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Effect of cyclodextrin complexation on the chemical stability of doxorubicin and daunorubicin in aqueous solutions

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Summary

The influences of cyclodextrins on the chemical stability of the antineoplastic drugs doxorubicin and daunorubicin in aqueous media have been studied using a stability-indicating high-performance liquid chromatographic method. Various parameters, such as cyclodextrin structure, pH, structure of the anthracycline, cyclodextrin concentration and the presence of a co-solvent, were investigated. In the acidic region, the degradation rates of both doxorubicin and daunorubicin decrease in the presence of γ-cyclodextrin, whereas α- and β-cyclodextrin show no effect in acid as well as in alkaline media. Above pH 4 the degradation of daunorubicin is accelerated by γ-cyclodextrin, while for doxorubicin this effect is only observed in strong alkaline solutions. On complexation, the order of the reactions as well as the degradation mechanisms of the anthracyclines do not change. In the presence of acetonitrile the anthracycline-γ-cyclodextrin complexes decompose because the organic co-solvent is embedded in the cavity of the cyclodextrin host.

Introduction

Cyclodextrins (CyD) (Fig. 1) are cyclic oligosaccharides, containing D(+)-glucose units attached by α-1,4 linkages. α-, β- and γ-CyD are naturally occuring CyD consisting of six, seven and eight glucose molecules, respectively, forming cavities with diameters of about 5–9 Å. CyD can form inclusion complexes with various molecules (Uekama and Otagiri, 1987; Szejtli, 1988). This complexation may lead to the use of CyD as an excipient in pharmaceutical applications, for example, to improve the aqueous solubility (Szejtli, 1987), chemical stability (Duchêne et al., 1985) or bioavailability (Jones et al., 1984) of a drug.

The anthracycline group of antibiotics are potent agents which are widely used in cancer chemotherapy (Arcamone, 1984). Doxorubicin (Dx) and daunorubicin (Dr) are the main representatives of this class of cytostatic drugs. The chemical structure of the anthracycline molecule consists of a tetrahydronaphthacenequinone aglycone part, glycosidically linked to an amino-

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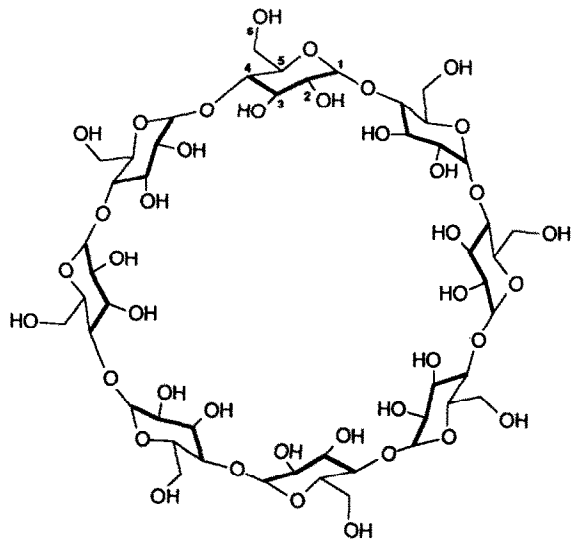

Fig. 1. Chemical structure of γ-cyclodextrin.

sugar (Fig. 2). This molecule is not stable in aqueous solutions (Beijnen et al., 1985; 1986a,b). Therefore, Dx and Dr are marketed as freeze-dried formulations which necessitates the drugs to be re-dissolved before administration. An injection fluid as the formulation has several advantages among which the ease of handling and administration. Stabilization of aqueous solutions of Dx, Dr and related anthracyclines may offer a new approach to develop a direct injectable formulation.

The present paper describes a systematic study of the influence of CyD complexation on Dx and Dr stability in aqueous media over a wide pH range.

