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Violacein/β-Cyclodextrin Inclusion Complex Formation Studied by Measurements of Diffusion Coefficient and Circular Dichroism

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Abstract. The formation of inclusion compounds between violacein and β-cyclodextrin was studied by diffusion and circular dichroism measurements. The present work was undertaken to explore the feasibility of the β-cyclodextrin in reducing the toxicity and enhancing the antitumoral efficacy of violacein by forming an inclusion complex. The results of the two experiments are in good agreement, suggesting the formation of 1 : 1 and 1 : 2 complexes. The diffusion coefficient measurements enabled estimates of the sizes of the complexes involved. From the circular dichroism and computational calculations it was possible to view a preference for inclusion of the most polar part of the molecule to form a 1 : 2 inclusion complex. We expect that this work proves the potential of these techniques to determining complex stoichiometry.

Key words: β-cyclodextrin, inclusion complex, stoichiometry, violacein, induced circular dichroism

1. Introduction

β-Cyclodextrins (β-CD) are toroidally cyclic oligosaccharides of seven glucose units [1–3]. Studies involving inclusion of solute into cyclodextrins are important due to the resulting improvement of aqueous solubility, stability against chemical degradation and to the possibility of controlled drug release, which presents many potential applications in drug formulations. In addition, the possibility of solute inclusion leads to many other interesting phenomena such as biomimetic systems, in relation to enzymes, in catalysis, separation of isomers (chiral recognition) among others [4, 5]. Recently an extensive review on cyclodextrin complexes was

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published predicting structure stability [6] and their future in drug formulation and delivery [7] and also their structural properties [8].

It is because of these reasons that the investigation of the formation of inclusion complexes has been the focus of great efforts in cyclodextrin chemistry. Many different approaches have been proposed to investigate the incorporation of small molecules into macromolecular systems. One of these involves measurements of the solute diffusion coefficient in the presence of the macromolecule, and its application has already been reported in studies involving biomimetic systems such as micelles and liposomes [9, 10]. More recently, the same technique was applied to investigate the incorporation of a series of alkyl p-hydroxybenzoates into β-CD [11]. Other studies involving CD and diffusion measurements have been reported, for instance, by Gafni and Cohen [12], Li et al. [13] and Manzanares et al. [14].

Inclusion complexation of various cinnamic acid derivatives with CDs in aqueous solution was studied by circular dichroism and this method was useful to examine the mode of interaction of cyclodextrin with drug molecules [15]. More recently several circular dichroism studies on inclusion of naphthalene [16–18], phenols [19, 20] and tyrosine [21] were published.

The 1 : 1 and 1 : 2 (guest/host) inclusion complexes are the most common types, whereas 1 : 3 inclusion complexes have not been widely reported in the literature, apart from the work by Shen et al. [22]. Several cyclodextrins were threaded on a single polyethylene glycol chain and trapped by capping the chain with bulky end groups This supramolecular assembly was denoted as a molecular necklace and described as self-assembly of double-stranded inclusion complexes by Harada et al. [23, 24].

Violacein (3-[1,2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ilydene]-1,3-dihydro-2H-indol-2-one) was isolated from *Chromobacterium violaceum* in the Amazon river, Manaus, Amazon State, Brazil [25], characterized and purified as recently described [26]. Violacein exhibited trypanocidal [27, 28], antibiotic [29] and antitumoral activities [30–33]. Its toxicity was also studied [32]. After *in vitro* testing in a panel cell line by the National Cancer Institute Developmental Therapeutics Program, the response parameter IG_{50} (50% growth inhibition) for ovarian cancer IGROV1 (0.62×10^{-8} M), non-small cell lung cancer NCI-H460 (0.58×10^{-9} M) and colon cancer KM12 (0.27×10^{-9} M) were found [30, 33]. In the case of the 2-methyl-5-hydroxy-1,4-naphthoquinone (Plumbagin) the formation of an inclusion complex with β-CD reduced its toxicity and enhanced its antitumor efficacy in mice bearing Ehrlich ascite carcinoma as reported by Singli and Udupa [34]. Also a methotrexate/β-CD complex had better bioavailability and showed better antitumor efficacy than methotrexate alone [35].

