International Journal of Pharmaceutics, 52 (1989) 239–248
Elsevier

IJP 01771

Stabilization of mitomycins on complexation with cyclodextrins in aqueous acidic media

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(Received 4 October 1988)
(Accepted 25 November 1988)

Key words: Mitomycin; Degradation; UV spectrophotometry; Cyclodextrin; Complexation; High-performance liquid chromatography; NMR spectroscopy

Summary

The effect of various cyclodextrins (α-, β-, dimethyl-β- and γ-cyclodextrin) on the acidic hydrolysis of several mitomycin antibiotics was investigated using a stability-indicating ultraviolet spectrophotometry assay. The mitomycins appear to complexate most prominently with γ-cyclodextrin. Although the degradation pattern does not change, complexation results in a decrease in degradation rate. The influences of the γ-cyclodextrin concentration as well as pH, ionic strength, buffer components and temperature on the complex stability were studied. The structure of the mitomycin C-γ-cyclodextrin inclusion complex has been proposed on the basis of nuclear magnetic resonance spectroscopic measurements.

Introduction

The mitomycins are a group of antibiotics exhibiting strong bactericidal and antitumour activity (Matsui et al., 1968). The compounds are produced by various *Streptomyces* strains (Wakaki et al., 1958). Mitomycin C (MMC) is the most important member and until now the only representative of the family of mitomycin antibiotics accepted in cancer chemotherapy practice (Crooke, 1981; Nagel et al., 1984). The chemical structures of other mitomycin representatives are given in Fig. 1. All these substances are unstable in aqueous solutions (Underberg and Lingeman, 1983; Beijnen and Underberg, 1985; Beijnen et al., 1985; 1986a). Degradation in acidic media leads to the formation of cytostatically inactive mitosenes (Tomasz and Lipman, 1979; Underberg and Lingeman, 1983; Beijnen and Underberg, 1985a) (Fig. 2).

Cyclodextrins (CyD) (Fig. 3) or cycloamyloses are cyclic oligosaccharides, containing a minimum of six D-(+)-glycopyranose units attached by α-1,4 linkages, produced by the action of *Bacillus macerans* amylase on starch. The most common natural CyD, α-, β- and γ-CyD, consisting of 6, 7 and 8 glucose units, respectively, as well as chemically modified forms, recently attracted consid-

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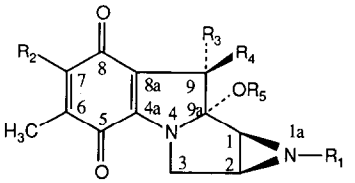
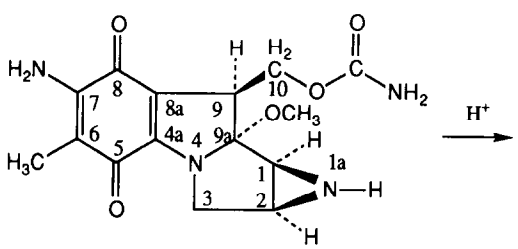
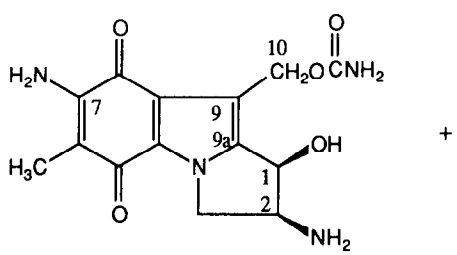


Fig. 1. Structures of several mitomycins.

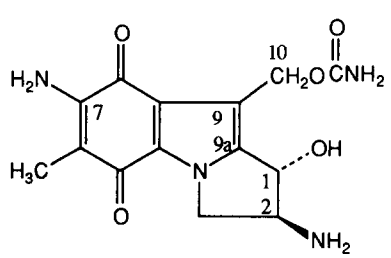
	R_1	R_2	R_3	R_4	R_5
Mitomycin A	H	H_3CO	H	CH_2OCONH_2	CH_3
Mitomycin B	CH_3	H_3CO	CH_3OCONH_2	H	H
Mitomycin C	H	H_2N	H	CH_2OCONH_2	CH_3
Porfiromycin	CH_3	H_2N	H	CH_2OCONH_2	CH_3
7-N-(p-hydroxyphenyl) mitomycin C	H	$4\text{-}OHC_6H_4NH$	H	CH_2OCONH_2	CH_3
1a-Acetylmitomycin C	$COCH_3$	N_2H	H	CH_2OCONH_2	CH_3



mitomycin C



cis - mitosene



trans - mitosene

Fig. 2. Degradation of mitomycin C in acidic solution.