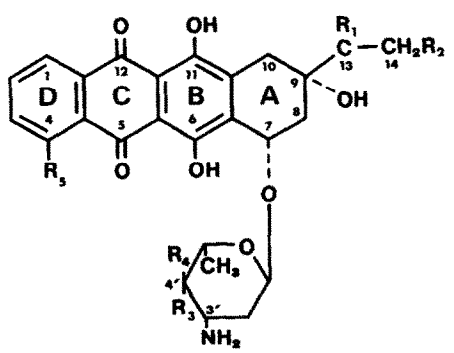

Fig. 2. Structures of some anthracycline antibiotics.

	R1	R2	R3	R4	R5
doxorubicin	=O	OH	OH	H	OCH_3
4′-epidoxorubicin	=O	OH	H	OH	OCH_3
4′-deoxydoxorubicin	=O	OH	H	H	OCH_3
daunorubicin	=O	H	OH	H	OCH_3
4-demethoxyduanorubicin	=O	H	OH	H	H
carubicin	=O	H	OH	H	OH

Experimental

Chemicals

Dx, 4′-epidoxorubicin (4′-epiDx), 4′-deoxydoxorubicin (4′-deoxyDx) and 4-demethoxydaunorubicin (4-demethoxyDr) were generous gifts from Dr S. Penco (Farmitalia, Milan, Italy). Dr was kindly provided by Rhône-Poulenc Nederland BV (Amstelveen, The Netherlands) and carubicin was obtained from Bristol Myers (New York, U.S.A.). The anthracyclines were supplied as hydrochloride salts. The CyD came from Nihon Shokukin Kako Co. Ltd (Tokyo, Japan) and were used as received. All other chemicals were of analytical grade and deionized water was obtained by filtration through a Milli-Q-Water Purification System (Millipore, Bedford, MA, U.S.A.).

Buffer / CyD solutions

For the kinetic studies the following aqueous solutions were used: H_0/pH 0.5–3: perchloric acid, pH 3–6: acetate, pH 6–9: phosphate, pH 9–11: carbonate. The pH values between 1 and 11 were measured at the temperature of study with a glass reference electrode and a pH meter (Metrohm, E512, Titriskop, Herisau, Switzerland). H_0 values below 1 were calculated by using the Hammet acidity function (Bates, 1973).

A constant ionic strength ($\mu = 0.3$), adjusted with sodium chloride, was maintained for each degradation solution. For the anthracycline degradation studies, where the influences of CyD and acetonitrile (ACN) were investigated, various amounts of CyD and/or ACN were added to the present buffer solution and the pH was adjusted to the desired value. The buffer/CyD solutions were always prepared freshly before use.

Kinetic measurements

The kinetic experiments were performed at a constant temperature of 50 ± 0.2°C whereby the reaction vials were protected from light. The reactions were initiated by adding 100 μl of an anthracycline stock solution (1 mg/ml) in water, to 5 ml of a pre-heated buffer/CyD solution to give an initial concentration of 3.5×10^{-5} M.

In order to obtain a reproducible degradation pattern it is important that some precautions are taken in preparing and handling of the degradation media (Beijnen et al., 1986a). Disodium edetate (4×10^{-5} M) was added to complexate traces of metal ion impurities which may catalyze the degradation. Oxygen catalyzes the anthracycline degradation at pH > 8. Therefore, during pre-heating, the buffer/CyD solutions were purged with nitrogen for 5 min and after addition of the anthracycline the reaction flasks were closed quickly, under a nitrogen atmosphere, with a rubber septum and cap. Adsorption of anthracyclines and their degradation products onto glass walls also introduces a complicating factor, especially in the pH region 4–9. This problem could be eliminated by silanization of the glassware with trimethylsilane in toluene (3%, v/v) and subsequent rinsing with methanol.

The influences of different CyD on anthracycline degradation were investigated at a CyD concentration ([CyD]) of 1.6×10^{-2} M. For the construction of the log k_{obs}-pH profiles, [γ-CyD] was kept constant at 1.6×10^{-2} M, whereas for the study of the influence of [γ-CyD] on the degradation the concentration varied from 0 to 4.8×10^{-2} M.

