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Effect of cyclodextrins on the chemical stability of mitomycins in alkaline solution*

O.BEKERS,† J.H. BEIJNEN,‡ M.J.T.KLEIN TANK,† A. BULT† and W.J.M. UNDERBERG†§

† *Department of Pharmaceutical Analysis, Faculty of Pharmacy, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*
‡ *Slotervaart Hospital/Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam, The Netherlands*

Abstract: The effects of cyclodextrins on the chemical stability of several mitomycin antibiotics in an alkaline medium have been investigated. A stability-indicating high-performance liquid chromatographic method was used to determine the overall degradation rate constants. The influence of various parameters such as structural variations of the cyclodextrins and mitomycins, temperature and pH was studied. It appears that complexation is most favourable with γ-cyclodextrin. All mitomycin–γ-cyclodextrin complexes degrade at lower rates than those of the free drugs. Moreover, it was shown that γ-cyclodextrin influences the equilibrium between mitomycin C and its zwitterion mesomer.

Keywords: *Mitomycins; cyclodextrins; inclusion complexes; stabilization; high-performance liquid chromatography.*

Introduction

Cyclodextrins (CyDs) are cyclic oligosaccharides composed of different numbers of α-1,4 linked glucopyranose residues. Naturally occurring CyDs consist of six, seven and eight glucose molecules and are designated α-, β- and γ-CyD, respectively; these possess cavity diameters of 5, 6 and 8 Å, respectively. The CyD molecule is conically shaped with a hydrophilic outer surface and a hydrophobic cavity. This property turns CyDs into typical host molecules; thus CyDs may form non-covalent inclusion complexes with various lipophilic compounds within a hydrophilic surrounding structure [1–3]. Encapsulation of a guest molecule will affect its physico-chemical properties and can result in increased aqueous solubility and chemical stability [4, 5]. Recently, semi-synthetic CyDs, such as hydroxypropyl-β-CyD (HP-β-CyD), have received considerable attention in the pharmaceutical field because of their low toxicity and favourable complexation properties [6].

Mitomycins (Fig. 1) are antibiotic drugs that exhibit strong bactericidal and antineoplastic activity. Mitomycin C (MMC) is the only member of the mitomycin family used in the current practice of cancer chemotherapy [7]. From a pharmaceutical point of view MMC has attracted much attention because the compound is unstable in aqueous media. The mechanism and kinetics of the degradation of MMC in acidic [8–10] as well as in alkaline [11, 12] solution have been described extensively. Degradation in acid leads to the formation of mitosenes whereas in alkaline solution the 7-substituent is cleaved leading to the formation of a 7-hydroxymitosane (Fig. 1). The same mechanism is valid for the 7-methoxymitosanes, mitomycin A (MMA) and mitomycin B (MMB) [13].

The chemical instability of MMC demands special precautions to be taken in the preparation and storage of its pharmaceutical preparations because degradation is accompanied by loss of antitumour activity. The present project is a sequel to earlier studies [14, 15] where the effects of CyDs on the chemical stability of MMC and related mitomycins in acidic media have been investigated with the purpose of designing a new, more stable parenteral formulation of this class of compound.

Experimental

Chemicals

MMC was donated by Bristol Myers (Weesp, The Netherlands); MMA and MMB

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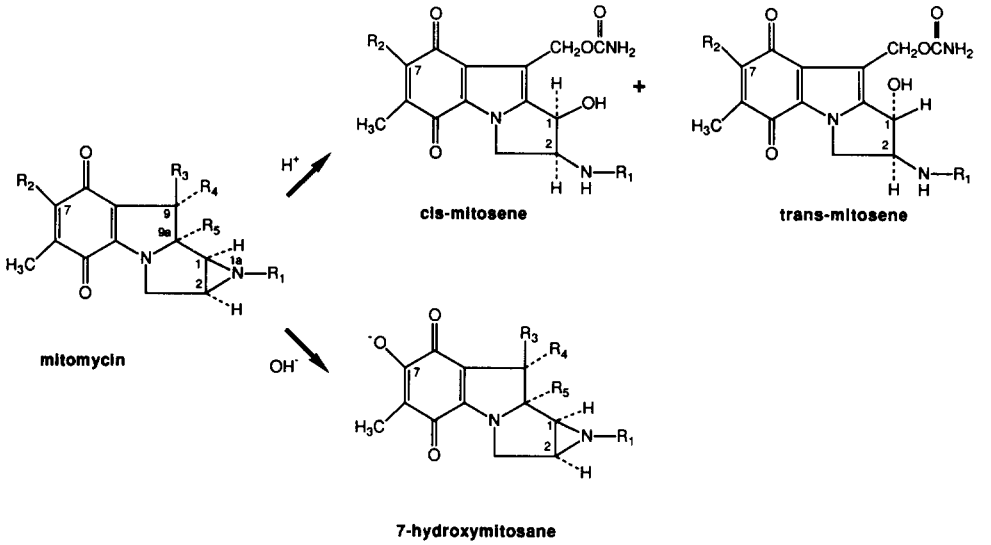
§ Author to whom correspondence should be addressed, at P.O. Box 80.082, 3508 TB Utrecht, The Netherlands.



