mol dm^{-3} pH 4.6; Phosphate: 1.0×10^{-1} mol dm^{-3} pH 7.0
k_1 is the first order rate constant for initial degradation and k_2 is the first order rate constant for final degradation

(b) Effect of clavulanate initial concentration on the rate of clavulanate degradation: First-order rate constant values of clavulanate at constant amoxycillin initial concentration of 1.29×10^{-3} mol dm^{-3}, μ= 0.5 and 55°C

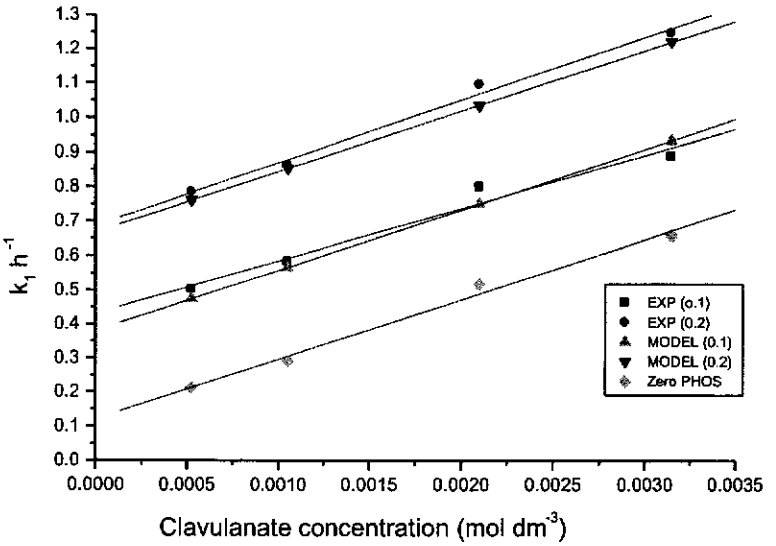
*BUFFER	CLAV mol dm^{-3}	CLAV h^{-1}	CLAV-COMB h^{-1}
Acetate	5.3×10^{-4}	2.20×10^{-1}	2.12×10^{-1}
	1.05×10^{-3}	2.08×10^{-1}	2.00×10^{-1}
	2.10×10^{-3}	2.18×10^{-1}	2.19×10^{-1}
	3.15×10^{-3}	1.95×10^{-1}	1.88×10^{-1}
Phosphate	5.3×10^{-4}	3.60×10^{-1}	3.30×10^{-1}
	1.05×10^{-3}	3.36×10^{-1}	3.29×10^{-1}
	2.10×10^{-3}	3.39×10^{-1}	3.23×10^{-1}
	3.15×10^{-3}	3.30×10^{-1}	3.34×10^{-1}

*Acetate: 2.2×10^{-1} mol dm^{-3} pH 4.6; Phosphate: 1.0×10^{-1} mol dm^{-3} pH 7.0

The above model (Equation 3.4) was tested by incorporating in Equations 3.5 and 3.6 various rate constant values predicted from the experimental results and the k_1 values obtained from the model was compared with that obtained directly from the experimental runs (Table 3.5b). The results indicate an acceptable agreement between the two data sets as illustrated in Figure 3.12a. Thus the testing of the proposed kinetic model demonstrates that the model is consistent with the experimental data.

The data in acetate buffer (Table 3.4) indicate that the rate of degradation of amoxycillin at the initial stage (k_1) is directly enhanced by clavulanate concentration (Fig 3.12b). The catalytic effect of clavulanate in acetate was less prominent than in phosphate. This made it difficult to estimate k_1 values particularly at lower concentrations and temperatures, which resulted in considerable error arising under these conditions. However the fact that y intercept in Figure 3.12b is 4.3×10^{-3} h^{-1} which is close to the rate constant for amoxycillin in the absence of clavulanate in acetate, reinforces the evidence of catalysis of amoxycillin by clavulanate in this buffer system.

(a)



(b)

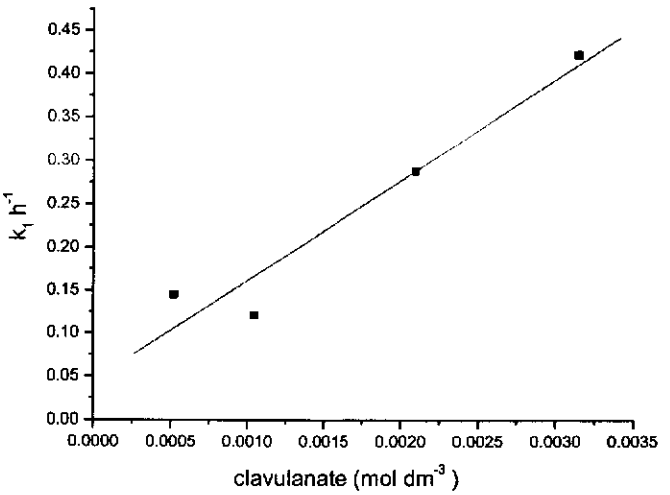


Figure 3.12: Effect of clavulanate concentration on the initial rate of amoxycillin at constant ionic strength μ = 0.5 and temperature 55°C.

(a) In phosphate buffer pH 7.0.

(b) In acetate buffer pH 4.6.

Exp [0.1,0.2] = experimental values in 1.0×10^{-1} & 2.0×10^{-1} mol dm^{-3} phosphate buffer; MODEL [0.1,0.2] = obtained from the model Eqn (3.4) in 1.0×10^{-1} & 2.0×10^{-1} mol dm^{-3} phosphate buffer; Zero PHOS = corrected by substracting the phosphate buffer catalytic effect (k_{phccv}); k_1= first order rate constant for amoxycillin at initial stage.

Table 3.5: (a) Effect of phosphate buffer concentration on clavulanate catalysis of amoxycillin: First-order rate constants for amoxycillin in presence of various concentrations of clavulanate at constant pH 7.0, μ = 0.5 and 55°C

CLAV	PHOSPHATE 1.0×10^{-1} mol dm^{-3}		PHOSPHATE 2.0×10^{-1} mol dm^{-3}	
mol dm^{-3}	$k_1(h^{-1})$	$k_2(h^{-1})$	$k_1(h^{-1})$	$k_2(h^{-1})$
5.3×10^{-4}	5.02×10^{-1}	9.4×10^{-2}	7.73×10^{-1}	1.45×10^{-1}
1.05×10^{-3}	5.83×10^{-1}	9.4×10^{-2}	8.66×10^{-1}	1.49×10^{-1}
2.10×10^{-3}	7.99×10^{-1}	9.2×10^{-2}	1.07×10^{0}	1.44×10^{-1}
3.15×10^{-3}	8.88×10^{-1}	1.03×10^{-1}	1.23×10^{0}	1.48×10^{-1}

Table 3.5: (b) Comparison between the initial first-order rate constant (k_1) values of amoxycillin obtained from the experimental runs in presence of clavulanate in phosphate buffer pH 7.0 and those calculated from the model Eqn (3.4)

CLAV	EXPERIMENTAL k_1 h^{-1}		MODEL k_1 h^{-1}	
mol dm^{-3}	1.0×10^{-1} moldm^{-3} *	2.0×10^{-1} moldm^{-3}*	1.0×10^{-1} moldm^{-3} *	2.0×10^{-1} moldm^{-3} *
5.3×10^{-4}	5.02×10^{-1}	7.85×10^{-1}	4.74×10^{-1}	7.61×10^{-1}
1.05×10^{-3}	5.83×10^{-1}	8.63×10^{-1}	5.66×10^{-1}	8.53×10^{-1}
2.10×10^{-3}	7.99×10^{-1}	1.10×10^{0}	7.50×10^{-1}	1.03×10^{0}
3.15×10^{-3}	8.88×10^{-1}	1.23×10^{0}	9.34×10^{-1}	1.22×10^{0}

*concentration of phosphate buffer

Thus from the above results it can be concluded, potassium clavulanate catalyses the rate of the reaction of amoxycillin in combination in the buffer systems, and that this catalysis is directly dependent upon the initial concentration of clavulanate and related to the rate of the degradation of clavulanate under these experimental conditions.

ii. Catalytic effects of amoxycillin on clavulanate: The effect of concentration of amoxycillin on clavulanate was studied according to the methods outlined under Section 2.3.1.5. The degradation of clavulanate in the presence of several concentrations of amoxycillin followed first order kinetics in both phosphate and acetate buffers. In phosphate buffer the rate plots for amoxycillin still showed some degree of curvature indicating the influence of the catalytic reaction of clavulanate. However as the ratio of concentration of amoxycillin was increased the degree of

curvature was decreased suggesting that the catalytic effect of clavulanate became less dominant due to the lower relative concentration of clavulanate (Figure 3.13). This observation could be due to the significant difference in the rate constants of the two drugs. The rate of degradation of clavulanate in acetate and phosphate buffer under this study was about 4.5 times and 3.7 times faster respectively than amoxycillin. However in combination runs due to the catalytic influence of clavulanate on amoxycillin, the rate of the degradation of clavulanate in acetate buffer was about 1.6 times the initial rate (k_1) of amoxycillin. This means that as 50% of amoxycillin degraded, 80% of clavulanate had already been degraded. When the concentration ratio of amoxycillin to clavulanate was increased, the catalytic influence of clavulanate was decreased leading to more linear runs similar to amoxycillin alone. Hence the rate of reaction of amoxycillin in the presence of clavulanate in acetate buffer appeared being monoexponential.

$$\text{i.e. } k_1 \approx k_2 = k_{obs}$$

Therefore for simplicity and consistency the rate constants obtained in these set of experiments were treated as first-order (Table 3.6b) since the rate plot with the maximum curvature (i.e. 1.29×10^{-3} mol dm^{-3} amoxycillin concentration) had still a satisfactory linearity $r > 0.99$.

Similarly in phosphate buffer the rate of clavulanate was 0.56 times the initial rate (k_1) of amoxycillin, which could well be the reason for greater curvature observed in these runs compared to the runs in acetate buffer. The result in Table 3.6 shows that the increase in concentration of amoxycillin led to an increase in rate of clavulanate in combination in phosphate buffer. Figure 3.14 illustrates the effect of the ratio of amoxycillin sodium to potassium clavulanate, on stability of potassium clavulanate in terms of t_{90} values. A similar type of catalytic effect of amoxycillin on clavulanate in combination has been reported in the literature (Ashwin, Lynn and Taskis 1987). These workers reported this effect at constant temperature 25°C by increasing the ratio of amoxycillin to clavulanate at constant clavulanate concentration. According to this report when the ratio of amoxycillin was increased from 5 to 10, the t_{90} of clavulanate was reduced from 235 minutes to 110 minutes which is a significant change in the shelf-life of clavulanate .

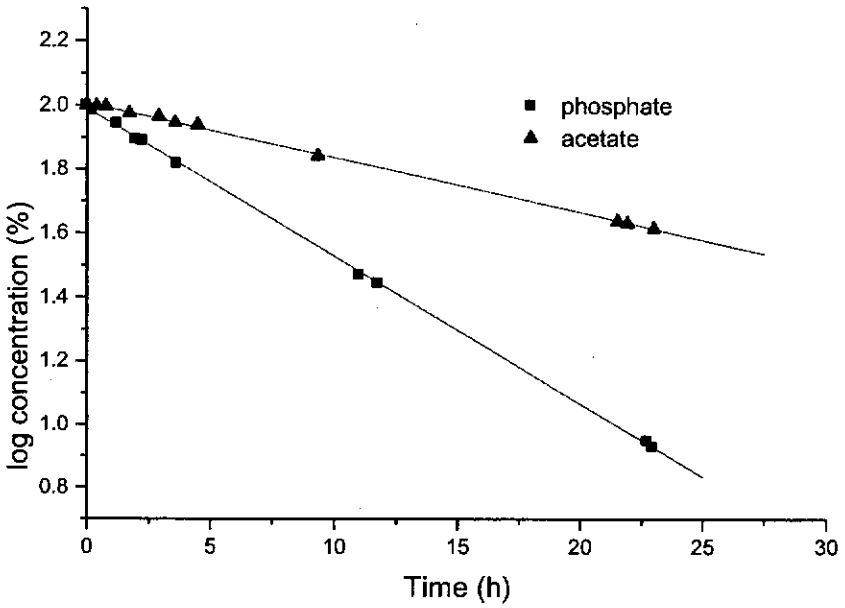


Figure 3.13: Effect of higher amoxycillin initial concentration on the rate of degradation of amoxycillin: First order plots of amoxycillin sodium in combination with clavulanate at constant ionic strength μ = 0.5 and temperature 55° C.

Phosphate = 1.0×10^{-1} mol dm^{-3} phosphate buffer pH 7.0; Acetate = 2.2×10^{-1} mol dm^{-3}acetate buffer pH 4.6 ; Amoxycillin concentration = 2.58×10^{-2} mol dm^{-3}.

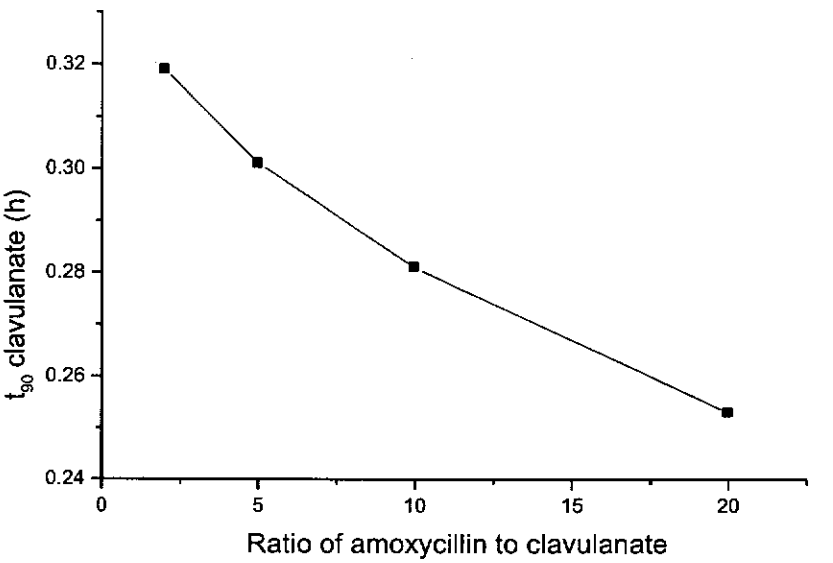


Figure 3.14: Effect of concentration of amoxycillin on t_{90} values of clavulanate at constant ionic strength $\mu = 0.5$ and temperature 55°C in phosphate buffer pH 7.0.

Table 3.6: (a) Effect of amoxycillin concentration on the rate of clavulanate degradation: First-order rate constants of clavulanate in the buffer systems at constant clavulanate initial concentration of 1.05×10^{-3} mol dm^{-3}, μ = 0.5 and 55°C

BUFFER*	AMOX (mol dm^{-3})	k_{obs} (h^{-1})
Acetate	1.29×10^{-3}	2.00×10^{-1}
	6.45×10^{-3}	2.10×10^{-1}
	1.29×10^{-2}	2.10×10^{-1}
	2.58×10^{-2}	2.04×10^{-1}
Phosphate	1.29×10^{-3}	3.29×10^{-1}
	6.45×10^{-3}	3.49×10^{-1}
	1.29×10^{-2}	3.74×10^{-1}
	2.58×10^{-2}	4.16×10^{-1}

(b) Effect of amoxycillin initial concentration on the rate of amoxycillin degradation: First-order rate constants of amoxycillin at constant clavulanate initial concentration of 1.05×10^{-3} mol dm^{-3}, μ = 0.5 and 55°C

BUFFER*	AMOX mol dm^{-3}	AMOX h^{-1}	AMOX-COMB h^{-1}
Acetate	$1.29\ 10^{-3}$	-	4.40×10^{-2}
	6.45×10^{-3}	-	4.20×10^{-2}
	1.29×10^{-2}	-	4.22×10^{-2}
	2.58×10^{-2}	-	3.90×10^{-2}
Phosphate	1.29×10^{-3}	9.06×10^{-2}	1.13×10^{-1}
	6.45×10^{-3}	1.01×10^{-1}	1.02×10^{-1}
	1.29×10^{-2}	1.01×10^{-1}	1.03×10^{-1}
	2.58×10^{-2}	1.02×10^{-1}	1.07×10^{-1}

* Acetate: 2.2×10^{-1} mol dm^{-3} pH 4.6; Phosphate: 1.0×10^{-1} mol dm^{-3} pH 7.0

From the data in Table 3.6(a) it can be concluded that a slight catalytic effect of amoxycillin on the rate of degradation of clavulanate is evident in pH 7.0 phosphate buffer and this catalysis is directly dependent upon the initial concentration of amoxycillin. In pH 4.6 acetate buffer however there is no significant change in the rate constant values of clavulanate, indicating that changes in amoxycillin initial concentration has no effect on the rate constant values of clavulanate.

Thus the investigation on effect of initial concentration on the rate of degradation of amoxycillin and clavulanate has demonstrated the rate of amoxycillin remained first-order in kinetics under the conditions of this study. Also the rate constant data for amoxycillin alone showed no significant change under all conditions of this study. This implies that the rate constant data were basically independent of initial concentration. However it is documented that amoxycillin apart from hydrolysis, undergoes other degradation pathways such as self-catalyzed degradation reactions (Chapter 1 Section 1.1.1.1.1). But the experimental results showed no evidence of this, since the rate constant values remained essentially the same. This can be explained by the fact that these reactions are both concentration and pH dependent. Reactions involving dimerization are expected (Bundgaard 1977a) to occur at higher concentrations such as 6.0×10^{-2} to 3.0×10^{-1} mol dm^{-3} and pH of about 7.6 and above where the a-amino group is largely in the nucleophilic form. The self-catalyzed hydrolysis reaction is also expected to occur at higher concentrations such as those mentioned for dimerization reactions and higher pH values such as pH of about 9.6 and above where the ionization of the aromatic hydroxyl group has taken place (Bundgaard 1977a). Therefore since under the present study the maximum concentration of amoxycillin used was 2.6×10^{-2} mol dm^{-3}, and the highest pH value used was 7.0, there were unfavorable conditions for these reactions to occur. Hence the rate constants for the degradation of amoxycillin alone and in combination were independent of the initial concentration of amoxycillin under the conditions of this investigation.

CHAPTER 4

STABILITY IN THE FROZEN STATE

Kinetics of the degradation of amoxycillin sodium and potassium clavulanate individually and in combination were studied in the frozen state in accordance with the methods specified in Chapter 2, Section 2.3.2. The concentrations of intact reactants were determined by the HPLC assay as explained previously (Section 2.4). The data used for estimations of the rate constants were treated similarly as described in Chapter 3. As the rate of reactions were generally quite slow, standard solutions freshly prepared from both drugs were used constantly to check the reproducibility of the column several times during each run. Typical HPLC chromatograms obtained are presented in Figures 4.1(a), 4.1(b) and 4.1(c).

4.1 Kinetics of Reactions

In this study as described earlier (Sections 2.3.2 and 2.8), experiments were performed in three systems, namely the hydrochloric acid system which contained sodium chloride (μ = 0.5), the acetate and phosphate buffer systems with no ionic strength control. Hence sodium chloride was not included in the buffer systems due to prolonged reactions times.

The data obtained from experimental runs demonstrated a linear relationship when plots of log concentration (%) remaining with respect to time were obtained over 2-3 half-lives of reaction or until a maximum of 10 days of reaction had occurred. Although some reactions were very slow where only about 20% of degradation was obtained, data were treated as first-order kinetics based on faster reactions in the frozen state. Figure 4.2 and Table 4.1 illustrate the rate constant data at various sub-zero temperatures and systems. The plots exhibited a fair linearity with correlation coefficients of $r > 0.94$. Therefore all the reactions were treated as first-order kinetics. General features of the mechanism of the reaction are produced in Chapter 1, Section 1.1.4.

The evidence for catalysis of amoxycillin by clavulanate, exhibited as curvature in the rate plots of the combination runs in buffer systems of the liquid state was undetectable in these systems. Because the rate of reaction of amoxycillin did not proceed beyond 2 half-lives where the clavulanate component was almost diminished.

