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Macromolecular prodrugs IV. Kinetics of hydrolysis of metronidazole monosuccinate dextran ester conjugates in aqueous solution and in plasma — sequential release of metronidazole from the conjugates at physiological pH

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Summary

The kinetics of hydrolysis of metronidazole monosuccinate dextran ester conjugates in aqueous solution over the pH range 6.61–7.80 (37°C) has been investigated. As demonstrated by HPLC the degradation of the dextran conjugates proceeds through parallel formation of metronidazole and metronidazole monosuccinate, respectively. The regeneration rates of the latter compounds followed first-order kinetics. The pH dependence of the various rate constants showed almost parallel straight line portions with slopes varying from 0.93 to 0.95, indicating that the hydrolysis reactions were subject to specific base catalysis. A 5-fold increase in the hydrolysis rate of the metronidazole-spacer arm ester bond compared to the stability of metronidazole monosuccinate, per se, was observed. Similarly, the carbohydrate succinic acid ester linkage showed greater susceptibility to undergo degradation at neutral pH in proportion to corresponding aliphatic ester derivatives, suggesting participation of intramolecular catalysis in the hydrolysis of the conjugate ester bonds by the neighbouring carbohydrate hydroxy groups. Almost identical stability of the conjugates was observed after incubation in 0.05 M phosphate buffer pH 7.40 and in 80% human plasma ($t_{1/2}$ ~ 32 h at 37°C) revealing that the hydrolysis in plasma proceeds without enzymatic catalysis.

Introduction

In order to optimize drug action new drug formulations have been developed based on advanced technological delivery systems (Juliano, 1980; Chien, 1982; Langer and Wise, 1984) or the prodrug approach (Stella et al., 1980; Bundgaard, 1985). The increasing interest in the field of macromolecular prodrugs should be seen in the light of the still growing need for delivery systems which may enable one to transport active agents selectively to the target and consequently release the drug over a desired period of time (Poznansky and Cleland, 1980; Hurwitz, 1983; Wingard, 1983; Edwards, 1983; Goldberg, 1983; Duncan and Kopecek, 1984; Poznansky, 1984; Poznansky and Juliano, 1984; Sezaki and Hashida, 1984; Larsen and Johansen, 1985a).

In the past only little attention has been paid to the design of macromolecular conjugates with effi-

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cient and predictable delivery characteristics. The present study was undertaken to provide basic information about the release pattern of drug compounds possessing an alcohol functional group linked to dextran carriers through a dicarboxylic acid spacer arm.

Materials and Methods

Metronidazole and dextran T-70 ($M_w = 75{,}200$; $M_n = 39{,}000$) were obtained from Dumex, Copenhagen and Pharmacia, Sweden, respectively. Metronidazole monosuccinate and the corresponding dextran ester conjugates were synthesized according to Vermeersch et al. (1985) with slight modifications. The conjugates were characterized as previously described (Johansen and Larsen, 1985; Larsen et al., 1986). Other chemicals and solvents were of analytical grade or the best possible quality.

General procedures

Ultraviolet spectral measurements were performed with a Shimadzu UV-190 recording spectrophotometer, using 1 cm quartz cells. Infrared spectra were determined on a Perkin-Elmer 457 Grating IR spectrophotometer. High-performance liquid chromatography was done with a Waters Associates Model 6000 A constant-flow pump equipped with a Pye Unicam PU 4020 variable wavelength detector and a Rheodyne Model 7125 injection valve with a 20-μl loop. A column, 250 × 4 mm, packed with LiChrosorb RP-8 (7 μm particles) was eluted with a mobile phase consisting of methanol – 0.05 M citrate buffer pH 2.7 (4:6 v/v). Optical rotation measurements were carried out using a Perkin Elmer Model 141 polarimeter. Readings of pH were done with a Radiometer Type pH M 26 meter at the temperature of study.