	R_1	R_2	R_3	R_4	R_5
MMA	H	H_3CO	H	CH_2OCONH_2	OCH_3
MMB	CH_3	H_3CO	CH_2OCONH_2	H	OH
MMC	H	NH_2	H	CH_2OCONH_2	OCH_3

Figure 1
Structures and degradation patterns of several mitomycin antibiotics.

were kindly provided by Kyowa Hakko Kogyo Chemical Co. (Tokyo, Japan). The natural CyDs originated from Nihon Shokukin Kako Co. Ltd (Tokyo, Japan); heptakis(2,6-di-*O*-methyl)β-CyD (DM-β-CyD) and HP-β-CyD were both gifts from Dr J. Mesens (Janssen Pharmaceutica, Beerse, Belgium). The CyDs were used as received. All other materials were of analytical grade and de-ionized water was obtained by filtration through a Milli-Q-Water Purification System (Millipore, Bedford, MA, USA).

Buffer–CyD solutions

For the kinetic studies the following aqueous buffer solutions were used: pH 7–9, phosphate (0.01 M); pH 9–12, carbonate (0.01 M); pH > 12, sodium hydroxide. The desired pH was adjusted with perchloric acid or sodium hydroxide and was measured with a pH meter (Metrohm, E512 Titriskop, Herisau, Switzerland). Solutions of pH > 12 were prepared as described by Licht [16]. A constant ionic strength (μ = 0.3 M) was maintained for each test solution by the addition of an appropriate amount of sodium chloride, except for the solutions where the hydroxyl concentration exceeded 0.3 M.

In the experiments where the effects of various CyDs on the degradation rate of MMC were studied, the CyD concentration was 2×10^{-2} M. For evaluation of the complex constants of the mitomycin–γ-CyD complexes, the γ-CyD concentration varied from 0 to 5×10^{-2} M. During all other experiments where the influence of γ-CyD was studied the concentration was 2×10^{-2} M. In all these experiments the pH of the solution was adjusted to the desired value after the addition of the CyD. The buffer–CyD solutions were always prepared shortly before use. No significant change of pH was observed during the course of the degradation processes.

Kinetic measurements

The buffer–CyD solutions, in polypropylene reaction vessels, were equilibrated to the temperature of the study in a thermostated water-bath and protected from light. The reactions were initiated by adding 20 µl of a mitomycin stock solution in methanol to a pre-heated buffer–CyD solution, yielding an initial concentration of 4×10^{-5}, 3×10^{-5} and 6×10^{-5} M for MMC, MMA and MMB, respectively. At appropriate time intervals, 10-µl samples were taken and analysed directly for

the content of undegraded mitomycin by a stability-indicating high-performance liquid chromatographic (HPLC) assay. Samples originating from test solutions above pH 12 were adjusted to pH 7 before injection on to the HPLC column.

Liquid chromatography

The liquid chromatograph comprised a Model 6000 solvent delivery system, a U6K injector (both from Waters Assoc., Milford, MA, USA) and a PU 4020 variable UV detector (Pye Unicam Ltd, Cambridge, UK), operating at 365 nm for MMC and at 313 nm for both MMA and MMB. For the analysis of the zwitterionic form of MMC, UV detection at 340 nm with a UV M440 absorbance detector (Waters Assoc., Milford, MA, USA) was also used. Separations were achieved on a home-packed column (12.5 × 4.0 mm i.d.) containing 5-μm Hypersil ODS (Shandon Southern Products Ltd, Cheshire, UK). The mobile phases used for MMC, MMA and MMB were: (I) methanol–water (20:80, w/w); (II) methanol–water (50:50, w/w); and (III) methanol–water (35:65, w/w), respectively; in all cases 1% (v/v) of 0.5 M sodium phosphate (pH 7) was added to the aqueous phase. The chromatographic analyses were carried out at ambient temperature. The flow rate was 1 ml min^{-1}. Quantitation of undegraded mitomycin was based on peak-height measurements. Standard solutions of the mitomycins at pH 7 were analysed and calibration curves in the concentration range of interest (0–6 × 10^{-5} M) showed linear responses ($r > 0.999$).

