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Kinetics of amoxicillin and clavulanate degradation alone and in combination in aqueous solution under frozen conditions

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Abstract

Kinetics of the reactions of amoxicillin sodium and potassium clavulanate alone and in combination were investigated in the frozen state at selected pH values of 2.0, 4.6 and 7.0.

Extrapolation of the rate constant values to the frozen state from the liquid state data indicated marked acceleration of the rates of amoxicillin and clavulanate degradation for the pH values investigated. The highest acceleration in rate recorded was 15.0-fold for clavulanate and the lowest value was 4.6-fold for amoxicillin at −7.3 °C in the hydrochloric acid system. The rate constant values obtained were interpreted in terms of the concentration model [Pincock, R.E., Kiovsky, T.E., 1966. Kinetics of reactions in frozen solution. J. Chem. Educ. 43, 358–360], phase–temperature relationship of the solutes, buffer catalysis, pH change and polymerization reactions. A kinetic model was deduced for the hydrochloric acid system providing adequate explanation of the experimental results. A large stabilizing effect of sodium chloride used for maintaining constant ionic strength ($\mu = 0.5$) was evident in this system. The shelf-life of amoxicillin was increased from 2.2 to 58.7 h at −7.3 °C when sodium chloride was included in the hydrochloric acid system.

Keywords: Amoxicillin; Clavulanate; Stability; Frozen state; Kinetics

1. Introduction

This study forms the second part of an evaluation of the stability of amoxicillin in combination with clavulanate in aqueous solution. Amoxicillin alone and in combination with clavulanate is still widely used in many parts of the world (Dufour et al., 2005; Quach et al., 2005) as a treatment of choice for a number of infections. Although freezing a dosage form in solution has been shown to improve the stability of many formulations however there are reports that for some (Concannon et al., 1986; McDonald et al., 1989; Mcquade et al., 2004) including aminopencillins such as amoxicillin and ampicillin it has been counterproductive. Since most formulations are multicomponent systems, development of frozen formulations requires a thorough understanding of the phase changes and concentration variation of each component that occurs during freezing. These changes could affect the stability of each formulation. Therefore it is essential to understand the kinetics of the reactions in each system under frozen conditions.

A theoretical model for the kinetics of reactions in the frozen state has been developed by Pincock and Kiovsky (1966) based on a solute concentration framework. When the volume of a liquid phase changes due to freezing or thawing, there would be changes in the molar concentrations of reactants accordingly. Hence for many frozen reactions, in order to obtain meaningful rate constants, the rate definition has to be modified (Pincock, 1969) to: $\text{rate} = (1/V)(\mathrm{d}M/\mathrm{d}t)$. Where V is the volume of the reactant phase and M is the moles of the reactant(s) in that phase.

On the basis that the rate of reaction in frozen solutions is related to the reactant concentrations measured in thawed solutions, the experimentally obtained rate of the reaction can be derived in terms of the concentration changes in frozen solutions. The rate however is influenced by the concentrations of all species including non-reactant species in solution. Frozen solutions at temperatures above the eutectic temperature increasingly concentrate as the temperature decreases. These concentrations are based on solution colligative properties.

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Although there are several reports on the stability of amoxicillin solutions (Concannon et al., 1986; Ashwin et al., 1987; McDonald et al., 1989) in the frozen state. These studies have primarily related to intravenous admixtures. There has been no evaluation of the kinetics of amoxicillin in the frozen state or on its stability in combination with clavulanate. In addition the kinetics of degradation of clavulanate in the frozen state has not been reported. This paper provides a detailed examination of the factors that influence the stability of a combination of amoxicillin with clavulanate in aqueous solution, at selected pH values of 2.0, 4.6 and 7.0 under frozen storage conditions.

2. Materials and methods

Amoxicillin sodium was obtained from SmithKline Beecham Pharmaceuticals Australia and potassium clavulanate was a gift kindly provided by SmithKline Beecham Pharmaceuticals UK. Buffers were prepared from AR grade reagents and water from a MilliQ apparatus was used for all solutions.

Kinetic studies were monitored by a stability indicating HPLC method, validated under stressed conditions. The stability indicating nature of the method was also verified by using a photo diode array detector to identify peak purity. The wavelength 228 nm was found to give a high response for both drugs when used in combination under these experimental conditions. The peak height rather than the peak area was concluded to give more consistent results in the presence of degradation products.

The validity of the method was ascertained by preparing standard solutions of the drug combination in water over the concentration range of 6.45×10^{-5} to 2.58×10^{-3} mol dm^{-3} (amoxicillin 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 drug compounds. The precision of the method was found by calculating the coefficient of variation ($n = 6$) to be 0.66% and 0.3% for amoxicillin (1.29×10^{-3} mol dm^{-3}) and clavulanate (1.05×10^{-3} mol dm^{-3}), respectively. All the experimental runs were performed in duplicate. The average deviation of the peak responses calculated as % errors (represented as error bars in the figures) were $\leq 1\%$. At the start of each specific experimental condition, a set of six replicates of each sample set were analysed to assess reproducibility. The R.S.D of all these replicates were $\leq 1\%$.