erable attention in chemical and pharmaceutical sciences (Jones et al., 1984a; 1984b; Pitha, 1984; Uekama, 1985; Szejtli, 1987). It is well known that CyD can form inclusion complexes with different types of drugs (Uekama and Otagiri, 1987). In general these inclusion complexes involve the entrapment of a single guest molecule in the cavity of one host molecule without any covalent bonds being formed. Because the outside of the CyD is hydrophilic and the inside of the cavity hydrophobic in nature, CyD can form water-soluble inclusion complexes with various lipophilic compounds (Uekama, 1979; Anderson and Bundgaard, 1984; Duchêne et al., 1985).

This paper describes a systematic study of the complexation of mitomycins with CyD in acidic media as well as the changes in chemical stability of the mitomycins after complexation with CyD. The structure of the MMC–γ-CyD complex in aqueous solution is postulated.

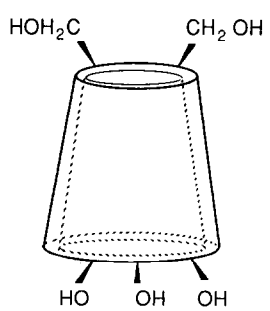


Fig. 3. Schematic representation of a natural cyclodextrin.

Materials and Methods

Chemicals

MMC was kindly provided by Bristol Myers (Weesp, The Netherlands); mitomycin A (MMA), mitomycin B (MMB) and 7-*N*-(*p*-hydroxyphenyl) mitomycin C (M-83) were gifts from Dr. K. Shirahata, Kyowa Hakko Kogyo Chemical Co. (Tokyo, Japan). 1α-Acetylmitomycin C (AcMMC) was prepared according to the procedure described by Stevens et al. (1965). Porfiromycin (PM) was prepared as described below. The various CyD came from Nihon Shokuhin Kako Co. Ltd. (Tokyo, Japan) and were used as received. All other chemicals were of analytical grade and deionized water was filtered through a Milli-Q Water Purification System (Millipore, Bedford, MA, U.S.A.) before use.

Synthesis of porfiromycin

For the synthesis of PM, 1.3 ml dry acetone and 50 mg potassium carbonate were added to 10 mg MMC and 50 μl methyl iodide. This mixture was allowed to stand for 12 h on a water bath at 70 °C. After filtration the solution was evaporated to dryness under a stream of nitrogen. The purity was checked with a HPLC system designed by Beijnen et al. (1985).

Degradation solutions

For the kinetic studies the following aqueous solutions were used Ho/pH −2 to 3: perchloric acid, and pH 3 to 5: acetate buffer (0.001 M). The pH values between 1 and 5 were measured at the temperature of study using a glass-reference electrode and a Metrohm 632 pH-meter (Herisau, Switzerland). pH values below 1 were calculated by using the Hammett acidity function (Bates, 1973). A constant ionic strength ($\mu = 0.3$), adjusted with sodium chloride, was maintained for each degradation solution, except for the solutions where the influence of ionic strength on the degradation was investigated as well as where the hydrogen concentration exceeded 0.3 M. For the degradation studies with CyD, various amounts of CyD were added to the acidic buffer solution and the pH was adjusted to the desired value. The buffer/CyD solutions were always prepared freshly before use.

Kinetics experiments

All kinetic degradation experiments were performed in the dark at 25 °C, except when stated otherwise. The degradation of all investigated mitomycins was quantified spectrophotometrically according to the procedures described earlier (Underberg and Lingeman, 1983; Beijnen et al., 1986a). The reactions were initiated by adding 10 μl of a methanolic stock solution of a mitomycin to 2.5 ml preheated buffer/CyD solution in a 1 cm quartz cell.

For the kinetic measurements of the MMA and MMB degradation the initial concentration was $2 \cdot 10^{-5}$ M and the degradation of both were followed by monitoring the increase in absorbance at 280 nm. The initial concentrations of MMC, PM and AcMMC were $3 \cdot 10^{-5}$ M and in these cases the decreases in absorbance at 363 nm were monitored continuously. The initial concentration of M-83 was $6 \cdot 10^{-5}$ M and the decrease in absorbance at 385 nm was registered.