At appropriate time intervals, samples of 20 μl were taken and analyzed directly for the content of undegraded anthracycline with a stability-indicating high-performance liquid chromatography (HPLC) method (Beijnen et al., 1985, 1986a). Samples from degradation solutions between pH values 4 and 10 were placed in polypropylene vessels and acidified to pH 3 with perchloric acid (70%, w/v). These samples were stored at −20°C until analysis. No anthracycline degradation was observed during storage under these conditions for a period of at least 3 months.

HPLC

The liquid chromatograph consisted of a Model 510 solvent delivery system, a U6K injector (both from Waters Assoc., Milford, MA, U.S.A.), a Spectroflow 773 absorbance detector (Kratos, Ramsey, U.S.A.), operating at 480 nm and a Merck-Hitachi F-100 fluorescence spectrophotometer (Tokyo, Japan) with λ(excitation) = 465 nm and λ(emission) = 550 nm. Injection of series thawed samples of 20 μl took place with a Gilson Auto-sampling injector Model 231-401 (Villiers-le-Bel, France). Quantitation of undegraded anthracycline was based on peak area measurements using an SP-4270 integrator (Spectra Physics, San José, U.S.A.). In the concentration range of interest (3.5×10^{-6}–3.5×10^{-5} M) standard curves of the anthracyclines showed linear responses.

Separations were achieved on a pre-packed stainless-steel column (12.5 cm × 4.0 mm i.d.), containing Lichrosorb RP8 (5 μm) material (Merck, Darmstadt, Germany). The eluent consisted of 0.01 M sodium chloride (pH 2.25)/ACN (70 + 30, v/v) and (60 + 40, v/v) for Dx and Dr analyses, respectively. The flow rate of the mobile phase was 1.0 ml/min. All chromatographic analyses were carried out at ambient temperature.

Results and Discussion

Analytical procedure

Anthracyclines tend to adsorb onto glass materials (Tomlinson and Malspeis, 1982), this process occurring especially at pH values between 4 and 9 where the compound exists, at least partially, as a neutral molecule. Beijnen et al. (1986a,b, 1987) have demonstrated that degradation products also show strong adsorption. In the presence of CyD (concentration $> 4 \times 10^{-3}$ M), the adsorption of the anthracyclines and their degradation products onto glass walls is strongly diminished. This was observed both visually, because the pink coating on the glass walls no longer occurred, and quantitatively, since the reproducibility of HPLC analysis of an anthracycline solution at pH 7.5 is greatly enhanced, the relative standard deviation obtained for ten replicate in-

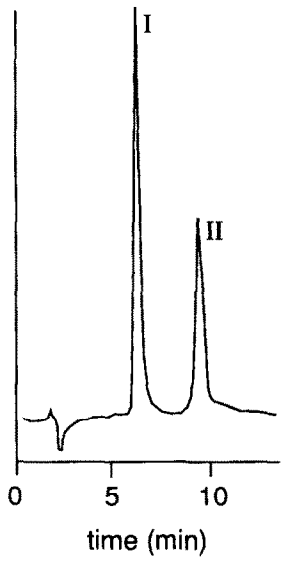

Fig. 3. HPLC chromatogram of a partially degraded doxorubicin-γ-CyD solution at pH 1. I, doxorubicin; II, doxorubicinone. For conditions see text.

jections decreasing from 12.2% to 2.6% in the presence of CyD, indicating that the anthracycline is set free from the glass. This means that silanization in these cases becomes unnecessary.

With the HPLC method the parent compounds are well separated from their degradation products (Figs 3 and 4).