Due to the medical importance of violacein such as antitrypanocidal, antibiotic and antitumoral activities, the aim of the present paper is to confirm the formation of an inclusion complex between violacein and β-CD by diffusion coefficient and induced circular dichroism measurements in order to apply this new formulation as an antitumor drug and to explore the feasibility of the carrier in reducing the

toxicity and enhancing the antitumor efficacy of violacein by forming this type of inclusion complex.

2. Experimental

β-Cyclodextrin was purchased from Sigma Chemical Co. and was used as received. Violacein was prepared and purified as previously published [29].

The Taylor dispersion technique for diffusion coefficient measurements consists in following the dispersion of a solute injected into a stream of solvent, flowing through a long and narrow tubing, without disturbance. The equipment used was previously described in more detail [10, 36]. Briefly, a 50% water–ethanol β-CD solution was pumped by using a peristaltic pump, at very low flow (1.5 μL h^{-1}) through a 10 m stainless steel tubing encased in a temperature controlled environment (at 25 $\pm$ 0.1 °C). Violacein, dissolved in the same β-CD solution at a concentration of 4.5 $\times$ 10^{-4} mol L^{-1}, was injected with a HPLC injector (20 mL loop) and the concentration profile after solute dispersion was followed with a UV/VIS detector interfaced to a computer. It was assembled to satisfy the theoretical requirements for the application of this technique [37]. The internal radius was calibrated as r = 0.605 mm, by accurately weighing a piece of tubing filled with water. This value was confirmed by measuring the diffusion coefficient of caffeine [38], toluene [39] and benzoic acid [40] in water. The reproducibility of the experimental data was better than 3% and no sign of solute adsorption onto the tubing wall [11] was detected. All the data shown represent the average of, at least, three measurements.

The concentration of violacein for the measurements of induced circular dichroism spectra was 8.8 $\times$ 10^{-6} mol L^{-1} in 0.1% ethanol–water solution (the stock solution was prepared in 0.003% DMSO and pure ethanol). Induced circular dichroism spectra were run on a JASCO J-720 spectropolarimeter. Spectroscopic measurements were performed at 25 $\pm$ 0.1 °C.

The minimum structure energy was calculated by the MM$^+$ force field, using HyperChem software.

3. Results and Discussion

Figure 1 shows the induced circular dichroism (ICD) spectra of 8.8 $\times$ 10^{-6} mol L^{-1} violacein in 0.1% ethanol in aqueous solution alone and containing β-CD, from 4.4 $\times$ 10^{-6} to 26.4 $\times$ 10^{-6} mol L^{-1}. From a ratio of 1 : 0.5 to 1 : 1.5 violacein/β-CD, violacein showed a positive band in the wavelength range of 500–720 nm with a maximum wavelength around 600 nm, and showed a small negative signal (at the 500 nm region) and at the 1 : 2 to 1 : 3 ratios violacein/β-CD showed negative signals. At ratios over 1 : 2.5 no significant changes were observed the maximum effect being at a 1 : 3 ratio. On the other hand, no large effect on the absorption bands which were responsible for the ICD spectra (not shown) were observed.