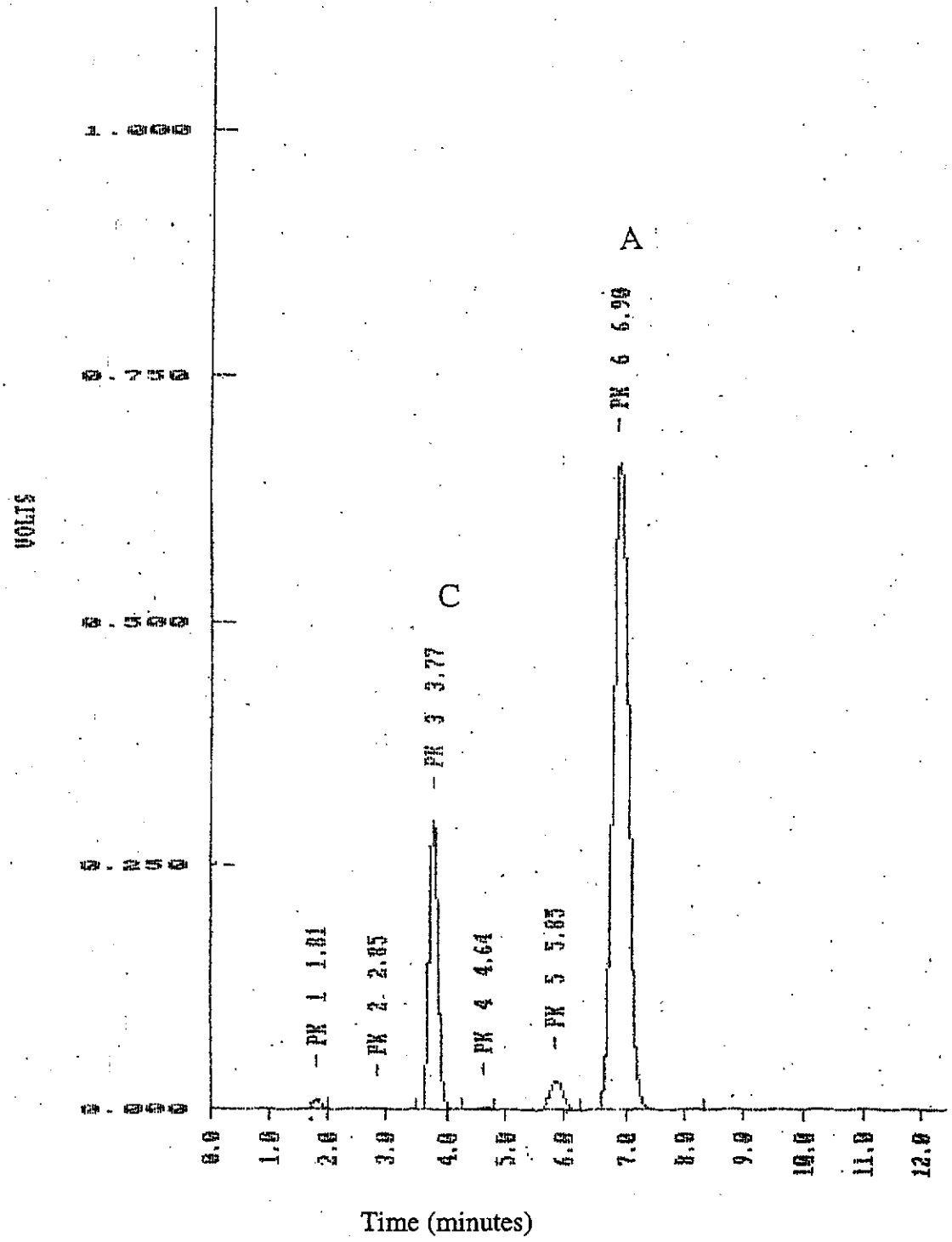


Figure 4.1: (a) Typical HPLC chromatograms of the partially degraded drug compounds in combination in hydrochloric acid system at –13.5°C, pH 2.0 and μ = 0.5 (NaCl), where A and C represent amoxycillin and clavulanate respectively.

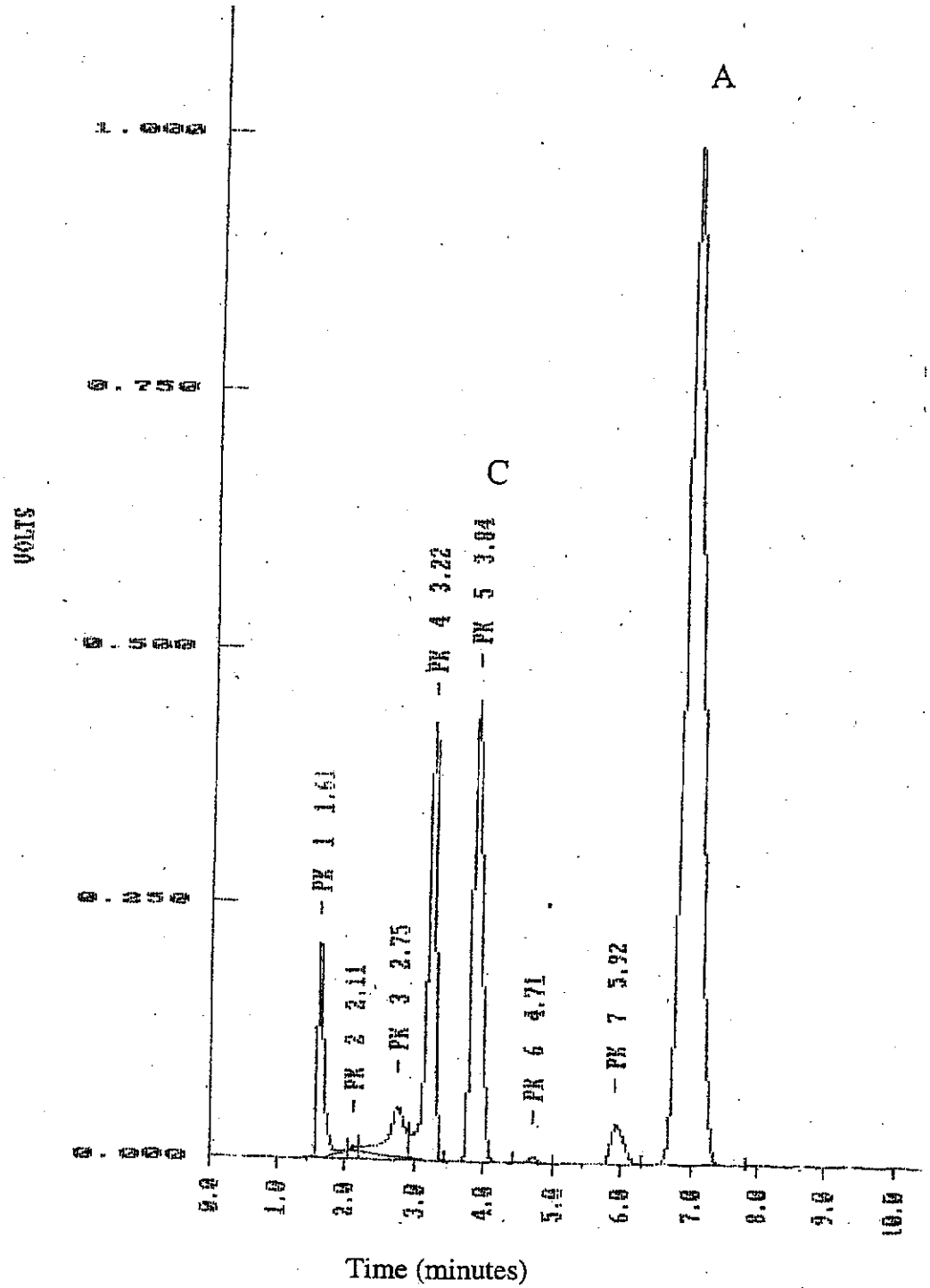


Figure 4.1: (b) Typical HPLC chromatograms of the partially degraded drug compounds in combination in acetate buffer at –13.5°C, pH 4.6 and $\mu = 0$ (NaCl), where A and C represent amoxycillin and clavulanate respectively.

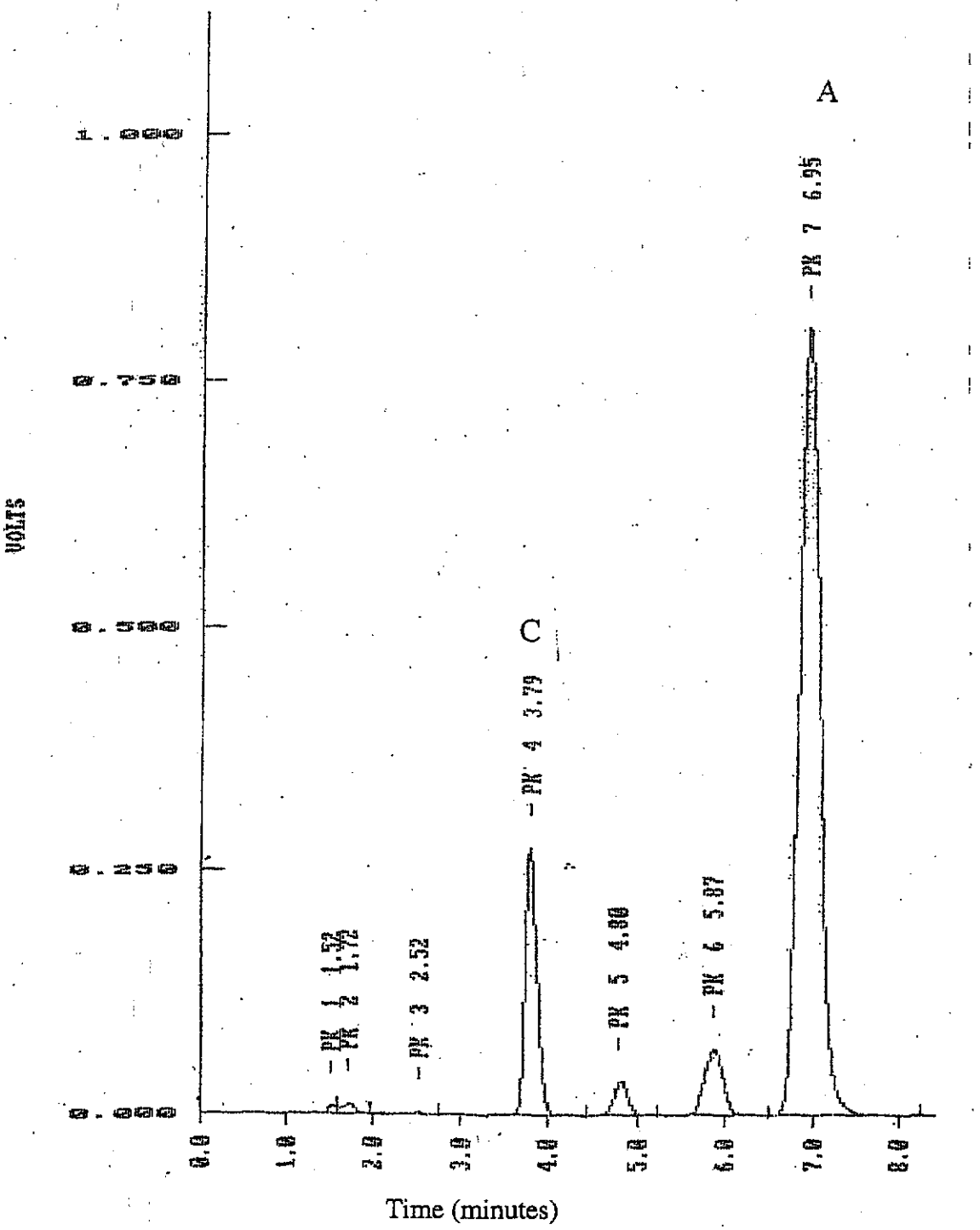
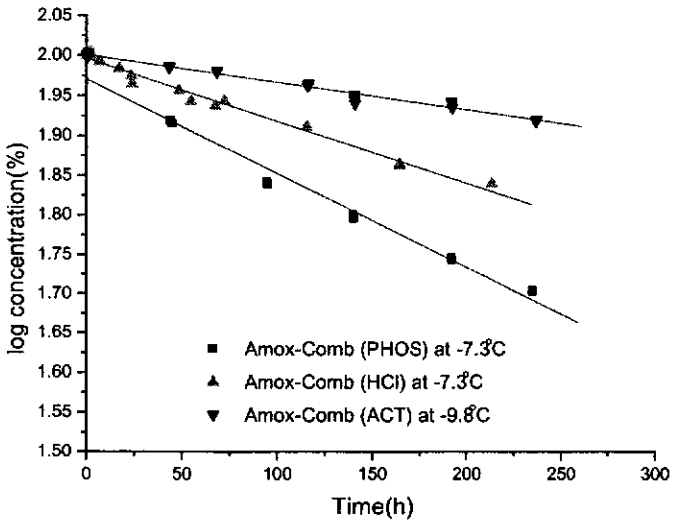


Figure 4.1: (c) Typical HPLC chromatograms of the partially degraded drug compounds in combination in phosphate buffer at –13.5°C, pH 7.0 and μ = 0 (NaCl), where A and C represent amoxycillin and clavulanate respectively.

(a)



(b)

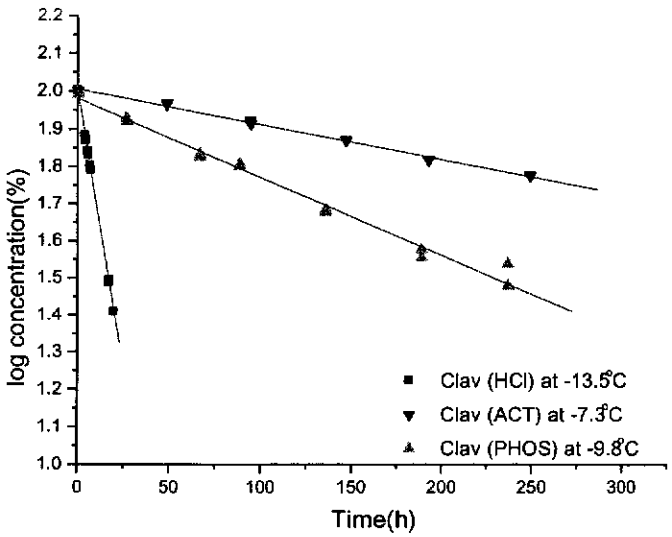


Figure 4.2: Representative first-order plots of amoxycillin and clavulanate in the frozen state.

(a) Sodium amoxycillin

(b) Potassium clavulanate

(HCl) = hydrochloric acid system; (ACT) = acetate buffer system; (PHOS) = phosphate buffer system.

The curvature in the other systems as described earlier (Section 3.6) occurred because clavulanate concentrations were diminishing as the reaction proceeded further. Since from the rate constants data presented in Table 4.1, it appears that the rate of amoxycillin degradation in combination in the buffer systems is significantly faster than that of amoxycillin alone. Therefore, clavulanate catalysis of amoxycillin as was observed in the liquid runs can be inferred.

Table 4.1: First-order k_{obs} rate constants of amoxycillin and clavulanate individually and in combination at pH values and temperatures indicated.

*pH	t (°C)	AMOX h^{-1}	CLAV h^{-1}	AMOX-COMB h^{-1}	CLAV–COMB h^{-1}
2.0	-7.3	1.79×10^{-3}	6.48×10^{-2}	1.80×10^{-3}	5.33×10^{-2}
	-9.8	1.99×10^{-3}	7.20×10^{-2}	1.93×10^{-3}	5.76×10^{-2}
	-13.5	1.86×10^{-3}	6.80×10^{-2}	1.65×10^{-3}	5.15×10^{-2}
4.6	-7.3	5.15×10^{-4}	2.14×10^{-3}	7.62×10^{-4}	2.32×10^{-3}
	-9.8	6.05×10^{-4}	2.50×10^{-3}	7.86×10^{-4}	2.66×10^{-3}
	-13.5	2.81×10^{-4}	1.46×10^{-3}	4.03×10^{-4}	1.60×10^{-3}
7.0	-7.3	7.87×10^{-4}	6.18×10^{-3}	2.73×10^{-3}	5.49×10^{-3}
	-9.8	6.86×10^{-4}	4.83×10^{-3}	2.02×10^{-3}	4.06×10^{-3}
	-13.5	1.69×10^{-4}	3.70×10^{-3}	9.53×10^{-4}	3.14×10^{-3}

*pH values measured at 20°C. pH 2.0 = 1.24×10^{-2} mol dm^{-3} hydrochloric acid and $\mu = 0.5$ (NaCl); pH 4.6 = 2.2×10^{-1} mol dm^{-3} acetate buffer (no NaCl); pH 7.0 = 1.0×10^{-1} mol dm^{-3} phosphate buffer (no NaCl).
Amoxycillin sodium initial concentration was 1.29×10^{-3} mol dm^{-3} in the buffers and 9.03×10^{-4} mol dm^{-3} in hydrochloric acid system.
Initial concentration of potassium clavulanate was1.05×10^{-3} mol dm^{-3} in the buffers and 7.38×10^{-4} mol dm^{-3} in hydrochloric acid system.

4.2 pH Effect

The effect of pH was studied by undertaking experimental runs at three pH values as described under Section 2.3.2. The corresponding rate constants obtained at each pH value are listed in Table 4.1.

4.2.1 Hydrochloric acid system pH 2.0: In this system the overall rate of reaction of amoxycillin whether alone or in the presence of clavulanate did not change significantly over all the temperatures studied. However, the rate of reaction of clavulanate showed an average increase of 23% when compared to its combination with amoxycillin. This increase in reaction rates similarly had been observed in the liquid state (Section 3.3.1) but was not such a marked difference. The extent of increase was 2-3 times greater in the frozen state. The average rate of clavulanate

degradation is estimated to be approximately 36 fold faster than that of amoxycillin alone and 30 times faster than of amoxycillin in combination, as it is evident from Table 4.1. This clearly maintains the role of clavulanate in the determination of shelf-life of the combination.

4.2.1.1 Kinetics of reactions in the hydrochloric acid system: The rate of degradation of amoxycillin or clavulanate in the presence of hydrochloric acid can be illustrated as follows,

$$A + H^+ \xrightarrow{k_2} products$$

The rate of reaction within the liquid regions of the frozen system can be denoted as,

$$-\frac{d[Total\ drug]}{dt} = k_2[H_l^+]\,[A_l] \qquad (4.1)$$

where k_2 is a second-order rate constant and A is the concentration of the antibiotic. The subscript l denotes the liquid regions of the frozen system.

Since $[H^+]$ was about 10 fold in excess of A, then $[H^+]$ was essentially constant throughout the reaction and the reaction was therefore conducted under first-order conditions. Hence integration of the above equation and subsequent conversion to logarithmic form gives,

$$\log A = \log A_0 - \frac{k_{obs}\,t}{2.303} \qquad (4.2)$$

where $k_{obs} = k_2\,[H^+]$

Under frozen conditions as stated in Chapter 1, Equation 1.31, the rate equation can be written as,

$$-\frac{dA}{dt} = k_2 C_l \frac{[H_s^+][A_s]}{C_s} \qquad (4.3)$$

where subscript s denotes concentration in the thawed solution, following the reaction in the frozen system. In this study C_s was dominated by $[Na^+]$ and $[Cl^-]$. Therefore the rate in the frozen state measured in the thawed condition can be denoted as:

$$\log A = \log A_0 - \frac{k_2 C_l t}{2.303} \frac{[H_s^+]_0}{[C_s]_0} \quad (4.4)$$

where under pseudo first-order conditions,

$$k_{obs} = k_2 C_l \frac{[H_s^+]_0}{[C_s]_0} \quad (4.5)$$

In Equation 4.5, k_2 is dependent on temperature and can be estimated by extrapolation of the reaction rates obtained in the liquid state to that of the frozen state, using the Arrhenius equation (see Chapter 2 Equation 2.7). The value of C_s can be estimated by adding the concentrations of various species present in the thawed solution and C_l values can be obtained from the phase diagram of sodium chloride in the literature (Cocks and Brower 1974; Seidel 1940; Rodebush 1918; Hall and Sherrill 1928). The term concentration of H^+ was ignored in the calculation for estimation of k_{obs} by prediction, because both C_s and C_l contained this term and also the estimated k_2 from extrapolation of the Arrhenius plot was in fact equal to k_{obs} / $[H^+]$. Hence $[H^+]$ was eliminated from the equation for all calculations of the predicted rate constants under these experimental conditions.

4.2.1.2 Factors effecting the rate of the reaction in the hydrochloric acid system

The overall rate of reaction in the frozen state has been accelerated significantly (Table 4.3) compared to the liquid state. The following factors can influence this change in the rate.

i. Concentration: According to Equation 4.5 the observed rate of hydrolysis of amoxycillin and clavulanate is dependent upon the concentration factor C_l / C_s. It has been proposed (Pincock and Kiovsky 1966) that under ideal conditions, at any frozen temperature the total concentration of solutes C_l is constant and independent of their nature or initial concentration. Considering that sodium chloride was the major constituent of this system, the concentration of sodium chloride at each particular temperature was obtained from the phase diagram data in the literature (Cocks and Brower 1974; Patel and Hurwitz 1972; Seidel 1940; Rodebush 1918; Hall and Sherrill 1928). This value of C_l was found to be in close agreement with the theoretical C_l value obtained by assuming ideal behaviour of sodium chloride from the following relationship (Martin 1993e)

$$C_I = \frac{\Delta T_f}{iK_f} \qquad (4.6)$$

where ΔT_f is the freezing point depression, and K_f is the cryoscopic constant which is 1.86 for water and i is the Van't Hoff factor which is 2 for sodium chloride. Hence sodium chloride has behaved almost ideally in this system. Table 4.2 lists the calculated values of C_I at the temperatures studied. The first-order rate constant values obtained by incorporating the concentration factor are presented in Table 4.3 and Figure 4.3, indicating that the concentration factor has significantly influenced the rate of the reactions.

Table 4.2: Comparison of C_I values of sodium chloride (in terms of $[Na^+]$ + $[Cl^-]$) estimated from the literature phase diagram with the ideal value obtained from Equation 4.6.

t (°C)	C_I (mol dm^{-3})	
	LITERATURE	IDEAL
-7.3	4.10	3.92
-9.8	5.34	5.27
-13.5	7.20	7.26

Literature = obtained from the phase diagram; Ideal = obtained from Equation 4.6.

Data in Table 4.3 indicate that incorporation of the concentration factor increased the rate of the reaction of this system significantly yet there still remains some differences between the rate constant values of those estimated by prediction, inclusive of the concentration factor, and the experimental results. This discrepancy between the data could be due to other factors which are discussed below or can arise from the fact that there could be error in C_I values and that the extrapolation of rate constant from the liquid state could induce a significant error in estimation of k_2 in Equation 4.5.

