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Estramustine: hydrolysis, solubilization, and stabilization in aqueous solutions

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Summary

The stability of estramustine, estradiol 3-bis(2-chloroethyl)carbamate, in buffered aqueous solutions was investigated over the pH range 1.0–11.2. The hydrolytic rate was unaffected by pH up to pH approx. 10 but increased at higher pH values The suggested unimolecular hydrolysis mechanism of the pH-independent region of the pH-rate profile, involving acyl-oxygen cleavage, is supported by the activation parameters, the solvent isotope effect and other data obtained during this investigation. The main hydrolysis product was estradiol. The effect of six cyclodextrin (CD) derivatives on the aqueous solubility of estramustine was investigated. All the CDs increased the solubility, but heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DMβCD) and a mixture of maltosyl- and dimaltosyl-β-cyclodextrin (M/DM-βCD) had the greatest effects. In the plateau region of the pH-rate profile, the hydrolysis of estramustine was about 2-times slower in a 1% aqueous M/DM-βCD buffer solution than in a solution containing no CD.

Introduction

Estramustine, estradiol 3-bis(2-chloroethyl)-carbamate, is a nitrogen mustard type bifunctional alkylating agent. Estramustine is practically insoluble in water, but its ester derivative, estramustine 17-phosphate (Estracyt), is soluble in water and used in the treatment of prostate cancer (Hauser and Merryman, 1984). Estramustine is one of the main active metabolites of estramustine 17-phosphate (Andersson et al., 1981) and, therefore, it is logical to investigate the possibility of using estramustine itself as the drug instead of its 17-phosphate ester. In fact, since the phosphate moiety appears to be lost in the gastro-intestinal tract and liver after oral administration (Hauser and Merryman, 1984), estramustine 17-phosphate can be considered a prodrug of estramustine.

The biological alkylation of the nitrogen mustards proceeds by an S_{Ni} mechanism via a highly strained intermediate ethyleneimmonium ion (Stenlake, 1979). Unfortunately, this intermediate, which is essential for the pharmacological activity, is also readily formed in aqueous solutions where it is attacked by nucleophiles such as water. Thus, most nitrogen mustards, e.g. chlorambucil and melphalan, which are bis(2-chloro-

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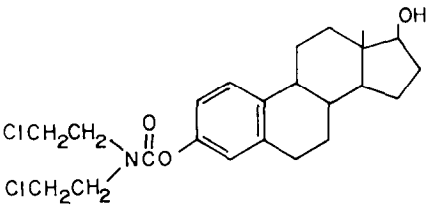
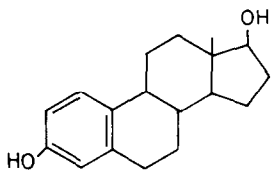


ESTRAMUSTINE



ESTRADIOL

ethyl)aminoaryl derivatives, have a short shelf-life in aqueous solutions (Loftsson et al., 1989). Estramustine, which is a bis(2-chloroethyl)carbamate derivative, has a weaker alkylating and antineoplastic activity than most other alkylating agents (Martindale, 1989). This decreased alkylating activity results in greater stability of the drug in aqueous solutions.

Materials and Methods

Materials

Estramustine was supplied by courtesy of Pharmacia LEO Therapeutics AB (Sweden). Estradiol was obtained from Sigma Chemical Co. (U.S.A.). The following cyclodextrins (CDs) were used as supplied without further purification: 2-hydroxypropyl-β-cyclodextrin (HPβCD, Pharmatec Inc., U.S.A.), 2-hydroxyethyl-β-cyclodextrin (HEβCD, Aldrich Chemical Co., U.S.A.), mixture of maltosyl- and dimaltosyl-β-cyclodextrin(3 : 7) (M/DM-βCD, Ensuiko, Japan), heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DMβCD, Sigma), 2-hydroxypropyl-γ-cyclodextrin (HPγCD, Pharmatec) and a mixture of glucosyl-maltosyl-α,β- and -γ-cyclodextrins (G/M-CD, Ensuiko). All other chemicals were commercially available products of special reagent grade.

