J. Pharm. Pharmacol. 1997, 49: 617–621
Received August 26, 1996
Accepted October 20, 1996

Influence of Hydroxypropyl β-Cyclodextrin on the Stability of Benzylpenicillin in Chloroacetate Buffer

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Abstract

Hydroxypropyl β-cyclodextrin (HPβCyD) has been shown to stabilize a wide variety of chemically distinct pharmaceutical entities through inclusion-complex formation between drug and cyclodextrin. The effect of HPβCyD on the acid-catalysed hydrolysis of benzylpenicillin (penicillin G) was evaluated in chloroacetate buffer at pH 2·20.

At penicillin G : cyclodextrin molar concentration ratios from 1:1 to 1:10, HPβCyD effected stabilization of penicillin G by 1·56- to 5·21-fold. At all temperatures, the observed first-order rate constant (k_{obs}) values assumed a non-linear, Michaelis–Menten type decrease as a function of increasing HPβCyD concentration. Degradation of penicillin G complexed with HPβCyD (penicillin G–HPβCyD), was approximately ninefold slower than uncomplexed penicillin G. The proportion of penicillin G degrading in either of these forms was, in turn, determined by the equilibrium constant for the complexation. The apparent thermodynamic and activation parameters for the complexation between penicillin G and HPβCyD have also been evaluated. The negative standard enthalpy change ($\Delta H°$) for the complexation implied that the penicillin G–HPβCyD complex would be predisposed towards enhanced stability, and thus the k_{obs} value for the hydrolysis of penicillin G decreased with reduction of temperature in these systems. The lack of difference between the enthalpies of activation ($\Delta H^{\ddagger}$) for the hydrolysis of uncomplexed and complexed penicillin G seemed to be compensated by the significant difference between the entropies of activation ($\Delta S^{\ddagger}$) for these hydrolytic reactions.

The results indicate that HPβCyD represents a viable means of stabilization of penicillin G solutions at the pH employed in this study.

Penicillin G undergoes hydrolysis of the β-lactam ring in aqueous solution. Complex formation between penicillin G and β-cyclodextrin (β-CyD) at acidic pH has been shown to stabilize penicillin G (Mizukami et al 1978). Hydroxypropyl β-cyclodextrin (HPβCyD), a chemically modified derivative of β-CyD in which some of the hydroxyl groups on the cyclodextrin molecule are substituted by hydroxypropyl groups (Irie et al 1988; Brewster et al 1991), has the advantage over β-CyD of much higher water-solubility (Yoshida et al 1988; Brewster et al 1989; Bekers et al 1991; Loftsson 1995) and an extended surface area for complexation with molecules (Yoshida et al 1988; Uekama & Irie 1990; Brewster et al 1991). Recent literature (Loftsson 1995) has reported the increased stability of a variety of chemically distinct pharmaceutical compounds as a result of complex formation with HPβCyD. For instance, the hydrolysis of cephalothin complexed with HPβCyD, was slower than that of the uncomplexed cephalosporin by 4·8-fold at pH 6·6 (Loftsson 1995).

It is the aim of this study to evaluate the effect of HPβCyD on the acid-catalysed hydrolysis of penicillin G in chloroacetate buffer at pH 2·20 over the temperature range 5 to 15°C. Temperature-dependence data for this hydrolytic reaction in the absence and presence of HPβCyD, and the equilibrium reaction of complex formation, will be determined from the kinetic data obtained.

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Materials and Methods

The chemicals used were acetic acid, glacial (Univar AR, Ajax Chemicals), acetonitrile (HPLC Grade, Unichrom, Acetonitrile-190, Ajax Chemicals), benzylpenicillin sodium, British Pharmacopoeia Chemical Reference (BPCR) Standard (British Pharmacopoeia Commission Laboratory), benzylpenicillin for injection, containing benzylpenicillin sodium (Commonwealth Serum Laboratories), glycine-hydrochloric acid buffer pH 1·0 (Merck), citrate-hydrochloric acid buffer pH 2·0 (Merck), 0·05 M potassium hydrogen phthalate buffer pH 4·0 (BDH Chemicals), chloroacetic acid (Ajax Chemicals), D-(+)-glucose (AG, Riedel-de-Haën), hydroxypropyl β-cyclodextrin, average degree of molar substitution ≈7 (experimental use, American Maize Products), methyl 4-hydroxybenzoate (LR, BDH Chemicals), methanol (HPLC Grade, Unichrom C2314, Ajax Chemicals), sodium chloride (Univar AR, BDH Chemicals), water (deionized by passage through a Milli Q-apparatus, specific conductivity less than $5{\cdot}5 \times 10^{-8}\ \Omega^{-1}\ cm^{-1}$ at 25°C, Millipore Corporation).