Another type of concentration effect that can be considered is due to the fact that in these systems amoxycillin and clavulanate make up only a proportion of the total number of species. Hence changes in temperature will lead to changes in concentration of the reactants. For instance the concentrations of amoxycillin and clavulanate anions were increased from 9.03×10^{-4} mol dm^{-3} and 7.38×10^{-4} mol

dm^{-3} initial concentrations in the liquid state to 4.81×10^{-3} mol dm^{-3} and 3.93×10^{-3} mol dm^{-3} at –9.8°C respectively. Similarly the concentrations of the said species were calculated to have increased by 7.2 fold at –13.5°C when compared to the initial concentration in the liquid state. These increased concentrations need to be considered, particularly whenever self-aminolysis or autocatalyzed hydrolysis reactions of amoxycillin take place. However under the conditions of this study and at the pH values used in these systems the likelihood of the self-catalytic degradation route is considered to be negligible (Bundgaard 1977a). Therefore from Equation 4.3 it can be concluded that the rate of reaction in this frozen system is dependent on the product of the rate constant and total concentration of all the species present and that this rate of reaction is independent of any one reactant concentration. Hence, as in the case of first-order reaction the rate constant is independent of initial concentration of the reactants.

Table 4.3: Comparison of the observed first – order rate constant values by incorporating the concentration factor in the hydrochloric acid system

t (°C)	COMPOUND	k_{Exp} h^{-1}	k_{Pred} h^{-1}	IDEAL k_{Pred} $h^{-1} \times C_l/C_s$	LITERATURE k_{Pred} $h^{-1} \times C_l/C_s$
-7.3	Amox	1.71×10^{-3}	3.89×10^{-4}	1.53×10^{-3}	1.60×10^{-3}
-9.8	Amox	1.99×10^{-3}	2.84×10^{-4}	1.50×10^{-3}	1.52×10^{-3}
-13.5	Amox	1.86×10^{-3}	1.77×10^{-4}	1.29×10^{-3}	1.28×10^{-3}
-7.3	Amox-Comb	1.80×10^{-3}	3.64×10^{-4}	1.43×10^{-3}	1.49×10^{-3}
-9.8	Amox-Comb	1.93×10^{-3}	2.65×10^{-4}	1.40×10^{-3}	1.42×10^{-3}
-13.5	Amox-Comb	1.65×10^{-3}	1.64×10^{-4}	1.19×10^{-3}	1.18×10^{-3}
-7.3	Clav	6.48×10^{-2}	1.18×10^{-2}	4.64×10^{-2}	4.86×10^{-2}
-9.8	Clav	7.20×10^{-2}	8.06×10^{-3}	4.26×10^{-2}	4.32×10^{-2}
-13.5	Clav	6.80×10^{-2}	4.55×10^{-3}	3.31×10^{-2}	3.29×10^{-2}
-7.3	Clav-Comb	5.33×10^{-2}	1.03×10^{-2}	4.64×10^{-2}	4.85×10^{-2}
-9.8	Clav-Comb	5.76×10^{-2}	7.02×10^{-3}	4.26×10^{-2}	4.31×10^{-2}
-13.5	Clav-Comb	5.15×10^{-2}	3.92×10^{-3}	3.31×10^{-2}	3.28×10^{-2}

k_{pred} = rate constant predicted from the Arrhenius plot ; k_{Exp} = rate constant obtained experimentally; Ideal = Predicted rate constant where C_l is from Eqn 4.6; Literature = Predicted rate constant where C_l is obtained from the phase diagram of sodium chloride.

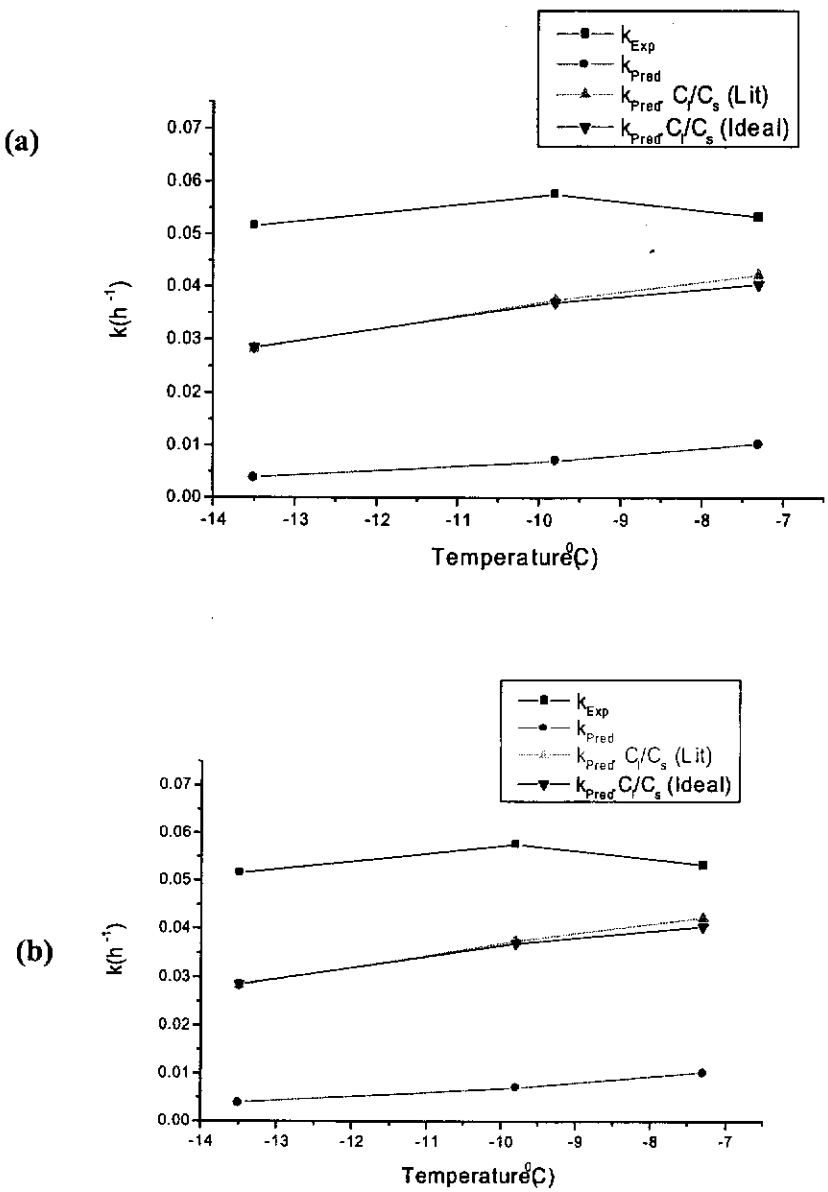


Figure 4.3:Comparison of first-order rate constant values of amoxycillin and clavulanate in the hydrochloric acid system.

(a) Amoxycillin in the presence of clavulanate.

(b) Clavulanate in the presence of amoxycillin.

Where, k_{Exp} = k_{obs} values obtained from experimental result; k_{Pred} = k_{obs} values obtained by extrapolation from the Arrehenius plot; k_{Pred} (Ideal) = k_{obs} values obtained by incorporating the concentration factor in Equation 4.5 where C_l is calculated by assuming the ideal behaviour of sodium chloride; k_{Pred} (Lit) = k_{obs} values obtained by incorporating the concentration factor where C_l is obtained from the literature.

ii. General catalysis: One of the major types of catalysis predicted in this system is acid catalysis owing to presence of hydrogen ion. As stated previously under ideal solute behaviour the total concentration of a frozen system at a particular temperature is constant. This implies that when a frozen system consists of number of solutes, the concentration of H^+ present becomes a proportion of the total concentration. Hence the concentration of H^+ varies with the change in temperature. Table 4.4 illustrates the effect of temperature on concentration of H^+. It is evident from these data that as the temperature falls the concentration of H^+ increases significantly potentially resulting in pH changes which leads to increased acid catalysis.

Table 4.4: Effect of temperature on the concentration of hydrogen ion in the hydrochloric acid system containing amoxycillin sodium at pH 2.0 and μ = 0.5 (NaCl)

t (°C)	$[H^+]$ mol dm^{-3}
20	1.24×10^{-2}
-7.3	5.07×10^{-2}
-9.8	6.60×10^{-2}
-13.5	8.90×10^{-2}

Following similar calculations for the concentration change of H^+ in solution for combinations of amoxycillin and clavulanate, it is understood that the H^+ is less concentrated in the combination solution than in individual drug solution owing to the dilution factor caused by the other antibiotic components. Hence the concentration of H^+ is expected to increase by 2.0×10^{-4} mol dm^{-3} in the systems containing clavulanate only compared to the combination. Although this estimated value seems negligible however owing to the enormous catalytic effect of H^+ on clavulanate observed in both the liquid and frozen states, this apparent small change in concentration could have resulted in the difference in the rate of reaction between the two systems. It is also evident from the data in Table 4.1 that the difference in rate of degradation for clavulanate in individual runs and that in combination runs is directly dependent on temperature which is in consistent with the above argument.

iii. Influence of pH: As stated above the change in concentration of hydrogen ion results in changes of pH which in turn can influence the rate of the reaction. Another

factor, which is responsible for the change in pH, is the ionic strength factor that is discussed in the following paragraphs. Also the degradation products of amoxycillin and clavulanate could influence a change in pH since the concentration of these degraded products can increase in like manner to the concentration of the antibiotic itself. Multiple degradation products would tend to moderate this effect.

iv. Influence of ionic strength: The rate of a reaction is influenced by ionic strength of the solution through primary or secondary salt effects.

When a solution of sodium chloride is frozen, the first solid phase formed is ice. Hence the remaining liquid becomes more concentrated in the remaining solute e.g. NaCl. As cooling is continued the concentration effect is increased until the concentration of solute reaches its saturation and precipitation of the solid phase occurs. For instance in a simple binary system H_2O-NaCl the equilibrium ice formation in solution containing a physiologic salt concentration (0.9% of NaCl) begins at –0.55°C (Cocks and Brower 1974). The eutectic temperature of sodium chloride is well documented through its phase diagram to be around –21.2°C (Cavatur and Suryanarayanan 1998; Milton and Nail 1996; Ramirez, Cavanaugh and Purcell 1974; Cocks and Brower 1974) where the eutectic composition is ~3.76 mol dm^{-3} of sodium chloride. Thus as illustrated in its phase diagram sodium chloride is present in its crystalline form below its eutectic temperature. Above the eutectic temperature up to -17°C, there appears to be some evidence of a crystalline form of sodium chloride dihydrate. But above -16°C, no crystalline phases have been reported which indicates the liquidus phase boundary. Hence under the conditions of this study, sodium chloride existed in a concentrated supercooled solution. Therefore the concentration of sodium chloride has increased significantly from its initial concentration 4.88×10^{-1}mol dm^{-3}.

It is documented (Harned and Owen 1950a) that the activity coefficient of hydrochloric acid in sodium chloride solution is a function of μ. Harned and co-workers (Harned and Owen 1950a; Harned and Mannweiler 1935; Harned 1920; Harned and Brumbaugh 1922) have studied the activity coefficient of hydrochloric acid in various concentrations of sodium chloride solution in the temperature range of 0°C to 60°C. These data indicate that the mean activity coefficient of hydrochloric acid increases and passes above unity as the concentration of sodium chloride increases and reaches values close to those obtained from the phase diagram. This

implies that there could be a slight decrease in the activity of the hydrogen ion. It should be noted that in this study the presence of sodium chloride used for constant ionic strength has had a significant rate slowing effect. This is evident from the experimental data (Section 4.4 and Table 4.7). Theoretically this effect can be predicted using Equation 4.5 by assuming the ideal behaviour of the solutes. For instance if sodium chloride is absent from the system, C_s is dominated by $[H^+]$ + $[Cl^-]$. It is evident that the value of C_s (2.48×10^{-2} mol dm^{-3}) is substantially lower in the system with no sodium chloride than with sodium chloride where C_s is dominated by $[Na^+]$ + $[Cl^-]$. Hence considering the concentration factor C_I/C_s in Equation 4.5 the numerator value (C_I), would be the same (under ideal conditions) for both the systems but the denominator value (C_s) is substantially greater in the system with sodium chloride (9.76×10^{-1} mol dm^{-3}) than the other system. Thus this will result in approximately 40 fold increase in the rate of the system with no sodium chloride (μ = 0) compared to the system of ionic strength of 0.5. This induced rate is too fast to follow experimentally.

4.2.2 Acetate buffer pH 4.6

In this system it is evident that the overall rate of reaction of amoxycillin and clavulanate (refer to data in Table 4.1), increases slightly as the temperature is decreased from –7.3 to –9.8 °C and then decreases significantly as the temperature decreases further to –13.5 °C. The data also demonstrate a notable increase in rate of amoxycillin degradation in the combination in comparison to the runs containing amoxycillin only. This increase in rate can be related to the catalytic effects of clavulanate on amoxycillin as noted at the initial stage of the liquid runs and explained earlier. However, as in the liquid state the rate of clavulanate did not change significantly as a result of combination with amoxycillin.

There was no significant change in pH during the course of the experiment. However extrapolation of the Arrhenius plot from the liquid state data indicates that the overall rate of the reaction of sodium amoxycillin and potassium clavulanate have been increased significantly when the compounds are stored at the frozen temperatures.

4.2.3 Phosphate buffer pH 7.0

The rate constant data in Table 4.1 indicate that the rate of reaction of amoxycillin and clavulanate in this system were slowed in general with a decrease in

temperature. The rates of degradation of amoxycillin in combination runs were significantly enhanced in comparison to those of amoxycillin alone. The extent of the increase in rate was greater than in acetate buffer thus reconfirming similar results in the liquid state. Unlike in the liquid state the rates of degradation of clavulanate were slightly slower in the combination run than in the runs containing clavulante only (about 12% at –7.3 °C, 17% at –9.8 °C and 16% at –13.5 °C).

There were no significant change in pH when the initial and final pH of each run was compared. Extrapolation of data from the liquid state by means of the Arrhenius equation indicates a significant increase in the rate of amoxycillin and clavulanate when stored under these frozen temperatures.

As the buffers were prepared according to the Henderson-Hasselbalch equation (Chapter 2 Equation 2.10) the type of concentration factor effect, discussed under hydrochloric acid system was not considered to influence the rate of reaction in buffer systems. However, the results in phosphate buffer system demonstrated similar trends of changes in rate with respect to temperature, to that reported by McDonald *et al.* (1989b), although the extent of change in the rate is significantly smaller in this study because of different experimental conditions used here. These workers used pH 8.68 with an amoxycillin initial concentration of 2.58×10^{-2} mol dm^{-3}. They studied the degradation of amoxycillin sodium in normal saline and glucose solutions and reported the maximum degradation rate of amoxycillin to be in the temperature range of –7.5 °C to –6.5 °C. The rapid rate of degradation of amoxycillin reported at these temperatures by these workers has been attributed to the significant increase in concentration of amoxycillin in the liquid vesicles of the frozen temperatures studied. They have also reported that this rate of change of concentration of amoxycillin with temperature was diminished as the temperature fell further in the sub-zero range. Therefore this observation is in agreement with the results of this study. Although the experimental conditions were different from the literature report, since the pH of this study was 7.00, there could be a concentration dependent dimerization reaction (see polymerization 4.2.4.4) occurring, which could accelerate the rate of amoxycillin degradation.

Since the above argument is not expected to bring about a major rate enhancing effect and is not applicable for clavulanate, therefore there should be

other factors influencing the rate of the reaction of these compounds in the frozen state.

4.2.4 Factors affecting the rate of the reaction of amoxycillin and clavulanate in the buffer systems

As stated previously the rates of degradation of amoxycillin and clavulanate have been accelerated significantly when stored at frozen temperatures. The following factors can be accountable for this change in rate in the buffer systems studied.

4.2.4.1 Buffer effects

As stated in Chapter 1, buffers can influence the rate of reactions through several ways. These are discussed sequentially.

i. Catalysis: In the liquid state it was demonstrated that both acetate and phosphate buffers had catalytic effects on the rates of degradation of amoxycillin and clavulanate. It was also illustrated that the buffer catalysis effect was dependent on the total buffer concentration. Thus as the buffer concentration is expected to increase at the frozen temperatures so also is the buffer catalysis effect.

ii. Concentration: As stated above, change in buffer concentration can lead to change in rate of reaction. To estimate the change in buffer concentration at each frozen temperature, the total concentrations of various buffer species in the liquid state were summed and then assuming ideal behaviour of solutes in the frozen state, the proportion of the buffer solutes present at a particular frozen temperature was calculated. Thus from the buffer concentration–rate constant plot of the liquid state data, the rate constant for that particular buffer concentration was estimated and the change in rate was compared with the liquid state data. Applying this principle the rate of amoxycillin was expected to increase 9.8 times at –7.3, 13.2 and 18.1 times at –9.8°C and –13.5°C respectively in acetate buffer. Similarly the rate of reaction of clavulanate was predicted to increase by 7.1, 9.4 and 12.7 times at –7.3°C, -9.8°C and –13.5°C respectively. If the same procedure is applied for the phosphate buffer system the rate of degradation of amoxycillin should be expected to increase for amoxycillin by 12.3, 16.5 and 22.6 times at –7.3°C, -9.8°C and –13.5°C respectively. Similarly for clavulanate the increase in rate would be expected to be 12.8, 17.1 and 23.5 times at –7.3 °C, -9.8°C and –13.5°C respectively.

Since the total change in the rate of reaction is an additive property, it is important to consider all other factors (discussed below) which could influence the rate of reaction and evaluate those, which tend to exert a greater influence on rate.

iii. Precipitation: Depending on the eutectic temperature of a compound, buffer constituents can selectively crystallize or precipitate under frozen conditions. Since the reported eutectic temperatures of acetic acid and sodium acetate are –26.4 °C and –16.6 respectively (Inoue, Shima and Inazu 1984; Ramirez and Purcell 1974), no precipitation of either buffer components are predicted at or above the frozen temperatures studied in acetate buffer. However the eutectic temperatures of potassium dihydrogen phosphate and disodium phosphate are reported (Murase, Echlin and Franks 1991; Van den Berg and Rose 1959) to be –2.7 °C and –0.5 °C respectively. Hence, in phosphate buffer the precipitation of buffer components would likely to occur under the frozen temperatures studied. Thus changes in buffer constituents need to be carefully considered as it can affect the rate of the reaction mainly by bringing about a change in pH and electrolyte concentration.

It has been well documented in the literature (Cavatur and Suryanarayanan 1998; Murase and Franks 1989) that when a dilute solution containing disodium hydrogen phosphate is cooled, first ice crystallizes, then due to the low solubility of disodium phosphate, it readily crystallizes as the dodecahydrate i.e. $Na_2HPO_4.12H_2O$ at –0.5°. In a solution containing a number of salts, the eutectic crystallization of each compound is governed by nucleation and also the growth of the crystalline phase from the concentrated freeze solution, the growth of crystals also depends on cooling rate. For instance Cavatur and Suryanaranan (1998) reported that in a ternary buffer system containing both disodium and monosodium hydrogen phosphate when the concentration of disodium hydrogen phosphate was kept constant while the concentration of sodium dihydrogen phosphate was altered, it appeared that at higher concentrations of sodium dihydrogen phosphate such as 1.4 mol dm^{-3}, crystallization of disodium hydrogen phosphate was inhibited. Whereas at lower concentration of sodium dihydrogen phosphate such as 3.6×10^{-1} mol dm^{-3} , the crystallization of disodium hydrogen phosphate was not completely inhibited. Van den Berg (1959) has determined the freezing point of a mixture of solutions of potassium dihydrogen phosphate and disodium hydrogen phosphate at pH 5.5 to be –4.3 °C. The ratio of dihydrogen phosphate to monohydrogen phosphate

taken in this study is ~6.9 times smaller than the one used by Van den Berg. Therefore, it can be said, under the experimental conditions the sequence of eutectic precipitation would be ice, disodium hydrogen phosphate and potassium dihydrogen phosphate respectively. Hence at the highest frozen temperature studied (i.e. –7.3°C), both the phosphate buffer constituents have been precipitated, since both disodium hydrogen phosphate and potassium dihydrogen phosphate are reported to be the least soluble form of phosphate salt which easily crystallize from the frozen solution. Also their melting heat eutectic is hardly dependent on the cooling rate (Murase and Franks 1989), indicating that the rate of nucleation and crystal growth of disodium hydrogen phosphate and potassium dihydrogen phosphate are high compared to that of cooling. Therefore one would expect an increase in rate of degradation of amoxycillin and clavulanate under these conditions, because once the buffer salts have precipitated, the relative concentrations of the antibiotics in the liquid region increases significantly. On the other hand however Murase and Franks (1989) have demonstrated that the salt precipitation of the phosphate buffer solution depends on the initial salt concentration. Thus in case of the potassium salt of phosphate, below approximately 1 mol dm^{-3} the fraction of salts which precipitates decreases with decrease in the initial concentration. This behavior has been explained by these workers as in a dilute solution, initially ice crystallization is fast which can determine the morphology of the freeze mixture hence resulting in small domain of salt concentrated freeze mixture. This small salt concentrated freeze mixture may become subject to an enormous supercooling and supersaturation which can eventually result in amorphous state formation as the temperature cools further. Considering that the initial concentration of phosphate buffer used in this study fell into the dilute solution category, one may expect occurrence of supercooling and supersaturation as a possibility especially at higher frozen temperatures. However, the freezing method (Chapter 2) used in this study was rapid cooling to temperatures well below –30°C, one would assume that the phosphate salts would then be in the solid state. Therefore when the temperature was increased to even the highest frozen temperature –7.3°C one would expect that most of the phosphate salts would be in the solid state, since the eutectic temperature of the mixture of the salts would be about -4°C. If this is the case, changes in pH and

concentrations are expected to occur leading to substantial changes in rates of the reaction in this system.