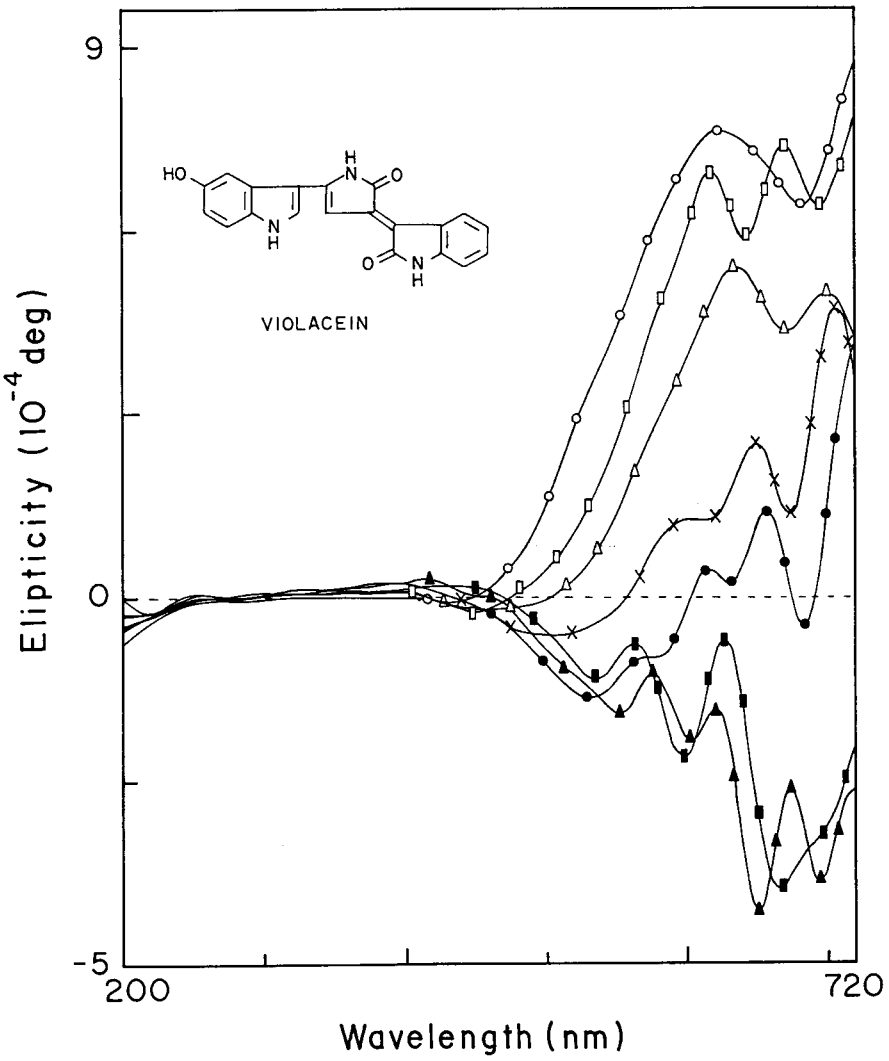

Figure 1. The circular dichroism spectra of the β-cyclodextrin-violacein complex. The molar ratios of violacein/β-cyclodextrin in the presence of 8.83×10^{-6} mol L^{-1} violacein (-○-) were: 1 : 0.5 (-□-), 1 : 1 (-△-), 1 : 1.5 (-×-), 1 : 2 (-●-), 1 : 2.5 (-■-) and 1 : 3 (-▲-).

Due to the irregular ICD spectra (see Figure 1), calculations of association constants [41] did not produce reliable results.

When the violacein concentration was increased up to 4.3×10^{-4} mol L^{-1} (ethanol/water) the same behavior was observed in the presence of β-CD at the same ratios described before.

The Cotton effect of violacein, which is observed in Figure 1 in the absence of β-CD, is explained by preliminary AM1 calculations in which the rotation barrier of the 5-hydroxy-indole moiety was around 10.5 kJ mol^{-1}. Apparently this bar-