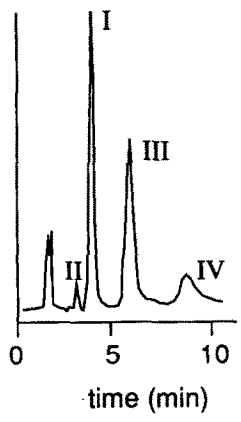

Fig. 4. HPLC chromatogram of a partially degraded daunorubicin-γ-CyD solution at pH 10. I, daunorubicin; II–IV are degradation products. For conditions see text.

Degradation products

At pH < 4, Dx and as Dr initially degrade into doxorubicinone and daunorubicinone, respectively, and the amino-sugar daunosamine (Beijnen et al., 1985). This is illustrated in Fig. 3 with Dx as an example. At pH values > 4, the degradation pattern of anthracyclines is more complex with the formation of different products (Beijnen et al., 1986a,b, 1987). Fig. 4 shows a typical chromatogram of a partly degraded Dr solution at pH 10. It il obvious that the degradation leads to a number of products. In previous studies performed by Beijnen et al. (1986a, 1987) the structures of these degradation products were elucidated. It is noteworthy that the anthracycline degradation products show, in the presence of CyD, distinct and well-shaped peaks in the HPLC chromatograms, which has hitherto not been reported in the literature.

Degradation kinetics

The degradation of both anthracyclines follows a pseudo first-order kinetic pattern. The rate equation for Dx can be written as:

$$\ln[\mathrm{Dx}]_t = \ln[\mathrm{Dx}]_0 - k_{\mathrm{obs}}t \qquad (1)$$

where $[\mathrm{Dx}]_t$ represents the concentration of Dx at time t, $[\mathrm{Dx}]_0$ the initial concentration and k_{obs} the pseudo first-order observed rate constant for the Dx degradation. The order of the reaction is indicated by the linearity ($r > 0.999$) in the $\ln[\mathrm{Dx}]_t$ vs time plots over at least three half-lives. Addition of CyD does not change this kinetic behaviour, since the linearity of $\ln[\mathrm{Dx}]_t$ vs time plots obtained in the presence of various CyD is essentially the same. Because the presence of CyD alters neither the number nor the kind of degradation products, it can be concluded that the degradation mechanism for both anthracyclines is the same whether or not CyD is present.

Standard deviation in k_{obs}

The standard deviation (SD) in k_{obs} has been determined for Dr at pH 7.5 and a buffer concentration of 5×10^{-3} M in the presence ([γ-CyD] $= 1.6 \times 10^{-2}$ M) and absence of γ-CyD. The values for $k_{\mathrm{obs}} \pm$ SD are $6.3 \pm 0.2 \times 10^{-7}$ s^{-1} ($n = 6$)

TABLE 1

Influence of various CyD ([CyD] = 1.6×10^{-2} M) on the degradation of doxorubicin (Dx) and daunorubicin (Dr) at pH 10; T = 50°C; μ = 0.3

CyD	k_{obs} (s^{-1})	
	Dx	Dr
-	1.3×10^{-3}	2.1×10^{-5}
α	1.5×10^{-3}	2.4×10^{-5}
β	1.5×10^{-3}	2.8×10^{-5}
γ	1.4×10^{-3}	2.3×10^{-4}

and $2.0 \pm 0.3 \times 10^{-7}$ s^{-1} ($n = 6$), respectively. All other rate constants are mean values of duplicate measurements.

Effect of cyclodextrin structure

The effects of α-, β- and γ-CyD on the degradation rates of Dx and Dr have been studied at pH 10. From Table 1 it can be concluded that, under the chosen experimental conditions, α-, β- and γ-CyD have no effect on Dx stability. On the other hand, the degradation of Dr is accelerated in the presence γ-CyD, whereas α- and β-CyD show no effect in this case. This observation and earlier results demonstrating that in acidic media the anthracyclines complex only with γ-CyD (Bekers et al., 1988) rationalize a detailed study on the effects of γ-CyD.