In the experiments where the influences of various CyD on the mitomycin degradation rate was studied, the CyD concentration was $2 \cdot 10^{-2}$ M. The CyD concentration varied from $0–8 \cdot 10^{-2}$ M for the determination of the apparent complex stability constants of the mitomycin-γ-CyD complexes. The studies of the influences of ionic strength, buffer concentration and temperature of the degradation media were all performed with MMC and γ-CyD, the γ-CyD concentration being $2 \cdot 10^{-2}$ M throughout.

The influence of CyD on the stereoselective degradation of MMC has been studied by using a high performance liquid chromatographic (HPLC) assay. These experiments were carried out in the absence of CyD and in the presence of $2 \cdot 10^{-2}$ M β-CyD and $5 \cdot 10^{-2}$ M γ-CyD in the pH range 2–5. 3 ml buffer/CyD solution was spiked with 15 μl of an MMC stock solution to give an initial concentration of about $4 \cdot 10^{-5}$ M. At appropriate time intervals aliquots were withdrawn and analyzed for their degradation products using the HPLC assay.

Ultraviolet-visible (UV-Vis) spectrophotometry

UV-Vis absorbance spectra were recorded on a Perkin Elmer Lambda 5 UV-VIS spectrophotome-

ter (Perkin Elmer, Oak Brook, IL, U.S.A.). Absorbance measurements were performed with a UV-140 Double Beam Spectrophotometer equipped with an ACP-140 Thermostated Cell-positioner (Shimadzu Corp., Kyoto, Japan).

High performance liquid chromatography (HPLC)

HPLC analysis was performed using a Model 510 solvent delivery system, a U6K injector and a Model 440 dual wavelength UV detector (all from Waters Assoc., Milford, MA, U.S.A.) operating at 254 nm and 313 nm. Quantitation was based on peak area measurements using a SP 4270 integrator (Spectra Physics, San José, U.S.A.). The analytical column (300 × 2.9 mm) was filled with Lichrosorb RP 18 (10 μm) material (Merck, Darmstadt, F.R.G.). The eluens consisted of methanol-water (25 + 75, w/w) with 1% (v/w) 0.5 M sodium phosphate solution pH 7.0. The flow rate was 1.0 ml/min.

Nuclear magnetic resonance (NMR) spectroscopy

400 MHz ^{1}H-NMR (JEOL-JNM-Gx 400 Spectrometer) spectra were measured in D_2O at 30 °C. An average of 200 accumulations with 32768 data points were made at a sweep width of 6000 Hz. The ^{1}H-chemical shifts were assigned values based on the external standard sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) with a accuracy of ±0.0015 ppm.

Results and Discussion

Absorption spectrophotometry

The mitomycins show profound spectral changes on degradation in acidic media. These changes are suitable to monitor the acid degradation and to calculate degradation rate constants. The initial degradation step of the mitomycins is illustrated in Fig. 2 for MMC. In the presence of CyD no significant differences were found in the spectra of the mitomycins. Fig. 4 illustrates the spectral changes during the degradation of a MMC-γ-CyD solution, as an example.

Order of reaction

In the investigated pH range the mitomycins degraded following a pseudo-first order pattern

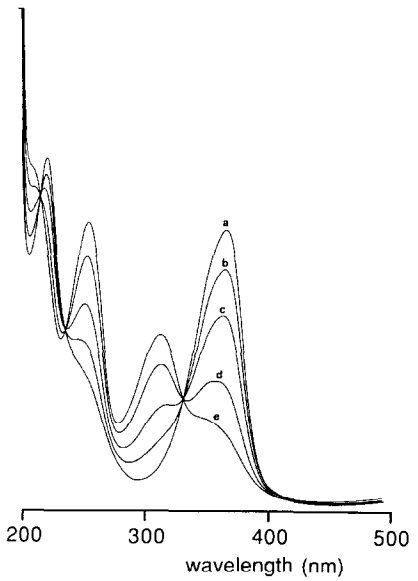


Fig. 4. UV-spectra of MMC–γ-CyD during degradation of mitomycin C in acidic solution; a, $t = 0$ min; b, $t = 10$ min; c, $t = 25$ min; d, $t = 60$ min; e, $t = 110$ min.

over at least 4 half-lives. The observed rate constants (k_{obs}) are calculated by least-squares linear regression analysis from the slopes of plots of the natural logarithm of the remaining concentration mitomycin, [mitomycin]$_t$, against time as represented by Eqn. 1:

$$\ln[\text{mitomycin}]_t = \ln[\text{mitomycin}]_0 - k_{obs} \cdot t \qquad (1)$$

where [mitomycin]$_0$ is the initial mitomycin concentration.

The pseudo-first order kinetic behaviour of the mitomycins is unaffected by the addition of CyD.