A commercial sample of penicillin G was analysed against a BPCR standard (99·3% w/w), and a concentration of $4{\cdot}21 \times 10^{-3}$ M of this sample in chloroacetate buffer, adjusted to a constant value of ionic strength, $\mu = 0{\cdot}50$, by use of sodium chloride, was used for kinetic studies. When required, HPβCyD at concentrations 4·21 to 42·1 mM, corresponding to molar concentration ratios of penicillin G to HPβCyD from 1:1 to 1:10, was added to these solutions. The solutions were equilibrated at temperatures appropriate to the kinetic studies,

in water-baths maintained to within ±0·1°. The pH values of solutions were measured initially and during the course of reaction.

The stability of penicillin G was monitored by HPLC assay of the amount of penicillin G remaining with time over 3 to 4 half-life ($t_{\frac{1}{2}}$) periods of reaction; methyl 4-hydroxybenzoate was added as the internal standard. The mobile phase consisted of 32·0% v/v acetonitrile and 1% v/v glacial acetic acid in water. A 20-μL Rheodyne loop injector was used in conjunction with a 3·9 mm × 300 mm μBondapak C_{18} reversed-phase column (Waters Associates). At a flow rate controlled at 1·8 mL min^{-1}, typical retention times were 7·3 and 4·9 min for the penicillin G and methyl 4-hydroxybenzoate, respectively. Areas under the curves (AUC) were recorded on a model HP3396A Hewlett-Packard integrator in conjunction with a variable-wavelength UV detector (Waters Associates single channel, tuneable UV–Vis absorbance detector, Model 484).

The HPLC assay was verified at $\lambda = 234$ nm using standard solutions of the same sample of penicillin G as was used in kinetic studies over the concentration range 0·0337 to 1·68 mM, where linearity ($r > 0.999$) was established. The precision of the assay was described by a coefficient of variation ($n = 6$), of ±1·20% or lower. The stability-indicating nature of the assay was established under experimental conditions by inducing degradation of penicillin G in water, acid and alkali at elevated temperatures of 45 to 50°C, in which addition of penicillin G to the completely degraded samples restored the peak area at the initial retention time and concentration.

In kinetic studies, the penicillin G remaining was calculated by dividing the peak-area ratio of penicillin G to methyl 4-hydroxybenzoate obtained at each sampling station, by the ratio obtained at time zero, which was arbitrarily designated as 100%, and expressing this quotient as a percentage. Where the actual concentration of the penicillin in a sample was desired, this was obtained by referring to the penicillin G sodium salt standard curve.

Samples containing HPβCyD, at the highest concentration used in kinetic studies, did not interfere with the areas under the curves of the penicillin G and methyl 4-hydroxybenzoate (and thus the peak area ratio) or with the retention times of such peaks when analysed after dilution and addition of the internal standard described previously. Linearity of response ($r = 0.999$) was obtained for standard solutions of penicillin G containing HPβCyD at 1·68 mM. Analysis of variance for the slopes obtained in the absence and presence of HPβCyD showed a significant difference ($P = 0.05$). However, the magnitude of these slopes differed by only 1·79%, and did not therefore affect the calculation of concentration of penicillin G remaining, because these were all referred to the value at time zero.

All reactions were studied under pseudo first-order conditions of reaction in respect of penicillin G. Plots of the logarithm of penicillin G concentration as a function of time were fitted by least-squares analysis. Where applicable, comparisons of the observed first-order rate constant (k_{obs}) values were made at the 95% confidence interval of the rate constants or mean values of replicate determinations thereof. Student's *t*-tests were also performed on these parameters, and comparisons made at the 0·05 level of significance (i.e. $P = 0.05$). The Bonferroni adjustment (Anderson et al 1994) was used where multiple comparisons of rate constants were made.