4.2.4.2 pH effect

As discussed above the precipitation of phosphate salts would lead to significant pH change and hence result in changes in the rate of the reaction. There are several reports (Murase, Echlin and Franks 1991; Gomez, Hornedo and Pikal 1994; Van den Berg 1966; Van den Berg and Rose 1959) indicating that the pH of phosphate buffers can change in the frozen state. Gomez and co-workers have studied the changes in pH of sodium phosphate buffer solution subjected to sub-zero temperatures. According to these workers solutions having an initial pH of 7.4 may experience a 3.6 unit decrease in pH when stored at the temperature range of 0°C-10°C. These workers also demonstrated that the change in pH was dependent on initial buffer total concentration. That is the change in pH is 3.4 for 1.00×10^{-1} mol dm^{-3} and 5.0×10^{-2} mol dm^{-3} buffer, 3.0 for 2.0×10^{-2} mol dm^{-3} buffer and 2.4 for 8×10^{-3} mol dm^{-3} buffer. The change in pH of the phosphate buffer is explained by Van den Berg (1966) as follows.

When a solution of phosphate containing disodium hydrogen phosphate and potassium dihydrogen phosphate is frozen, a marked decrease in pH is observed as the result of ice formation which starts at the freezing point, the decrease in pH is accelerated when disodium hydrogen phosphate precipitates as the result of further cooling. As the temperature further cools, potassium dihydrogen phosphate precipitates resulting in a further decrease of pH, but less rapidly. The pH-temperature phase relations of phosphate buffer indicates that in multisalt solutions, the changes in pH and eutectic points of the solution are governed by the type of salt precipitating. Also the change in pH is dependent on the sequence of the salts precipitating. This sequence depends on the relative solubility of the salts. Van den Berg (1966) has also observed that supersaturation occurs frequently and can last for long periods affecting the pH of the solution temporarily. The reported data by Van den Berg showed a decrease of about 2 pH unit in a system containing 3.9×10^{-2} mol dm^{-3} of potassium phosphate and 2.9×10^{-2} mol dm^{-3} of disodium phosphate. As the quantity of disodium phosphate used in this study was 6.29×10^{-2} mol dm^{-3} a larger pH shift could be expected. In the investigation carried out by Murase and Franks (1989) the buffer composition used by them was not the same as used in this study.

However their investigation on sodium phosphate buffer is in agreement with Van den Berg (1966), that the precipitation of disodium hydrogen phosphate resulted in a dramatic change in pH towards acidic values. This pH change as described previously has been reported by Gomez and coworkers (1994) to be approximately 3 pH units. Therefore, based on these reports it can be concluded that the pH of phosphate buffer used in this study could have decreased from 7.00 to about 4.00, which is a substantial fall in pH. Accordingly the rate of reactions could be affected in the following manner. However in this study pH measurements were not attempted owing to the very marked difficulties in obtaining useable results.

The pH-rate profile of amoxycillin (Zia, Shalchian and Borhanian 1977) indicates that a decrease of up to 1.5 unit in pH would reduce the rate of degradation of amoxycillin, however further reduction of 1 unit in pH would not bring any significant change in rate with respect to pH 7.0. Below pH about 4.5 the rate of hydrolysis of amoxycillin increases with any reduction in pH. For instance the rate of degradation of amoxycillin would increase by approximately 2 fold when pH falls from 7.0-to 4.0. Similarly in the case of clavulanate the pH-rate profile data provided by Haginaka and co-workers (1981) indicates that the rate of hydrolysis of clavulanate would not increase (rather stabilizes) for up to a 1.5 unit fall in pH. However, the rate of the reaction of clavulanate increased directly with any further fall in pH. It was estimated there would be about 10 fold increase in rate of clavulanate when the pH falls from 7.0 to 4.0. From the data presented in Table 4.6, it is evident that the rate of degradation of clavulanate in phosphate buffer system has increased in the range of 7.3 to 9.2 fold. Therefore it appears that the fall in pH due to phosphate precipitation can induce greater rate acceleration on clavulnate than amoxycillin. This is based on differences in the characteristics of their pH-rate profile.

Since in acetate buffer no precipitation of the buffer components is predicted, little pH change is expected. However the change in pK_a with respect to temperature in acetate buffer is worthy of consideration. The temperature dependence of the dissociation constant of acetic acid is well documented (Fisher and Barnes 1972; Harned and Ehlers 1933; Harned and Owen 1950b). Table 4.5 illustrates this relationship.

From Figure 4.4 it is evident although the dependence of temperature of acetic acid on pK_a is non-linear over wide range of temperature, but linearity is

observed when dealing with the fraction at the higher or lower ends of the curve. Therefore one should be careful in extrapolation of pK_a data. Sunderland (1983a) has studied the suitability of acetate data for extrapolation over temperature range of 20-150°C and determined the use of Equation 4.7 which takes account of the non-linearity.

$$pK_a = \frac{A}{T} + B \ln T + C \qquad (4.7)$$

The author found the model to be suitable within this temperature range and predicted the pK_a of acetic acid at 150°C in close agreement with the experimental result. However, this model was tested for the temperature range below 20°C, a poor degree of fitness was observed and was found to be not suitable especially for the temperature range of 0-10°C. As it is evident from Figure 4.4 the temperature pK_a relationship is linear within this temperature range. Hence a linear fit was attempted for the temperatures below 10°C using the least square method where correlation coefficient was 0.975.

Table 4.5: The effect of temperature on ionization constant of acetic acid.

t (°C)	pK_a *
0	4.780
5	4.763
10	4.756
20.0	4.756
30.0	4.757
40.0	4.769
50.0	4.787
60.0	4.81
70.0	4.84
80.0	4.87
90.0	4.91
100	4.94
110	5.01
120	5.06
130.5	5.11

* Data obtained from Fisher and Barnes1972; Harned and Ehlers 1933 and Harned and Owen 1950b.

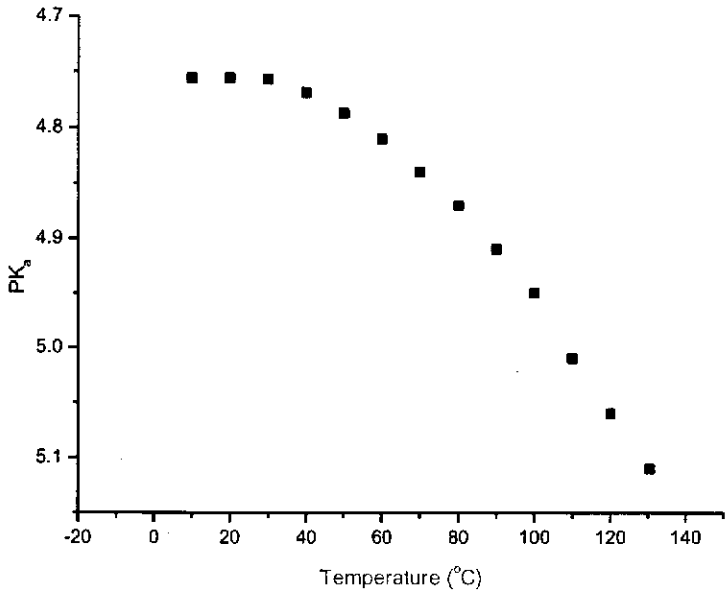


Figure 4.4: Effect of temperature on ionization constant of acetic acid.

Data obtained from Fisher and Barnes 1972; Harned and Ehlers 1933 and Harned and Owen 1950b

Thus extrapolating these data to the temperatures used in this study resulted in pK_a values of 4.80, 4.80 and 4.81 corresponding to temperature of –7.3 °C, -9.8°C and –13.5 °C respectively. Accordingly the pH of the buffer can be calculated from the following Equation 4.8.

$$pK_a = pH + \log \frac{[NaAc]}{[HAc]} \qquad (4.8)$$

where *[NaAc]* is the concentration of sodium acetate and *[HAc]* is the concentration of acetic acid.

Under frozen conditions the ratio of the concentration of sodium acetate to acetic acid remains similar to the liquid state. Therefore placing this value in Equation 4.8, provides the pH values of 4.95, 4.95 and 4.96 for temperatures –7.3 °C, -9.8°C and –13.5°C respectively. Hence in course of freezing the pH of the acetate buffer has changed from the room temperature pH of 4.60 to 4.96 at –13.5 °C, that is about 0.36 unit rise in pH. There could also be some change in activities of these species on concentration. This is difficult to predict at high electrolyte concentrations. Thus according to the pH-rate profiles analysis of amoxycillin and clavulanate described previously, this small change in pH (0.36 unit rise in pH) should have an stabilizing effect on the rates of reaction of both antibiotics.

The effect of temperature on the second dissociation constant of phosphoric acid has been documented in the literature (Bates and Acree 1945; Bates and Acree 1943) over the temperature range of 0-60°C. However, the pK_a data indicate that the changes in pK_{a2} of phosphoric acid with respect to temperature are smaller than found for acetic acid (Sunderland 1983b). Hence as other effects discussed earlier have greater influence on pH of phosphate buffer, the impact of a small variation of pK_a of phosphate buffer with temperature has not been considered important in these studies.

4.2.4.3 Catalysis effects

The catalytic effect of buffer has already been discussed under the section on buffer effects. Another factor, which can influence the rate of reaction when the compounds are frozen, is the possibility of enhancement of catalytic effect of one reacting species upon another due to increase in the proportional concentration of the catalyst. Hence the catalysis of amoxycillin by clavulanate or amoxycillin can be considered. There are no reports in the literature on the catalytic effect of

clavulanate on amoxycillin. However the investigation of this study in the liquid state suggested the catalysis of amoxycillin by clavulanate in acetate and phosphate buffer as discussed in Chapter 3, Section 3.6. It was also demonstrated in the same section that as the concentration of clavulanate increased this effect was more prominent. The change in concentration of clavulanate is supported by the theory stated earlier (Pincock and Kiovsky 1966) that reactions in the frozen state occurs in liquid vesicles of the apparently frozen solvent (ice). Hence in the combination runs, as the temperature reaches sub-zero, increase in concentration of clavulanate within the liquid vesicles of the frozen solvent could result in an increase of its catalytic effect on amoxycillin. Thus the results in Table 4.1 indicates a notable increase in rate of amoxycillin in combination runs in both the buffer systems, compared to its individual runs. The data also indicates that the rate of degradation of amoxycillin in combination with clavulanate in phosphate buffer is far greater than in acetate buffer, thus reconfirming the results of the liquid state. The catalytic effect of phosphates on clavulanate catalysis of amoxycillin was illustrated in Chapter 3 Section 3.6 and could also be another factor influencing the increase in rate of amoxycillin in combination runs in the phosphate system. This should be particularly true where supercooling and supersaturation has occurred, resulting in phosphate buffer species remaining soluble in the liquid pockets of the frozen system. The data in Table 4.1 support this explanation by showing a greater rate constant at –7.3 °C than other lower temperatures. Since as the temperature decreases there would be more likelihood for the phosphate components to either precipitate or solidify as described under the section on buffer effects (4.2.4.1).

Another type of catalysis may be considered is general acid catalysis due to the presence of protonated side-chain amino group in amoxycillin. Since the pK_a of the carboxyl group of amoxycillin is 2.63 at 23°C, it means that in acetate buffer (pH 4.6) the ionization of carboxyl group is almost complete and the amino group is in protonated form. In phosphate buffer (pH 7.0) however some of the protonated amino (NH_3^+) group (about 22%) is converted to the unionized form (NH_2). Bundgaard (1976, 1977a) has demonstrated that the presence of NH_3^+ amino group in ampicillin has exerted a marked catalysis effect on dimerization of ampicillin at pH range of 7.3-9.1 at 35°C. The rate constant due to this type of catalysis on ampicillin reported by Bundgaard (1976) is 8.1×10^{-1} $(mol\ dm^{-3})^{-2}$ h^{-1} at 35°C. The

author (Bundgaard 1977a) has also suggested that this type of general catalysis could operate in dimerization reactions of amoxycillin as well. However, owing to the limited solubility of amoxycillin at the pH values where the concentration of the protonated amino group would be significant, he did not attempt to investigate it experimentally. Hence in the present study it may be predicted that as the concentration of all the solute species is markedly increased, the presence of highly concentrated protonated amino group could theoretically exert a general acid catalysis on the rate of reactions.

4.2.4.4 Polymerization

The concentration dependent degradation of amoxycillin is well documented (Bundgaard 1977a; Connors and Stella 1986). As indicated previously the dimerization reaction of amoxycillin could occur to a limited extent at pH 7.0. In the liquid regions of the frozen solution, the concentration of amoxycillin increases significantly as the temperature decreases below sub-zero. Under supersaturation and supercooling conditions which is expected to occur frequently in the frozen state (thereby preventing the precipitation of buffer species and subsequently the fall in pH) the free side-chain amino group of one amoxycillin species can take part in dimerization reaction with another amoxycillin moiety. Thus as the apparent pK_a of the amino group is reported to be 7.55 at 23 °C, at pH 7.0 there should be about 22 % of free amino group available. Therefore the presence of some unionized amino group in an atmosphere of concentrated solution of amoxycillin could induce the polymerization reaction. Also as stated before the presence of highly concentrated protonated amino group could possibly impart a general catalysis of the dimerization reaction. These arguments are supported following the findings of precipitation at higher amoxycillin concentrations in the acetate system, which revealed evidence of polymerization reactions (Section 4.5). The likelihood of such precipitation in phosphate buffer at higher amoxycillin concentration would be possible. However owing to the difficulties in measuring kinetic runs under these conditions would not ensure reliable results and were therefore not pursued.

4.3 Temperature Effects

The effect of temperature on the rate of reaction of amoxycillin and clavulanate and their combination was investigated as outlined in Chapter 2 Section 2.3.2. Table 4.6 provides data for the rate constants at various temperatures studied

along with Figures 4.5(a) to 4.5(c) illustrate extrapolation of Arrhenius plots from the liquid state. These data demonstrate a significant acceleration of the reaction rates of both amoxycillin and clavulanate in the pH values studied under the frozen conditions. The linear extrapolations represent liquid state conditions at the frozen temperatures. It is evident from data in Table 4.6 that the highest acceleration in rate has occurred in the case of clavulanate in hydrochloric acid system where the rate of clavulanate has increased 14.95 times in the frozen state compared to the extrapolated liquid state.

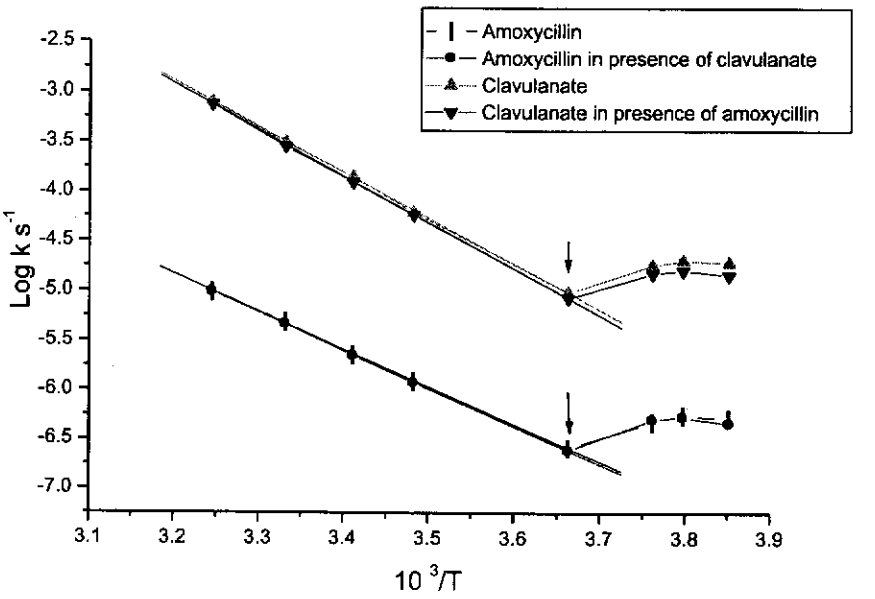
To further evaluate the effect of freezing on the rate of the reaction of amoxycillin and clavulanate and compare these data with the existing literature data, Figure 4.6 is presented in terms of reciprocal of shelf-life values versus temperature. From these illustrations it is evident that in buffer systems the rate-temperature profiles obtained for amoxycillin and clavulanate are similar with the literature data for benzyl penicillin (Larsen 1971a) and amoxycillin (McDonald *et al.* 1989b; Concannon *et al.* 1986). However the rate of change of reaction rates recorded in this study is not as large as those of the other reported studies. This is because the buffer systems used in this study have greatly stabilized the system.

4.4 Effect of Sodium Chloride

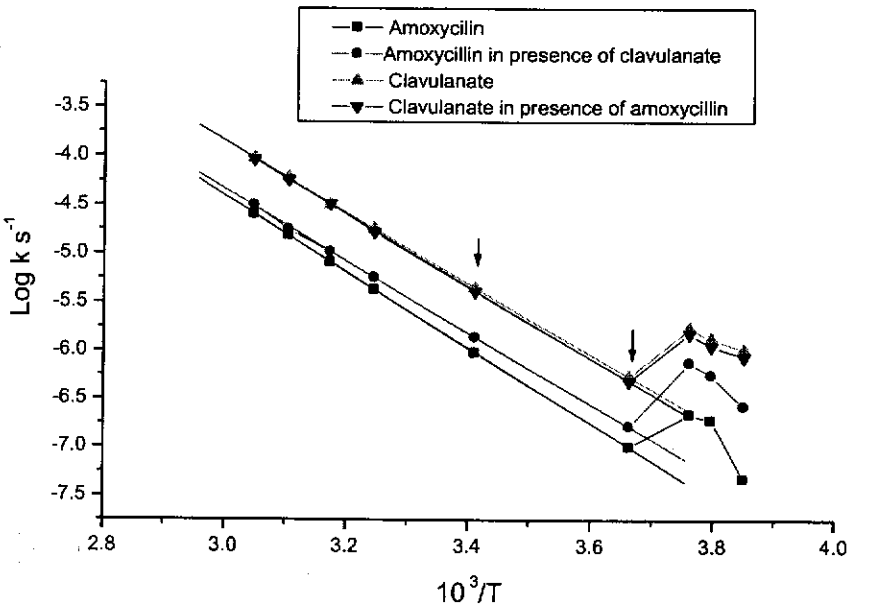
Sodium chloride, which was used to maintain constant ionic strength in the liquid state runs, was found to have an enormous stabilizing effect in the frozen state. Therefore as stated previously its use was restricted to the hydrochloric system only because the presence of buffer salts in the buffer systems had already enhanced the stability of these solutions. Preliminary studies in the buffer systems with sodium chloride (μ = 0.5) indicated no significant degradation ($\leq$ 10%) up to 10 days of reaction for amoxycillin, thus sodium chloride was not incorporated in the buffer runs. Table 4.7 and Figure 4.7 demonstrate these effects on amoxycillin sodium.

While the pH of all the runs did not change significantly, when measured at the beginning and towards the end of each runs, there appeared a rise of 0.27 units in pH of the run in water only.

(a)


Amoxycillin
Amoxycillin in presence of clavulanate
Clavulanate
Clavulanate in presence of amoxycillin
Log k s^{-1}
-2.5
-3.0
-3.5
-4.0
-4.5
-5.0
-5.5
-6.0
-6.5
-7.0
3.1
3.2
3.3
3.4
3.5
3.6
3.7
3.8
3.9
10^{3}/T

(b)


Amoxycillin
Amoxycillin in presence of clavulanate
Clavulanate
Clavulanate in presence of amoxycillin
Log k s^{-1}
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-4.0
-4.5
-5.0
-5.5
-6.0
-6.5
-7.0
-7.5
2.8
3.0
3.2
3.4
3.6
3.8
4.0
10^{3}/T

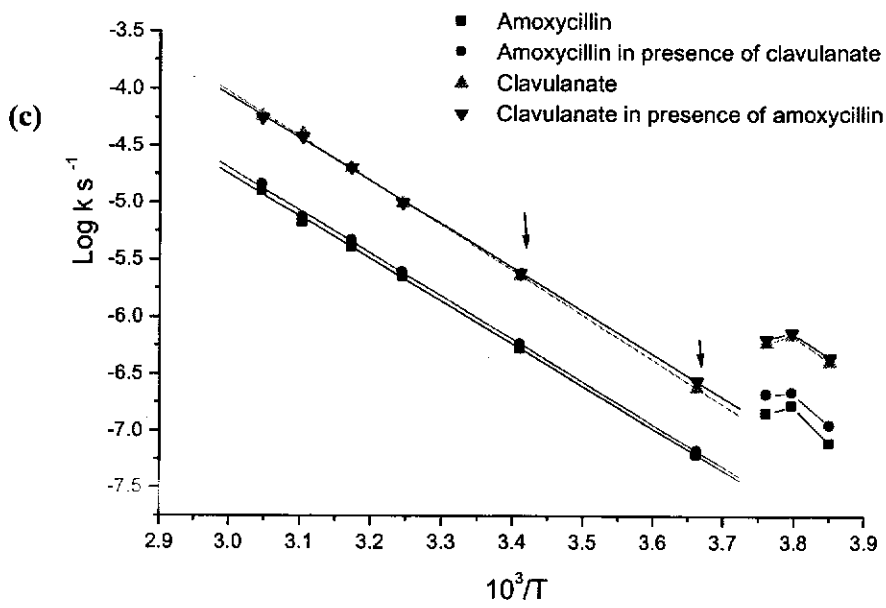


Figure 4.5: Comparison of the effect of temperature on the rate of reaction of amoxycillin and clavulanate in the liquid and the frozen states.