Circular dichroism (CD) and UV–vis spectrophotometry

CD spectra were obtained with a Dichrograph III (Jobin Yvon, Longjumeau, France). Sensitivity calibration was performed with isoandosteron. The bandwidth was programmed automatically. Absorption spectra were recorded on a Perkin–Elmer Lambda 5 UV–vis spectrophotometer (Perkin–Elmer, Oak Brook, IL, USA).

Results and Discussion

Liquid chromatography

The initial degradation step of the three mitomycins in alkaline media is the replacement of the 7-substituent by a hydroxyl group to yield their respective 7-hydroxymitosanes [11, 12]. This is illustrated in Fig. 1. The stability-indicating capability of the HPLC systems used in this study has been demonstrated earlier [13]. The capacity factors of the mitomycins and the 7-hydroxymitosanes remain unchanged in the presence of CyDs; thus the HPLC systems are also suitable for the analysis of mitomycins in these media. Figure 2 shows typical chromatograms of partly degraded MMC–γ-CyD solution.

CD and UV–vis spectra

At pH 7 neither the CD nor the UV–vis

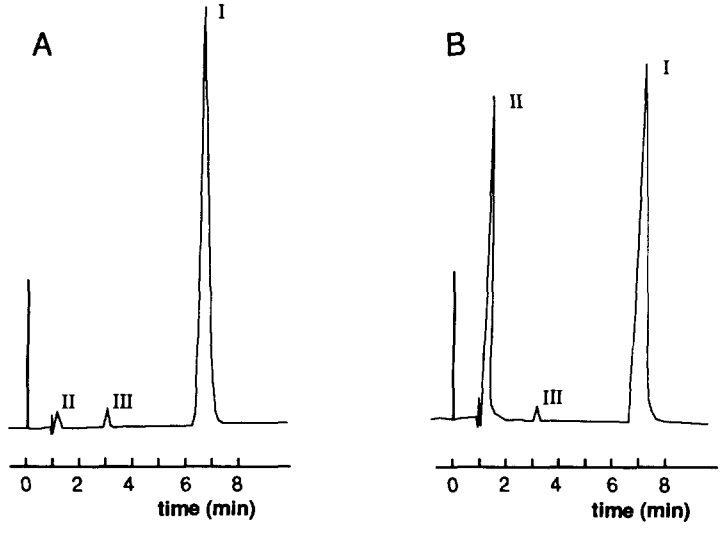


Figure 2
HPLC chromatograms of partly degraded MMC–γ-CyD solutions at pH 11 and 25°C at various degradation times (t) I = MMC; II = 7-hydroxymitosane; III = zwitterion. (A) t = 30 min; (B) t = 16 h.

spectra of MMC change after addition of the various CyDs. This indicates that, if a MMC molecule is included in the CyD cavity, no difference or only a very small difference in molecular orientation occurs.

Chemical stability of mitomycins

Degradation kinetics and mechanism. Degradation of the mitomycins follows pseudo-first-order kinetics. The presence of CyDs does not change this kinetic behaviour. Identical degradation products are formed in the presence and absence of a CyD; it can be concluded that the degradation mechanism of the mitomycins is not influenced by complexation with CyDs.

Determination of the observed rate constant (k_{obs}). The k_{obs} and its standard deviation (SD) for the degradation of MMC was determined at 25°C and pH 11. The value of k_{obs} and the SD, calculated from six observations, is $1.24 \pm 0.13 \times 10^{-5}$ s^{-1}. All other rate constants are mean values of duplicate determinations.

Influence of CyD structure. The influence of CyD structure on the degradation of MMC has been investigated at pH 11 and 25°C. Table 1 summarizes the results of these experiments in terms of k_{obs} values. It is obvious that α- and β-CyD do not affect the rate of MMC degradation. HP-β- and DM-β-CyD exert a stabilizing effect while the strongest effect is obtained with γ-CyD. It appears that the bulky structure of MMC fits best in the large γ-CyD cavity. All further experiments were, therefore, conducted with γ-CyD.

In acidic media the degradation of MMC results in opening of the aziridine ring system (Fig. 1). This process is also retarded by γ-CyD [14]. On the basis of kinetic measurements and NMR data a structure for the MMC–γ-CyD complex could be proposed [15], in which the aziridine-ring is included in the γ-CyD cavity. The kinetic data obtained in the present study indicate that the 7-aminoquinoid moiety of MMC is also involved. Further investigations, deploying molecular modelling, may possibly demonstrate the existence of two different complex structures.