The HPLC system consisted of a reversed phase HPLC column (C_{18} Altima 5μ; Guard-column C_{18} Alltech), a Varian Vista 5500 HPLC pump, Varian UV detector with an integrator connected to a Delta chromatography digital system for data manipulation and a 20μl loop Rheodyne injector. The mobile phase was a mixture of aqueous phosphate buffer and methanol (95:5) adjusted to pH 4.4 ± 0.1. The detection wavelength was set at 228 nm and the flow rate was set at 1.5 ml/min. Experiments were carried out at three sub-zero temperatures, -7.3 ± 0.2, -9.8 ± 0.2, -13.5 ± 0.1 °C and at room temperature 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 0.5 using sodium chloride.

Solutions of amoxicillin 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 2 ml 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 1 h. The tubes were then transferred into a glycol–water bath mixture (∼50% ethanediol (w/v), at constant density = 1.067) set at the specific temperature and left for about 45 min to equilibrate. At the set time the first tube was removed and thawed at room temperature by placing it in a bath of lukewarm water with occasional shaking (usually about 5 min was required to reach room temperature) and an aliquot was immediately injected onto the HPLC column. The time for the first sample was taken as time zero. Subsequently the remaining samples were injected by the same procedure at specified time intervals until about two to three half-lives of the reaction was completed or until a minimum of 8 days 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 degradation of the drug during the sample preparation and after thawing. Therefore 1 ml of the double strength drug solution was added to 1 ml 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 remainder of the procedure was as stated above.

All the samples were injected onto the HPLC column within 2 min of attaining room temperature.

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

All data were fitted by a least squares program.

3. Results and discussion

The data obtained from experimental runs demonstrated a linear relationship when plots of log concentration (%) remaining with respect to time indicating first-order kinetics. Although some reactions were slow where about 20% of degradation was obtained, these data were treated as first-order kinetics based on faster analogous reactions providing greater levels of degradation in the frozen state. Fig. 1 and Table 1 illustrate the rate constant data at various sub-zero temperatures and systems. All plots exhibited linearity with correlation coefficients of $r > 0.94$.

A comparison of these results with the extrapolated values obtained from the liquid state data (Vahdat, 2000) indicated that

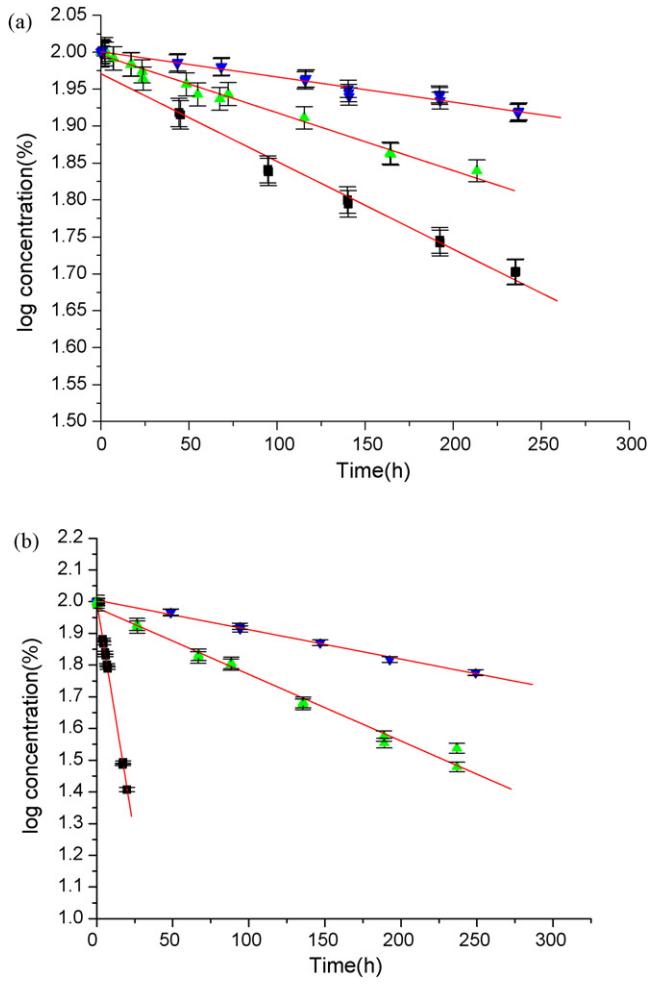

Fig. 1. Representative first-order plots of amoxicillin and clavulanate in the frozen state. (a) amoxicillin (b) clavulanate. (a) ■, Amoxicillin in combination with clavulanate in phosphate buffer pH 7.0 at −7.3 °C; ▲, amoxicillin in combination with clavulanate in hydrochloric acid system pH 2.0 at −7.3 °C; ▼, amoxicillin in combination with clavulanate in acetate buffer pH 4.6 at −9.8 °C. (b) ■, Clavulanate in hydrochloric acid pH 2.0 system at −13.5 °C; ▲, clavulanate in phosphate buffer pH 7.0 at −9.8 °C; ▼, clavulanate in acetate buffer pH4.6 at −7.3 °C.

freezing significantly increased the overall rates of the reactions of amoxicillin and clavulanate (see Table 2).

