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Stabilization of an anthrapyrazole antitumour agent, DuP 937, on complexation with heptakis(2,6-di-O-methyl)-β-cyclodextrin in aqueous solution

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Abstract: DuP 937, an anthrapyrazole antitumour agent that is chemically unstable in aqueous solution, was shown, by absorption spectroscopy, to form an inclusion complex with heptakis(2,6-di-O-methyl)-β-cyclodextrin (DMβCD) in aqueous solution. Proton nuclear magnetic resonance spectroscopy was used to determine the stoichiometry and association constant of the complex. The 1:1 stoichiometry of the complex was established by the continuous variation method by following changes in the chemical shifts of aromatic protons of DuP 937. The complex association constants determined by different techniques used in this study were in the same order of magnitude. The kinetics of degradation of DuP 937 in aqueous solution were investigated as a function of DMβCD concentration at pH 5.5 and 60°C. The results indicated about a seven-fold increase in the stability of DuP 937 in the presence of DMβCD in aqueous solution.

Keywords: *Stability; cyclodextrins; inclusion complex; ^{1}H-NMR.*

Introduction

The anthracycline antibiotics, doxorubicin and daunorubicin, have proven clinical applications with activity against a wide range of human neoplasms, including a variety of solid tumours [1]. Unfortunately, their clinical value is limited by their dose-limiting cardiotoxicity [1]. The cardiotoxicity of these antibiotics has been linked to the daunosamine sugar moiety [2]. It was hypothesized that replacement of the daunosamine sugar moiety with an alkylamino side chain whilst retaining the planar ring system that is capable of intercalating with DNA, should lead to a new generation of antitumour agents with modes of action similar to the anthracyclines but without their untoward cardiac effects [2]. The result of such an effort was mitoxantrone, an amino-anthracenedione that is clinically as effective as doxorubicin in breast cancer but with an apparently reduced cardiotoxicity [1]. DuP 937 (Fig. 1), which is structurally related to mitoxantrone, is an anthrapyrazole antitumour agent with a broad spectrum of activity against many solid tumours but with reduced cardiotoxicity. However, it is susceptible to hydrolytic and oxidative degradation in aqueous solution. A viable solution to enhance the chemical stability of DuP 937 in aqueous solution is complexation with a suitable compound.

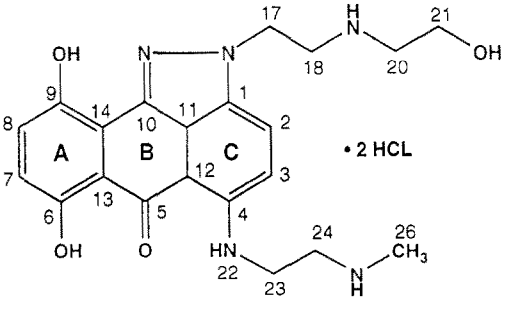

Figure 1
DuP 937.

Cyclodextrins (CD) are cyclic, non-reducing oligosaccharides, and form inclusion complexes with a number of drugs affecting many of their physicochemical properties such as chemical stability, aqueous solubility, dissolution rate, bioavailability, etc. and, in many

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cases, bringing about an improvement in these properties [3]. For example, testosterone, which suffers from a low and variable oral bioavailability, when complexed with 2-hydroxypropyl-β-cyclodextrin (HPβCD) is readily absorbed sublingually resulting in a rapid increase in plasma levels [4]. The stability of the formed complex depends on how well the guest molecule fits into the CD cavity and the strength of the mostly hydrophobic interactions between the guest and host molecules.

The inherent chemical instability of anticancer compounds imposes constraints in formulating them as liquid dosage forms. It has been demonstrated that the rate of degradation of a drug within the drug–CD inclusion complex is slower than that of the free drug in solution, and addition of CD to aqueous drug formulations can in many cases improve the shelf-life of the drug [5]. For example, cyclodextrins have been shown to stabilize chlorambucil and melphalan [6], lomustine [7] and doxorubicin [8]. In the present study the effectiveness of cyclodextrins, in particular, heptakis(2,6-di-O-methyl)-β-cyclodextrin (DMβCD), in preventing the degradation of DuP 937 in aqueous solution was investigated with the aim of improving the chemical stability of DuP 937.