Kinetic measurements

Phosphate and carbonate buffers were used. Each buffer was adjusted to an ionic strength of 0.5 by use of potassium chloride. The reaction solutions were kept at a constant temperature of 37 ± 0.2°C. In the case of the carbonate buffer the degradation was followed at 60 ± 0.2°C. The reactions were initiated by adding an accurately weighted amount of the conjugate to preheated solutions of the individual buffers. The initial concentration of the conjugate corresponded to approximately 2×10^{-3} M with respect to metronidazole monosuccinate (MMS). Samples were withdrawn at suitable intervals and analyzed immediately by using the HPLC procedure, which allowed baseline separation of metronidazole and the monosuccinate ester derivative. Conjugates with degrees of substitution (DS) of 0.32, 4.61, 6.20 and 8.41 have been employed in this study (DS is expressed as the percentage of mg MMS released per mg of the conjugate).

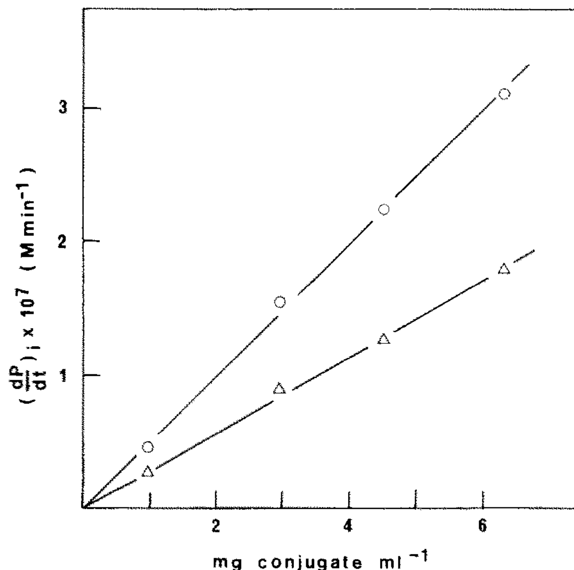


Fig. 1. The influence of the concentration of dextran metronidazole monosuccinate ester (DS 4.61) on the initial rates of product formation $(dP/dt)_i$ in 0.05 M phosphate buffer pH 7.50 (37°C and $\mu = 0.5$), △, metronidazole; ○, metronidazole monosuccinate.

The pH dependent first-order rate constants were determined exploiting the initial rate method (Connors, 1973). The presence of pseudo-first-order degradation kinetics was established by linear plots of the initial rates of product formation versus the concentration of the dextran conjugate (Fig. 1).

The release kinetics of the dextran conjugates in 80% human plasma has been determined by transferring a known amount of the conjugate to a pre-equilibrated plasma sample at 37°C to give a

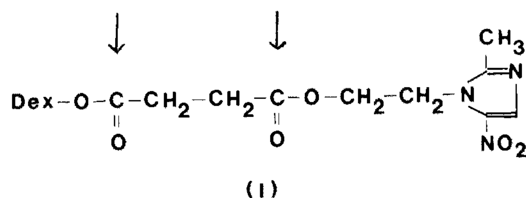
final concentration of MMS corresponding to 8×10^{-3} M. The experimental conditions were as described above, but 500 μl samples were withdrawn and deproteinized with 1500 μl of methanol. The mixture was vortexed and after centrifugation (2 min at $10{,}000 \times g$) 20 μl of the clear supernatant was applied to column. The simultaneous formation of metronidazole and MMS was followed for a time period resulting in an overall degradation of the conjugate of less than 3%.