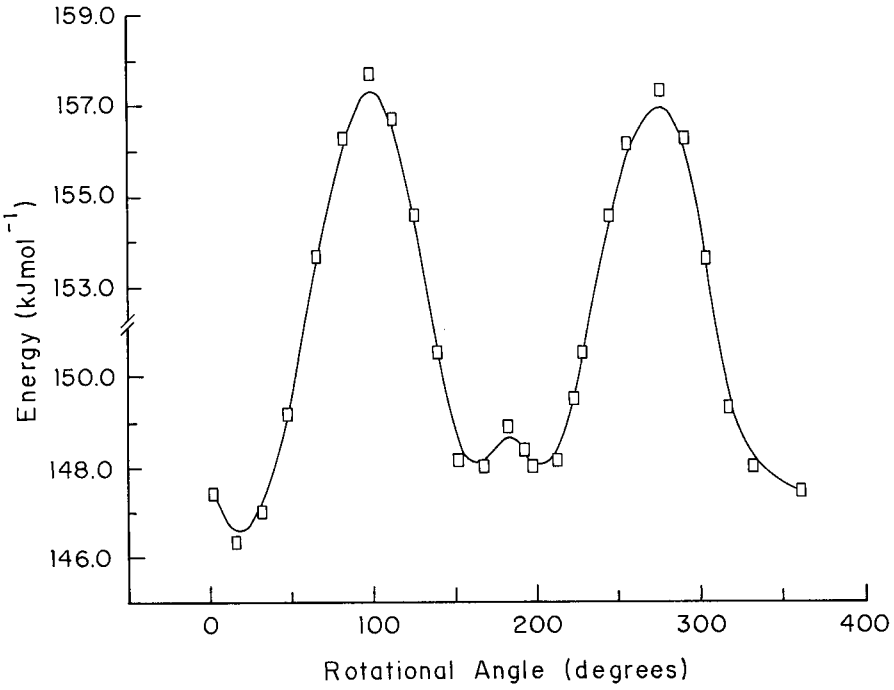

Figure 2. AMI calculation for violacein related to the rotational angle (degrees).

rier is enough to maintain the non-planarity of the violacein molecule, showing a positive ICD spectrum (Figure 2). The lower energy conformations have no planar structure. This non-planar structure must be responsible for the Cotton effect of violacein.

The diffusion coefficients for 4.5×10^{-4} mol L^{-1} violacein in aqueous–ethanol (50%) solutions at 298 K are shown in Figure 3. As the drug is incorporated into the macromolecule, its diffusion coefficient is reduced due to the larger complex formed. Therefore, the observed decrease in violacein diffusion confirms the drug incorporation as the β-CD concentration increases. The plateaux represent the diffusion when all the solute is incorporated, that is, the diffusion coefficients of the complexes. No change in viscosity which could cause such a change is observed within the studied range of β-CD and solute concentrations. Two well-defined plateaux were observed, reflecting the formation of 1 : 1 and 1 : 2 complexes, with an indication of a possible third one of 1 : 3 (solute: β-CD), determined by the decrease after the second plateaux (Figure 3). Following the Stokes–Einstein relationship [11] the first plateaux with $D = 2.56 \times 10^{-10}$ m^2 s^{-1} represents a hydrodynamic radius of 9.57 Å, and the second one with $D = 0.97 \times 10^{-10}$ m^2 s^{-1} represents a hydrodynamic radius of 25.25 Å. These sizes are in good agreement with the other studies on cyclodextrin inclusion complexes [11].

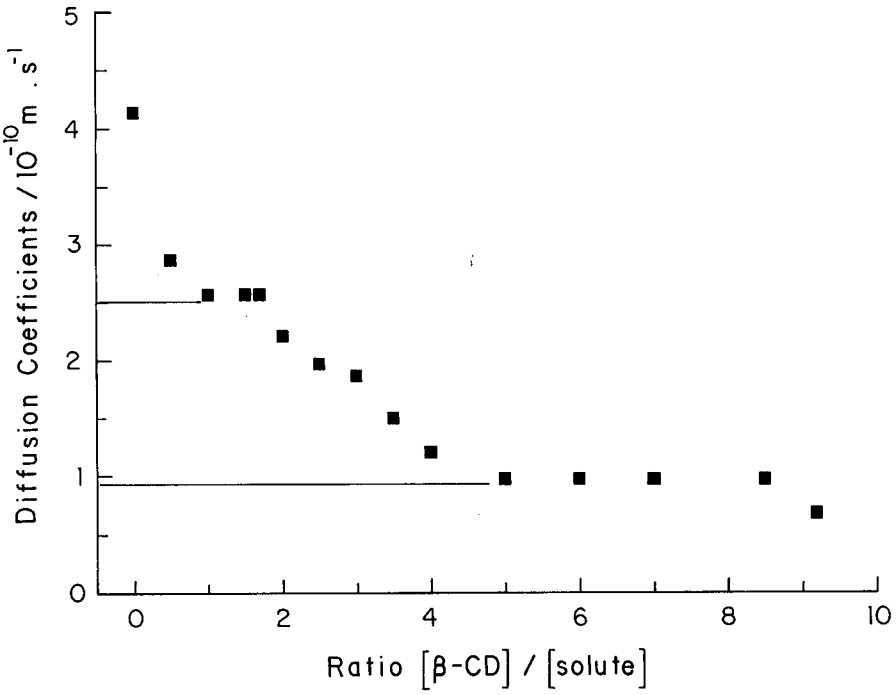

Figure 3. Diffusion coefficient for 4.5×10^{-4} mol L^{-1} violacein in aqueous-ethanol (50%) of β-cyclodextrin solutions. The concentration ratio, [β-CD]/[solute] represents the injected solutions (see text for discussion).