Influence of pH

The pH profiles in the region 0.5–11 of Dx and Dr degradation, corrected for buffer influences (Beijnen et al., 1986a,b) yielding k' values, were compared with similar profiles for the drug in the presence of γ-CyD. As can be seen from Figs 5 and 6, for Dx and Dr, respectively, in the pH range 0–3.5, both anthracyclines show identical degradation profiles whereas γ-CyD also shows similar stabilizing effects. Plots of k_{obs} vs $[H^+]$ yield straight lines ($r > 0.99$) for both anthracyclines irrespective of whether CyD are present. The slopes of these lines provide the second-order rate constant for the proton-catalyzed degradation (k_H). Table 2 lists these values. The presence of

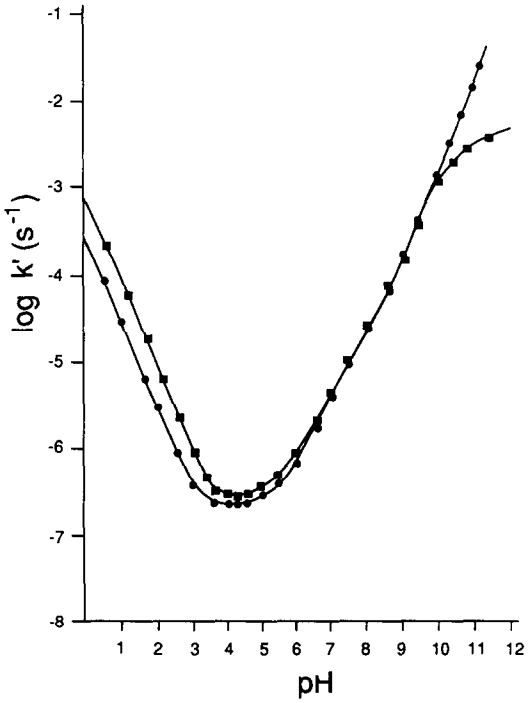

Fig. 5. Log k_{obs}-pH profiles for degradation of free doxorubicin (■) and in the presence of γ-CyD (●); $T = 50°C$; $\mu = 0.3$.

γ-CyD results in lower k_H values, indicating that the anthracyclines are protected from the attack of hydrogen atoms.

At pH > 4 differences between both anthracyclines occur. In the pH region of 4–10 the effect of γ-CyD on Dx degradation is negligible, while the degradation of Dr is accelerated. The acceleration of degradation of both anthracyclines at pH > 10 might be explained by the fact that the CyD molecule becomes deprotonated ($pK_a = 12.1$; Griffiths and Bender, 1973; Gelb et al., 1982) and the negative charge on the CyD molecule, which probably acts as a proton acceptor, has a catalyzing effect on the degradation. The differences in the profiles of both anthracyclines in the pH range 4–10 can possibly be caused by the differences in molecular structure and, moreover, a difference in degradation mechanism between Dx and Dr. Since Dx and Dr only differ at the R_2 position (a hydroxyl function instead of a hydrogen atom),