Nuclear magnetic resonance (NMR) spectroscopy has been used to investigate the stoichiometries and association constants of pharmacologically important drug–CD complexes [9]. This technique provides direct evidence for the existence of true inclusion complexes in solution, as well as in the solid state. In addition, it is one of the most useful techniques in the analysis of structural and dynamic properties of complexes both in aqueous solution and in the solid state [10]. In the present study, proton magnetic resonance spectroscopy (^{1}H NMR) was used to characterize the DuP 937–DMβCD complex in terms of stoichiometry, association constant, and to obtain information on the possible inclusion modes of DuP 937 and DMβCD in aqueous solution.

Materials and Methods

Chemicals and reagents

DuP 937 was prepared by the Chemical Processing Division of The DuPont Merck Pharmaceutical Company. DMβCD (~30%, remainder primarily higher and lower O-methyl homologs), HPβCD (average molar substitution = 0.6) and β-cyclodextrin (β-CD) were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). α-Cyclodextrin (α-CD) and γ-cyclodextrin (γ-CD) were obtained from Fluka Chemical Corp., NY, USA. Deuterium oxide (99.9% atom % D) was purchased from Isotec Inc., Miamisburg, OH, USA.

Kinetic studies

The kinetic experiments were performed at a constant temperature of 60°C. Sodium citrate buffer (0.1 M, pH 5.5, μ = 0.3) was used in all kinetic studies. The studies were initiated by adding a stock solution of DuP 937 in water to buffered cyclodextrin solutions yielding an initial concentration of 1×10^{-4} M. The cyclodextrin concentrations were as follows: β-CD (1×10^{-2} M); γ-CD (1×10^{-2} M); HPβCD (1×10^{-2} M); DMβCD (1×10^{-2} M to 5×10^{-2} M). All cyclodextrins were freely soluble in the buffer at the concentrations specified above. A solution of DuP 937 (1×10^{-4} M) in the buffer containing no cyclodextrin was used as the control. At appropriate time intervals, samples were withdrawn and analysed for DuP 937 using liquid chromatography (LC). The pseudo-first-order rate constants were determined from the DuP 937 concentration vs time plots.

Liquid chromatography

Liquid chromatography was performed on a system consisting of a pump programmed by a system controller (Model 600E), an autoinjector (Model 712 Wisp), a tunable ultraviolet–visible spectrophotometric detector (Model 486) operated at 500 nm, and a column oven programmed by a temperature control module and operated at 35°C, all from Waters, USA. All separations were performed on a Zorbax Rx C-8 column (4.6 mm × 250 mm) using a mixture of acetonitrile and aqueous phase containing hexanesulphonic acid sodium salt (10 mM) and pH adjusted to 3.00 with glacial acetic acid (22.5:77.5 v/v) as mobile phase at a flow rate of 1.5 ml min^{-1}. The chromatographic data was acquired and analysed on a VG multichrom system.

Spectroscopic studies

Ultraviolet–visible absorption spectra were recorded on a Hewlett–Packard 8451A diode array spectrophotometer. All spectral

measurements were carried out at ambient temperature using hydrochloric acid (pH 1.03, 0.1 M, $\mu = 0.3$), sodium citrate buffer (pH 5.5, 0.1 M, $\mu = 0.3$), sodium phosphate (pH 7.0, 0.1 M, $\mu = 0.3$) and sodium carbonate buffer (pH 10.0, 0.1 M, $\mu = 0.3$). DuP 937 concentration was 6.2×10^{-5} M and α-, β-, γ-, HPβCD concentrations were 1×10^{-2} M and DMβCD was varied from 5×10^{-2} M to 2×10^{-3} M.