The solute/β-CD ratio indicated in Figure 3 refers to the injected solution. Since the solute is diluted as it disperses into the β-CD carrier solution, this ratio increases up to 65 times before reaching the detector. Similar plateaux were observed for 1 : 1 and 1 : 2 (solute/β-CD) complexes of alkyl *p*-hydroxybenzoates and β-CD by the same method [11]. The occurrence of CD complexes with different stoichiometries has been reported in many different studies, and an extreme example is the rotaxane-like 1 : 3 complex formed by iodotrimethyl-(2-*p*-hexylaminophenyl)-3,3-dimethyl-5-carboethoxy-3H-indole ammonium and β-CD [22].

Theoretical calculations using the MM^+ HyperChem force field, in a similar way as described in the literature [41], showed that two β-CD molecules can easily accommodate one violacein molecule (Figure 4); however, the presence of a third one, as suggested by the two methods used, seems to be quite difficult to get the necessary arrangement, in contrast to the case of the tubular arrangement [23, 24].

Probably both methods are indicating a rapid equilibrium process with two β-CD molecules and not really a third β-CD molecule interacting with violacein.

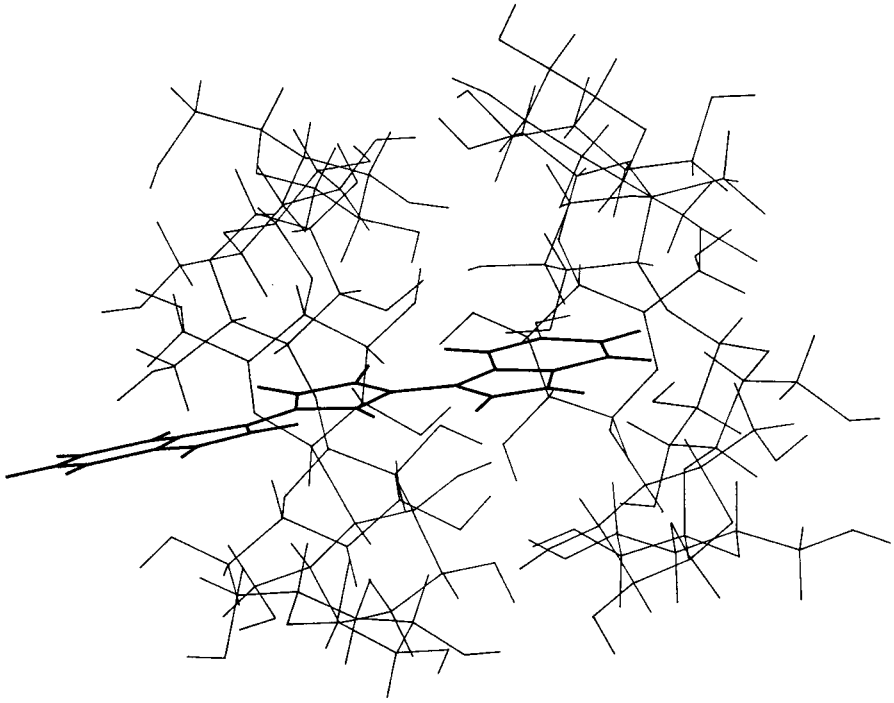

Figure 4. Theoretical calculations by HYPERCHEM force field for violacein inserted in β-cyclodextrin.

4. Conclusion

In summary, the 1 : 1 violacein/β-CD complex was shown to coexist, at least, with 1 : 2 and maybe with 1 : 3 ratios, which could be important to the toxicity and antitumoricidal activities, since it is known, e.g., that for Plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) the toxicity was reduced and the antitumor efficacy was enhanced upon complexation with β-CD [35].

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