In the hydrochloric acid system, the overall rate of reaction of amoxicillin whether alone or in the presence of clavulanate did not change significantly. However, the rate of reaction of clavulanate showed an average increase of 23% when compared to its combination with amoxicillin. This increased reaction rate has been reported in the liquid state (Vahdat, 2000) to a smaller degree. The extent of the increase was two to three times greater in the frozen state. The average rate of clavulanate degradation was estimated to be approximately 36-fold faster than that of amoxicillin alone and 30 times faster than that of amoxicillin (Table 1) in combination. This clearly demonstrates the role of clavulanate in controlling the shelf-life of the combination.

3.1. Kinetics of reactions in the hydrochloric acid system

The rate of degradation of amoxicillin or clavulanate in the presence of hydrochloric acid can be illustrated as follows:

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

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

$$-\frac{\mathrm{d[total\ drug]}}{\mathrm{d}t} = k_2[\mathrm{H}_l^+][A_l] \quad (1)$$

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

$$\log A = \log A_0 - \frac{k_{\mathrm{obs}}t}{2.303} \quad (2)$$

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

Under frozen conditions, considering the concentration effect model derived by Pincock and Kiovsky (1966), the rate equation

Table 1
First-order (k_{obs}) rate constants of amoxicillin and clavulanate individually and in combination at pH values and temperatures indicated

pH[a]	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}

[a]pH values measured at 20 °C. pH 2.0 is in 1.24×10^{-2} mol dm^{-3} hydrochloric acid and $\mu = 0.5$ (NaCl); pH 4.6 is in 2.2×10^{-1} mol dm^{-3} acetate buffer (no NaCl); pH 7.0 is in 1.0×10^{-1} mol dm^{-3} phosphate buffer (no NaCl). AMOX: amoxicillin sodium, initial concentration: 1.29×10^{-3} mol dm^{-3} in the buffers and 9.03×10^{-4} mol dm^{-3} in hydrochloric acid system. CLAV: potassium clavulanate, initial concentration: 1.05×10^{-3} mol dm^{-3} in the buffers and 7.38×10^{-4} mol dm^{-3} in hydrochloric acid system. AMOX–COMB: amoxicillin in combination with clavulanate; CLAV–COMB: clavulanate in combination with amoxicillin.

Table 2
Comparison of the first-order rate constants obtained experimentally with calculated values obtained from the extrapolation of Arrhenius plots

SYSTEM	COMPOUND	t (°C)	k_{Exp} (h^{-1})	k_{Pred} (h^{-1})	ACLER (fold)	t_{90} (h)
Acetate	AMOX	−7.3	5.15×10^{-4}	9.47×10^{-5}	5.44	203.88
		−9.8	6.05×10^{-4}	7.02×10^{-5}	8.62	173.55
		−13.5	2.81×10^{-4}	4.43×10^{-5}	6.35	373.67
	AMOX–COMB	−7.3	7.62×10^{-4}	1.03×10^{-4}	7.39	137.80
		−9.8	7.86×10^{-4}	7.63×10^{-5}	10.28	133.59
		−13.5	4.03×10^{-4}	4.43×10^{-5}	9.11	260.55
	CLAV	−7.3	2.14×10^{-3}	3.53×10^{-4}	6.06	49.07
		−9.8	2.50×10^{-3}	2.58×10^{-4}	9.69	42.00
		−13.5	1.46×10^{-3}	1.58×10^{-4}	9.25	71.92
	CLAV–COMB	−7.3	2.32×10^{-3}	4.21×10^{-4}	5.50	45.26
		−9.8	2.66×10^{-3}	3.05×10^{-4}	8.72	39.47
		−13.5	1.6×10^{-3}	1.88×10^{-4}	8.51	65.63
Phosphate	AMOX	−7.3	7.87×10^{-4}	1.50×10^{-4}	5.26	133.42
		−9.8	6.86×10^{-4}	1.09×10^{-4}	6.28	153.06
		−13.5	1.69×10^{-4}	6.59×10^{-5}	2.56	621.30
	AMOX–COMB	−7.3	2.73×10^{-3}	2.53×10^{-4}	10.78	38.46
		−9.8	2.02×10^{-3}	1.88×10^{-4}	10.77	51.98
		−13.5	9.53×10^{-4}	1.18×10^{-4}	8.05	110.18
	CLAV	−7.3	6.18×10^{-3}	8.46×10^{-4}	7.32	16.99
		−9.8	4.83×10^{-3}	6.26×10^{-4}	7.70	21.74
		−13.5	3.70×10^{-3}	4.03×10^{-4}	9.20	28.38
	CLAV–COMB	−7.3	5.49×10^{-3}	7.52×10^{-4}	7.32	19.13
		−9.8	4.06×10^{-3}	5.58×10^{-4}	7.29	25.86
		−13.5	3.14×10^{-3}	3.52×10^{-4}	8.92	33.44
HCl	AMOX	−7.3	1.79×10^{-3}	3.89×10^{-4}	4.60	58.66
		−9.8	1.99×10^{-3}	2.84×10^{-4}	7.00	52.76
		−13.5	1.86×10^{-3}	1.77×10^{-4}	10.51	56.45
	AMOX–COMB	−7.3	1.80×10^{-3}	3.64×10^{-4}	4.95	58.33
		−9.8	1.93×10^{-3}	2.65×10^{-4}	7.27	54.40
		−13.5	1.65×10^{-3}	1.64×10^{-4}	10.04	63.64
	CLAV	−7.3	6.48×10^{-2}	1.18×10^{-2}	5.49	1.62
		−9.8	7.20×10^{-2}	8.06×10^{-3}	8.93	1.46
		−13.5	6.80×10^{-2}	4.55×10^{-3}	14.95	1.54
	CLAV–COMB	−7.3	5.33×10^{-2}	1.03×10^{-2}	5.18	1.97
		−9.8	5.76×10^{-2}	7.02×10^{-3}	8.21	1.82
		−13.5	5.15×10^{-2}	3.92×10^{-3}	13.12	2.04