The ^{1}H NMR spectra were acquired at 400 MHz on a Varian Unity-400 spectrometer. All chemical shifts were referenced to residual HDO at 4.64 ppm. Typical conditions were as follows: 8000 Hz spectral width; 4.07 s acquisition time; 32 or 64 scans acquired with a relaxation delay of 6 s and a pulse angle of 90°. All spectra were obtained at 30°C. Steady state nuclear Overhauser enhancements (NOE) were obtained using selective irradiation for 30 s, and processed as difference spectra. Two dimensional-NOE (2D-NOE) spectra were obtained with a 300 ms mix time and a 3 s relaxation delay.

Complexation studies

The stoichiometry of the complex was determined by the continuous variation method of Job [11]. The overall concentration of the two species was kept constant at 10 mM ([DMβCD] + [DuP 937] = 10 mM), and the ratio r = [DuP 937]/([DuP 937] + [DMβCD]) was varied from 0.1 to 1.0. The value $\Delta\delta_{obs}$[DuP 937] was plotted against r. The value, $\Delta\delta_{obs}$, is the difference between the chemical shift of free DuP 937 and the observed value for a mixture of DuP 937 and DMβCD.

The association constant (K_s) was determined by holding the concentration of DuP 937 constant at 5 mM while the concentration of DMβCD was varied from 0 to 25 mM. The value $\Delta\delta_{obs}$ was plotted against the concentration of DMβCD, and fitted to equation (1) [12] using a nonlinear least squares method.

$$\Delta\delta_{bs} = \Delta\delta_{\infty} \times \tfrac{1}{2}\left(\frac{[H]}{[G]} + \frac{1}{K_S[G]} + 1\right) - \left\{\tfrac{1}{4}\left(\frac{[H]}{[G]} + \frac{1}{K_S[G]} + 1\right)^2 - \frac{[H]}{[G]}\right\}^{1/2} \quad (1)$$

In equation 1, $\Delta\delta_{obs}$ is the change in the chemical shift of a proton of DuP 937 due to the addition of a certain concentration of DMβCD, and $\Delta\delta_{\infty}$ is the limiting value of this change for infinite DMβCD concentration, H and G are concentrations of DMβCD and DuP 937, respectively.

Results and Discussion

The influence of DMβCD on the absorption spectrum of DuP 937 was studied. As cyclodextrin concentration increased at constant pH and ionic strength the absorption spectrum changed significantly, bringing about an enhancement in absorption upon addition of DMβCD. This change was observed over the entire pH range studied, with the location of the maxima shifting with the pH, suggesting that DMβCD complexes with DuP 937 irrespective of its ionization state at a pH apparently without altering the pK_a of the groups involved. The spectra of DuP 937 in a 50% v/v mixture of methanol–citrate buffer (pH 5.5) and dioxane–citrate buffer (pH 5.5) were also compared with those in citrate buffer (pH 5.5) with and without added DMβCD. The absorption characteristics of DuP 937 in the presence of DMβCD were observed to be similar to those in buffers containing methanol and dioxane, suggesting that the hydrophobic nature of DMβCD cavity was responsible for the interaction with DuP 937.

The absorptivity change of DuP 937 in the presence of different DMβCD concentrations was utilized in calculating the association constant at a particular pH using the Benesi–Hilderbrand equation 2:

$$\frac{b}{\Delta A} = \frac{1}{S_t K_s \Delta_\epsilon [\text{DM}\beta\text{CD}]} + \frac{1}{S_t \Delta_\epsilon} \quad (2)$$

where b is the path length, ΔA the absorbance change, S_t the total substrate concentration, K_s the association constant, Δ_ϵ the molar absorptivity change and [DMβCD] the ligand concentration [13]. Equation 2 is the linearized form of the 1:1 binding isotherm for spectroscopic data. A representative double reciprocal plot of the UV-Vis absorption data is shown in Fig. 2, and the K_s value was calculated to be 54 M^{-1} at pH 5.5 and 22°C. The K_s values obtained were similar in magnitude over the pH range studied suggesting that the mode of complexation is the same at all pH.