Results and Discussion

The hydrolysis of penicillin G followed first-order kinetics in respect of penicillin G over three to four $t_{\frac{1}{2}}$ of reaction at all temperatures investigated. This hydrolysis was not catalysed by the buffer. Introduction of HPβCyD did not affect the observed kinetic order. First-order plots at all temperatures studied typically exhibited linearity, giving high correlation coefficients ($r = 0.998$–0.999); some typical plots at 5°C are shown in Fig. 1. The pH values of solutions were maintained constant throughout the course of kinetic studies at all temperatures, in the absence and presence of HPβCyD.

The influence of HPβCyD was evaluated by comparing k_{obs} values determined in the presence of the cyclodextrin with that in its absence, in chloroacetate buffer, at each temperature. The degree of stabilization was dependent on the concentration of HPβCyD used and on the temperature, as is shown in Table 1. At all temperatures k_{obs} values assumed a non-linear, Michaelis–Menten type decrease as a function of increasing HPβCyD concentration, as shown in Fig. 2. Larger increases in stability were observed up to a molar ratio of penicillin G to HPβCyD of 1:5. This observation was consistent with reports of the retardation of the hydrolytic degradation of a number of pharmaceuticals by HPβCyD (Loftsson et al 1989; Backensfeld et al 1990; Pop et al 1991; Bekers et al 1993; Choudhury & Mitra 1993; Loftsson 1995; Roy & Guillory 1996). A maximum increase in stability of penicillin G, of 5·21-fold, was observed at 5°C, at a penicillin to cyclodextrin molar ratio of 1:10.

On the other hand, a study conducted at 15°C on the effect on penicillin G hydrolysis of D-(+)-glucose at concentrations of 14·7 to 294·6 mM, concentrations identical with those that would be obtained on complete hydrolysis of 2·10 to 42·1 mM HPβCyD, showed that D-(+)-glucose did not stabilize penicillin G (k_{obs}, $P = 0.05$). These results are shown in Fig. 2. Hydrolysis of HPβCyD would not occur under the current experimental conditions (Bekers et al 1991). The current results, and reports of the negligible influence of glucose on the degradation of penicillin G in acidic media below pH 5·4

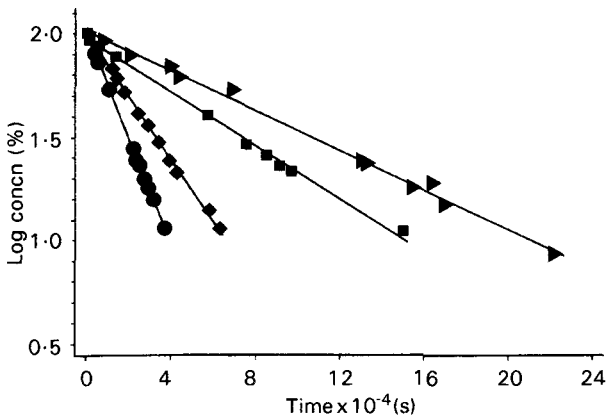

FIG. 1. Typical first-order plots for the hydrolysis of penicillin G in chloroacetate buffer (0·1 M, pH 2·20, $\mu = 0.50$) at 5°C. ● Penicillin G only, ◆ penicillin G:HPβCyD, 1:1 (molar ratio), ■ penicillin G:HPβCyD, 1:8·5, ▲ penicillin G:HPβCyD, 1:10.

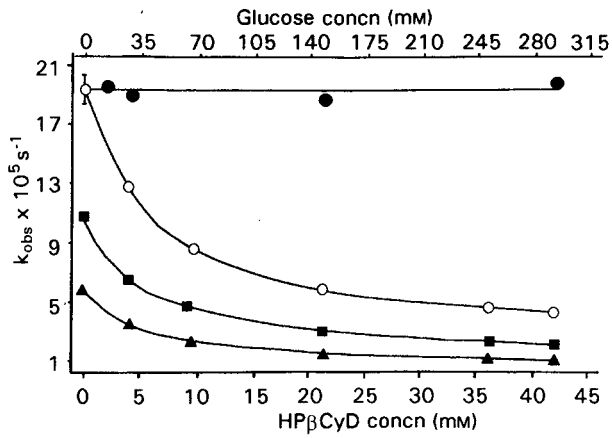

FIG. 2. Plots of k_{obs} for the hydrolysis of penicillin G in chloroacetate buffer (0·1 M, pH 2·20, $\mu = 0{\cdot}50$) as a function of HPβCyD concentration at 5°C (▲), 10°C (■) and 15°C (○) and showing the influence of glucose on k_{obs} at 15°C (●).