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 $\mu = 0.5$ (NaCl). AMOX: amoxicillin, CLAV: clavulanate, AMOX–COMB: amoxicillin in combination with clavulanate, CLAV–COMB: clavulanate in combination with amoxicillin.

can be written as

$$-\frac{dA}{dt} = k_2 C_l \frac{[H_s^+][A_s]}{C_s} \quad (3)$$

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:

$$\log A = \log A_0 - \frac{k_2 C_l t}{2.303} \frac{[H_s^+]_0}{[C_s]_0} \quad (4)$$

where under pseudo first-order conditions:

$$k_{obs} = k_2 C_l \frac{[H_s^+]_0}{[C_s]_0} \quad (5)$$

In Eq. (5), k_2 is dependent on temperature and can be estimated by extrapolation of the reaction rates obtained in the liquid state (Vahdat, 2000) to that of the frozen state, using the Arrhenius equation. The value of C_s can be estimated by summation of 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 (Rodebush, 1918; Hall and Sherrill, 1928; Seidell, 1940; Cocks and Brower, 1974). 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. 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.

According to Eq. (5) the observed rate of hydrolysis of amoxicillin or 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

Table 3
Comparison of C_l values of sodium chloride (in terms of $[Na^+]+[Cl^-]$) estimated from the literature phase diagram with the ideal value obtained from Eq. (6)

t (°C)	C_l (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 Eq. (6).

of sodium chloride at each particular temperature was obtained from the phase diagram data in the literature (Rodebush, 1918; Hall and Sherrill, 1928; Seidell, 1940; Patel and Hurwitz, 1972; Cocks and Brower, 1974). The 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, 1993):

$$C_l = \frac{\Delta T_f}{iK_f} \quad (6)$$

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 3 lists the calculated values of C_l at the temperatures studied. The first-order rate constant values obtained by incorporating the concentration factor are presented in Table 4 and Fig. 2, indicating that the concentration factor has significantly influenced the rate of the reactions.

Table 4 compares the rate of the reaction in the frozen systems with that of extrapolated values from the liquid state (Vahdat, 2000). The data indicates that incorporation of the concentration factor increased the rate of the reaction of this system significantly yet there still remain some differences between the rate constant values of those estimated by prediction, inclusive of the concentration factor, and the experimental results. This could be due to other factors which are discussed below or could arise from errors in the values and from extrapolation of rate constant data from the liquid state which could induce a significant error in the estimation of k_2 values used in Eq. (5).

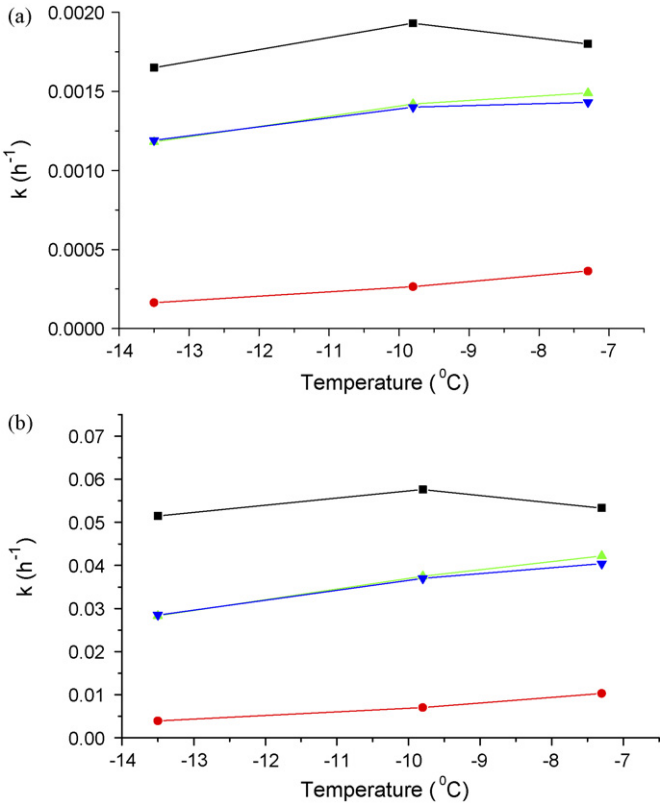

Fig. 2. Comparison of first-order rate constant values of amoxicillin and clavulanate in the hydrochloric acid system. (a) Amoxicillin in the presence of clavulanate. (b) Clavulanate in the presence of amoxicillin. Where ■, k_{Exp}: k_{obs} values obtained from experimental results; ●, k_{Pred}: k_{obs} values obtained by extrapolation from the Arrehenius plot; ▼, k_{Pred} (ideal): k_{obs} values obtained by incorporating the concentration factor in Eq. (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.