Influence of CyD structure

The influences of 4 different CyD on the degradation of the 6 mitomycins have been studied. It can be assumed that at the pH values and temperatures of study no noticeable degradation of the used CyD will occur (Uekama and Otagiri, 1987).

The measured k_{obs} values for the MMC degradation in the absence and presence of several CyD at pH 2.8 are documented in Table 1. From Table 1 it is clear that α- and β- as well as heptakis-(2,6,-di-*O*-methyl)-β-CyD (dimethyl-β-

TABLE 1

Influences of some CyD ([CyD] = $2 \cdot 10^{-2}$ M) on the degradation of MMC ([MMC] = $3 \cdot 10^{-5}$ M) at pH = 2.8 and t = 25 °C.

CyD	k_{obs} (s^{-1})
–	7.6×10^{-4}
α	7.9×10^{-4}
β	6.9×10^{-4}
DM-β	6.7×10^{-4}
γ	4.1×10^{-4}

CyD, DM-β-CyD) have very little if any influences on the degradation of MMC. However, γ-CyD has a significant stabilizing effect. This may indicate that the labile moiety of the MMC molecule fits best in γ-CyD, which has the largest cavity of the 4 CyD used (Uekama and Otagiri, 1987). The same stabilizing effect for the various CyD occurs for the other mitomycins. All further experiments were therefore carried out with γ-CyD.

Influence of mitomycin structure

The influences of structural differences in the guest mitomycin molecule on the mitomycin-γ-CyD complex stability and degradation have been studied with the 6 mitomycin analogues (Fig. 1) at pH 0.8, 2.8 and 4.8. These 3 pH values were selected because the aziridine pK_a values of the investigated mitomycins are all around 2.8 except for the pK_a of AcMMC which is below 0 (Beijnen et al., 1986a). This means that at pH 0.8 the amino-group of the aziridine-ring is almost quantitatively protonated while at pH 4.8 the mitomycins are almost exclusively present in the deprotonated form and at pH 2.8 about 50% is deprotonated. It will be clear that AcMMC is present in its non-protonated form at all pH values mentioned.

The complexation of a mitomycin with γ-CyD and the degradation reactions of both free and the included mitomycin are illustrated in Scheme 1.

$$\begin{array}{ccc} \text{mitomycin} + \gamma\text{-CyD} & \overset{K_s}{\rightleftharpoons} & \text{mitomycin-}\gamma\text{-CyD} \\ \downarrow k_0 & & \downarrow k_{cat} \\ \text{degradation products} & & \text{degradation products} \end{array}$$

Scheme 1

In this scheme K_s represents the apparent stability constant of the inclusion 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 included drug. The relationship between the observed pseudo-first order rate constant, k_{obs}, and the pseudo-first order rate constants k_0 and k_{cat} in the presence of γ-CyD is given by Eqn. 2:

$$k_{obs} = k_0 \cdot f + k_{cat} \cdot (1 - f) \tag{2}$$

where f and $(1 - f)$ are the fractions of the free and complexed mitomycin, respectively.

The apparent stability constant, K_s, for the mitomycin-γ-CyD inclusion complex is defined by Eqn. 3:

$$K_s = \frac{(1 - f)}{f \cdot [\gamma - \text{CyD}]_f} \tag{3}$$

Combination of Eqns. 2 and 3 leads to the expression for the k_{obs} of mitomycin in the presence of γ-CyD:

$$k_{obs} = \frac{k_0 + k_{cat} \cdot K_s \cdot [\gamma - \text{CyD}]_f}{1 + K_s \cdot [\gamma - \text{CyD}]_f} \tag{4}$$

In the presence of a large excess of γ-CyD the amount complexed with mitomycin can be considered to be negligible in comparison to the concentration free γ-CyD. Under this assumption the concentration free γ-CyD, $[\gamma\text{-CyD}]_f$, equals the total γ-CyD concentration $[\gamma\text{-CyD}]_{tot}$ (Eqn. 5):

$$k_{obs} = \frac{k_0 + k_{cat} \cdot K_s \cdot [\gamma - \text{CyD}]_{tot}}{1 + K_s \cdot [\gamma - \text{CyD}]_{tot}} \tag{5}$$

Rearrangement of Eqn. 5 leads to the Lineweaver-Burke equation (Eqn. 6) (Uekama et al., 1981; Hirayama et al., 1987)

$$\frac{[\gamma - \text{CyD}]_{tot}}{k_0 - k_{obs}} = \frac{1}{k_0 - k_{cat}} [\gamma - \text{CyD}]_{tot} + \frac{1}{K_s (k_0 - k_{cat})} \tag{6}$$