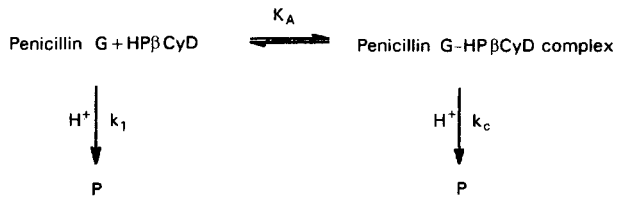

FIG. 3. Schematic representation of 1:1 stoichiometric complexation between penicillin G and HPβCyD, and the pathways of degradation of penicillin G in the uncomplexed and complexed forms. k_1 = observed pseudo first-order rate constant for hydrolysis of penicillin G in the absence of HPβCyD, k_c = pseudo first-order rate constant for hydrolysis of penicillin G complexed with HPβCyD, K_A = equilibrium constant for the complex formation between penicillin G and HPβCyD, P = degradation products formed from the hydrolysis of uncomplexed and complexed penicillin G, assuming these to be similar.

(Lundgren & Landersjö 1970), serve to support the observation of stabilization arising from the particular interactions of HPβCyD with penicillin G.

Stabilization effects of HPβCyD on penicillin G were further analysed through a model shown in Fig. 3, which is descriptive of the reversible, non-covalent interaction of penicillin G in the ionized and unionized forms with HPβCyD to give a penicillin G–β-cyclodextrin complex in solution. Such a model is based on the assumption of 1:1 complexation stoichiometry of penicillin G and HPβCyD. The observed rate constant for the hydrolysis of penicillin G in the absence of HPβCyD, is denoted k_1, whereas the slower rate constant for penicillin G degradation in the complexed form is given by k_c. The experimentally determined k_{obs} values at each cyclodextrin concentration, shown in Table 1, were therefore weighted means of k_1 and k_c (Loftsson 1995), the proportion of penicillin G in the uncomplexed and complexed forms being determined by the equilibrium constant for the complexation reaction, K_A. Mathematically, the relationship between the pseudo first-order rate constants in the model (Fig. 3) can be represented as in equation 1 shown below:

$$k_1 - k_{obs} = \{K_A[CD](k_1 - k_c)\}/(1 + K_A[CD]) \quad (1)$$

The applicability of the model was tested through linearization of equation 1 according to modified Scott, Lineweaver–Burk or Scatchard equations for studies of molecular complexes previously described in the literature (Connors & Mollica 1966; Cohen & Connors 1970; Connors 1987). Linearity ($|r| > 0{\cdot}999$) of these analyses indicated 1:1 complexation stoichiometry as assumed, over the concentrations of HPβCyD used, as illustrated in Fig. 4. Values of K_A and k_c obtained from the three linear variants of equation 1 at each temperature were similar ($P = 0{\cdot}05$). These values obtained through application of the modified Scott equation are given in Table 2. The smaller magnitudes of k_c compared with k_{obs}, indicated protection of penicillin G by complex formation. Degradation of penicillin G in the complexed form was thus approximately ninefold slower than that of uncomplexed penicillin G.

The temperature-dependence of K_A was analysed using the van't Hoff equation (Martin 1993a), where linearity ($r = 0{\cdot}999$) was obtained. The negative enthalpy change ($\Delta H°$) of $-13{\cdot}13 \pm 0{\cdot}88$ kJ mol^{-1} for K_A indicated that the complexation process was exothermic. This implied that penicillin G in the complexed form with HPβCyD (penicillin G–HPβCyD) would be predisposed to enhanced stability, with a resulting decrease in the k_{obs} value for the hydrolysis of penicillin G with reduction of temperature in these systems (Loftsson 1995). The negative $\Delta H°$ (Table 2) is consistent with reports in the literature on the complexation of HPβCyD and β-CyD with carbenicillin (Mizukami et al 1978) and several chemically distinct pharmaceuticals, in which retardation of hydrolysis was effected by these cyclodextrins (Loftsson et al 1989; Bettinetti et al 1991; Pop 1991; Hoshino et al 1993). The small negative entropy change ($\Delta S°$) of $-3{\cdot}14 \pm 3{\cdot}09$ J mol^{-1} K^{-1}, can be interpreted as a loss in entropy as a result of the reduced vibrational, rotational and translational degrees