In the buffer systems the rate of degradation of amoxicillin and clavulanate decreased with temperature reduction except in

Table 4
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	Literature
				k_{Pred} (h^{-1} × C_l/C_s)	k_{Pred} (h^{-1} × C_l/C_s)
−7.3	AMOX	1.79×10^{-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 (Vahdat, 2000); k_{Exp}: rate constant obtained experimentally; ideal: predicted rate constant where C_l is from Eq. (6); literature: predicted rate constant where C_l is obtained from the phase diagram of sodium chloride.

acetic acid buffer at −9.8 °C (Table 1). The data also demonstrates a notable increase in rate of amoxicillin degradation in the combination in comparison to the runs containing amoxicillin alone. This increase in rate which was more prominent in phosphate buffer can be related to the catalytic effects of clavulanate on amoxicillin as noted at the initial stage of the liquid state runs (Vahdat, 2000). However, as in the liquid state the rate of clavulanate degradation did not change significantly as a result of combination with amoxicillin. There was no significant change in pH during the course of the experiment. As the buffers behave according to the Henderson–Hasselbalch equation, the type of concentration factor effect, discussed under the hydrochloric acid system was not considered to influence the rate of reaction in buffer systems.

3.2. Possible factors affecting the rate of the reaction in the frozen systems

3.2.1. Hydrochloric acid system

3.2.1.1. Specific acid catalysis. The hydrogen ion concentration in the hydrochloric acid system can impose specific acid catalysis. Based on the lowering of freezing point theory the total concentration of species in a frozen system at a particular temperature is constant. Under ideal conditions the concentration of H^+ present becomes a proportion of the total species concentration at each sub-zero temperature. Hence the concentration of H^+ varies with a change in frozen state temperature. Table 5 illustrates this effect on concentration of H^+. It is evident from these data that as the temperature falls the concentration of H^+ increases significantly resulting in pH changes which leads to increased acid catalysis.

3.2.1.2. Influence of pH. As stated above the change in hydrogen ion concentration results in changes of pH which in turn can influence the rate of the reaction. Degradation products of amoxicillin and clavulanate could influence a change in pH since the concentration of these degraded products can increase in like manner, based on the concentration of the antibiotic itself. Multiple degradation products would tend to moderate this effect. Species added to influence the ionic strength influence the pH of hydrochloric acid by reducing the frozen state concentration of H^+.

3.2.1.3. Influence of ionic strength. The rate of a reaction is influenced by the 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 as water freezes. Hence the dissolved species become more concentrated in the remaining water, e.g. NaCl. As cooling is continued the concentration effect is increased until the concentration of solute reaches its saturation concentration and precipitation occurs. The eutectic temperature of sodium chloride is −21.2 °C (Cocks and Brower, 1974; Ramirez et al., 1974; Milton and Nail, 1996; Cavatur and Suryanarayanan, 1998) where all the sodium chloride precipitates and the eutectic composition is ∼3.76 mol dm^{-3} of sodium chloride. Hence under the conditions of this study, sodium chloride existed in a concentrated solution. Therefore the concentration of sodium chloride has increased significantly from its initial concentration of 4.88×10^{-1} mol dm^{-3}.

Table 5
Effect of temperature on the concentration of H^+ in the hydrochloric acid system containing amoxicillin sodium at pH 2.0 and $\mu = 0.5$ (NaCl)

Temperature (°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}

It is documented (Harned and Owen, 1950) that the activity coefficient of hydrochloric acid in sodium chloride solution is a function of μ. Harned et al. (Harned, 1920; Harned and Brumbaugh, 1922; Harned and Mannweiler, 1935; Harned and Owen, 1950) have studied the activity coefficient of hydrochloric acid in various concentrations of sodium chloride solution in the temperature range of 0–60 °C. These data indicates 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. Theoretically this effect can be predicted using Eq. (1) by assuming the ideal behaviour of the solutes. For instance if sodium chloride is absent from the system, C_s is dominated by $[H^+]$ and $[Cl^-]$. 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 $[Na^+]$ and $[Cl^-]$. Hence considering the concentration factor C_l/C_s in Eq. (5) the numerator value (C_l), would be the same (under ideal conditions) for both of 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 without. Thus this will result in approximately a 40-fold increase in reaction rate of the system with no sodium chloride compared to the system with sodium chloride ($\mu = 0.5$). This increased rate was too fast to follow experimentally.

3.2.2. Buffer effects

Buffers can influence the rate of reactions through several ways. These are discussed sequentially.

3.2.2.1. Catalysis. The experimental results obtained from the liquid state (Vahdat, 2000) demonstrated that both acetate and phosphate buffers had catalytic effects on the rates of degradation of amoxicillin 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 an additional buffer catalysis effect would be predicted.