Table 1
Influences of various CyDs on MMC degradation at pH 11 and 25°C

CyD	k_{obs} (s^{-1})
—	1.24×10^{-5}
α-	1.29×10^{-5}
β-	1.31×10^{-5}
HP-β-	5.5×10^{-6}
DM-β-	4.3×10^{-6}
γ-	2.9×10^{-6}

Influence of mitomycin structure. The influence of structural differences in the guest molecule on the stability of the mitomycin–γ-CyD complex was studied at pH 10.5 and/or pH 11 and at 25°C with three mitomycin analogues. The complexation of mitomycin with γ-CyD and the degradative reactions of both free and complexed drug are shown in the following scheme:

$$\text{mitomycin} + \gamma\text{-CyD} \overset{K_s}{\rightleftharpoons} \text{mitomycin–}\gamma\text{-CyD}$$

$$\text{mitomycin} \xrightarrow{k_0} \text{degradation products} \qquad \text{mitomycin–}\gamma\text{-CyD} \xrightarrow{k_{cat}} \text{degradation products}$$

where K_s is the equilibrium constant of the complex, k_0 the pseudo-first-order rate constant for degradation of the free drug and k_{cat} the pseudo-first-order rate constant for degradation of the drug trapped in the CyD cavity. The relationship between these constants is in accordance with equation (1) [15, 17],

$$\frac{[\gamma\text{-CyD}]_{tot}}{k_0 - k_{obs}} = \frac{1}{k_0 - k_{cat}}[\gamma\text{-CyD}] + \frac{1}{K_s(k_0 - k_{cat})}. \quad (1)$$

Table 2
Equilibrium constants (K_s) and degradation rate constants (k_{cat}) of various mitomycin–γ-CyD complexes at 25°C

pH	Mitomycin	k_0* (s^{-1})	k_{cat} (s^{-1})	K_s (M^{-1})	$k_0\,k_{cat}$
11.0	MMA	1.87×10^{-4}	1.04×10^{-4}	155	1.8
11.0	MMB	1.04×10^{-4}	4.73×10^{-5}	83	2.2
10.5	MMC	3.20×10^{-6}	3.50×10^{-7}	275	5.8
11.0	MMC	1.24×10^{-5}	2.20×10^{-6}	235	5.6

* Rate constant for degradation of the free drug.

In Table 2 the characteristics of various mitomycin–γ-CyD complexes are included. All complexes degrade with lower rates compared with those of the free drugs, indicating that complexation with γ-CyD protects the labile 7-substituent of the molecule. The MMC complex possesses the highest equilibrium constant and is also best protected against hydroxyl-catalysed degradation, as can be seen from the high k_0/k_{cat} ratio. Although at pH 11, the K_s value is only an estimate in the case of MMC owing to the presence of an acidic function (pK_a 12.4), this value approaches closely the real K_s value for uncharged MMC [15, 18]. This is concluded from the fact that at pH 10.5, within experimental error, the same value for K_s has been found, indicating that the degree of deprotonation of MMC at pH 11 has only a limited influence on the overall K_s value. As a consequence, the values for the rate and equilibrium constants at pH 11 for MMC are, to a large extent, comparable to those found for MMA and MMB.

The apparent straightforward relationship between complexation and degradation seen for MMC could not be observed for MMA and MMB. The higher equilibrium constant of the MMA complex compared to that of MMB does not lead to a higher k_0/k_{cat} ratio. This result suggests that structural changes in the mitomycin molecule may alter not only the stability of the guest molecule but also the mode of complexation. NMR studies may cast more light on this matter.

Influence of temperature. The temperature dependence of MMC degradation in the presence of γ-CyD was compared with the degradation of the free drug. These experiments were performed at pH 10 over the temperature range 25–60°C. The Arrhenius relationship was obeyed (equation 2).

$$\ln k_{obs} = \ln A - (E_a/RT), \qquad (2)$$

where A represents the frequency factor, E_a the activation energy, R the molar gas constant and T the temperature in Kelvins.

From a plot of $\ln k_{obs}$ versus the reciprocal of T, A and E_a can be calculated. The results are summarized in Table 3. The fact that changes in the K_s value of MMC–γ-CyD occur on changing the temperature is not taken into account; this means that the real value of E_a for the degradation of MMC in the presence of γ-CyD may differ from the experimentally determined value. However, the higher E_a value obtained in the presence of γ-CyD suggests that on complexation some protection against MMC degradation occurs.