By plotting $[\gamma\text{-CyD}]_{tot}/k_0 - k_{obs}$ versus the γ-CyD concentration straight lines ($r > 0.99$) were obtained. The k_{cat} and K_s values of mitomycin–γ-CyD inclusion compounds were calculated from slopes and intercepts, respectively, of these straight lines. In Table 2 the characteristics of the various mitomycin-γ-CyD complexes at the 3 different pH values of study are summarized. At pH 0.8 k_{obs} at every concentration γ-CyD, is equal to the degradation constant k_0 in the absence of γ-CyD, except for AcMMC, so the suggestion can be made that no inclusion complexes are formed. It appears that at the pH values 2.8 and 4.8 complexation of the mitomycins with γ-CyD occurs. All complexes degrade with lower velocity compared to the free drugs. This indicates that protection of the labile part of the molecule is achieved on complexation with γ-CyD.

However, although the apparent stability constants K_s of most inclusion complexes are in the same order of magnitude, the k_0/k_{cat} ratios, indicating the protective capability, are by no means the same. A clear correlation between the apparent stability constants and the k_0/k_{cat} ratios has not been found yet.

TABLE 2

Degradation rate constants and apparent stability constants of various mitomycin–γ-CyD complexes at different pH values, calculated from Eqn. 6 (see text)

		k_0 (s^{-1})	k_{cat} (s^{-1})	K_s (M^{-1})	k_0/k_{cat}
pH 0.8	MMA	3.4×10^{-3}	N.S.	–	–
	MMB	1.4×10^{-3}	N.S.	–	–
	MMC	2.5×10^{-3}	N.S.	–	–
	PM	3.3×10^{-3}	N.S.	–	–
	M-83	2.1×10^{-3}	N.S.	–	–
	AcMMC	1.7×10^{-2}	2.3×10^{-3}	144	7.4
pH 2.8	MMA	1.2×10^{-3}	1.9×10^{-4}	96	6.4
	MMB	6.5×10^{-4}	3.4×10^{-4}	34	1.9
	MMC	7.6×10^{-4}	1.2×10^{-4}	107	6.5
	PM	7.1×10^{-4}	1.5×10^{-4}	169	6.9
	M-83	7.6×10^{-4}	2.3×10^{-4}	201	3.3
	AcMMC	1.8×10^{-4}	2.6×10^{-5}	120	6.9
pH 4.8	MMA	1.9×10^{-5}	3.4×10^{-6}	233	5.7
	MMB	1.0×10^{-5}	5.2×10^{-6}	154	2.0
	MMC	1.7×10^{-5}	2.1×10^{-6}	249	8.0
	PM	4.7×10^{-6}	4.9×10^{-7}	375	9.6
	M-83	1.2×10^{-5}	1.7×10^{-6}	382	7.0
	AcMMC	9.2×10^{-7}	1.2×10^{-7}	124	7.4

k_0, degradation rate constant for free MMC; k_{cat}, degradation rate constant for MMC-γ-CyD complex; K_s, apparent stability constant of MMC-γ-CyD complex; N.S., no stabilization occurs.

TABLE 3

Ratio between apparent stability constants K_s of mitomycin–γ-CyD complexes at pH 4.8 and pH 2.8

	$K_s^{pH4.8}/K_s^{pH2.8}$
MMA	2.4
MMB	4.5
MMC	2.3
PM	2.2
AcMMC	1.1

Another indication for complexation between the mitomycins and γ-CyD around the aziridine moiety is illustrated with the data of Table 3. Except for AcMMC, which is present in its non-protonated form over the whole pH range investigated, all mitomycins show a greater K_s at pH 4.8 compared to pH 2.8. The ratios between K_s at pH 4.8 and 2.8 for all complexes is around 2.3 except for MMB-γ-CyD (Table 3). The fact that MMB differs structurally from the other mitomycins at C9 (stereochemically) and C9a (OH instead of OCH_3) should account for this different ratio, indicating that the functional groups at C9 and C9a play an important role in the inclusion of the mitomycins in γ-CyD. The fact that K_s for MMB is significantly lower at all pH values investigated may be explained by the greater hydrophilicity of MMB as a result of the presence of a hydroxyl group at C9a instead of a methoxy function.