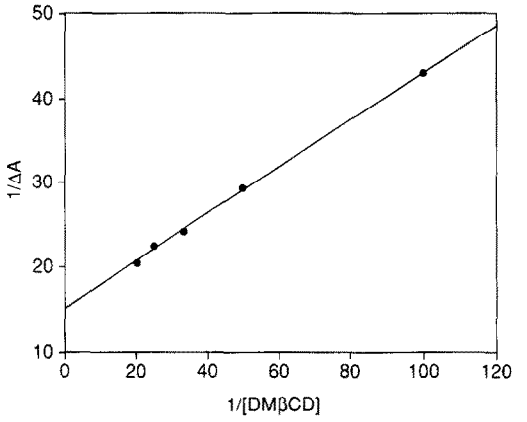


Figure 2
Representative double-reciprocal plot of the UV–visible absorption data for DuP 937 at pH 5.5 (μ = 0.3) and 22°C.

The absorption measurements were also performed with α-, β-, γ-, and HPβCD. Over the entire pH range, α-, β- and γ-CD did not cause any significant change compared with the spectra of DuP 937. The location of the maxima as well as the molar extinction of the compound did not change. However, HPβCD caused significant change over the entire pH range in a manner similar to that observed with DMβCD. These results coupled with the earlier observations on the spectral behaviour of DuP 937 in organic–aqueous buffer mixtures suggest that hydrophobicity of the cyclodextrin cavity plays a more significant role than the size of the cavity itself in their interaction with DuP 937.

The degradation of DuP 937 followed pseudo-first-order kinetics in aqueous buffer solutions at constant pH and temperature. The presence of cyclodextrin did not affect this kinetic behaviour. The influence of various cyclodextrin derivatives on the degradation of DuP 937 was studied at pH 5.5 (μ = 0.3) and 60°C. Table 1 summarizes the k_{obs} values. The results indicate that none of the cyclodextrin derivatives other than DMβCD exert significant stabilizing effect on DuP 937. All subsequent studies were therefore conducted using DMβCD as the host molecule.

The rate of degradation of DuP 937 decreased with increasing concentration of DMβCD and a non-linear relationship was obtained between the pseudo-first-order rate constants and the DMβCD concentration (Fig. 3). The results are consistent with a kinetic system where the free drug degrades at a faster rate than when it is encapsulated within the cyclodextrin cavity.

Table 1
Influence of various cyclodextrin derivatives on the degradation of DuP 937 at pH 5.5 (μ = 0.3) and 60°C

CD	k_{obs} (day^{-1})
Control	0.120
β-CD	0.103
HP-β-CD	0.102
DM-β-CD	0.088
γ-CD	0.117

[DuP 937] = 1×10^{-4} M; [CD] = 1×10^{-2} M.

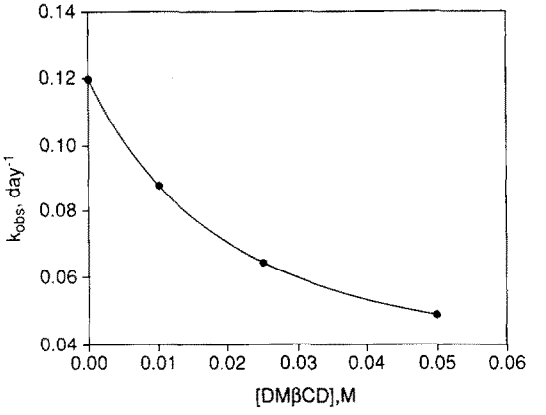
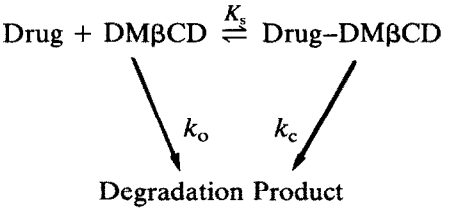


Figure 3
Influence of DMβCD on the observed rate constants for the degradation of DuP 937 at pH 5.5 (μ = 0.3) and 60°C. The initial concentration of DuP 937 was 1×10^{-4} M.