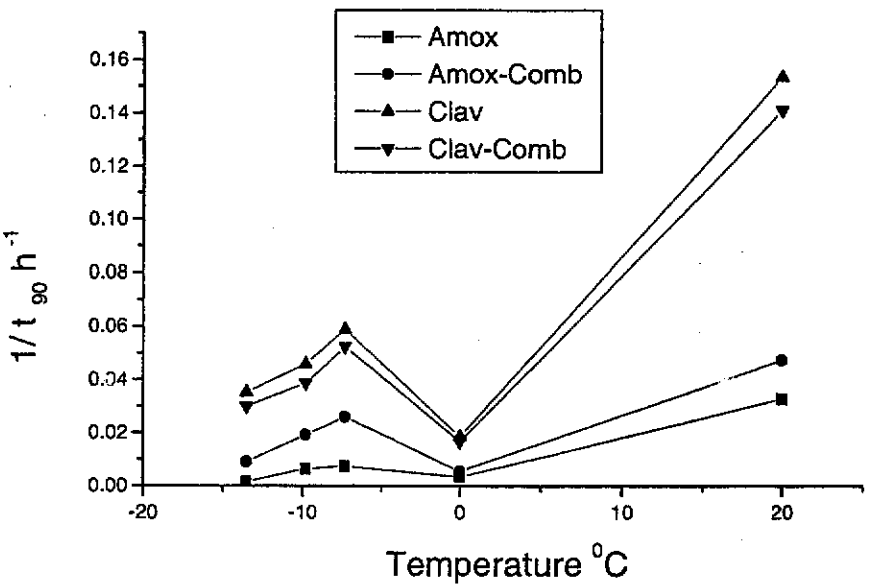
(a) Hydrochloric acid system pH 2.0.

(b) Phosphate buffer pH 7.0.

(c) Acetate buffer pH 4.6.

Arrow indicates extrapolated values.

(a)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Amox-Comb
Clav
Clav-Comb
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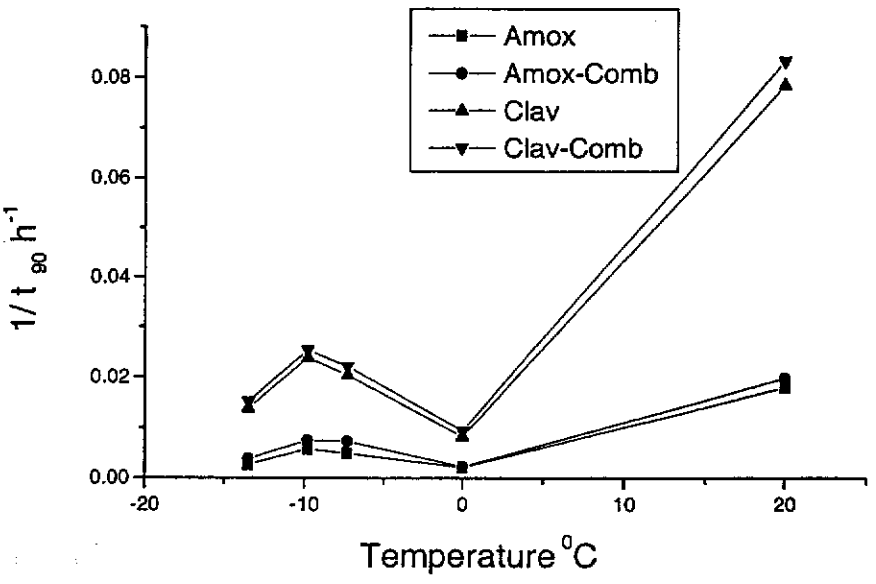
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
1/t 90 h-1
-20
-10
0
10
20
Temperature 0C

(b)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Amox-Comb
Clav
Clav-Comb
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0.02
0.00
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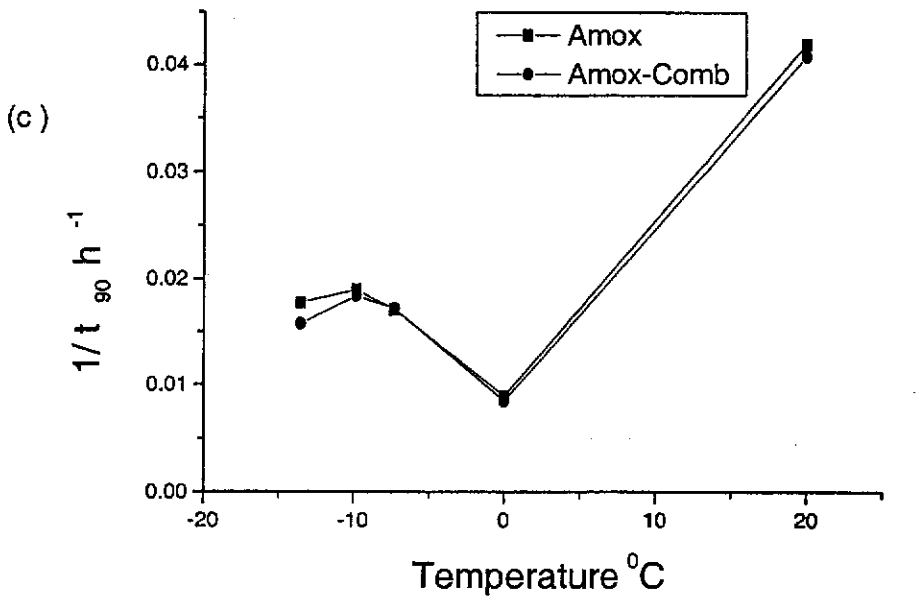
-20
-10
0
10
20
Temperature 0C



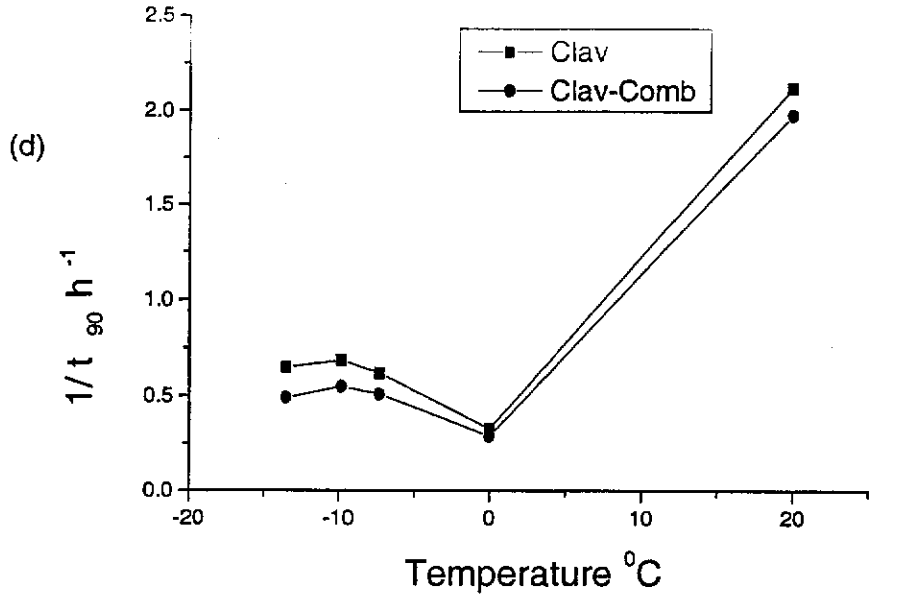


Figure 4.6: The effect of freezing on the reciprocal values ($1/t_{90}$) of shelf-lives of amoxycillin and clavulanate in various systems.

(a) In phosphate buffer pH 7.0.
(b) In acetate buffer pH 4.6.
(c) & (d) In hydrochloric acid system pH 2.0.

Table 4.6: Comparison of the first-order rate constants obtained experimentally with calculated values obtained from the extrapolation of the Arrhenius plot

SYSTEM	COMPOUNDS	t (°C)	k_{Exp} h^{-1}	k_{Pred} h^{-1}	ACLER (fold)
Acetate	Amox	-7.3	$5.15x10^{-4}$	$9.47x10^{-5}$	5.44
		-9.8	$6.05x10^{-4}$	$7.02x10^{-5}$	8.62
		-13.5	$2.81x10^{-4}$	$4.43x10^{-5}$	6.35
	Amox in combination	-7.3	$7.62x10^{-4}$	$1.03x10^{-4}$	7.39
		-9.8	$7.86x10^{-4}$	$7.63x10^{-5}$	10.28
		-13.5	$4.03x10^{-4}$	$4.43x10^{-5}$	9.11
	Clav	-7.3	$2.14x10^{-3}$	$3.53x10^{-4}$	6.06
		-9.8	$2.50x10^{-3}$	$2.58x10^{-4}$	9.69
		-13.5	$1.46x10^{-3}$	$1.58x10^{-4}$	9.25
	Clav in combination	-7.3	$2.32x10^{-3}$	$4.21x10^{-4}$	5.50
		-9.8	$2.66x10^{-3}$	$3.05x10^{-4}$	8.72
		-13.5	$1.6x10^{-3}$	$1.88x10^{-4}$	8.51
Phosphate	Amox	-7.3	$7.87x10^{-4}$	$1.50x10^{-4}$	5.26
		-9.8	$6.86x10^{-4}$	$1.09x10^{-4}$	6.28
		-13.5	$1.69x10^{-4}$	$6.59x10^{-5}$	2.56
	Amox in combination	-7.3	$2.73x10^{-3}$	$2.53x10^{-4}$	10.78
		-9.8	$2.02x10^{-3}$	$1.88x10^{-4}$	10.77
		-13.5	$9.53x10^{-4}$	$1.18x10^{-4}$	8.05
	Clav	-7.3	$6.18x10^{-3}$	$8.46x10^{-4}$	7.32
		-9.8	$4.83x10^{-3}$	$6.26x10^{-4}$	7.70
		-13.5	$3.70x10^{-3}$	$4.03x10^{-4}$	9.20
	Clav in combination	-7.3	$5.49x10^{-3}$	$7.52x10^{-4}$	7.32
		-9.8	$4.07x10^{-3}$	$5.58x10^{-4}$	7.29
		-13.5	$3.14x10^{-3}$	$3.52x10^{-4}$	8.92
HCl	Amox	-7.3	$1.79x10^{-3}$	$3.89x10^{-4}$	4.60
		-9.8	$1.99x10^{-3}$	$2.84x10^{-4}$	7.00
		-13.5	$1.86x10^{-3}$	$1.77x10^{-4}$	10.51
	Amox in combination	-7.3	$1.80x10^{-3}$	$3.64x10^{-4}$	4.95
		-9.8	$1.93x10^{-3}$	$2.65x10^{-4}$	7.27
		-13.5	$1.65x10^{-3}$	$1.64x10^{-4}$	10.04
	Clav	-7.3	$6.48x10^{-2}$	$1.18x10^{-2}$	5.49
		-9.8	$7.20x10^{-2}$	$8.06x10^{-3}$	8.93
		-13.5	$6.80x10^{-2}$	$4.55x10^{-3}$	14.95
	Clav in combination	-7.3	$5.33x10^{-2}$	$1.03x10^{-2}$	5.18
		-9.8	$5.76x10^{-2}$	$7.02x10^{-3}$	8.21
		-13.5	$5.15x10^{-2}$	$3.92x10^{-3}$	13.12

Acler = Acceleration in the rate of the reaction in number of times.
Acetate = Acetate buffer pH 4.6
Phosphate = Phosphate buffer pH 7.0
HCl = Hydrochloric acid system pH 2.0 and μ = 0.5 (NaCl)

Table 4.7: Effect of various solutions on shelf-life of amoxycillin at –7.3 °C

SOLVENT	t_{90} (h)
Water	3.0
HCl system, without NaCl (pH 2.0)	2.2
HCl system with NaCl (pH 2.0)	58.3
Phosphate buffer, without NaCl (pH 7.0)	133.4
Acetate buffer, without NaCl (pH 4.6)	203.9

There is no report of this type of stabilizing effect of sodium chloride on amoxycillin or clavulanate in the frozen state in the literature. Since the total concentration of a frozen solution at a particular temperature is constant, addition of sodium chloride to the system will increase the number of solute species present in that system significantly (because under all the conditions of this study, the relative ratio of sodium chloride to other solutes has been significantly large), hence diluting the concentrations of the reactants and other solutes present. The low eutectic temperature of sodium chloride, makes it a suitable protective agent by diluting the concentration effects of the solute in the frozen state.

4.5 Effect of Initial Concentration

A preliminary study was carried out in acetate buffer at –7.3°C where the initial concentrations of amoxycillin and clavulanate were increased to 1.29×10^{-2} mol dm^{-3} and 1.05×10^{-2} mol dm^{-3} respectively. This change revealed a significant acceleration in the rate of amoxycillin degradation. Although there was some acceleration in the rate of clavulanate, but the extent of increase was not significant when compared to amoxycillin. The rate of amoxycillin degradation in combination with clavulanate was increased by about 3-fold compared to its lower initial concentration as found in Table 4.1. However this type of investigation was not further pursued due to technical problems. One being the frozen solution exhibited a white precipitate that could not be re-dissolved into the solution upon thawing. The precipitate obtained in these runs was filtered and dried at room temperature and then dissolved in DMSO and analyzed by Proton Nuclear Magnetic Resonance Spectroscopy (NMR), see Figure 4.8(a) to 4.8(c).

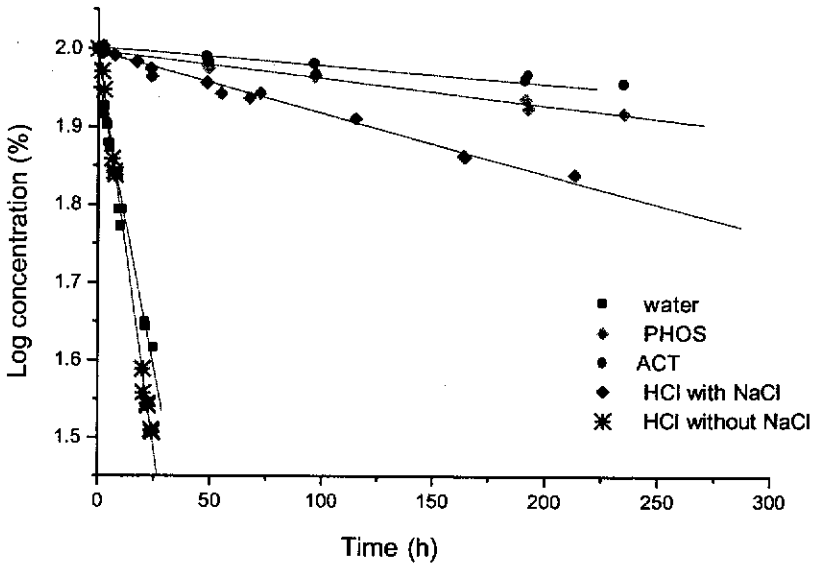


Figure 4.7: Degradation of amoxycillin sodium in various solutions at –7.3°C.

ACT = 2.2×10^{-1} mol dm^{-3}acetate buffer pH 4.60; PHOS = 1.0×10^{-1} mol dm^{-3} phosphate buffer pH 7.0; HCl with NaCl = 1.24×10^{-2} mol dm^{-3} hydrochloric acid with sodium chloride ($\mu = 0.5$), pH2.0.

Amoxycillin concentration = 9.03×10^{-4} mol dm^{-3} in hydrochloric acid and 1.29×10^{-3} mol dm^{-3} in the rest.

The multiplets in the 5.5-5.3 ppm region of NMR (Figure 4.8b) instead of doublets, which are present in amoxycillin spectrum, perhaps suggests the evidence of polymerization. One possible explanation to this effect is that in acetate buffer as explained before the presence of highly concentrated protonated amino group could catalyze the dimerization of amoxycillin. But an essential component in the polymerization reaction is the presence of free amino group, which occurs, in higher pH. Although at pH 4.6 there is only a small proportion (< 0.1%) for formation of deprotonated amino group. However, considering at higher initial concentration, the initial pH of the run was increased to 4.76 and also correcting for the pK_a-temperature effect of acetate discussed under Section 4.2.4.2, the pH of the system is expected to rise to 5.1. Hence this would increase the concentration of deprotonated amino group to about 0.4% which could be significant particularly at higher concentrations.

Thus the results and descriptive analysis presented here raises the complexities of stability studies of amoxycillin and clavulanate in the frozen state. In studying a drug admixture that is relevant for such storage conditions provides a number of factors that can significantly influence the rate and in this case is too complex to be identified and examined in this study.

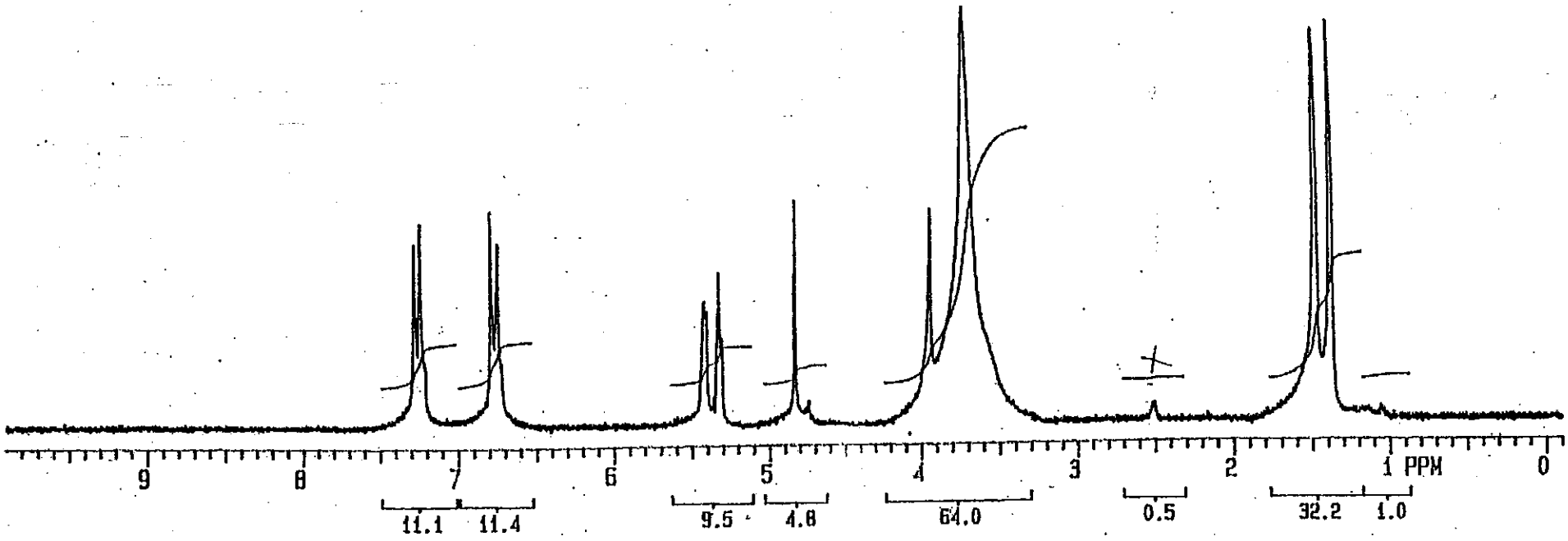


Figure 4.8: (a) 200 MHz Proton NMR spectrum of the precipitate in DMSO

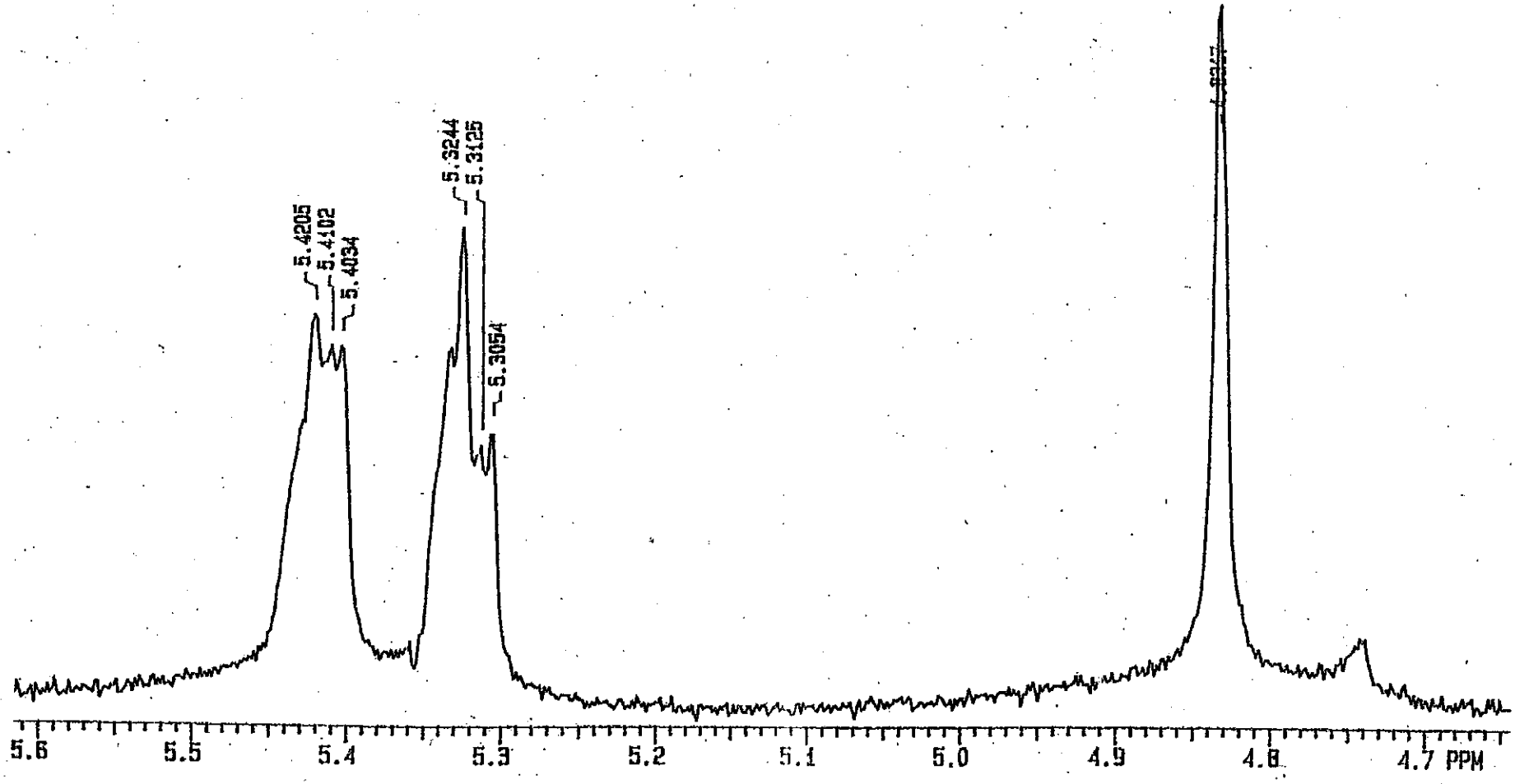


Figure 4.8: (b) 200 MHz Proton NMR spectrum of the precipitate in DMSO (Expanded)