3.2.2.2. Concentration. 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 con-

centrations 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 rates of amoxicillin degradation were expected to increase 9.8 times at −7.3, and 13.2 and 18.1 times at −9.8 and −13.5 °C, respectively, in acetate buffer. Similarly for clavulanate the increase was by 7.1, 9.4 and 12.7 times at −7.3, −9.8 and −13.5 °C, respectively. If the same procedure was applied to the phosphate buffer system the increase for amoxicillin was 12.3-, 16.5- and 22.6-fold at −7.3, −9.8 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, −9.8 and −13.5 °C, respectively.

3.2.2.3. Precipitation. Depending on the eutectic temperature of a species, buffer constituents can selectively crystallize or precipitate under frozen conditions. This could lead to changes in pH and electrolyte concentration and subsequently affect the rate of the reaction. For example, the eutectic temperatures of acetic acid and sodium acetate are −26.4 and −16.6 °C (Ramirez et al., 1974; Inoue et al., 1984), respectively. So precipitation of either buffer component was not predicted at or above the frozen temperatures studied in acetate buffer. However, since the eutectic temperatures of potassium dihydrogen phosphate and disodium phosphate are reported (Van den Berg and Rose, 1959; Murase et al., 1991) to be −2.7 and −0.5 °C, respectively, precipitation of buffer components is likely to occur under the frozen temperatures studied. Supercooling rather than precipitation, can also occur.

3.2.2.4. pH effects. There are a number of reports (Van den Berg and Rose, 1959; Van den Berg, 1966; Murase et al., 1991; Gomez et al., 1994) on the precipitation of phosphate salts at sub-zero temperatures leading to significant pH change. For example a phosphate buffer of initial pH of 7.4 is reported to undergo a 3.6 pH unit decrease when stored at the temperature range from 0 to −10 °C. 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 as a function of solubility of each salt. Frozen solutions also can experience supersaturation that can last for long periods maintaining close to the initial pH of the solution until precipitation. Considering the buffer concentrations used in this study, disodium phosphate is expected to precipitate first inducing a 3 units pH decrease. Therefore 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. However, in this study pH measurements in the frozen state were not attempted owing to the very marked difficulties in obtaining useable results.

The pH-rate profile of amoxicillin (Zia et al., 1977) indicates that a decrease of up to 1.5 units in pH would reduce the rate of degradation of amoxicillin, however, a further reduction of one pH unit would not bring any significant additional change in rate with respect to pH 7.0. Below pH about 4.5 the rate of hydrolysis of amoxicillin increases with any reduction in pH. For instance the rate of degradation of amoxicillin would increase by approximately twofold when pH falls from 7.0 to 4.0. Similarly in the case of clavulanate the pH-rate profile data reported by Haginaka et al. (1981) indicated that the rate of hydrolysis of clavulanate would not increase (rather stabilize) for up to a 1.5 units fall in pH. However, the rate of the reaction of clavulanate increases directly with any further fall in pH. It was estimated there would be about 10-fold increase in rate of clavulanate degradation when the pH falls from 7.0 to 4.0.

Table 6
Effect of solution composition on the shelf-life of amoxicillin 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)	58.7
Phosphate buffer, without NaCl (pH 7.0)	133.4
Acetate buffer, without NaCl (pH 4.6)	203.9

From the data presented in Table 6, it is evident that the rate of degradation of clavulanate in phosphate buffer system has increased in the range of 7.3–9.2-fold. Therefore it appears that the fall in pH due to phosphate precipitation induced a greater rate of acceleration on clavulanate than amoxicillin. 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 warrants consideration. Data (Vahdat, 2000) suggests that in the course of freezing the pH of acetate buffer changes from the room temperature pH of 4.60 to 4.96 at −13.5 °C. 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 amoxicillin and clavulanate described previously, this change in pH (0.36 units rise in pH) should have an stabilizing effect on the rates of reaction of both antibiotics.

Similar analysis of the pK_a data available in literature (Bates and Acree, 1943, 1945; Sunderland, 1983) indicates that the changes in pK_{a2} of phosphoric acid with respect to temperature are smaller than found for acetic acid. Hence as other effects discussed earlier have greater influence on pH of phosphate buffer, the impact of a small variation of pK_a of $H_2PO_4^-$ with temperature was ignored in these studies.

3.2.3. Catalysis effects

Enhancement of the catalytic effect of one reacting species upon another due to proportional increased concentration of the catalyst is possible. Hence the catalysis of amoxicillin by clavulanate or amoxicillin should be considered. There are no reports in the literature on the catalytic effect of clavulanate on amoxicillin. However, the investigation in the liquid state

(Vahdat, 2000) suggested catalysis of amoxicillin by clavulanate in acetate and phosphate buffers. It was also demonstrated 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 occur 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 could result in an increase of its catalytic effect on amoxicillin. Thus the results in Table 1 indicate a notable increase in rate of amoxicillin in combination runs in both the buffer systems, compared to its individual runs. The data also indicates that the rate of degradation of amoxicillin in combination with clavulanate in phosphate buffer is far greater than in acetate buffer, thus confirming the results of the liquid state (Vahdat, 2000). The catalytic effect of phosphates on clavulanate catalysis of amoxicillin has been demonstrated (Vahdat, 2000) and would also be another factor influencing the increased rate of amoxicillin degradation 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 1 support this explanation by showing a greater rate constant at −7.3 °C than other lower temperatures. As the temperature decreases there would be more likelihood for the phosphate components to precipitate.