Standard deviation in k_0, k_{cat} and K_s

The standard deviations (S.D.) in k_0, k_{cat} and K_s, were determined at pH 2.8 for the degradation of MMC with γ-CyD as complexing agent. The value of k_0 obtained with a γ-CyD free MMC solution, is $7.6 \pm 0.3 \times 10^{-4}$ s^{-1} ($n = 6$) whereas the values of $k_{cat} \pm$ S.D. and $K_s \pm$ S.D. for the complexed MMC are $1.2 \pm 0.5 \times 10^{-4}$ s^{-1} and 107 ± 16 M^{-1} ($n = 5$), respectively. Other rate and stability constants are mean values of duplicate determinations.

Influence of pH

From Table 2 it is obvious that the pH has a distinct influence on the formation of the mitomycin–γ-CyD complexes. As stated earlier, at pH < 1 no stabilizing effect of γ-CyD on the degradation of MMA, MMB, MMC, PM and M-83 has been found. At pH > 1 an increase in stabilization for these mitomycins occurs with pH. This observation finds its explanation in the deprotonation at these pH values of the cationic forms of the mitomycins of which the aziridine pK_a values are all around 2.8.

No influence of the pH was found in the case of AcMMC, since the pK_a of AcMMC is < 0. It seems attractive to conclude from these experiments that only the non-protonated, neutral forms of the mitomycins are included in γ-CyD while the cations cannot be included in the γ-CyD cavity. The hydrophobic atmosphere in the γ-CyD cavity may prevent the relatively hydrophilic, charged mitomycin molecule from entering.

Influence of CyD concentration

Fig. 5 shows the effect of the γ-CyD concentration on the reaction rate constant of MMC. This effect is most prominent in the concentration range $0–2 \cdot 10^{-2}$ M γ-CyD. On increasing the γ-CyD concentration above the level of $2 \cdot 10^{-2}$ M γ-CyD, only a slight effect on the stability of MMC is observed. This observation can be explained by assuming that after addition of a sufficient γ-CyD concentration almost all MMC has been captured.

Influence of ionic strength

The influence of the ionic strength of the medium on the degradation of MMC has been studied at pH 2.8 in the absence and in the presence of γ-CyD at constant buffer concentration and temperature. The ionic strength was varied by adding various amounts of sodium chloride to the degradation media. With a k_{obs} of 8.0×10^{-4} s^{-1} for the free drug the k_{obs} decreases to 4.0×10^{-4} s^{-1} in the presence of γ-CyD in the ionic strength range of 0.01 M to 1.0 M. No changes in both k_{obs} values were observed on changing the ionic strength in this range. So no noticeable influence of the ionic strength on the degradation rate of free as well as complexed MMC could be observed.

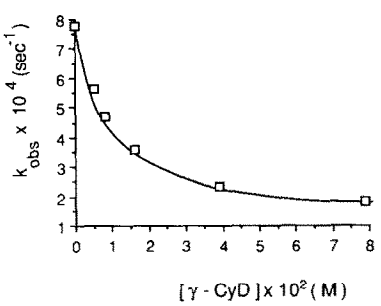


Fig. 5. Influence of the γ-CyD concentration on MMC degradation at pH 2.8.

Influence of buffer components

It is well known that buffer components, such as acetate and phosphate, exert catalytic effects on the degradation of MMC in acidic medium (Beijnen et al., 1986b). The influence of an acetate buffer on the degradation of free MMC and the MMC-γ-CyD complex has been studied at pH 2.8 and 4.8. The pK_a of acetic acid is 4.8, so at pH 2.8 acetate solutions consist almost exclusively of acetic acid while at pH 4.8 half of the acetic acid is deprotonated.

The influence of the total buffer concentration on the overall degradation of the free and complexed drug is given in Table 4. It appears from Table 4 that the influence of the total buffer concentration on the observed degradation rate constant, k_{obs}, is greater for the complexed than the free MMC. A possible explanation for this observation is that the large excess of uncharged acetic acid forms a complex with γ-CyD in competition with MMC, which results in a dissociation for the MMC-γ-CyD complex. Confirmation for this explanation is given by the fact that at pH 4.8 the ratio between k_{obs} for the free and complexed drug decreases less strongly by increasing acetate concentration than at pH 2.8 where 99% uncharged acetic acid is present.