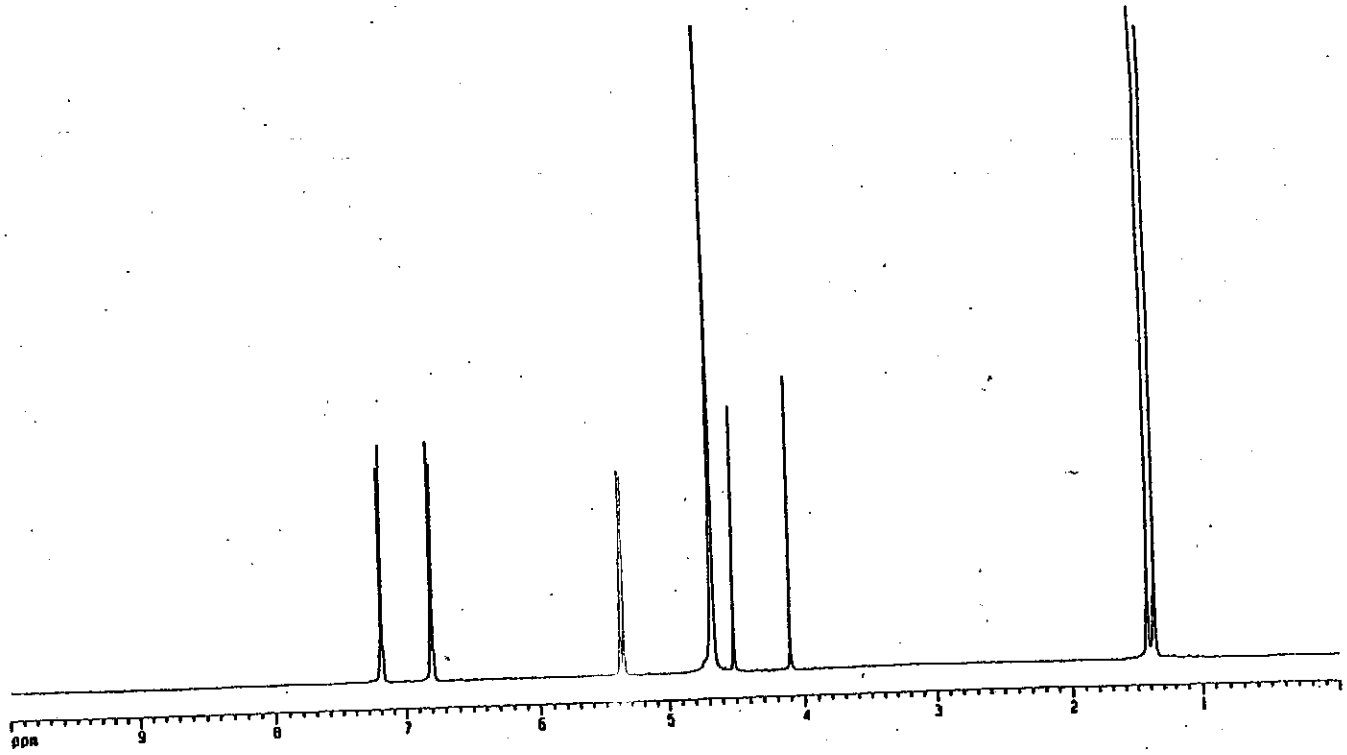


Figure 4.8: (c) 400 MHz Proton NMR spectrum of amoxycillin in D_2O (Bird 1992)

CHAPTER 5

THE EFFECT OF HYDROXYPROPYL β- CYCLODEXTRIN ON THE DEGRADATION RATES OF AMOXYCILLIN AND CLAVULANATE

The degradation rates of amoxycillin and clavulanate individually and in combination, in the presence of hydroxypropyl β-cyclodextrin (HPβCD) were investigated according to the methods stated in Section 2.3.3 of Chapter 2.

The quantitative determination of the antibiotic concentrations was performed by the HPLC assay method described in Chapter 3 and the rate constant data were obtained by similar methods as described in Chapter 3. A control sample each of amoxycillin and clavulanate without HPβCD was run under the same experimental condition with each set of runs.

Acetate buffer was selected from the three systems used in this study as the medium for the experimental runs, since both amoxycillin and clavulanate showed a greater stability in this system. At this pH (i.e. 4.6) amoxycillin would be largely in its zwitterionic form which may be the most suitable form for complexation.

5.1 Kinetics of the Reactions in the Liquid State

5.1.1 At the standard reactants concentration

These set of experiments were performed at the amoxycillin and clavulanate concentrations previously adopted with the molar concentration of (amox or clav):HPβCD, from 1:2 to 1:10 mol dm^{-3} (refer Chapter 2 Section 2.3.3.A) in acetate buffer (pH 4.6) at 55°C. In this set of experiments lithium clavulanate was used instead of potassium clavulanate.

The hydrolysis of amoxycillin and clavulanate whether individually or in combination followed first-order kinetics over approximately 3 half-lives of reaction, under these experimental conditions. The results presented in Table 5.1 indicate that introduction of HPβCD did not change the observed order of the reactions. Thus the plots of log concentration remaining versus time were linear with correlation coefficients r=0.996-0.999. The data indicated no significant change in the rates of amoxycillin in runs containing amoxycillin alone or combination runs upon introduction of HPβCD. However, a small decrease (about 10%) in rate was

observed in the case of clavulanate in combination with amoxycillin when a molar ratio of 1:10 of clavulanate: HPβCD was investigated.

Typical HPLC chromatograms are presented in Figure 5.1 and the typical first-order plots are illustrated in Figure 5.2.

The rate stabilizing effect of HPβCD on clavulanate apparent in Figure 5.3, showed a linear relationship with increasing concentration of HPβCD. This effect was tested by the Lineweaver-Burk plot (refer Chapter 1 Section 1.1.5) and the rate constant value within the cyclodextrin complex (k_c) and the stability constant of the complex (K_c) were determined. The plot is presented in Figure 5.4. The values of k_c $=1.54\times 10^{-1}$ h^{-1} and K_c = 74.2 (mol dm^{-3})$^{-1}$ were obtained using the Equation 1.39 (Chapter 1).

Table 5.1: Observed first-order rate constant values obtained in presence of various concentrations of HPβCD in acetate buffer of pH 4.6 and at constant (μ = 0.5) ionic strength at 55°C.

DRUG	k_{obs} (h^{-1})			
	ANTIBIOTIC : HPβCD RATIO (mol dm^{-3})			
	1: 0	1: 2	1: 5	1:10
Amox	4.4×10^{-2}	4.3×10^{-2}	4.3×10^{-2}	4.2×10^{-2}
Amox-Comb	-	4.8×10^{-2}	4.7×10^{-2}	4.6×10^{-2}
Clav	1.90×10^{-1}	1.87×10^{-1}	1.83×10^{-1}	1.80×10^{-1}
Clav-Comb	-	1.85×10^{-1}	1.81×10^{-1}	1.71×10^{-1}

Initial concentration of amoxycillin and clavulanate were 1.29×10^{-3} and 1.05×10^{-3} mol dm^{-3} respectively

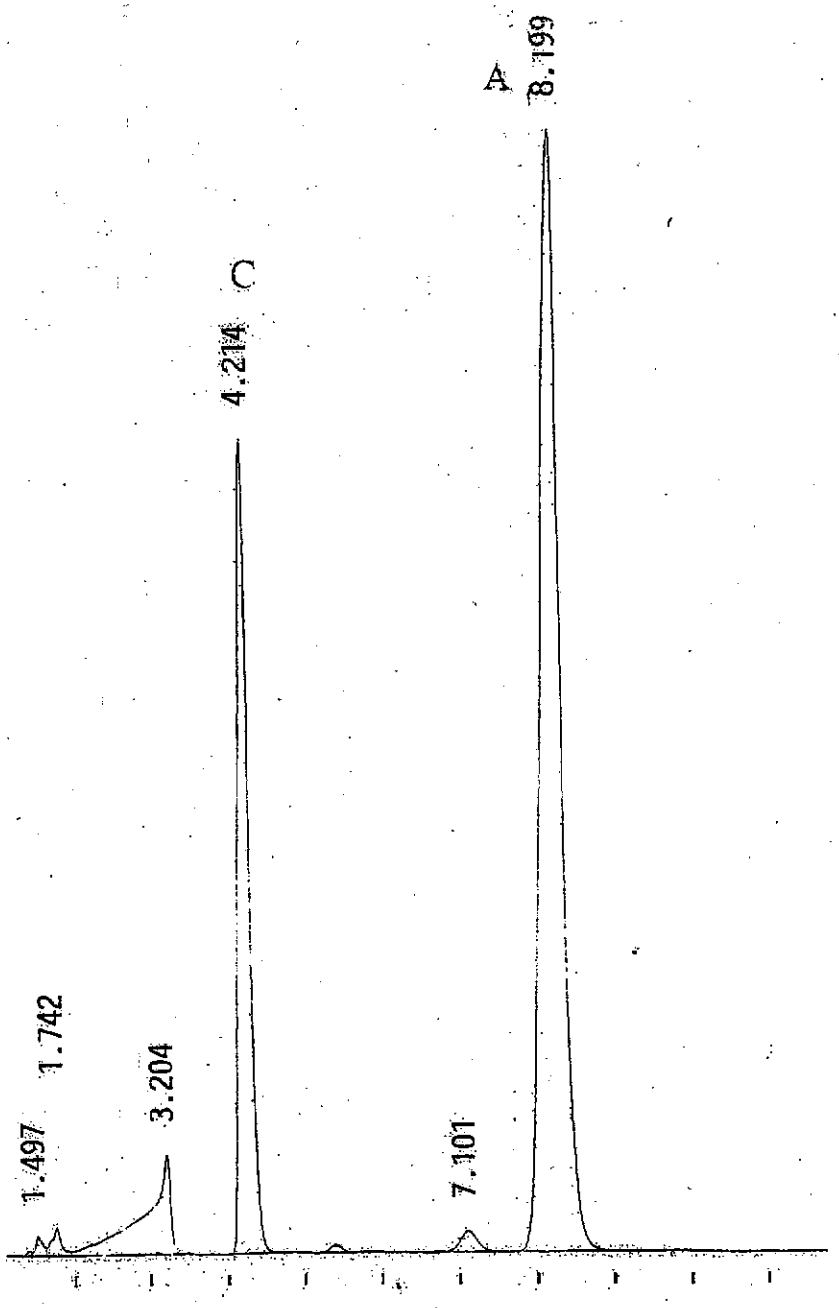


Figure 5.1: (a) Typical HPLC chromatogram of a zero time sample of amoxycillin and clavulanate in combination in the presence of HPβCD.
At (amox or clav): HPβCD of 1: 10 mol dm^{-3} in acetate buffer pH 4.6, μ = 0.5, at 55°C, where A and C represent the response due to amoxycillin and clavulanate respectively.

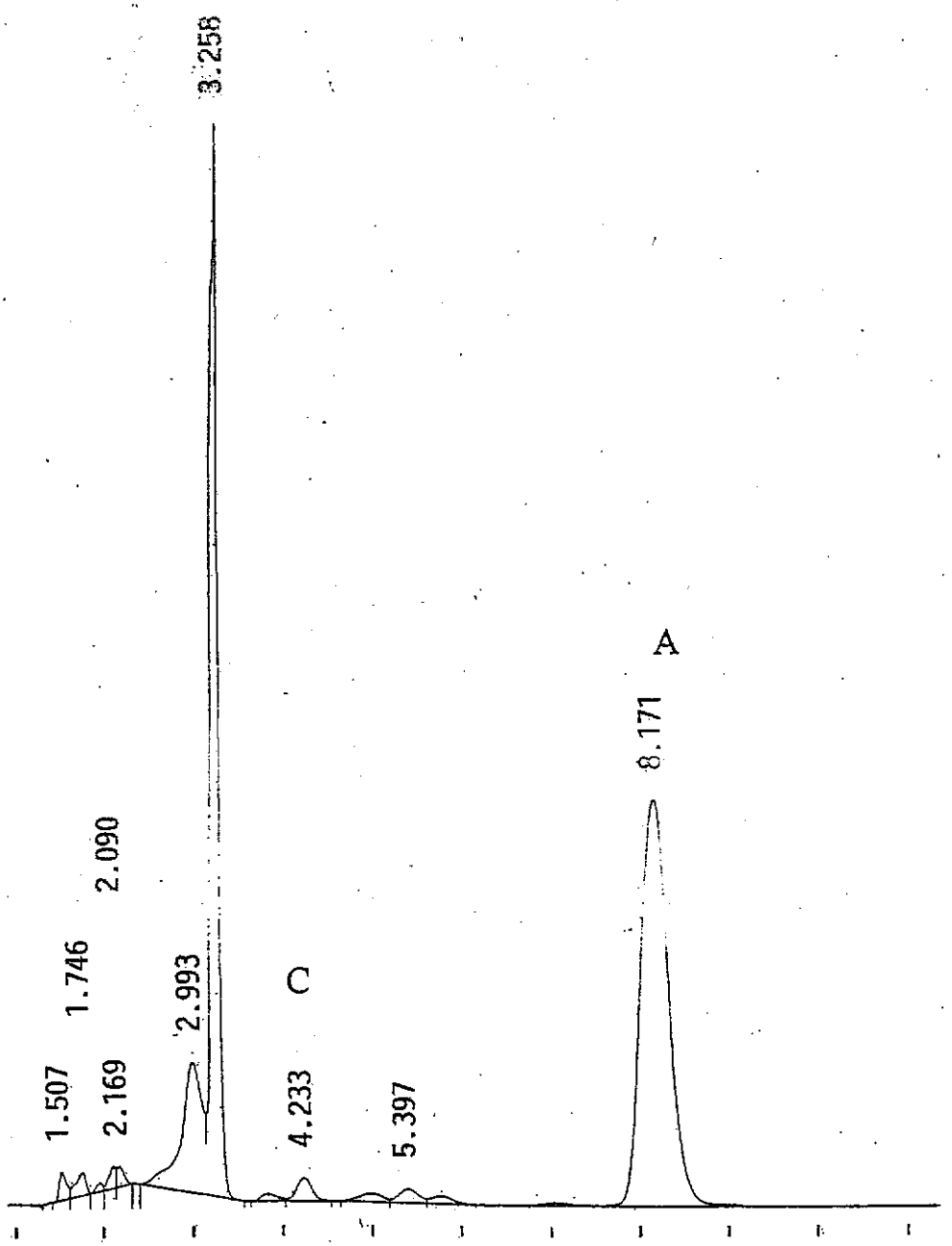


Figure 5.1: (b) Typical HPLC chromatogram of a degraded sample of amoxycillin and clavulanate in combination in the presence of HPβCD.
At (amox or clav): HPβCD of 1: 10 mol dm^{-3} in acetate buffer pH 4.6, μ = 0.5, at 55°C, where A and C represent the response due to amoxycillin and clavulanate respectively.

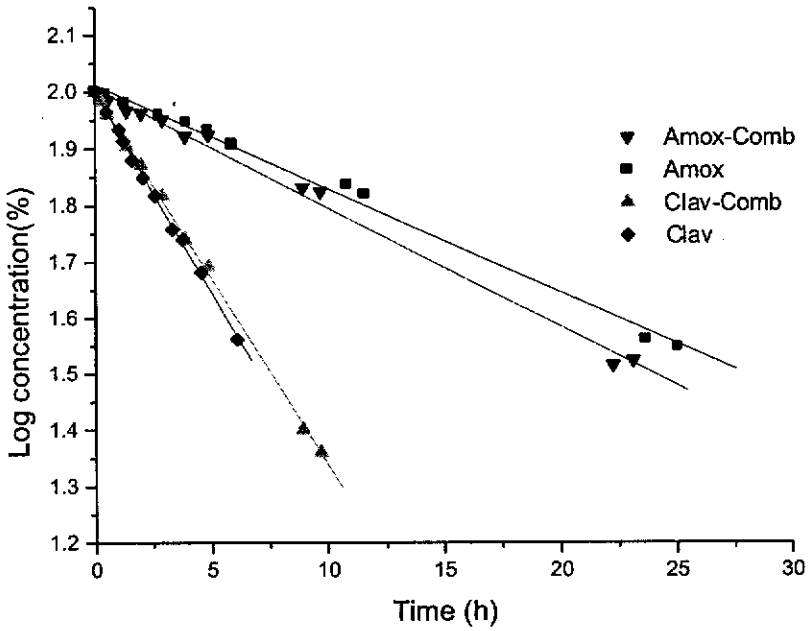


Figure 5.2: Representative first-order plots of amoxycillin and clavulanate in the presence of HPßCD at 1:10 mol dm^{-3} of amox or clav: HPßCD in acetate buffer pH 4.6, μ = 0.5 at 55°C.

Initial concentration of amoxycillin and clavulanate were 1.29×10^{-3} and 1.05×10^{-3} mol dm^{-3} respectively.

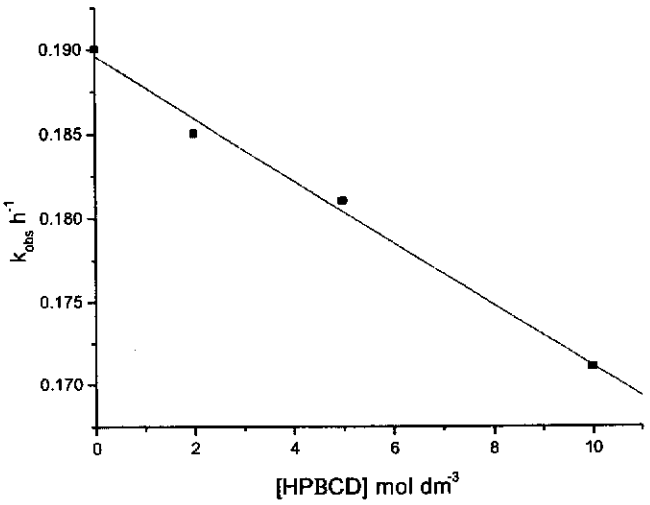


Figure 5.3: Effect of HPβCD concentration on the rate of hydrolysis of clavulanate in combination with amoxycillin in acetate buffer pH 4.6, μ = 0.5 at 55°C.

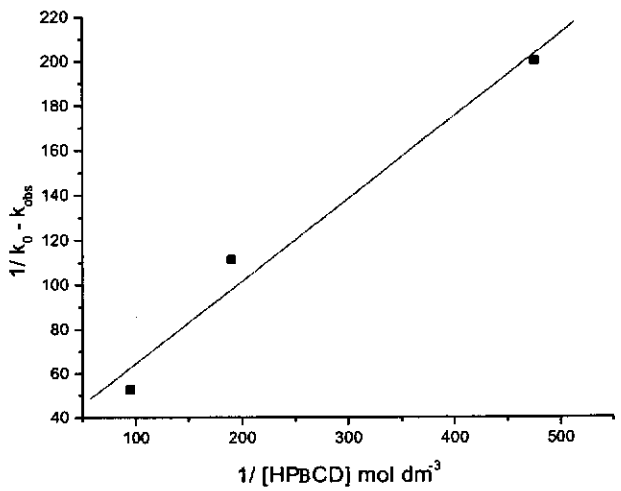


Figure 5.4: Lineweaver-Burk plot for effect of HPβCD on the rates of clavulanate in combination with amoxycillin (data taken from Table 5.1) at pH 4.6 , μ = 0.5 and 55°C.