Another type of catalysis to consider is general acid catalysis due to the presence of the protonated side-chain amino group in amoxicillin. Since the pK_a of the carboxyl group of amoxicillin 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 the 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, 1977) have demonstrated that the presence of the NH_3^+ amino group in ampicillin has exerted a marked catalysis effect on its dimerization at a pH range of 7.3–9.1 at 35 °C. The rate constant for this type of catalysis was 8.1×10^{-1} (mol dm^{-3})$^{-2}$ h^{-1} at 35 °C. Bundgaard (1977) also suggested this type of general catalysis could operate in dimerization reactions of amoxicillin as well. But, due to the limited solubility of amoxicillin at the pH values where the concentration of the protonated amino group would be significant, the investigation was not pursued. 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.

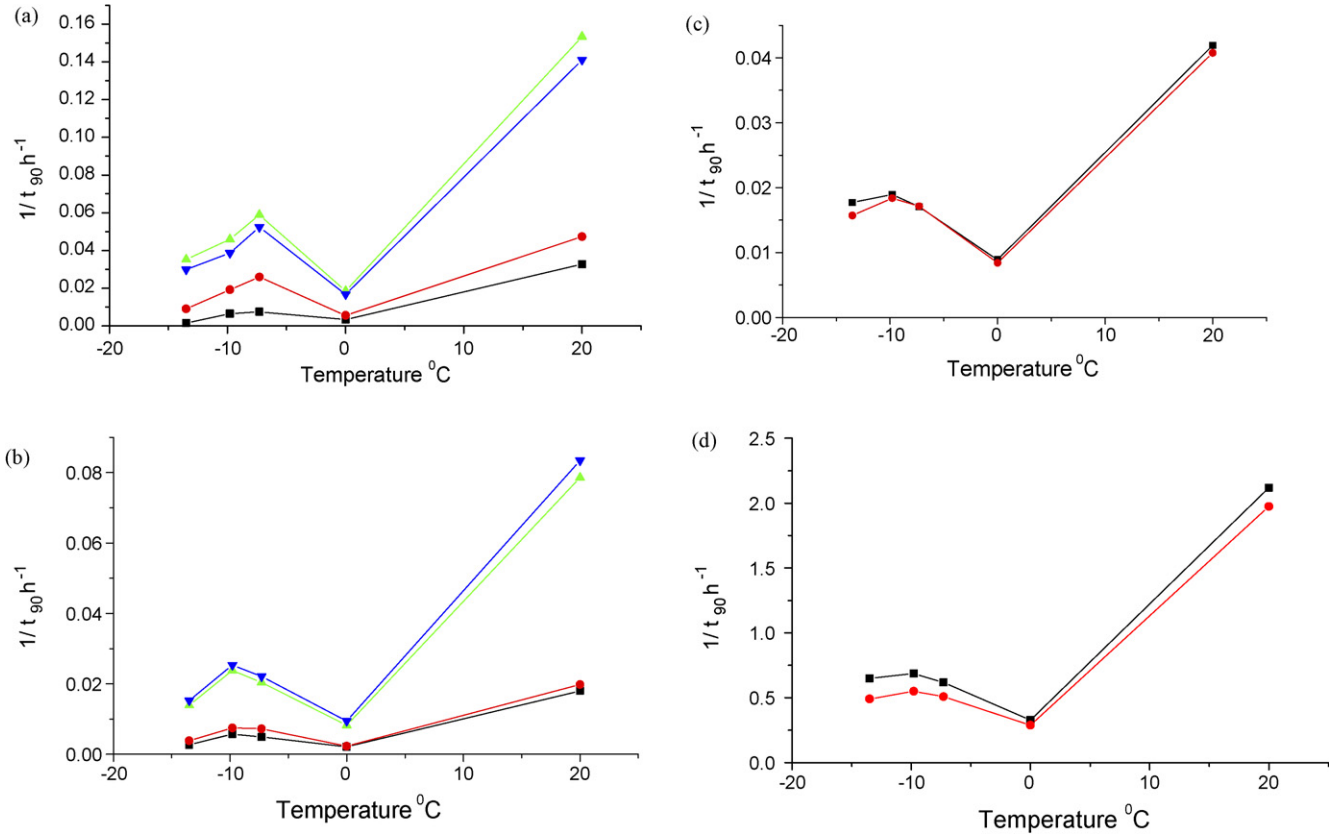

Fig. 3. Effect of freezing on the reciprocal values ($1/t_{90}$) of shelf-lives of amoxicillin and clavulanate in various systems. (a) In phosphate buffer pH 7.0, (b) in acetate buffer pH 4.6, (c) and (d) in hydrochloric acid system pH 2.0. *Note*: The liquid state data are obtained from the extrapolation of the liquid state data (Vahdat, 2000). (a) and (b) ■, AMOX: amoxicillin; ●, AMOX–COMB: amoxicillin in combination with clavulanate; ▲, CLAV: clavulanate; ▼, CLAV–COMB: clavulanate in combination with amoxicillin. (c) ■, AMOX: amoxicillin; ●, AMOX–COMB: amoxicillin in combination with clavulanate. (d) ■, CLAV: clavulanate; ●, CLAV–COMB: clavulanate in combination with amoxicillin.