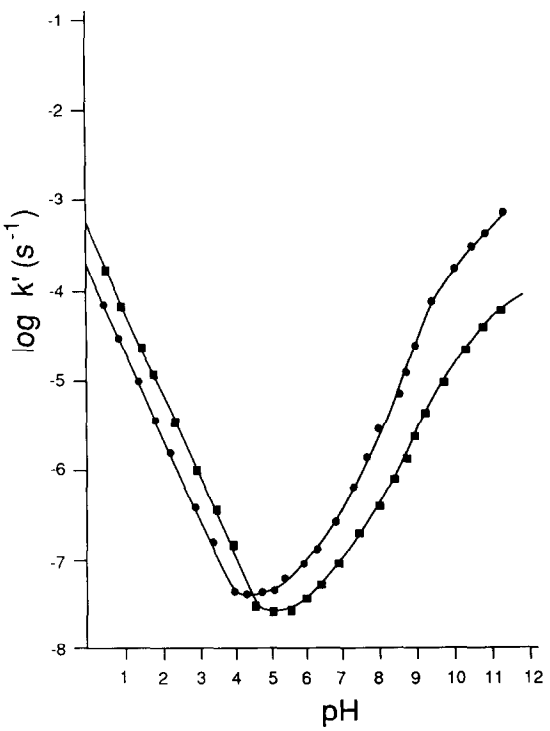

Fig. 6. Log k_{obs}-pH profiles for degradation of free daunorubicin (■) and in presence of γ-CyD (●); $T = 50°C$; $\mu = 0.3$.

the influence of γ-CyD on the stability of some Dx and Dr congeners has been studied to gain more insight into the importance of the R_2 functional group in the difference between the degradation of Dx and Dr in the presence γ-CyD.

Influence of anthracycline structure

The influence of structural modifications in the aglycone or sugar parts of the anthracycline guest molecule on the complexation with CyD has been studied at pH 10 with a number of Dx and Dr derivatives (Fig. 2). The results have been documented in Table 3. From these data it is clear that the degradation of Dr and congeners with a C_9 acetyl substituent is accelerated in the presence of γ-CyD. On the other hand, γ-CyD shows no significant influence on the degradation of the Dx derivatives possessing an α-ketol group at C_9, 4′-epi-Dx, 4′-deoxy-Dx.

The present data and the observation that the inclusion modes of Dx and Dr with γ-CyD are identical in the pH region 1.5–10 (Bekers et al., 1990) support the hypothesis that the difference in effect on the degradation rate in the presence of γ-CyD is due to a different mechanism of degradation for both drugs. As stated earlier, the only structural difference between Dx and Dr is a C_{14} hydroxyl vs a C_{14} proton, respectively. Beijnen et al. (1986a,b) previously proposed degradation mechanisms for Dx and Dr at pH > 4. The α-ketol side chain in Dx favours enolization while in Dr this tautomerization is not likely to occur. The degradation reaction in Dr is initiated by abstraction of a, weakly acidic, C_{10} benzylic proton (Arcamone, 1978), followed by splitting off the C_9 acetyl or hydroxyl function. Possibly, γ-CyD favours the abstraction of the C_{10} proton, since the apparent pK_a value of this proton is nearly the same as that of γ-CyD. This indicates that these protons are easily exchangeable (Van der Houwen et al., 1991), resulting in the acceleration of degradation. The apparent pK_a value of the α-ketol substituent in Dx, however, is significantly lower (Beijnen et al., 1986b) as compared to

TABLE 2

Second order rate constants for the proton-catalyzed degradation of doxorubicin (Dx) and daunorubicin (Dr) at $T = 50°C$ in the absence (k_H) and presence of 1.6×10^{-2} M γ-CyD (k_H^{CyD})

	k_H ($mol^{-1}\ s^{-1}$)	k_H^{CyD} ($mol^{-1}\ s^{-1}$)
Dx	7.3×10^{-4}	2.7×10^{-4}
Dr	6.5×10^{-4}	2.4×10^{-4}

TABLE 3

Degradation rate constants of some anthracycline antibiotics ([anthracycline] = 3.5×10^{-5} M) at pH 10; $T = 50°C$; $\mu = 0.3$, in the absence (k_{obs}) and presence of 1.6×10^{-2} M γ-CyD (k_{obs}^{CyD})

	k_{obs} (s^{-1})	k_{obs}^{CyD} (s^{-1})
Daunorubicin	2.1×10^{-5}	2.3×10^{-4}
4-Demethoxydaunorubicin	8.6×10^{-5}	3.7×10^{-4}
Carubicin	5.7×10^{-5}	2.3×10^{-4}
Doxorubicin	1.3×10^{-3}	1.4×10^{-3}
4′-Epidoxorubicin	1.0×10^{-3}	1.1×10^{-3}
4′-Deoxydoxorubicin	1.0×10^{-3}	1.1×10^{-3}