Chromatographic conditions

The quantitative determination of estramustine was performed on a high-performance liquid chromatographic (HPLC) equipment consisting of a Milton Roy ConstaMetric 3000 solvent delivery system, a Rheodyne 7125 injector, a Beckman Ultrasphere ODS 5 μm (150 × 4.6 mm) column and a Spectra-Physic SP8450 UV/Vis detector operated at 210 nm (estramustine) and 270 nm (estradiol). The mobile phase used for quantitative determination of estramustine consisted of acetonitrile, ethanol and water (79 : 1 : 20) and that of estradiol comprised acetonitrile, ethanol and water (54 : 1 : 45). The retention time of estramustine was 2.4 min and that of estradiol 2.0 min at 1.50 ml/min flow rate.

Buffers

Chloroacetate (pH 2.5), formate (pH 2.7), acetate (pH 3.5–5.6), phosphate (pH 1.6 and 6.4–7.6), Tris (pH 8.1), and carbonate (pH 9.2–9.6) buffers were prepared by mixing aqueous solutions of the acid with aqueous solutions of the corresponding salt. The concentration of the buffer salts ranged from 0.02 to 0.3 M. Hydrochloric acid solutions were used at pH about 1 and sodium hydroxide solutions at pH about 11. Unless otherwise indicated, the ionic strength of the buffer solutions was adjusted to 0.5 by addition of sodium chloride. The water used for the buffer preparation was distilled in all-glass apparatus.

Cyclodextrin (1.0% w/v) was added to the buffer solutions when the effects of different cyclodextrins were investigated. Water was replaced by deuterium oxide (D_2O, purity 99.8%, from Merck, Germany) when the solvent isotope effect was determined. The effect of the solvent dielectric constant on the reaction rate was investigated at 80.0°C in aqueous buffer solutions containing up to 30% v/v dioxane.

Kinetic studies

The studies of estramustine degradation were carried out by adding stock solution (15 μl) of the

drug in methanol to aqueous buffer solution (3 ml), previously equilibrated at the desired temperature in a water bath, and mixed thoroughly. The initial estramustine concentration was 9×10^{-6} M. The pH of the final reaction mixture was determined at the end of each experiment with a pH-meter (PW 9420; Philips, U.K.) standardized at the appropriate temperature. All reactions were run under pseudo-first-order conditions. Aliquots (20 μl) were injected into the column at various time intervals, and the pseudo-first-order rate constants (k_{obs}) determined from the disappearance of the drug by linear regression of natural logarithm of the peak height vs time plots. The correlation coefficient was calculated for each run.

The enthalpy of activation ($\Delta H^{\ddagger}$) and the entropy of activation ($\Delta S^{\ddagger}$) were determined from linear plots of $\ln(k/T)$, where k is k_{obs}, versus $1/T$ based on the Eyring equation:

$$\ln(k/T) = \ln(k_B/h) + \Delta S^{\ddagger}/R - (\Delta H^{\ddagger}/R) \cdot 1/T \quad (1)$$

where T is the absolute temperature, k_B denotes Bolzmann's constant, h is Planck's constant and R represents the gas constant.

Solubility studies

Solubilities were determined by adding an excess amount of estramustine to aqueous solutions containing various concentrations of cyclodextrins. The suspensions formed were sonicated in an ultrasonic bath (Kerry, U.K.) for 2 h and then placed in a 30.0 ± 0.1°C constant-temperature water bath. After equilibration for 5 days, an aliquot was filtered through a 0.45 μm membrane filter unit (Millex-HV; Millipore, U.S.A.), diluted with 70% aqueous methanol solution and analyzed by HPLC.