Although the data presented in Table 5.1 demonstrated no major effect on the rate of reaction of both antibiotics upon addition of HPβCD, it seems it has marginally decreased the rate of the reaction of these antibiotics in the combination runs. A small reduction in rate of clavulanate at clavulanate: HPβCD concentration of 1:10 mol dm^{-3} occurred, which would increase the shelf life of the mixture by 10% because clavulanate is the stability limiting component of the mixture. The other is the fact that the rate plots of the mixture runs (Figure 5.2) in the presence of HPβCD show no detectable curvature unlike those observed in the buffer systems, discussed previously (Section 3.2.1 Chapter 3). This had caused acceleration in the rate of amoxycillin at the initial stage. Hence the lack of the initial rapid degradation would stabilize the initial rate (k_1) of amoxycillin significantly. For instance the initial rate (k_1) of amoxycillin under the same conditions, in the absence of HPβCD (reported earlier, Table 3.1) was about 2.5 times faster than the rate of amoxycillin at the later (k_2) stage. This stabilizing effect could arise due to the protecting effect of HPβCD by forming inclusion complexes with the reacting compounds thereby shielding amoxycillin from the catalytic effect of clavulanate.

The overall pH of the runs did not change significantly during the course of each run and across the HPβCD concentration change.

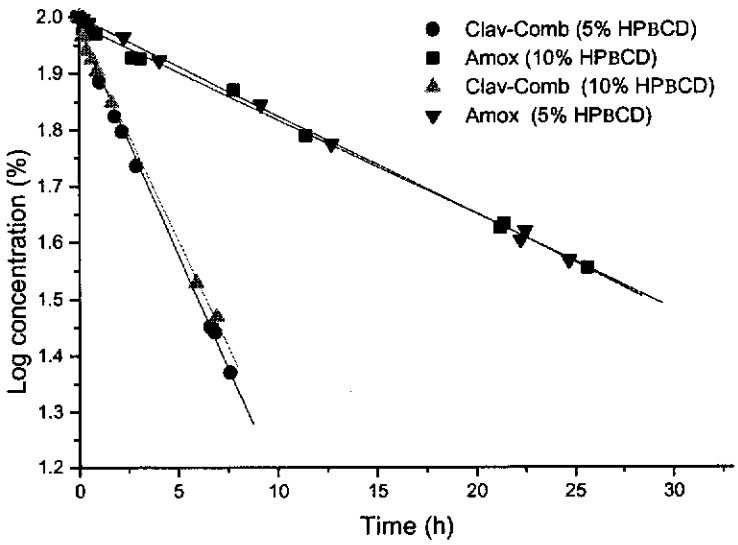
5.1.2 At higher antibiotics concentration

These set of runs were performed at an earlier stage with a different HPβCD lot number (P-104-29-1) and at higher antibiotics concentration with the objective of extending the experiment to the frozen state where HPβCD could influence the solubility of the antibiotics which could be an issue at higher concentration. The concentration of the reacting antibiotics was increased 10-fold and HPβCD was added at 2.5%-10% w/v keeping the concentration of the antibiotics constant (Section 2.3.3 B Chapter 2).

The data (Table 5.2) obtained from the reaction rates exhibit similar results as stated under 5.1.1. Thus the rates of the reactions with respect to amoxycillin and clavulanate showed first-order in kinetics (Figure 5.5a). It appeared that HPβCD had no significant effect on the rate of reaction of the antibiotics even at higher concentration. However the slight decrease in rate of clavulanate degradation especially at higher concentrations of HPβCD observed earlier was also evident.

An interesting observation made in this set of data was in the case of the combination runs. Here because the initial concentration of the mixture was increased 10-fold, the type of curvature in the rate plots (Figure 5.5b) due to clavulanate catalytic effect discussed earlier (Chapter 3) was more prominent in these runs which is evident from the large k_1 values (Table 5.2) including the control sample with no HPβCD. Thus the data from these combination runs were treated by use of a first-order biexponential model described previously (Section 3.2.1, Chapter 3).

Therefore unlike the experiments at low concentrations (5.1.1), addition of HPβCD seemed to have no stabilizing effect on the rate of the amoxycillin in the mixture runs except for the slight stabilizing effect on the rate of the clavulanate discussed earlier. To analyze the reason behind this difference it is better to consider the complexing properties of HPβCD in terms of molar ratios. Hence from the data provided in Table 5.1 it appears that as the molar ratio of HPβCD to antibiotic (amox or clav) is increased from 2 mol dm^{-3} to 10 mol dm^{-3}, keeping the concentration of the reacting antibiotic constant at 1 mol dm^{-3}, the stabilizing effect of HPβCD appeared to have improved to a small extent.

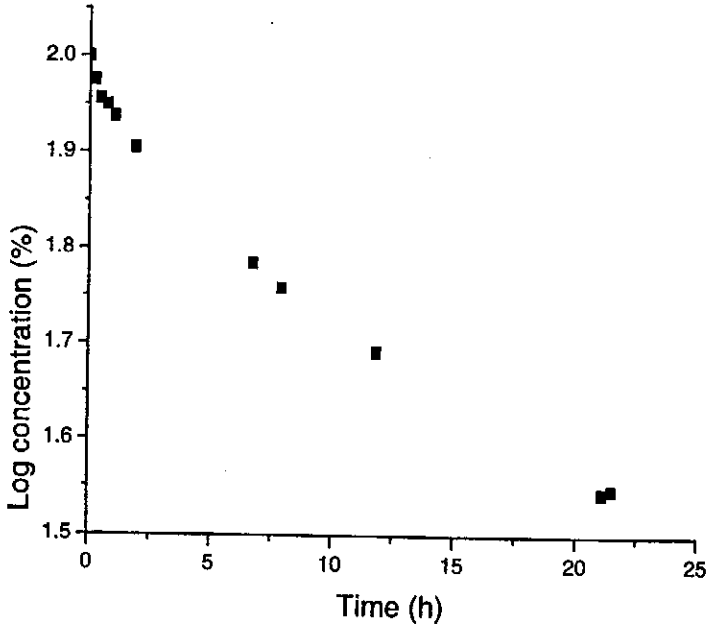


(a)

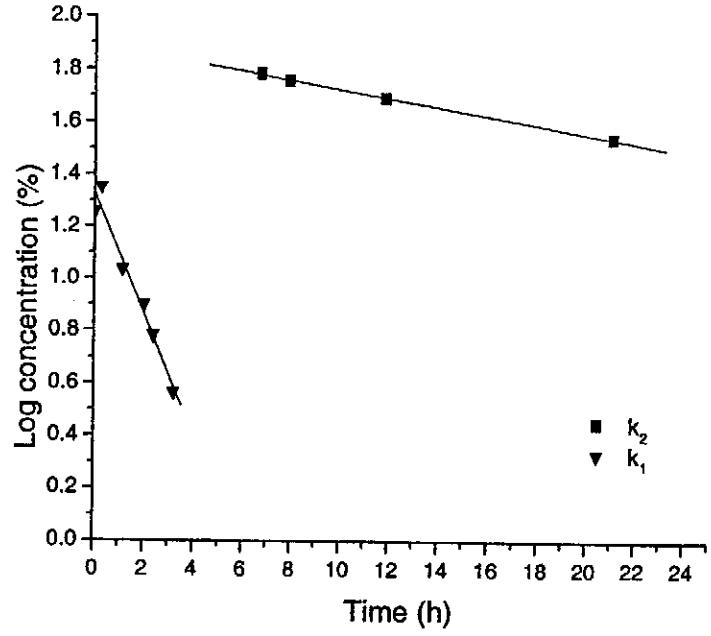
Figure 5.5: (a) First-order plots of amoxycillin and clavulanate in the presence of 10% HPßCD in acetate buffer pH 4.75, μ = 0.5 at 55°C.

Clav-Comb(5% HPβCD) = Clavulanate in combination with amoxycillin in presence of 5% w/v HPβCD; Amox (5% HPβCD) = Amoxycillin in presence of 5%w/v HPβCD.

Initial concentrations of amoxycillin and clavulanate were 1.29×10^{-2} and 1.05×10^{-2} mol dm^{-3} respectively.



(b)



(c)

Figure 5.5: (b) First-order plots of amoxycillin in combination with clavulanate in the presence of 10% HPβCD in acetate buffer pH 4.75, $\mu = 0.5$ at 55°C.

(c) Solved by biexponential method for k_1 and k_2.

Table 5.2: Observed first-order rate constant values at higher antibiotics concentration, with various concentrations of HPβCD (in % w/v) in acetate buffer, pH 4.75 at constant ionic (μ = 0.5) strength and at 55°C

HPβCD (g% W/V)	AMOX h^{-1}	AMOX-COMB k_1 h^{-1}	AMOX-COMB k_2 h^{-1}	CLAV h^{-1}	CLAV-COMB h^{-1}
0	3.9×10^{-2}	4.69×10^{-1}	4.2×10^{-2}	1.80×10^{-1}	1.78×10^{-1}
2.5	3.9×10^{-2}	5.00×10^{-1}	3.9×10^{-2}	1.75×10^{-1}	1.72×10^{-1}
5	3.8×10^{-2}	4.97×10^{-1}	4.1×10^{-2}	1.69×10^{-1}	1.66×10^{-1}
10	3.7×10^{-2}	4.88×10^{-1}	3.7×10^{-2}	1.65×10^{-1}	1.60×10^{-1}

Initial concentration of amoxycillin and clavulanate were 1.29×10^{-2} and 1.05×10^{-2} mol dm^{-3} respectively

Converting the concentration of HPβCD to the molar scale for these set of runs, will result in 1:1.5, 1:3.1 and 1: 6.2 mol dm^{-3} corresponding to (amox or clav): HPβCD concentration of 1:2.5%, 1:5% and 1:10% respectively. This is comparatively a lower molar concentration of HPβCD than the previous data (Section 5.1.1). The concentration of HPβCD became substantially lower particularly in case of the mixture runs, where the (amox or clav): HPβCD were 1: 0.75, 1:1.55 and 1:3.1 corresponding to 2.5%, 5% and 10% HPβCD respectively. This is because unlike the previous set of experiment (Section 5.1.1), in the mixture runs the quantity of HPβCD added was not proportional to the total antibiotic: HPβCD. Therefore the overall concentration of HPβCD in the mixture runs would be significantly lower compared to Section 5.1.1. Thus, in the case of 1:0.75 (amox or clav): HPβCD there was insufficient HPβCD concentration to achieve a 1:1 ratio for either of reactants.

The initial pH of the control sample as well as samples with HPβCD was increased by 0.15 of pH units owing to higher concentration of reacting antibiotics used in these runs. However, the pH of all the sample solutions with and without HPβCD did not change within 4.75 ± 0.05 throughout the experiments. No measurable pH change was observed across the solutions with various HPβCD concentrations. Hence the small stabilizing effect seen in clavulanate was concluded

to be due to HPβCD, not a pH effect. The lack of change in amoxycillin degradation rate also supports this conclusion.

There are no reports on the stabilizing or destabilizing effect of HPβCD on amoxycillin or clavulanate. The stabilizing effect of betacyclodextrin on ampicillin has been reported (Hysu et al. 1984) in pH 1-4 at 25°C to 37°C where ampicillin is mainly present in protonated form. These authors however did not provide the concentration of the beta cyclodextrin added. In another report (Aki et al. 1990) betacyclodextrin was reported to have an inhibitory effect on the polymerization of ampicillin at pH 8.4 at 60°C. These data suggest that HPβCD could have stabilizing effects on amoxycillin under similar conditions.

5.2 Kinetics of the Reactions in the Frozen State

Since the data in the liquid state showed no major change in the rates of reactions of amoxycillin and clavulanate upon addition of HPβCD, a preliminary run at the higher concentration of the antibiotics at –7.3°C was performed with 2.5% w/v HPβCD along with a control sample in absence of HPβCD. The method is described under Section 2.3.3 Chapter 2 and the result is listed in Table 5.3.

The data were treated in similar way as stated above for the liquid state. The first-order rate constants were obtained from the plot of log concentration versus time, where the linearity was obtained by calculating the correlation coefficients (r) to be at the range of 0.964-0.990.

Table 5.3: Observed first-order rate constants of amoxycillin and clavulanate in combination in acetate buffer at –7.3°C

HPβCD (g% W/V)	AMOX-COMB h^{-1}	CLAV-COMB h^{-1}
0	2.7×10^{-3}	3.3×10^{-3}
2.5	2.3×10^{-3}	2.1×10^{-3}

Initial concentration of amoxycillin and clavulanate were 1.29×10^{-2} and 1.05×10^{-2} mol dm^{-3} respectively

The data in Table 5.3 indicate a marginal rate slowing influence (about 16%) due to HPβCD on amoxycillin but a significant (about 44%) rate stabilizing effect

on clavulanate. However as stated in Chapter 2 the sample solutions (both with and without HPβCD) from these experiments exhibited an off-white precipitate which did not dissolve in the solution on shaking. Therefore the samples were filtered prior to assay and the precipitate obtained upon drying at room temperature was evaluated by NMR. As was discussed in Section 4.5 Chapter 4, the precipitate was suspected to be a polymer derivative of amoxycillin.

This precipitation and the complex nature of the frozen state made the interpretation of data difficult. Also due to the slow reacting nature of the frozen state experiments these runs were not further pursued.

Since addition of HPβCD did not affect the rate of the reaction of amoxycillin and induced only a slight decrease in rate of clavulanate, the experimental design was not expanded further.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The kinetics of hydrolysis of amoxycillin sodium an aminopenicillin and potassium clavulanate a β-lactamase inhibitor, was investigated as functions of pH and temperature in the liquid and frozen states. The study revealed several novel findings. However, results show that there is still some scope for further investigations in this field.

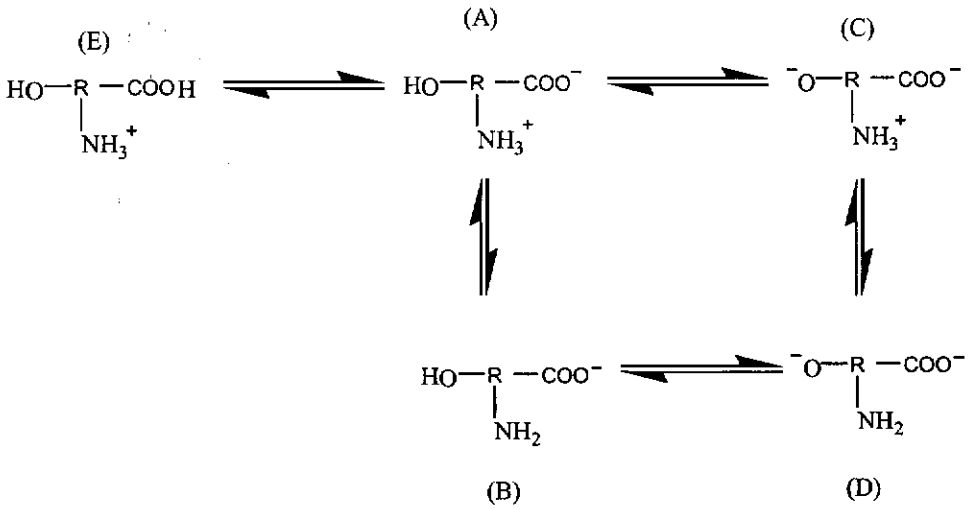
This chapter deals with the general assessment of the experimental design in generating kinetic data with its application to the stability of amoxycillin in combination with clavulanate in liquid and frozen dosage forms. It further includes a general discussion on the results and the overall conclusions from the results. Finally some suggestions for further investigations have been discussed.

6.1 General Discussion

6.1.1 Assessment of the experimental design

Under the conditions of this study amoxycillin existed in three different ionized forms (species E, A and B in Scheme 6.1). In each experimental run when solutions of amoxycillin were prepared in water, the pH of the solution was approximately 8.0 at room temperature. Hence, in water amoxycillin could have existed in four ionic species B, A, C and D (Scheme 6.1 and Figure 1.1, Chapter 1) with their relative concentrations decreasing from B to D. When this solution was rendered acid in hydrochloric acid (pH 2.0), protonation (proton gain) of the zwitterion species A caused the formation of cation, species E (Scheme 6.1). Thus in hydrochloric acid system the predominant (about 80%) species would be the cation form E with some (about 19%) zwitterion form A. Similarly in acetate buffer (pH 4.6) the predominant ionic form (about 99%) of amoxycillin was the zwitterion species A, with small quantities of species B (about 0.1%) and the remainder form E. In phosphate buffer (pH 7.0) proton dissociation of the zwitterion species would lead into the formation of some (about 22%) anion form B. The percentage quantities stated are estimated from the pK_a of the carboxyl group (2.63) and the a-amino group (7.55) at 23°C, refer to Table 1.1 in Chapter 1.

Scheme 6.1: Various ionized species of amoxycillin in pH values of 2.0-8.0



Hence the pH values used in this study provided a selection of relevant ionized forms of amoxycillin allowing determination of their contribution to the kinetic studies carried out under the conditions of this study. For instance the cationic form of amoxycillin present in the hydrochloric acid system would be relevant clinically in terms of the drug stability in gastro-intestinal fluid. The zwitterionic form of amoxycillin present in the acetate system is an important species in terms of formulation stability and clinically. Because the pH of maximum stability of amoxycillin happens to be at the region around the isoelectic point. Also the zwitterionic form is a clinically relevant species because it has been suggested (Hou and Poole 1969b) that the broad antimicrobial activity of the amphoteric penicillins including amoxycillin, ampicillin and cyclacillin is increased when these compounds are largely present in the zwitterion form. The phosphate buffer system provided a neutral medium, which is relevant to some parenteral formulations. It was also considered a priori that the zwitterion may be a more favourable species for complexation with β-cyclodextrins.

As the pK_a of clavulanate is 2.4 (Haginaka, Nakagawa and Uno, 1981), in the hydrochloric acid system about 28.5% of clavulanate was ionized whereas in acetate and phosphate buffers clavulanate was completely ionized.

The HPLC assay method developed enabled simultaneous quantification of amoxycillin and clavulanate. The validity of the method was ascertained from standard solutions of each substance and estimating the correlation coefficient and coefficient of variation (Section 3.1). The method was proved (Section 3.1) to be stability indicating in nature (Section 3.1), providing well-resolved responses with respect to the concentration of the antibiotics (amox or clav) with no detectable interference from degraded products, induced under stressed conditions.

The wide range of temperatures used was essential for determining the effect of temperature on the rate of the reaction and was beneficial in two ways. Primarily new data have been recorded in this study that have considerably extended the understanding of factors influencing the degradation rates, and in providing results which enabled some comparisons with existing data. Temperature dependence data is fundamental to frozen state studies.

Experiments were designed in such a way to minimize the occurrence of the dimerization reaction of amoxycillin. Firstly because this region of the pH scale has been extensively studied by Bundgaard (1977a). Secondly, as it has been documented (Munro *et al.*, 1976; Dewdney, Smith and Wheeler 1970; Smith, Dewdney and Wheeler 1971) that ampicillin polymers are highly antigenic and capable of inducing cellular immunity, hence this is an inappropriate formulation region. Additionally complexation reactions for example with β-cyclodextrins would be rendered difficult to interpret in such systems.

Reactions in the frozen state were carried out under the same pH and solvent systems as in the liquid state. It was expected that due to the concentration effect, there would be significant acceleration on the rate of degradation of amoxycillin as has been indicated (McDonald *et al.*, 1989; Concannon *et al.*, 1986) in the literature.

6.1.2 Kinetic studies in the liquid state

Studies in the liquid state demonstrated that the degradation of amoxycillin and clavulanate followed first-order kinetics over 2-4 half-lives of the reaction at controlled ionic strength (μ = 0.5). Thus various species of amoxycillin behaved similarly in kinetics, under the conditions of this study. This is supported from the E_a values (Table 3.2) from temperature dependence studies of each species being within experimental error. In combination with clavulanate, and in the buffer

systems the degradation plots of amoxycillin showed marked curvature. This novel finding arose from catalysis by clavulanate.

Clavulanate generally was markedly less stable than amoxycillin hence controlling the shelf-life of amoxycillin when formulated in combination. This is consistent with the literature data (Ashwin, Lynn and Taskis 1987; Wildfeuer and Radar 1996) that clavulanate is the less stable component of the combination. Shelf-life data are summarized in Table 6.1 and clearly show the marked impact of clavulanate on the stability of the combination. Obviously these data indicate that formulating at the pH minimum for clavulanate seems to be the best option. However other data found in this study indicate that under specific conditions in phosphate buffer systems, the initial rate of amoxycillin (k_I) was markedly increased as the concentration ratio of clavulanate to amoxycillin was increased, (Chapter 3, Section 3.6) hence rendering (k_I) to control the shelf-life of the combination.

As with most β-lactam penicillins (Hou and Pool 1969a; Stjernstrom *et al.* 1978; Bundgaard and Hansen 1981; Tsuji *et al.* 1978) the presence of buffer species was shown to exert general catalysis on the degradation of amoxycillin and clavulanate, which was directly proportional to their total concentration. Phosphate buffer showed a greater rate acceleration effect than acetate buffer. This difference in buffer catalytic effect has been documented (Haginaka, Nakagawa and Uno 1981) for clavulanate.

Literature data (Hou and Pool 1969a; Bundgaard and Hansen 1981; Zia, Shalchian and Borhanian 1977; Haginaka, Nakagawa and Uno 1981) on the catalytic effect of phosphate on β-lactam antibiotics have shown that the HPO_4^{2-} species is the primary catalytic species. The non-buffer-catalyzed degradation rate constants were determined. These data along with the estimated total buffer effects were found to be within acceptable agreement (Section 3.5) with the literature data for amoxycillin (Zia, Shalchian and Borhanian 1977) and clavulanate (Haginaka, Nakagawa and Uno 1981).