Table 1. Observed pseudo first-order rate constants (k_{obs}) for the hydrolysis of penicillin G in chloroacetate buffer (0.1 M, pH 2·20, $\mu = 0{\cdot}50$) in the absence and presence of hydroxypropyl β-cyclodextrin at 5, 10 and 15°C·

System	Pseudo first-order rate constant $k_{obs} \times 10^5$ (s^{-1})		
	5°C	10°C	15°C
Penicillin G	5·85 ± 0·13*	10·78 ± 0·15*	18·90 ± 0·59*
Penicillin G + hydroxypropyl β-cyclodextrin, 1:1†	3·48	6·40	12·12
Penicillin G + hydroxypropyl β-cyclodextrin, 1:2·25	2·44	4·90	8·60
Penicillin G + hydroxypropyl β-cyclodextrin, 1:5	1·57	3·09	5·66
Penicillin G + hydroxypropyl β-cyclodextrin, 1:8·5	1·35	2·45	4·53
Penicillin G + hydroxypropyl β-cyclodextrin, 1:10	1·12	2·23	4·15

*Mean of three determinations with errors expressed at the 95% confidence interval. † Molar concentration ratio of penicillin G : hydroxypropyl β-cyclodextrin.

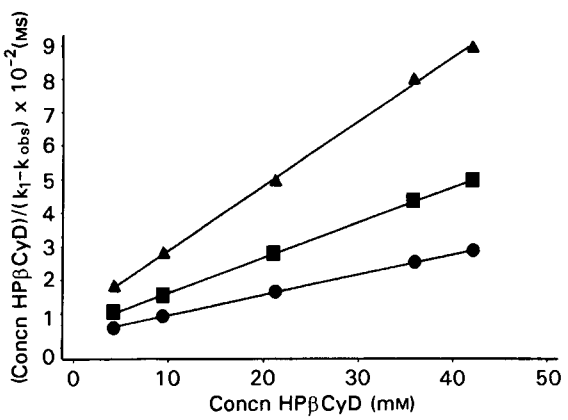

FIG. 4. Scott plots using kinetic data for the hydrolysis of penicillin G in the presence of HPβCyD in chloroacetate buffer (0·1 M, pH 2·20, $\mu = 0{\cdot}50$) to determine the complexation constant, K_A, between the penicillin and HPβCyD, and rate constant, k_c, for the hydrolysis of this complex at 5°C (▲),10°C (■) and 15°C (●).

of freedom in the association of two molecules (Gelb et al 1979; Szejtli 1988; Bekers et al 1991; Fromming & Szejtli 1993), giving a state of higher order (Loftsson et al 1989). The orientation of water of hydration molecules around the penicillin G–β-cyclodextrin complex, has also been proposed as contributing towards a more ordered state (Lewis & Hansen 1973; Hardee et al 1978; Szejtli 1988; Fromming & Szejtli 1993). The small magnitudes of $\Delta H°$ and $\Delta S°$ values were consistent with reports in the literature (Mizukami et al 1978; Bettinetti et al 1991; Hoshino et al 1993), and are indicative of the rather non-specific nature of the complex formed between penicillin G and HPβCyD via weak forces of interaction such as van der Waals forces, hydrophobic and hydrogen bonding (Cramer et al 1967; Lewis & Hansen 1973; Saenger 1988; Szejtli 1988; Bekers et al 1991; Otero-Espinar et al 1992; Fromming & Szejtli 1993), and the changes in hydration associated with this complexation (Hardee et al 1978). The $\Delta G°$ values calculated at 5, 10 and 15°C were negative, showing that the equilibrium of complex formation was spontaneous and that the unfavourable $\Delta S°$ was compensated for by the $\Delta H°$ (Martin 1993b).

The Arrhenius temperature-dependencies for the hydrolysis of penicillin G in the absence and presence of HPβCyD, were evaluated over 5 to 15°C. Activation parameters for these reactions, employing the transition-state theory of temperature-dependence of reaction rates (Martin 1993c), have also been determined using the Eyring equation. Values of the activation

Table 2. Pseudo first-order rate constants (k_c) for the hydrolysis of hydroxypropyl β-cyclodextrin-complexed penicillin G and the equilibrium constants, K_A, for the complexation reaction at 5, 10 and 15°C.