Results and Discussion

Hydrolysis

The hydrolysis of estramustine followed first-order kinetics in aqueous buffer solutions at constant pH and temperature. The pseudo-first-order rate constants were determined by HPLC, following the disappearance of the drug as a function of time at 80.0°C (Fig. 1). At pH 1.6, 7.5 and 9.6, the main hydrolysis product was determined to be estradiol (see representative Fig. 2) and addition of chloride ions to the reaction medium did not affect the rate of hydrolysis. Most nitrogen mustards, e.g. chlorambucil and melphalan which are bis(2-chloroethyl)aminoaryl derivatives, degrade through a cyclic ethyleneimmonium ion which is

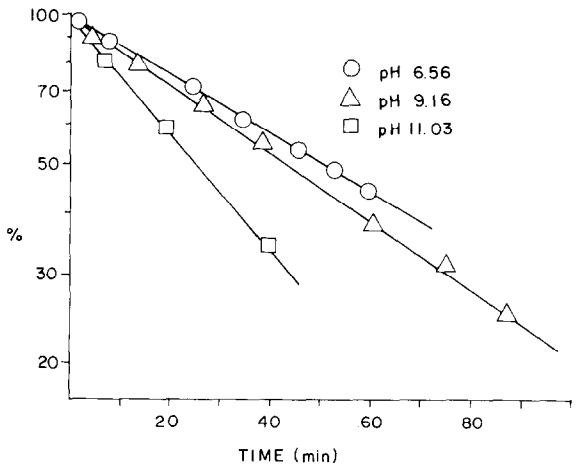


Fig. 1. Representative first-order plots (log% remaining against time) for the degradation of estramustine in aqueous buffer solutions at 80.0°C. (○) pH 6.56, (△) pH 9.16, (□) pH 11.03.

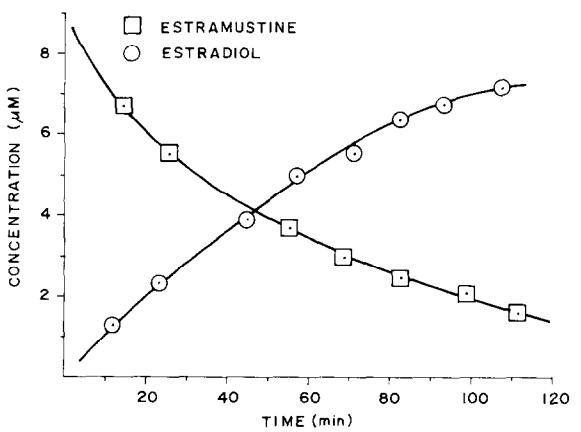


Fig. 2. Kinetics of degradation of estramustine in a 0.135 M aqueous phosphate (pH 7.5) buffer solution at 80.0°C.

formed by reversible intramolecular displacement of chloride by nitrogen (Ehrsson et al., 1980; Stout and Riley, 1985; Loftsson et al., 1989). This cyclic intermediate is highly susceptible to substitution by nucleophiles such as water and, thus, their main degradation products in aqueous solutions are the inactive hydroxyethylamino derivatives. Due to the reversible formation of the cyclic intermediate, which is the rate-determining step, addition of chloride ions to the reaction medium slows down the rate of degradation of this type of nitrogen mustard. Estramustine is a bis(2-chloroethyl)carbamate derivative and the adjacent electron-withdrawing carboxyl ester group probably prevents formation of a cyclic ethyleneimmonium ion intermediate, as indicated by the inability of chloride ions to slow down its degradation rate.