Influence of temperature

The temperature dependence of the degradation of free and complexed MMC was studied at pH 2.8 over the range 20–60 °C. The ionic strength and the buffer concentration were maintained constant. The relationship between k_{obs} and the

TABLE 4

Influence of the total acetate buffer concentration on the degradation rate constant of free MMC (k_{obs}^{MMC}) and MMC in the presence of 2×10^{-2} M γ-CyD ($k_{obs}^{MMC/\gamma\text{-}CyD}$) at pH 2.8 and 4.8

pH 2.8				pH 4.8		
[NaAc] (M)	k_{obs}^{MMC} (s^{-1})	$k_{obs}^{MMC/\gamma\text{-CyD}}$	$\frac{k_{obs}^{MMC}}{k_{obs}^{MMC/\gamma\text{-CyD}}}$	k_{obs}^{MMC} (s^{-1})	$k_{obs}^{MMC/\gamma\text{-CyD}}$	$\frac{k_{obs}^{MMC}}{k_{obs}^{MMC/\gamma\text{-CyD}}}$
0.01	8.5×10^{-4}	4.0×10^{-4}	2.1	1.0×10^{-5}	3.8×10^{-6}	2.6
0.1	9.0×10^{-4}	4.8×10^{-4}	1.9	1.4×10^{-5}	5.6×10^{-6}	2.5
0.2	9.6×10^{-4}	5.8×10^{-4}	1.7	1.7×10^{-5}	7.0×10^{-6}	2.4
0.4	10.5×10^{-4}	7.2×10^{-4}	1.4	2.3×10^{-5}	1.0×10^{-5}	2.3
0.6	11.1×10^{-4}	8.7×10^{-4}	1.3	2.7×10^{-5}	1.3×10^{-5}	2.1
0.8	11.5×10^{-4}	9.2×10^{-4}	1.3	2.9×10^{-5}	1.5×10^{-5}	1.9
1.0	11.8×10^{-4}	9.6×10^{-4}	1.2	3.1×10^{-5}	1.6×10^{-5}	1.9

reciprocal of absolute temperature follows the Arrhenius relationship (Eqn. 7):

$$\ln k_{obs} = \ln A - \frac{E_a}{RT} \quad (7)$$

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

After construction of Arrhenius plots, it appears that the activation energy for the free MMC is 73.4 kJ · Mol^{-1} and for MMC in the presence of γ-CyD 81.5 kJ · Mol^{-1}. The somewhat higher activation energy obtained for MMC in the presence of γ-CyD suggests that some protection against the influence of heat on the degradation occurs. The fact that changes in K_s occur on changing the temperature of the degradation experiments in the presence of γ-CyD is not taken into account, so that the intrinsic value of E_a for the degradation process of MMC in the presence of γ-CyD may differ from the experimentally determined value, mentioned above.

Degradation mechanism

It is well known that the degradation of MMC in acidic medium results in the formation of two degradation products, *cis* and *trans* 2,7-diamino-1-hydroxymitosene (Fig. 2). This *cis*/*trans* ratio is pH dependent (Beijnen and Underberg, 1985; Underberg and Beijnen, 1987). The influences of

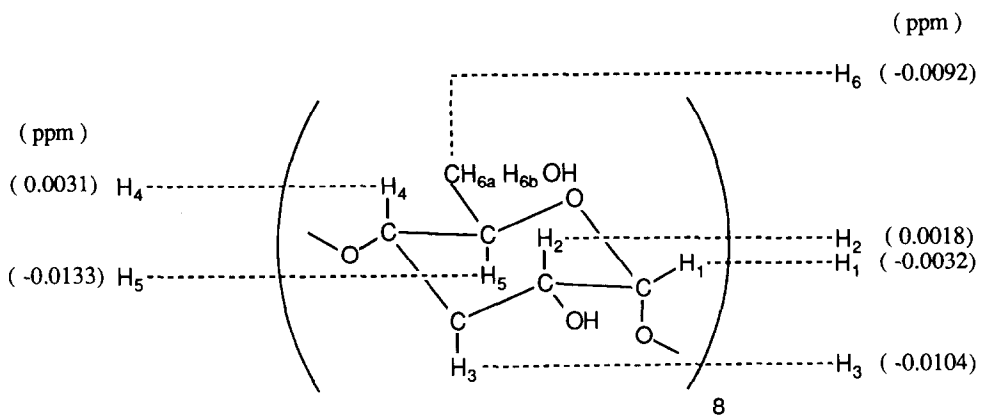


Fig. 6. Influence of MMC on the ^1H chemical shifts δ of γ-cyclodextrin at pH 6.3. The values in parentheses are calculated by the expression: $\Delta\delta = \delta_{Complex} - \delta_{\gamma\text{-}CyD}$.