Table 3
Influence of temperature on the degradation of free and γ-CyD-complexed MMC at pH 10 in terms of frequency factors (A) and activation energies (E_a)

	A (s^{-1})	E_a (kJ mol^{-1})
MMC	3.0×10^8	81.8
MMC–γ-CyD	8.1×10^{11}	105

Influence of pH. At pH values of 7–11 accelerated stability tests were carried out since degradation of MMC in this pH range is slow. By application of the Arrhenius equation (equation 2) the k_{obs} values were extrapolated to 25°C. Figure 3 demonstrates a comparison of the log k_{obs}–pH plots of free MMC and MMC in the presence of γ-CyD at pH ≥ 7. The plots show that in the region 7–11.5 the shapes of the curves are similar and shifted over a constant distance, indicating that the stabilizing effect in this range is pH-independent. At pH values >11.5 the curves approach and coincide at pH 14. This observation indicates that, in contrast to the uncharged molecule, deprotonated MMC does not form complexes with γ-CyD. Figure 3 also demonstrates that the inflection, which indicates the pK_a of MMC, shifts upwards on complexation. This supports the hypothesis that the quinoid part of MMC may be involved in γ-CyD complexation, since an enolic function with an apparent pK_a of 12.4 occurs in this part of the

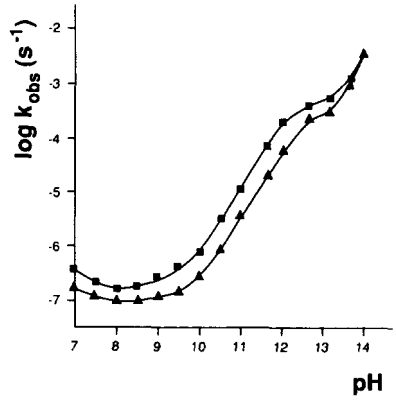


Figure 3
Plots of log k_{obs} against pH for the degradation of MMC in the absence (■) and in the presence of γ-CyD (▲) ([γ-CyD] = 2×10^{-2} M).

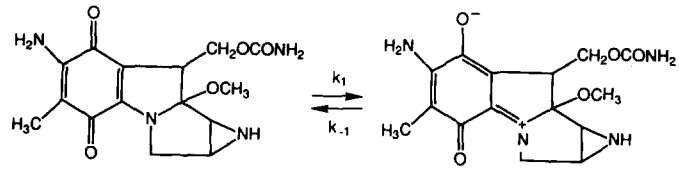


Figure 4
Equilibrium between the quinoid and zwitterionic forms of MMC.

molecule [8, 9] because of keto-enol tautomerism.

Influence of γ-CyD on the equilibriuim between the quinoid and the zwitterionic forms of MMC. Den Hartigh has presented evidence that in acidic as well as alkaline solution a zwitterionic form of MMC is present [19]. The equilibrium reaction is shown in Fig. 4. This process can be described by a simple reversible reaction mechanism [20]. Both the forward and reverse reaction steps are first-order processes with k_1 and k_{-1} as their respective rate constants. The expression for the equilibrium constant K_e is

$$K_e = k_1/k_{-1} = [Z]_e/[M]_e, \qquad (3)$$

where $[M]_e$ and $[Z]_e$ represent the equilibrium concentrations of MMC and the zwitterion, respectively. Measurements of $[Z]$ and $[M]$ at equilibrium enable values for K_e to be calculated. The studies were performed at pH 7 and 25°C where the degradation of MMC does not have to be taken into account owing to the low k_{obs} value at this pH value [11]. To calculate the equilibrium concentrations of both species, peak heights of both species in the HPLC chromatograms were used. Because no spectroscopic differences between MMC and the zwitterion were found [19], the assumption was made that the molar absorptivity of the zwitterion is the same as that of MMC.

The influence of γ-CyD on the equilibrium has been investigated. As can be seen from Table 4 γ-CyD stabilizes the MMC form as shown by a decrease in K_e. This result is in good accordance with literature data [3], which suggest that uncharged lipophilic molecules form complexes with CyDs considerably better than do ionic species.

Table 4
Influence of γ-CyD on the value of K_c for the equilibrium of MMC and its zwitterion at pH 7 and 25°C

	Wavelength of detection	K_c
MMC	340 nm	2.0
	365 nm	2.1
MMC + γ-CyD	340 nm	0.4
	365 nm	0.4

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