where k_o and k_c represent the pseudo-first-order rate constants for the degradation of the free drug and the drug in the complex, respectively, and K_s the association constant of the inclusion complex assuming 1:1 complexation. These constants can be extracted from equation 3,

$$\frac{k_o}{(k_o - k_{obs})} = \frac{k_o}{(k_o - k_c)\,K_s[\text{DM}\beta\text{CD}])} + \frac{k_o}{(k_o - k_c)} \qquad (3)$$

which is a double-reciprocal linear plotting form of the 1:1 binding isotherm for chemical reaction of a substrate in presence of a complexing agent [13]. Knowing k_o, k_c and K_s were obtained from a plot of $k_o/(k_o-k_{obs})$ vs 1/[DMβCD]. The values of k_c and K_s were calculated to be 0.0165 day^{-1} and 44.7 M^{-1}, respectively. The results reflect that DuP 937–DMβCD complex degrades with a lower velocity compared to the free drug by a factor of 7.3 (k_o/k_c = 7.3), indicating that protection of the labile part of the molecule was achieved by complexation. However, the ratio of k_{obs} in the absence of DMβCD versus the k_{obs} in the presence of the maximum concentration of DMβCD (0.05 M) was smaller (~2.4) compared with k_o/k_c of 7.3. This was not surprising considering that DuP 937–DMβCD is a weak complex; the small magnitude of the binding constant reflects this fact.

The stoichiometry of the complex was determined using the continuous variation method of Job [11]. A plot of the change in chemical shift of the H2 proton of DuP 937 versus the ratio r is presented in Fig. 4, and shows a maxima when the ratio r is 0.5, indicating that a complex with DuP 937:DMβCD stoichiometry of 1:1 is formed. Studies with less lipophilic cyclodextrins, such as α-cyclodextrin and γ-cyclodextrin, show no such maxima.

It has been shown [14] that the formation of inclusion complexes with DMβCD is accompanied by a significant downfield shift of the protons of the guest molecule due to the hydrophobic nature of the cavity. This effect was employed to determine the association

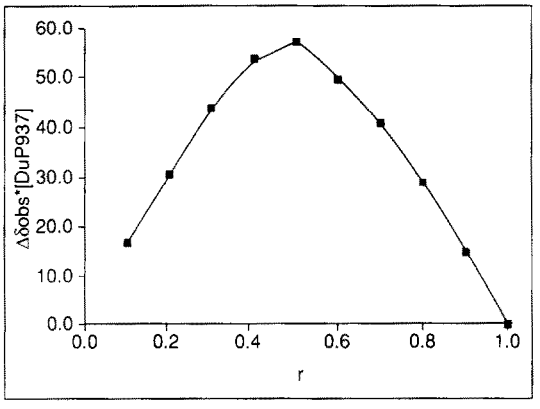


Figure 4
Continuous variation plot for change in chemical shift of the H2 proton of DuP 937 versus the ratio r.

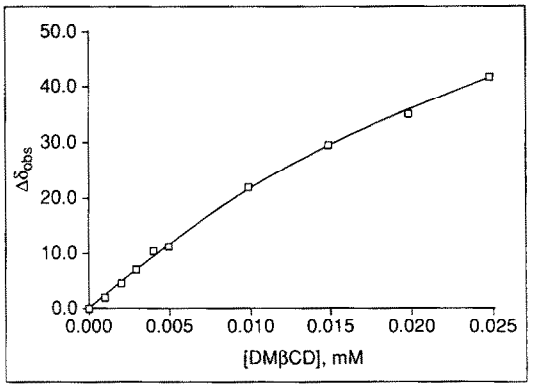


Figure 5
Plot of change in chemical shift of H2 proton of DuP 937 versus DMβCD concentration.

constant (K_s). The chemical shift of the H2 proton of DuP 937 was determined in a series of samples in which the concentration of DuP 937 was held constant at 5 mM and the concentration of DMβCD was varied from 0 to 25 mM. A plot of the change in chemical shift versus the concentration of DMβCD is shown in Fig. 5. The experimental data points were fitted to equation (1) to obtain a K_s of 30.2 M^{-1} for the inclusion of DuP 937 in DMβCD at 30°C. Values of K_s of similar magnitude were obtained for the other aromatic protons (H3, H7 and H8) and the H26 methyl group. The methyl group showed the least change in the chemical shift (8.36 Hz downfield shift at 25 mM DMβCD). All other protons of DuP 937 were obscured by the resonances of DMβCD.