Results and Discussion

Kinetics of hydrolysis of metronidazole monosuccinate dextran ester conjugates at neutral pH

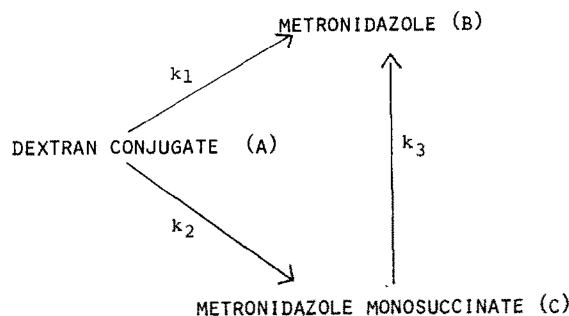
The degradation of metronidazole monosuccinate dextran ester conjugates (Dex-MMS) was investigated in aqueous phosphate buffer solutions over the pH range 6.61–7.80 at 37°C. At each pH value the pseudo first-order rate constants were derived as described above. No significant general acid–base catalysis of Dex-MMS hydrolysis by the phosphate buffers in the concentration range 0.05–0.2 M was observed.

Since Dex-MMS conjugates (I) possess two es-



Formule 1

ter groups susceptible to undergo hydrolytic cleavage the overall reactions may be described by the following scheme:



Scheme 1

where k_1–k_3 are pseudo-first-order rate constants for the depicted reactions. Letting k_{obs} represent the overall first-order rate constant for degradation of the dextran conjugate:

$$k_{obs} = k_1 + k_2 \tag{1}$$

and therefore the 3 compounds in the reaction mixture (A, B and C in Scheme 1) have a time dependence given by the following expressions:

$$A_t = A_0 \cdot e^{-k_{obs}t} \tag{2}$$

$$\frac{dB}{dt} = k_1 \cdot A_0 \cdot e^{-k_{obs}t} + k_3 \cdot C_t \tag{3}$$

$$\frac{dC}{dt} = k_2 \cdot A_0 \cdot e^{-k_{obs}t} - k_3 \cdot C_t \tag{4}$$

where A_0 is the initial concentration of the conjugate. By measurement of the formation of the compounds B and C for a time period in which only a negligible extent of the reaction has occurred ($A_t - A_0$) equations 3 and 4 may be written:

$$k_1 = \frac{\left(\frac{dB}{dt}\right)_i}{A_0} \tag{5}$$

$$k_2 = \frac{\left(\frac{dC}{dt}\right)_i}{A_0} \tag{6}$$

TABLE 1

Rate constants for degradation of a dextran metronidazole monosuccinate ester conjugate (DS 4.61) in 0.05 M aqueous phosphate buffer solutions (37°C and μ 0.5)

pH	$k_1 \times 10^3$ (h^{-1})	$k_2 \times 10^3$ (h^{-1})	$k_{obs} \times 10^3$ (h^{-1})	k_2/k_1
7.80	19.8	32.8	52.6	1.66
7.50	10.6	17.7	28.3	1.67
7.40	7.80	13.8	21.6	1.77
7.20	5.46	9.10	14.6	1.67
7.00	3.00	5.76	8.8	1.92
6.91	2.92	4.75	7.7	1.63
6.61	1.72	2.60	4.3	1.51
			average	1.69

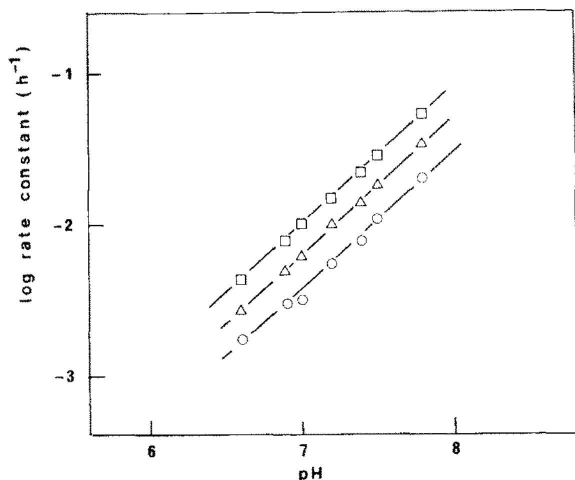


Fig. 2. pH dependence of the rate constants involved in the degradation of metronidazole monosuccinate dextran ester conjugate (DS 4.61) in 0.05 M phosphate buffer solutions at 37°C ($\mu = 0.5$). □, k_{obs}; △, k_2; ○, k_1.

where $(dB/dt)_i$ and $(dC/dt)_i$ refer to the initial rates of formation of compound B and C, respectively. In Table 1 are presented values of k_1 and k_2 determined at various pH values together with values of the overall degradation rate constants, k_{obs}, calculated from Eqn. 1.