β- and γ-CyD on the *cis/trans* mitosene ratio in the MMC degradation have been studied over the pH range 2 to 5. It appears that the presence of β-CyD as well as γ-CyD has no significant influence on the *cis/trans* ratio of the mitosenes in comparison with the free MMC. These results make it acceptable to assume that the degradation mechanism for the complexed mitomycin in acidic media is identical to that for the free drug. Confirmation for this suggestion is obtained by the fact that CyD has no influence on the kinetic behaviour (order of reactions) of the mitomycins.

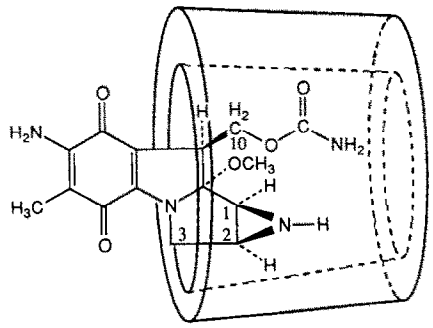


Fig. 8. Proposed structure of the inclusion complex of γ-CyD with MMC in acidic aqueous medium.

Structure of the MMC–γ-CyD complex

^{1}H-NMR spectra were analyzed to gain insight into the mode of inclusion of MMC in γ-CyD in aqueous solution. Assignment of the ^{1}H-NMR peaks of both MMC and γ-CyD was straight forwardly on the basis of previous work by Lown and Begleiter (1974) and Demarco and Thakker (1970). Fig. 6 shows the MMC-induced ^{1}H-chemical shifts of γ-CyD. The signals of protons located around the interior of the CyD cavity (e.g. H-3, H-5 and H-6) are shifted upfield, while the protons (H-2 and H-4) on the exterior of the cavity were shifted downfield, except for the H-1, as expected from the previous data of drug–cyclodextrin interaction. These data suggest that the MMC molecule interacts with γ-CyD at the entrance side of the torus. Fig. 7 shows the effects of γ-CyD on the ^{1}H-chemical shifts of MMC. Unfortunately, some proton signals were too weak to be quantitatively analyzed under the present experimental conditions, By the binding to γ-CyD, the signal changes for C_{10}-H, C_{9a}-OCH_3 and C_1-H were significantly larger, compared with other signals.

In this respect it is noteworthy that the C_{10}–H and C_{9a}–OCH_3 signals were shifted to downfield, and the C_1–H peak was shifted to upfield. This easily leads to the suggestion that the aziridine-ring and the side chain at C_9 of the pyrroline-ring involve the inclusion complexation with γ-CyD, rather than the pyrrolidine-ring and quinone part. The proposed structure of the complex is illustrated in Fig. 8. This structure is in good agreement with the obtained kinetic results, because these suggest that the aziridine-ring is protected for degradation in acidic aqueous media.

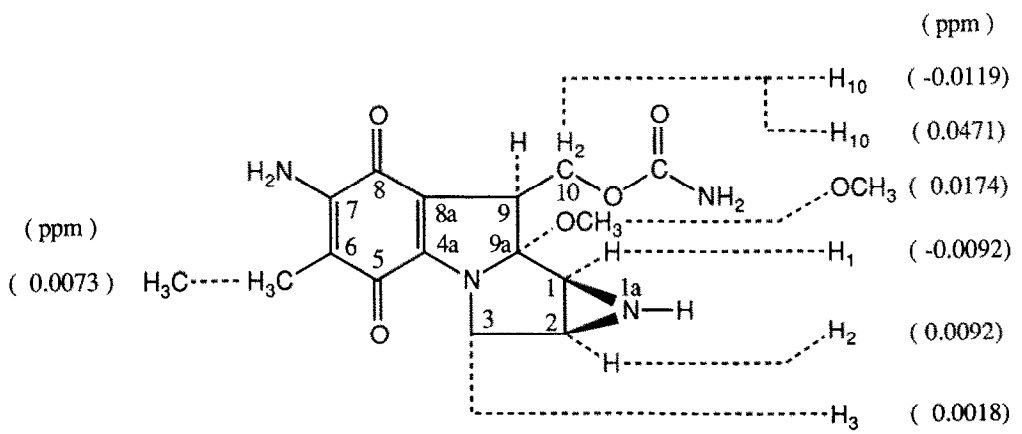


Fig. 7. Influence of γ-CyD on the ^{1}H chemical shifts of MMC at pH 6.3. The values in parenthesis are calculated by the expression: $\Delta\delta = \Delta\delta_{complex} - \delta_{MMC}$.

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