The observed rate of loss of estramustine, k_{obs}, may be given as

$$k_{obs} = k_O + k_{OH}[OH^-]$$

where k_O and k_{OH} are the constants for the uncatalyzed and the specific base-catalyzed reactions, respectively. From the pH-rate profile at 80.0°C (Fig. 3), the value of k_O was determined to be 1.40×10^{-2} min^{-1} and that of k_{OH} to be

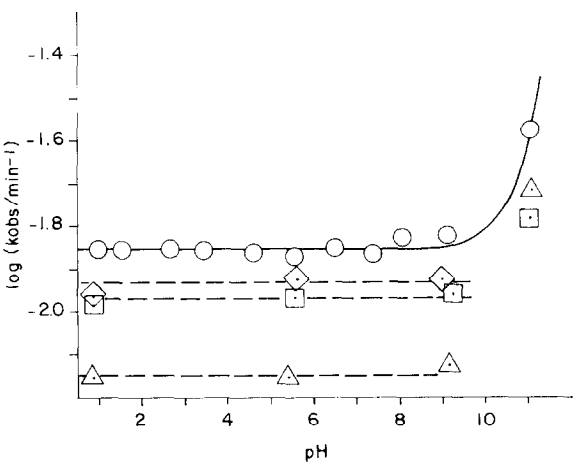


Fig. 3. The pH-rate profile for the observed first-order degradation of estramustine in aqueous buffer solution at 80.0°C. (○) No cyclodextrin, (◇) G/M-CD, (□) HPβCD, (△) M/DM-βCD.

TABLE 1

The observed first-order rate constants (k_{obs}) and the half-lives ($t_{1/2}$) for the degradation of estramustine in aqueous pH 7.5 phosphate buffer solution at various temperatures

Temperature (°C)	(K)	k_{obs} ($\times 10^4$) (min^{-1})	$t_{1/2}$ (days)
86	359	260	0.02
80	353	154	0.03
75	348	94.8	0.05
70	343	62.3	0.08
25	298	4.62 [a]	10 [a]
5	278	0.03 [a]	150 [a]

$\Delta H^{\ddagger}$ = 89.3 kJ/mol or 21.4 kcal/mol
$\Delta S^{\ddagger}$ = −62.0 J/mol per K or −14.8 cal/mol per K

[a] Calculated value.

0.51 M^{-1} min^{-1}. The pH-rate profile shows a large plateau up to pH about 10. Estramustine undergoes pH-dependent specific base-catalyzed hydrolysis at pH > 10. The plateau of pH-independent rate could be due to either a water attack on the estramustine molecule or a spontaneous, uncatalyzed decomposition of the molecule.

The enthalpy of activation ($\Delta H^{\ddagger}$) for the degradation rate in the plateau region was determined to be 89.3 kJ/mol and the entropy ($\Delta S^{\ddagger}$) of activation to be −62.0 J/mol per K (Table 1). The relatively high enthalpies and small negative entropies of activation are characteristic of a unimolecular reaction. The enhanced solvation due to the charge separation in the transition state results in a small negative entropy of activation. A bimolecular reaction involving water attack on the carbamate moiety, i.e. a molecule of water acting as a general base to assist the addition of a second molecule of water to the carbonyl group (a tetrahedral mechanism), would result in much larger negative entropy, typically about −170 to −200 J/mol per K (Kirby, 1972; Loftsson and Bodor, 1981).

The deuterium solvent isotope effect on the hydrolysis of estramustine in a pH 6.5 phosphate buffer solution was measured at 80.0°C. The hydrolysis rate constant in a buffered deuterium oxide solution (k_{D_2O}) was 1.23×10^{-2} min^{-1}

TABLE 2

Observed pseudo-first-order rate constants (k_{obs}) for the degradation of estramustine in various aqueous phosphate buffer (0.086 M, pH 7.4)-dioxane mixtures and at 80.0°C (no sodium chloride was added to the buffer solution)