The pH dependence of the rate constants k_1, k_2 and k_{obs} is shown in Fig. 2. In the investigated pH range the pH–rate profiles show almost parallel straight line portions with slopes varying from 0.93 to 0.95, indicating that the hydrolysis reactions are subject to specific base catalysis. Using the value of the hydroxide ion activities, calculated from the following equation (Harned and Hamer, 1933):

$$\log a_{OH} = \text{pH} - 13.62 \qquad (7)$$

the value of the overall second order rate constant for specific base catalysis (k_{OH}) amounts to 3.94 $\times 10^4$ $M \cdot h^{-1}$.

The rate constants for hydrolysis of Dex-MMS conjugates with varying degree of substitution in 80% human plasma at 37°C are presented in Table 2. A typical rate plot is shown in Fig. 3. From Tables 1 and 2 it appears that the hydrolysis rates of the conjugates in plasma and 0.05 M phosphate buffer pH 7.40 (37°C) are comparable

TABLE 2

Rate constants for hydrolysis of dextran metronidazole monosuccinate ester conjugates with varying degree of substitution (DS) in 80% human plasma (37°C)

Dex-MMS (DS)	$k_1 \times 10^3$ (h^{-1})	$k_2 \times 10^3$ (h^{-1})	$k_{obs} \times 10^3$ (h^{-1})	k_2/k_1
4.61	4.32	7.10	11.42	1.64
8.43	4.56	7.34	11.90	1.61

suggesting that the hydrolytic reactions in human plasma are not accelerated by catalysis of non-specific esterases.

In addition, it is apparent that the degradation rates are insensitive to variation of the degree of substitution of the conjugates.

Intramolecular catalysis of release of metronidazole and MMS from Dex-MMS conjugates by the polysaccharide hydroxy groups

Participation of intramolecular catalysis in the hydrolysis of various dextran esters has previously been suggested (Larsen and Johansen, 1985b; Larsen et al., 1986). The carboxylic acid compounds were linked directly to dextran through ester link-

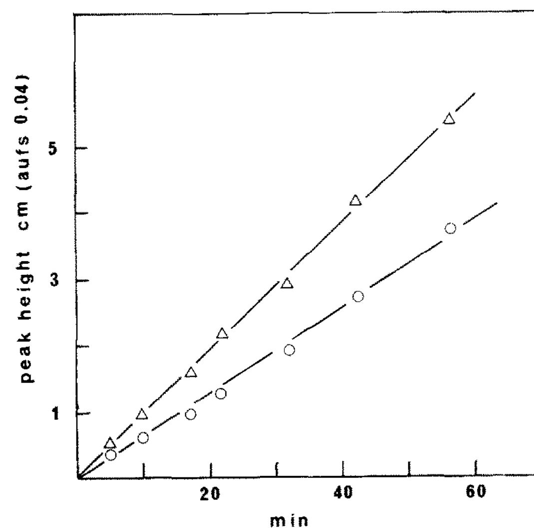


Fig. 3. Initial rates of formation of metronidazole (○) and MMS (△) in 80% human plasma from a Dex-MMS conjugate (DS 4.61 and 12.0 mg/ml) at 37°C as determined by HPLC.