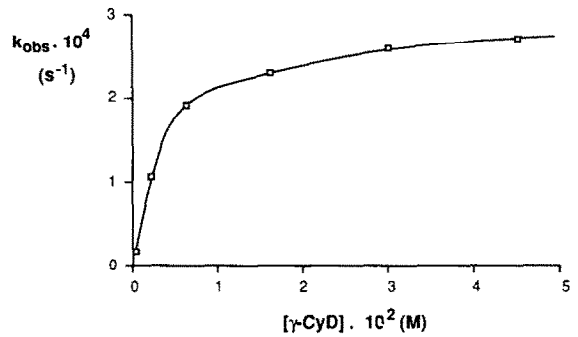

Fig. 7. Influence of the γ-CyD concentration on daunorubicin degradation at pH 10; $T = 50°C$; $\mu = 0.3$.

the benzylic proton pK_a in Dr as well as the pK_a of γ-CyD; this means that γ-CyD probably will show no effect on the deprotonation of this substituent in enolized Dx. The complexation mode of Dr and Dx supports this hypothesis, since the aglycone part of the anthracycline molecule is partly included in the γ-CyD, whereas the sugar moiety and the A- and B-rings (Fig. 2) of the aglycone point outward from the cavity (Bekers et al., 1990, 1991) and, thus, the enolization reaction is probably not affected by complexation. Therefore, the C_9 and C_{10} functions of the aglycone are close to the hydroxyl groups of the γ-CyD molecule, which may facilitate C_{10} proton abstraction and therefore hydroxyl-catalyzed Dr degradation.

Influence of cyclodextrin concentration

Fig. 7 shows the effect of [γ-CyD] on the reaction rate of Dr at pH 10, while under the experimental conditions no effect was noticed for Dx. The dependency of k_{obs} is most pronounced in the concentration range $0-2 \times 10^{-2}$ M γ-CyD. Further increase in γ-CyD concentration leads only to a slight accelerating effect of Dr degradation. This result may be explained by the complete capture of all Dr molecules in the host molecules under these conditions.

TABLE 4

Influence of the co-solvent acetonitrile (ACN) on daunorubicin (Dr) degradation at pH 10; $T = 50°C$; $\mu = 0.3$, in the presence ([γ-CyD] = 1.6×10^{-2} M) and absence of γ-CyD

	k_{obs} (s^{-1})
Dr	2.1×10^{-5}
Dr + γ-CyD	2.3×10^{-4}
Dr + γ-CyD + 5% ACN	9.0×10^{-5}
Dr + γ-CyD + 10% ACN	6.0×10^{-5}
Dr + γ-CyD + 25% ACN	2.5×10^{-5}
Dr + 25%ACN	2.3×10^{-5}
Dr + 50% ACN	9.6×10^{-6}

Influence of acetonitrile

The effect of a co-solvent on the degradation of Dr in the presence of γ-CyD was investigated at pH 10. As stated earlier anthracyclines have a tendency to adsorb onto glass walls; co-solvents, such as ACN, have been used to solve this problem (Tomlinson and Malspeis, 1982). Table 4 summarizes the influence of ACN concentration on the k_{obs} of the Dr degradation in the presence and absence of γ-CyD. A decrease in the degradation rate of Dr in the presence of γ-CyD with increasing ACN concentration is observed. In the presence of 25% (v/v) ACN the Dr-γ-CyD complex is probably completely dissociated. This result can be ascribed to competition for the inclusion site between the co-solvent and the guest molecules into the CyD cavity. When the co-solvent is present in excess probably all the Dr molecules are expelled from the cavity.

Increasing the ACN concentration up to 50% leads to a retardation of the Dr degradation rate, this can be assigned to the change in dielectrical constant of the medium. For Dx addition of ACN until about 25% did not affect the rate constant, whereas an increase in the concentration up to 50% resulted in stabilization.

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