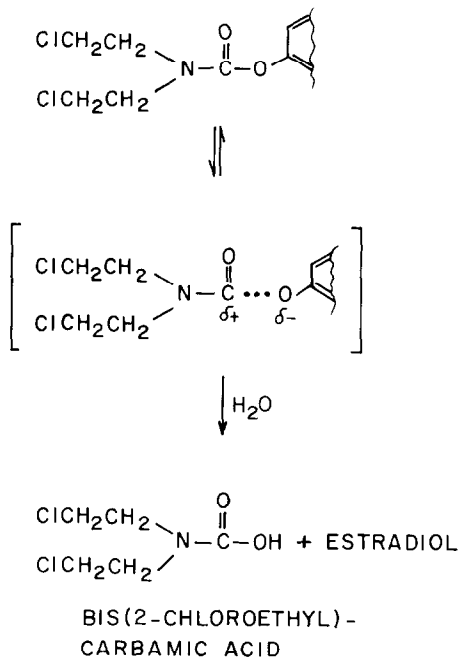
Dioxane (% v/v)	ϵ [a]	k_{obs} ($\times 10^2$) (min^{-1})
0	60.55	1.478
10	53.08	1.273
20	45.77	0.933
30	38.64	0.619

[a] The dielectric constant (ϵ) of each mixture was calculated at 80°C according to Owen and Harris (1958).

compared to (k_{H_2O}) 1.35×10^{-2} min^{-1} in aqueous buffer solution, which results in an isotope effect (k_{H_2O}/k_{D_2O}) of 1.10. This small solvent isotope effect could entirely be due to secondary effects such as solvation. Primary contribution, involving cleavage of the O-H bond in a water molecule during the formation of the activated complex, usually results in a much larger solvent isotope effect (Maskill, 1985). The hydrolysis of estramustine in the plateau region is dependent on the nature of the solvent, being about 2.4-times greater in water than in a 30% dioxane-water mixture (Table 2), again suggesting some charge development in the transition state.

All these results support the degradation mechanism shown in Scheme 1 for spontaneous, uncatalyzed decomposition of estramustine. It has been shown that acylium cations, formed by hydrolysis of *N,N*-dialkylated carbamoyl chlorides, are stabilized by electron donation from the nitrogen atom (Johnson, 1967):

This provides additional support for the proposed mechanism, since formation of a positive charge on the carbonyl carbon should also be facilitated by electron donation of the nitrogen atom. The *N*-substituted carbamic acid, bis(2-chloroethyl)-carbamic acid, formed during the hydrolysis is probably unstable and breaks down to carbon dioxide and the amine, bis(2-chloroethyl)amine (March, 1968).



Scheme 1. Proposed mechanism of degradation of estramustine at the pH-independent plateau region of the pH-rate profile.

Cyclodextrins

The effects of six CD derivatives on the aqueous solubility of estramustine were investigated. All the CDs tested increased the aqueous solubility of the drug, but DMβCD had the largest effect followed by M/DM-βCD, G/M-CD and HPβCD (Table 3). Methylated β-CDs are relatively lipophilic and act to destabilize biological membranes even at low concentration. This prop-

TABLE 3

Solubility of estramustine in aqueous unbuffered solutions, containing 0 or 1.6×10^{-2} M (approx. 2.5%) cyclodextrin, at 30.0°C

Cyclodextrin	Solubility (μg/ml)
No cyclodextrin	about 1
HPβCD	110
HEβCD	80
M/DM-βCD	1550
DMβCD	2460
HPγCD	50
G/M-CD	190

erty causes DMβCD to be hemolytic and also imparts a low i.v. LD_{50} value; only about 200 mg/kg (Müller and Brauns, 1985). Thus, DMβCD is unsuitable for i.v. preparations. The effects of the other three CDs, i.e. M/DM-βCD, G/M-CD and HPβCD, on the degradation rate of estramustine were investigated (Fig. 3). In the plateau region of the pH-rate profile the hydrolysis of estramustine was about 2-times slower in a 1% (w/v) aqueous M/DM-βCD buffer solution than in a solution containing no CD. The other two CD derivatives had less effect. The i.v. dosage of estramustine 17-phosphate is 150–300 mg (Martindale, 1989). Our results show that the solubilizing and stabilizing effects of this type of CD are not sufficient for formulation of estramustine i.v. solutions.

Acknowledgement

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