Temperature (°C)	$k_c \times 10^6$ (s^{-1})*	$K_A \times 10^{-2}$ (M)*
5	6·15 ± 3·19	1·99 ± 0·45
10	11·39 ± 4·07	1·82 ± 0·28
15	20·18 ± 8·27	1·64 ± 0·12

*Errors in k_c and K_A are expressed at the 95% confidence interval.

energy (E_a), enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) for the hydrolytic reactions are shown in Table 3. The E_a of 79·63 kJ mol^{-1} for the hydrolysis of penicillin G in the absence of HPβCyD agreed well with a value at pH 2·7 reported in the literature (Blaha et al 1976). There appeared to be a weak trend of an increase in E_a and $\Delta H^{\ddagger}$ on increasing the HPβCyD concentration. An increase in E_a and $\Delta H^{\ddagger}$ values of 9·42% and 9·70%, respectively, for the hydrolysis of penicillin G in the presence of HPβCyD at a 1:10 penicillin G : HPβCyD molar ratio, compared with that in the absence of the cyclodextrin, represented a significant change. This magnitude of increase in E_a was typically seen in kinetic studies, reported in the literature, on the hydrolysis of several pharmaceutical entities and substituted organic acids in the presence of β-CyD, HPβCyD and other cyclodextrins (Uekama et al 1981; Bekers et al 1989; Backensfeld et al 1990).

However, when these parameters were determined for the hydrolysis of the penicillin G–cyclodextrin complex from k_c data, no difference from those values obtained in the absence of HPβCyD was found. This might indicate that the mechanism of hydrolysis of penicillin G was probably unchanged in the presence of HPβCyD, as suggested in the literature (Vander Jagt et al 1970; Choudhury & Mitra 1993). On the other hand, a similar comparison made with $\Delta S^{\ddagger}$ showed an increased negative change. These results are indicative of the retardation of the hydrolysis of penicillin G being effected through the favourable reduction in $\Delta S^{\ddagger}$ (Martin 1993c) in the presence of HPβCyD. This result was in agreement with a number of studies in the literature whereby a lack of change in $\Delta H^{\ddagger}$ in the hydrolysis of the complexed pharmaceutical, had been compensated by a marked change in $\Delta S^{\ddagger}$, so that a resultant retardation of hydrolysis was effected (Loftsson et al 1989; Pop et al 1991). Protection of penicillin G by HPβCyD, might therefore be hypothesized as arising from a steric effect,

Table 3. Activation parameters for the hydrolysis of penicillin G in chloroacetate buffer (0·1 M, pH 2·20, $\mu = 0{\cdot}50$) in the absence and presence of hydroxypropyl β-cyclodextrin at 5, 10 and 15°C.

	E_a (kJ mol^{-1})*	$\Delta H^{\ddagger}$ (kJ mol^{-1})*	$\Delta S^{\ddagger}$ (J K^{-1} mol^{-1})*
Penicillin G	79·63 ± 2·16	77·29 ± 2·15	− 47·58 ± 7·67
Penicillin G + hydroxypropyl β-cyclodextrin, 1:1†	83·44 ± 4·08	80·82 ± 3·75	− 39·20 ± 13·25
Penicillin G + hydroxypropyl β-cyclodextrin, 1:2·25	83·91 ± 8·60	81·56 ± 8·63	− 39·27 ± 30·50
Penicillin G + hydroxypropyl β-cyclodextrin, 1:5	85·50 ± 4·15	83·14 ± 4·16	− 37·34 ± 14·70
Penicillin G + hydroxypropyl β-cyclodextrin, 1:8·5	80·80 ± 3·10	78·31 ± 3·23	− 56·08 ± 11·42
Penicillin G + hydroxypropyl β-cyclodextrin, 1:10	87·13 ± 3·20	84·79 ± 3·20	− 34·24 ± 11·32
Penicillin G − hydroxypropyl β-cyclodextrin, complex	79·62 ± 1·22	77·27 ± 1·23	− 66·26 ± 4·35

*Errors are expressed at the 95% confidence interval.

rendering the approach of catalytic H^+ species from the aqueous bulk solution to the β-lactam group, more difficult and less probable (Martin 1993c). In terms of the transition state theory of reaction rates (Martin 1993c), the hydrolytic reaction of the complexed penicillin G can be considered as proceeding through the formation of an activated state in which considerable rearrangement of the structure of reactant molecules was required. Because this transition state represented a less probable structure (Martin 1993c), there was a resultant retardation of the hydrolytic reaction to form degradation products of penicillin G.

Introduction of HPβCyD therefore represented a viable means of stabilization of penicillin G solutions at the pH employed in this study.

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