3.2.4. Polymerization effects

The concentration-dependent degradation of amoxicillin is well documented (Bundgaard, 1977; Connors et al., 1986). Dimerization reactions of amoxicillin could occur to a limited extent at pH 7.00. In the liquid regions of the frozen solution, the concentration of amoxicillin increases significantly as the temperature decreases. 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 amoxicillin species can take part in dimerization reaction with another amoxicillin moiety. Thus as the apparent pK_a of the amino group is reported to be 7.55 at 23 °C, at pH 7.0 about 22% of free amino group is available. Therefore the presence of some unionized amino group in a concentrated solution of amoxicillin 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 by precipitation occurring at higher amoxicillin concentrations in the acetate system, which revealed evidence of polymerization reactions (Vahdat, 2000). The likelihood of such precipitation in phosphate buffer at higher amoxicillin concentration would be possible. However, owing to the difficulties in measuring kinetic runs that would ensure reliable results under these conditions, these experiments were not pursued.

3.2.5. Temperature effects

Rate constant values obtained under these experimental conditions were compared with theoretical rate constant values obtained by extrapolation of the liquid state data. The results (Table 2) indicate acceleration in the rates of degradation of amoxicillin and clavulanate under frozen temperatures. Fig. 3 further compares these results with the existing literature data, in terms of the reciprocal of shelf-life values versus temperature. These indicate that in buffer systems the rate–temperature profiles obtained for amoxicillin and clavulanate are similar with the literature data for benzyl penicillin (Larsen, 1971) and amoxicillin (Concannon et al., 1986; McDonald et al., 1989). However, the rate of change of reaction rates recorded in this study was not as marked as those of the other reported studies. This is because the buffer systems used in this study have greatly stabilized the system.

3.2.6. Effect of sodium chloride

Sodium chloride, which was used to maintain constant ionic strength in the liquid state runs, was found to have a stabilizing effect in the frozen state. Its use in these studies was restricted to the hydrochloric system since the buffer systems had already enhanced the stability of these solutions. Preliminary studies in the buffer systems with sodium chloride ($\mu = 0.5$) indicated no significant degradation ($\leq$10%) for up to 10 days of reaction for amoxicillin, thus sodium chloride was not incorporated in the buffer runs. Table 6 and Fig. 4 demonstrate these effects on amoxicillin sodium.

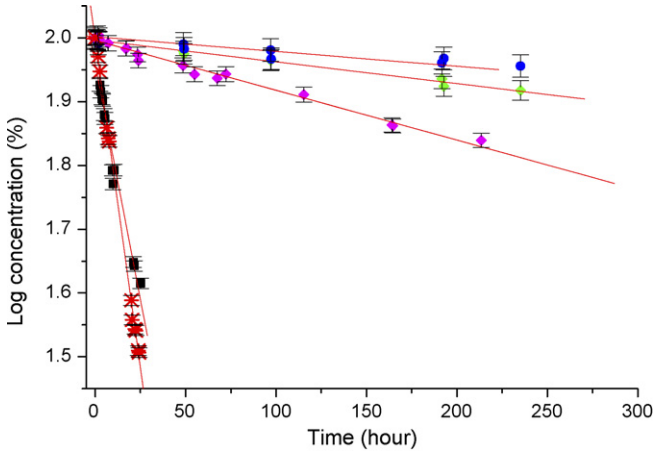

Fig. 4. Degradation of amoxicillin sodium in various solutions at −7.3 °C. Acetate: 2.2×10^{-1} mol dm^{-3} acetate buffer pH 4.6; phosphate: 10×10^{-1} mol dm^{-3} phosphate buffer pH 7.0; HCl with NaCl: 1.24×10^{-2} mol dm^{-3} hydrochloric acid with sodium chloride ($\mu = 0.5$), pH 2.0. Amoxicillin concentration: 9.03×10^{-4} mol dm^{-3} in hydrochloric acid and 1.29×10^{-3} mol dm^{-3} in the rest. ■, water; ◆, phosphate; ◆, acetate; ◆, hydrochloric acid with sodium chloride; ✳, hydrochloric acid without sodium chloride.

4. Conclusions

This study has identified several factors influencing the rate of amoxicillin and clavulanate decomposition in solution under frozen conditions. An evaluation of the experimental data obtained has provided possible explanations for the kinetic behaviour of this combination formulation.

Inclusion of sodium chloride commonly used for ionic strength control was found to have significant stabilizing effects. This effect was significant in the hydrochloric acid system where the shelf-life of amoxicillin was increased from 2.2 h in its absence to 58.7 h in its presence at −7.3 °C.

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

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