Similarly, inclusion of aromatic guests in the cyclodextrin cavity induce upfield shifts of the H3 and/or H5 protons of the cyclodextrin, due to the ring currents of the aromatic guest [15]. A study in which the concentration of DMβCD was held constant at 5 mM and the concentration of DuP 937 was varied from 0 to 25 mM showed minor or no significant shifts in the cyclodextrin protons. This result can be attributed to two possible causes. Firstly, the association constant is very low, so the amount of time DuP 937 spends in the cavity is extremely short. Secondly, because of the rapid exchange between complexed and free DMβCD, the ring current effect is averaged over all of the possible orientations of DuP 937 in the cavity. If the exchange rate between free and complexed DuP 937 is fast on the NMR time scale, the ring current effects on the proton of DMβCD would be averaged to near zero.

Attempts to obtain information on the conformation of the complex using both 2D-NOE and steady state NOE difference NMR were not successful.

In summary, DuP 937 was shown by absorption spectroscopy to form an inclusion complex with DMβCD in aqueous solution. The 1:1 stoichiometry of the complex was established by the continuous variation method, following changes in the chemical shifts of aromatic protons of DuP 937. The complex association constants determined by different techniques used in this study were in the same order of magnitude. The kinetics of degradation of DuP 937 in aqueous solution was investigated as a function of DMβCD concentration at pH 5.5 and 60°C. The results indicated about a sevenfold increase in the stability of DuP 937 in presence of DMβCD in aqueous solution, indicating that protection of the labile part of the molecule is achieved by complexation.

References

[1] P. Calabresi and R.E. Parks, Jr, in *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 7th Ed. (A.G. Gilman, L.S. Goodman, T.W. Rall and F. Murad, Eds), pp. 1283–1285. Macmillan, New York (1985) and the reviews cited therein.

[2] R.H. Adamson, *Cancer Chemother. Rep.* **58**, 293 (1974).

[3] J. Szejtli, in *Controlled Drug Bioavailability, Vol. 3, Bioavailability Control by Drug Delivery System Design*, (V.F. Smolen and L. Ball, Eds), pp. 365–420. John Wiley & Sons, New York (1985).

[4] J. Pitha, S.M. Harman and M.E. Michel, *J. Pharm. Sci.* **75**, 165–167 (1986).

[5] T. Loftsson, M.E. Brewster, H. Derendorf and N. Bodor, *Pharm. Ztg. Wiss.* **4/136**, 5–10 (1991).

[6] T. Loftsson, S. Björnsdóttir, G. Pálsdóttir and N. Bodor, *Int. J. Pharm.* **57**, 63–72 (1989).

[7] M.E. Brewster, T. Loftsson, K.S. Estes, J.L. Lin, H. Fridriksdóttir and N. Bodor, *Int. J. Pharm.* **79**, 289–299 (1992).

[8] T. Loftsson and H. Fridriksdóttir, *Int. J. Pharm.* **62**, 243–247 (1990).

[9] F. Djedaïni and B. Perly, *J. Pharm. Sci.* **80**, 1157–1161 (1991).

[10] G. Bettinetti, F. Melani, P. Mura, R. Monnanni and F. Giordano, *J. Pharm. Sci.* **80**, 1162–1169 (1991).

[11] P. Job, *Ann. Chim.* **9**, 113–134 (1928).

[12] G. Wenz and E. von der Bey, in *Proceedings of the Fourth International Symposium on Cyclodextrins*, (O. Huber and J. Szejtli, Eds), pp. 133–138. Kluwer Academic Publishers, Dordrecht (1988).

[13] K.A. Connors, *Binding Constants: The Measurement of Molecular Complex Stability*. John Wiley & Sons, New York (1987).

[14] A. Sunega, O. Bekers, J.H. Beijnen, W.J.M. Underberg, T. Tanimoto, K. Koizumi and M. Otagiri, *Int. J. Pharm.* **82**, 29–37 (1992).

[15] P.V. Demarco and A.L. Thakkar, *J. Chem. Soc.* **D(1)**, 2–4 (1970).

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