ages, but the increased susceptibility of the latter compounds to undergo alkaline hydrolysis, compared to the corresponding esters derived from aliphatic alcohols, does not entirely rule out the possibility that the increased hydrolytic sensitivity of the carbohydrate ester bond might be due to enhanced reactivity of the dextran hydroxy groups. In the case of Dex-MMS conjugates metronidazole is attached to the terminal carboxylic acid group of the spacer arm and therefore the rate constant k_1 (Scheme 1) represents the release rate of metronidazole from the conjugate, whereas the stability of MMS, per se, is expressed by the rate constant k_3. The degradation kinetics of MMS and several other aliphatic esters of metronidazole in aqueous solution has been investigated revealing almost equal stabilities of the compounds at neutral pH (Johansen and Larsen, 1984; Johansen and Larsen, 1985a). By comparison of the rate constant for hydrolysis of metronidazole (pH 7.40 and 37°C) reported in the latter studies and the corresponding k_1-value in Table 1 a 5-fold greater reactivity of the Dex-MMS ester bond can be calculated. The enhanced rate of release of metronidazole from the conjugate may most likely be attributed to intramolecular catalysis by the neighbouring hydroxy groups.

The reaction pathways illustrated in Scheme 1 and consequently the above suggestions might be at least partly substantiated by showing that k_3 actually is a measure of the hydrolysis rate of metronidazole monosuccinate, per se. Determination of k_3 might be carried out using Eqns. 1, 3 and 4 and the following expressions:

$$C_t = \frac{k_2 \cdot A_0}{k_{obs} - k_3}\left(e^{-k_3 t} - e^{-k_{obs} t}\right) \tag{8}$$

$$\frac{dB}{dt} = \left(k_1 \cdot A_0 - \frac{k_3 \cdot k_2 \cdot A_0}{k_{obs} - k_3}\right) e^{-k_{obs} t} + \frac{k_3 \cdot k_2 \cdot A_0}{k_{obs} - k_3} e^{-k_3 t} \tag{9}$$

When the degradation reaction has proceeded for a certain time period the former term in Eqn. 9 approaches zero (since $k_{obs} > k_3$) leading to:

$$\frac{dB}{dt} = \left(\frac{k_3 \cdot k_2 \cdot A_0}{k_{obs} - k_3}\right) e^{-k_3 t} \tag{10}$$

or

$$\ln(B_\infty - B_t) = \ln\left(\frac{k_2 \cdot A_0}{k_{obs} - k_3}\right) - k_3 t \tag{11}$$

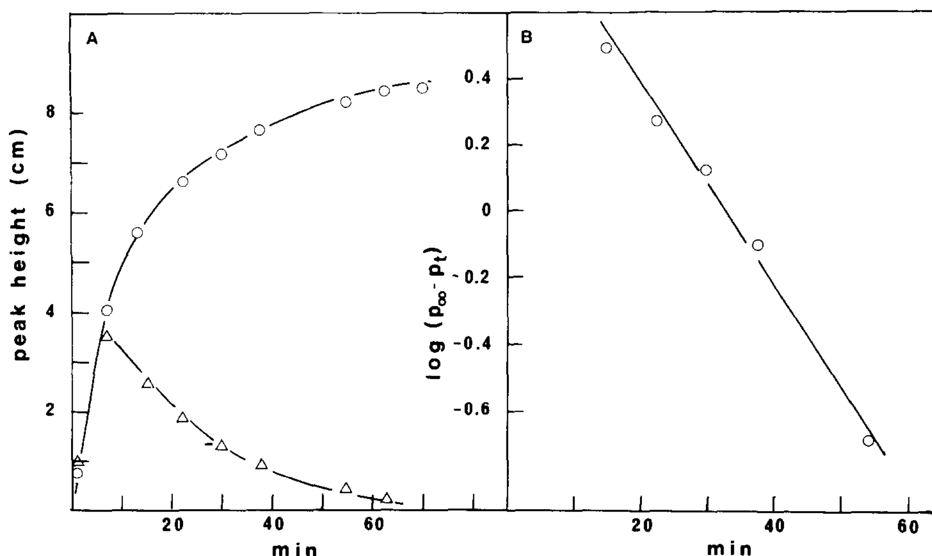


Fig. 4. Time courses for metronidazole (○) and MMS (△) after incubation of a Dex-MMS conjugate in 0.05 M carbonate buffer pH 10.00 (60°C and $\mu = 0.5$) as determined in HPLC (see text for details).