Table 6.1: Effect of temperature and pH on shelf-lives of amoxycillin and clavulanate

pH	t (°C)	AMOX t_{90} (h)	CLAV t_{90} (h)
2.0	35	3.05	0.04
	27	6.10	0.09
	20	12.84	0.21
	14	23.86	0.47
	-7.3	58.66	1.62
	-9.8	52.76	1.46
	-13.5	56.45	1.54
4.6	55	2.29	0.50
	49	4.43	0.73
	42	7.19	1.45
	35	13.03	2.92
	-7.3	203.88	49.07
	-9.8	173.55	42.00
	-13.5	373.67	71.92
7.0	55	1.16	0.31
	49	1.91	0.48
	42	3.61	0.92
	35	6.82	1.62
	-7.3	133.42	16.99
	-9.8	153.06	21.74
	-13.5	621.30	28.38

The effect of pH on the rate of hydrolysis of amoxycillin and clavulanate was evident from the rate constant data obtained in the three pH systems studied (Table 3.1). Both amoxycillin and clavulanate showed maximum stability at pH 4.60 in acetate buffer (except at –13.5°C for amoxycillin in phosphate) under all the conditions of this study. However from the pH-rate profile of amoxycillin (Zia, Shalchian and Borhanian 1977) and clavulanate (Haginaka, Nakagawa and Uno 1981), the pH of maximum stability for amoxycillin is 5.8 and for clavulanate is 6.4 in buffer free conditions. Therefore, formulating at pH around 6.0 would be the most stable system for the combination, although buffer effects would need to be considered.

The investigation of concentration effects of clavulanate and amoxycillin on the stability of the combination demonstrated that amoxycillin hydrolysis was catalyzed by the buffer species but was also dependent on the concentration of clavulanate. This finding was particularly prominent in phosphate buffer. Hence amoxycillin degradation was catalyzed by both phosphate and clavulanate. A kinetic model (Chapter 3, Equation 3.9) was proposed which described the experimental data within acceptable agreement with theoretical values. The rate of hydrolysis of clavulanate however did not change significantly upon increasing the concentration of amoxycillin, although a marginal increase in rate was observed in the phosphate buffer system. The latter effect has been reported by Ashwin and co-workers (1987) in an investigation of the stability of intravenous Augmentin® (Section 3.6 iii) however the extent of this effect was not as large as reported by Ashwin and co-workers. These workers found that increased concentration ratios of amoxycillin to clavulanate (from 5 to10) at constant clavulanate concentration decreased the shelf-life of clavulanate from 235 minutes to 110 minutes in water at 25°C.

These studies demonstrated that the rate of degradation of amoxycillin and clavulanate in runs containing a single antibiotic did not change significantly with change in initial concentration under the conditions of this study. However the stability of the combination formulation was affected by concentration ratios of amoxycillin to clavulanate. In this study the standard ratio used was 2:1. Increase in the ratio of amoxycillin to clavulanate indicated a decrease in clavulanate catalysis in the buffer systems. The standard clinical ratios used are 2:1, 4:1 (solid dosage form) and 5:1 (used as reconstituted powder for injection) of amoxycillin to

clavulanate (Martindale 1996; Ashwin, Lynn and Taskis 1987). Therefore a ratio of 5:1 amoxycillin to clavulanate would have the least clavulanate catalysis effect.

The temperature dependence of amoxycillin, clavulanate and their combinations were studied and the resulting Arrhenius plots showed linear relationships. The estimated E_a values (Table 3.2) were in acceptable agreement with the available literature data (Zia, Shalchian and Borhanian, 1977; Haginaka, Nakagawa and Uno, 1981). The proximity of E_a values obtained for amoxycillin from this study and the one reported by Hou and Poole (1969a) for ampicillin relates to a similar mechanism of β-lactam ring cleavage for both these penicillins (Section 3.4).

The dimerization reaction has been extensively studied (Bundgaard 1977a). This has been shown to occur at 6.0×10^{-2} mol dm^{-3} and higher concentrations of amoxycillin sodium. The self-catalyzed hydrolytic degradation is also concentration dependent and requires higher concentrations similar to those required for dimerization to occur. As this catalysis is dependent upon phenoxide ion concentration (Bundgaard 1977a) this reaction is only evident at pH around 9 and above.

The conditions of this study involved a maximum concentration of amoxycillin sodium of 2.6×10^{-2} mol dm^{-3} and a maximum pH of 7.0 were selected to minimize both of these reactions. In addition the kinetic studies showed no evidence of these reaction pathways. No significant change was evident in the experimental rate constants when amoxycillin initial concentrations were increased. The HPLC assay developed was proven to be stability indicating under high pH conditions hence would be specific for amoxycillin in alkaline media. It is concluded that the conditions used in the liquid state have not included either of these reactions.

6.1.3 Kinetic studies in the frozen state

The data obtained from the frozen temperature studies have indicated that the rate of reaction of amoxycillin and clavulanate followed first-order kinetics as in the liquid state over 2-3 half lives or until a maximum of 10 days of reaction. The rate of hydrolysis of clavulanate in all the conditions was significantly greater than amoxycillin indicating that frozen temperatures did not change the relative reaction rates of these antibiotics. Hence the shelf-life of the combination antibiotics was controlled by the rate of hydrolysis of clavulanate (Table 6.1).

The overall rate of reaction in the frozen state was slow compared to the liquid state, for instance the shelf- life of amoxycillin was 13 h at 35°C against 203.9 h at –7.3°C in acetate buffer. However extrapolation of the kinetic data from the liquid state to the frozen state using the Arrhenius equation indicated significant rate acceleration at the frozen temperatures. The highest acceleration recorded was 14.95 fold for clavulanate at –13.5°C and the lowest was 4.40 fold for amoxycillin at –7.3°C in the hydrochloric acid system. It is evident however that the conditions used in this study have provided less acceleration for amoxycillin compared to previous studies documented (McDonald *et al.* 1989; Concannon *et al.* 1986) where the rate of amoxycillin was reported (Concannon *et al.* 1986) to have been accelerated by about 60 fold at –7.5°C compared to the extrapolated value from liquid state data.

Evaluation of the experimental data in the hydrochloric acid system revealed that the rate of the reaction was dependent on an additional term in the rate equation (Equation.4.4, Chapter 4) that is, the concentration factor C_l / C_s. This effect has been explained by Pincock and Kiovsky (1966) using a concentration model which is described in Chapter 1, Equation 1.31. Based on this model an equation (Chapter 4, Equation 4.4) was developed which provided adequate explanation for the changes in rates of this system. The rate of the reaction in this system was significantly slowed by the presence of sodium chloride used for maintaining the ionic strength (μ=0.5). From the rate equation (Equation 4.4) deduced, in the hydrochloric acid system it can be concluded that the greater the concentration of sodium chloride the larger the C_s term, which in turn would result in a smaller concentration factor and consequently smaller rate constant values. The t_{90} value of amoxycillin in the presence of sodium chloride (μ=0.5) was 58.3 h against 2.2 h in absence of sodium chloride at –7.3°C. Other factors influencing the rate of the reaction of these antibiotics include, increased concentration of hydrogen ion (pH change) and the influence of ionic strength.

The data obtained from the phase diagram of sodium chloride indicated a close agreement between the theoretical C_l value and that obtained from the literature phase diagram. Thus sodium chloride has been shown to behave almost ideally in the hydrochloric acid system, see Table 4.2.

In the buffer systems the overall rates of degradation of the antibiotics were generally slow. Since inclusion of sodium chloride used for maintaining ionic

strength (μ=0.5) further stabilized the systems and made it difficult to measure the rates of the reactions even over 10 days, sodium chloride was removed from the buffer systems. This should have the effect of inducing a greater concentration effect, since under the frozen conditions the total concentration of the species should remain constant at a particular temperature (under ideal conditions) irrespective of their nature or initial concentrations (Pincock and Kiovsky 1966).

Various factors affecting the rates of degradation of amoxycillin and clavulanate in the buffer systems were analyzed and discussed under Section 4.2.3. Notable of these were increases in concentration of buffer species, precipitation of phosphate buffer components, general catalysis and pH changes as a result of buffer precipitation or change in the buffer pK_a values with respect to temperature.

The evidence of clavulanate catalysis of amoxycillin as described in the liquid state was evident owing to a notable increase in the rate constant data of amoxycillin in combination compared to that of amoxycillin alone (Table 4.1).

The effect of pH on the rate of degradation of amoxycillin and clavulanate was similar to that described in the liquid state. However in phosphate buffer it was previous studies prediction (Chapter 4 Section 4.2.4.2) that both disodium phosphate and potassium hydrogen phosphate would precipitate under the frozen temperatures studied resulting in marked reduction in pH (up to approximately 3 units of pH). This effect is expected to induce a greater influence on the rate of degradation of clavulanate than amoxycillin due to the characteristic pH-rate profile of each compound. Hence from the pH-rate profile it is evident that there could be approximately a 10 fold increase in the rate of clavulanate when the pH falls from 7.0 to 4.0, where as the rate of amoxycillin would increase only about 2 fold under the same conditions. Another aspect, which is important to consider, is the issue of supercooling and supersaturation which is expected to occur easily in any frozen system (Van Den Berg 1966; Van Den Berg and Rose 1959; Murase and Franks 1989). When the systems are subjected to these transitory changes, particularly at the higher frozen temperatures studied (-7.3 and –9.8°C) if the precipitation of disodium phosphate has occurred, potassium dihydrogen phosphate becomes further concentrated and supersaturated. Hence the fall in pH is expected to be smaller (approximately 1.5 unit). This would induce a stabilizing effect for amoxycillin and a smaller destabilizing effect or no significant effect (depending on the magnitude of

pH fall) on rate of clavulanate degradation. However if supercooling and supersaturation occurs before disodium phosphate has precipitated then due to the presence of concentrated HPO_4^{2-} species, catalysis of clavulanate by this species is expected to play a significant destabilizing effect, since the catalytic rate constant for HPO_4^{2-} is 7.7×10^{-1} (mol dm^{-3})$^{-1}$ h^{-1} (Haginaka, Nakagawa and Uno, 1981) which is far greater than for amoxycillin i.e. 2.9×10^{-1} (mol dm^{-3})$^{-1}$ h^{-1} (Zia, Shalchian and Borhanian, 1977) at 35°C.

The dimerization reaction involves nucleophilic attack by the NH_2 group of amoxycillin on the β-lactam ring. The NH_2 group is a powerful nucleophile however its protonation leads to a loss of its nucleophilicity. In acetate buffer only a small amount exists in the NH_2 form. However in the frozen state there is very significant concentration of amoxycillin and possible localized variations in pH. These conditions are favourable for the dimerization reaction. Hence although the formation of dimers and higher species was expected its occurrence could be explained by the conditions arising in the frozen state being conducive for this reaction pathway.

Since many parenteral admixtures are prepared at a physiological pH of about 7.0 or above, because of the higher solubility of amoxycillin at these pH, if the stability of the phosphate buffer system is improved, it can be a valuable system for the frozen formulations. The possible precipitation of the buffer species is of concern in providing only a pseudo stable product. Therefore a buffer salt with low eutectic temperature and appropriate composition which would not result in large pH changes is worthy of future consideration. As discussed in Chapter 4 Section 4.2.4.1 precipitation of the phosphate buffer salts are largely dependent on the type of salts used, their composition and their initial concentrations (Murase and Franks, 1989; Van den Berg, 1966). Among the potassium and sodium salts of phosphate, dipotassium hydrogen phosphate has the lowest eutectic temperature (-13.7°C). Murase and Franks (1989) have elucidated that at lower concentrations such as below 1 mol dm^{-3} this salt did not often precipitate even below -53°C (this may however be a metastable condition). For instance these authors, based on their experimental data, have suggested, that in the case of potassium salts (potassium dihydrogen phosphate and dipotassium hydrogen phosphate) of phosphate at pH 7.0, the ternary eutectic composition of the salts are very close to the composition of the

pH 7.0 buffer. Therefore precipitation of the potassium dihydrogen phosphate salt should not easily occur and if it occurs the pH change would be small and towards alkaline values. Consequently these salts at pH 7.0 composition should be evaluated for a frozen system for amoxycillin and other liquid formulation antibiotics.

Sodium chloride used for constant ionic strength ($\mu = 0.5$) was demonstrated to have significant stabilizing effect (Chapter 4, Section 4.4) on the degradation rate of amoxycillin and clavulanate. Amoxycillin showed little degradation $\leq$ 10% in presence of sodium chloride when stored at –7.3 °C in the buffer systems for 10 days. Upon removing sodium chloride the shelf life of amoxycillin reduced to 133.4 h in phosphate buffer and 203.9 h in acetate buffer systems at –7.3 °C. Therefore the results indicate that inclusion of sodium chloride has increased the shelf life of amoxycillin by a minimum of 10 days at –7.3°C in these systems. This can be beneficial in production of frozen formulations for home-based medical care. The stabilizing effect of sodium chloride was massive in the hydrochloric acid and water systems as indicated in Chapter 4, Table 4.7. The issue of administering sodium chloride in large amounts may be of concern. Glucose at double the molar concentration would be expected to have a similar effect.

The temperature effects on the stability of amoxycillin and clavulanate were unique to each system. In hydrochloric acid the rate of hydrolysis of amoxycillin as well as clavulanate was increased marginally with the decrease in temperature. The reason for this was described in Section 4.2.1 to be due to various concentration factors such as the increase in concentration of hydrogen ion. In the buffer systems however, the rate of degradation of the antibiotics was generally decreased notably (except at –9.8 °C in acetate system) with decreased temperature. The effect of freezing on reciprocal shelf-life values of amoxycillin and clavulanate is illustrated in Chapter 4, Figure 4.6. In the buffer systems the rate-temperature profiles obtained are in general concordance with the literature data for benzylpenicillin sodium (Larsen 1971a), amoxycillin (McDonald et al, 1989b; Concannon *et al.*, 1986) and several other frozen reaction systems (Shija, Sunderland and McDonald 1992; Pincock and Kiovsky 1965b). However the rate of change of reaction rates observed in this study although notable are not as large as those of the other reported studies. This is because in this study as buffers were used, the concentration effect was not

as significantly evident here. But other factors which were discussed previously were more likely to cause the acceleration in reaction rates.

Thus it is evident that the experimental conditions of this study have greatly stabilized the rate of degradation of amoxycillin compared to the previous reports (McDonald et al, 1989b; Concannon *et al.*, 1986).

6.1.4 The effect of hydroxypropyl β-cyclodextrin (HPβCD) on the rate of degradation of amoxycillin and clavulanate

An investigation of the effect of HPβCD on degradation rates of amoxycillin and clavulanate indicated HPβCD did not change the order of the reaction rates. The acetate system was selected for this study since amoxycillin and clavulanate both showed a greater stability in this system. The results indicated that HPβCD did not have a substantial effect on the reactant rates of reactions. No significant effect was observed on the rate of amoxycillin and only a marginal stabilizing effect (up to maximum 11.3%) was recorded for clavulanate hydrolysis. However since in this system, the stability of the combination was controlled by shelf-life of clavulanate, this small stabilizing effect was considered to be beneficial in prolonging the shelf-life of the combination by approximately 10%. Another beneficial effect of HPβCD observed in the combination runs was its effect in curbing the rapid degradation of amoxycillin by clavulanate catalysis in the initial stage (Section 5.1.1). Frozen state studies were not possible due to technical problems (Section 5.2).

The literature data have indicated both stabilizing (Ong, Sunderland and McDonald 1997; Loftsson and Olafsdottir 1991; Hsyu *et al.* 1984) and destabilizing (Loftsson 1995; Loftsson and Olafsdottir 1991) effects of HPβCD on β-lactam antibiotics. From these reports it appears that the complexation of HPβCD is pH dependent and is affected by the ionization states of the compounds. Hence the stabilization effects of ampicillin documented, was studied at low acidic pH (Hsyu *et al.* 1984) and in another report the inhibitory effect of β-cyclodextrin on polymerization of ampicillin (Aki *et al.* 1990) was investigated in alkaline pH. Thus in this study the zwitterionic form of amoxycillin was expected a priori to provide complexing ability with HPβCD compared with the charged species. However the data presented in this study are a contribution to the literature since there are no reports on the effects of HPβCD on amoxycillin or clavulanate in the literature.

6.2 Conclusions

The hydrolytic degradation of amoxycillin sodium and potassium clavulanate either alone or in combination followed first-order kinetics in both the liquid and frozen states at pH values (2.0, 4.6 and 7.0) studied.

In hydrochloric acid system the overall rate of clavulanate hydrolysis was approximately 60 fold faster than amoxycillin.

Buffer catalysis influenced the rate of degradation of amoxycillin and clavulanate. The second-order rate constants due to total phosphate were 5.84×10^{-1} (mol $dm^{-3})^{-1}$ h^{-1} and 2.33 (mol $dm^{-3})^{-1}$ h^{-1} at 55°C for amoxycillin and clavulanate respectively. Similarly the second-order rate constants due to total acetate were 1.53×10^{-1} (mol $dm^{-3})^{-1}$ h^{-1} and 4.4×10^{-1} (mol $dm^{-3})^{-1}$ h^{-1} at 55°C for amoxycillin and clavulanate. The non-buffer-catalyzed rate constants (k_{pH}) in phosphate and acetate buffers at 55°C were found to be 3.5×10^{-2} h^{-1}, 7.0×10^{-3} h^{-1} for amoxycillin and 1.1×10^{-1} h^{-1}, 1.0×10^{-1} h^{-1} for clavulanate at the same temperature. The acetate system was the most stable system examined in this study.

In addition the degradation of amoxycillin undergoes clavulanate catalysis and this catalysis was influenced by phosphate concentration. The second-order rate constant values for this novel finding was 1.75×10^{2} (mol $dm^{-3})^{-1}$ h^{-1} for clavulanate catalysis (k_{cvc}) and 2.87 (mol $dm^{-3})^{-1}$ h^{-1} for phosphate catalysis of clavulanate catalysis (k_{phccv}).

The temperature dependence data for amoxycillin sodium and potassium clavulanate gave E_a values of 72.5, 71.2 and 74.8 kJ mol^{-1} for amoxycillin and 88.1, 75.1 and 69.8 kJ mol^{-1} for clavulanate respectively at pH values of 2.0, 4.6 and 7.0 respectively.

The investigation on the effect of HPβCD on rate of hydrolysis of amoxycillin and clavulanate in acetate system indicated that inclusion of HPβCD did not influence the observed order of the reactions. The stability of the combination was improved by prolonging the shelf-life of clavulanate by approximately 10%. The rate constant within the cyclodextrin complex (k_c) and the stability constant of the complex (K_c) for clavulanate were estimated at 55°C to be 1.54×10^{-1} h^{-1} and 74.2 (mol $dm^{-3})^{-1}$ respectively.

Frozen state studies showed significant acceleration in the rate of the degradation of amoxycillin and clavulanate alone and in combination when based on

extrapolated liquid state data. The rate of hydrolysis of clavulanate was increased 15.0 fold in the hydrochloric acid system and that of amoxycillin in combination by 10.8 fold in phosphate buffer system. Various factors causing this acceleration in rates were identified, these include: concentration effect (in hydrochloric acid system), increased buffer catalysis, precipitation of buffer components, pH change, general catalysis, and polymerization.

Inclusion of sodium chloride commonly used for ionic strength control was found to have significant stabilizing effects. This effect was enormous in the hydrochloric acid system where the shelf-life of amoxycillin was increased from 2.2 h in absence of sodium chloride to 58.3 h in the presence of sodium chloride at –7.3°C.

The buffer systems stabilized the rate of degradation of amoxycillin when compared with previous studies (McDonald *et al.* 1989b; Concannon *et al.* 1986). The highest shelf life data recorded in this study were 621.3 h for amoxycillin in phosphate buffer at –13.5°C and 71.9 h for clavulanate in acetate buffer at –13.5°C. However, the rate of degradation of amoxycillin in combination with clavulanate in the buffer systems was increased notably. The shelf-life of amoxycillin in combination with clavulanate in phosphate buffer fell to 110.2 h at –13.5°C.

6.3 Scope for Future Work

As many intravenous preparations utilize higher concentrations of the antibiotics it could be worthwhile to design some experiments with greater concentrations of amoxycillin and clavulanate at frozen temperatures. Since the investigations of this study carried out at higher concentrations in acetate buffer resulted in precipitation further studies could also be limited by this. Higher pH values are a possibility where the solubility of amoxycillin would be improved however, the autocatalytic reactions would have pronounced effects. If neutral pH or low alkaline pH such as below 7.5 were selected, it would reduce the risk of dimerization reactions to some extent and self-catalyzed hydrolysis reaction of amoxycillin to a greater extent. Hence, potassium salts of phosphate in a buffer system of pH 7.0 with low concentration of potassium salts as suggested by Murase and Franks (1989) and described under 6.1.3 could be a possible system for further investigation.

Since the investigation on stabilizing effect of sodium chloride in the buffer systems indicated promising results, it may be worthwhile to perform long term frozen state studies of incubated samples for marketing and manufacturing purposes.

Another area, which could be of interest to investigate, is the effect of other cyclodextrins on rate of degradation of amoxycillin at higher pH values and higher concentrations not used in this study. Since it has been reported (Aki *et al.*, 1990) that HPβCD has an inhibitory effect on polymerization of ampicillin. This would have a possible application especially in parenteral preparations of the combination antibiotics where higher concentration and pH values need to be used. The study could be expanded to frozen temperatures under similar conditions of buffer salts described above. Although the influence of HPβCD on the polymerization reaction might be of interest, however this may be limited by low K_c values and possible HPβCD catalysis.

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PRESENTATION OF WORK FROM THESIS

L.Vahdat and V.B. Sunderland 1997, "Catalytic effect of potassium clavulanate on the rate of degradation of amoxycillin sodium in the liquid state", presented as a poster at the Scientific Meeting of the Australian Pharmaceutical Science Association, Sydney, NSW, 1997.

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