where B_∞ represents the concentration of metronidazole at infinite time. At the point when the term in $e^{-k_{obs}t}$ becomes negligible a plot of $\ln(B_\infty - B_t)$ versus time will be linear with the slope equal to $-k_3$. A value of k_3 corresponding to 0.067 min^{-1} has been calculated after incubation of a Dex-MMS conjugate in 0.05 M carbonate buffer pH 10.00 at 60°C (Fig. 4).

Under identical reaction conditions a value of k_{obs} for hydrolysis of metronidazole monosuccinate of 0.078 min^{-1} has been found (Johansen and Larsen, 1984) indicating that the degradation of Dex-MMS adequately may be described as presented in Scheme 1. Also the dextran succinic acid monoester bond is disrupted faster in aqueous solution pH 7.40 (represented by the first-order rate constant k_2 in Scheme 1) in proportion to aliphatic succinate monoester compounds. The increment of the former ester structure with respect to susceptibility to undergo hydrolysis amounts to a factor of approximately 10. This observation adds additional evidence for the proposed participation of intramolecular catalysis by the carbohydrate alcohol groups in the degradation of Dex-MMS conjugates in neutral to weakly alkaline aqueous solutions. The occurrence of intermolecular catalysis seems less likely, since k_3 and the similar rate constant for hydrolysis of MMS in the absence of dextran are of the same order of magnitude.

As all rate data are derived using the initial rate method, it cannot be totally excluded that the Dex-MMS conjugates contain small portions of particularly reactive ester structures responsible for the observed enhanced release rate of MMS from the conjugates. It has been suggested that the C-2 hydroxy group of glucose exhibits greater reactivity than the alcohol groups in position C-3 and C-4 (de Belder and Norrman, 1968). Unless this difference in sensitivity towards hydrolysis is pronounced, however, the rate of cleavage of all 3 ester structures contribute to the determined k_2-values, since MMS is expected to be attached equally among the secondary hydroxy groups due to acyl migration (de Belder and Norrman, 1968). Substituents on the anomeric C-1 OH-group of the polysaccharide conjugates are presumably not present due to the extreme instability of such acylal-type derivatives (Hussain et al., 1979). It might therefore be concluded that the data of the present study strongly suggest the involvement of intramolecular catalysis of release of metronidazole and MMS by the neighbouring carbohydrate hydroxy groups. Presently, experiments are being carried out in this laboratory in order to provide unequivocal evidence for the above proposal.

Sequential liberation of drugs from macromolecular prodrugs

The application of the spacer arm technique in the macromolecular prodrug approach has hitherto predominantly been employed in order to enable drug attachment in cases where the functional groups of the active agent and/or the carrier have not allowed direct fixation (for reviews, see Poznansky and Cleland, 1980; Baurain et al., 1983; Sezaki and Hashida, 1984; Larsen and Johansen, 1985a). However, selective activation of daunorubicin by lysosomal hydrolases has been reported (Trouet et al., 1982) by intercalating between the drug and serum albumin a tetrapeptidic spacer arm.

As evident from the present study of hydrolysis of Dex-MMS conjugates the pharmacokinetic profile of the active compound is dependent on the lability of both the spacer arm drug bond and the linkage between the spacer and the polymeric matrix. Sequential release of the drug substance may proceed as shown for Dex-MMS. Another situation may occur if the pH-dependent hydrolysis of the conjugate only regenerates the spacer arm drug derivative (the corresponding low molecular weight prodrug). After parenteral administration sequentially labile prodrugs might therefore act as a depot releasing a low molecular weight derivative which subsequently is activated at the target tissue. The potential utility of this principle seems especially attractive when selective drug action has to be exerted after passage across the capillary membrane.

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