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Determination of the Formation Constant for the Inclusion Complex of Methyl-β-cyclodextrin with Anticoagulant Drugs Warfarin and 8-Chlorowarfarin in Aqueous Solution

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Abstract. Inclusion of anticoagulant drugs within the cavity of methyl-β-cyclodextrin in aqueous solution was studied by ultraviolet absorption, fluorimetry and reversed-phase liquid chromatography. Formation constants were obtained for complex formation of methyl-β-cyclodextrin with warfarin and 8-chlorowarfarin.

Key words: Cyclodextrin, complex, fluorescence, ultraviolet, warfarin, chromatography.

1. Introduction

The interactions of cyclodextrins with drugs have received considerable attention because the act of complexation increases the aqueous solubility of the drug, and it may increase its chemical stability [1]. Drugs have usually been found to form one-to-one complexes with β-cyclodextrin (β-CD). Cyclodextrins form inclusion complexes ('host–guest chemistry') with organic solutes in aqueous solutions. The binding force between cyclodextrins and organic solutes has been assumed to be hydrogen bonding, van der Waals force, or hydrophobic interaction [2]. The purpose of the present work is to examine the interaction of warfarin (W_f) and 8-chlorowarfarin, in aqueous methanolic solution, with modified cyclodextrin: methyl-β-cyclodextrin (Me-β-CD).

Warfarin (α-3-acetonylbenzyl-4-hydroxycoumarin) (Figure 1) is a widely used oral anticoagulant drug. In humans, the hypothrombinemic effect of *S*-(−)warfarin is two to five times more potent than *R*-(+)warfarin. A number of research groups [3–6] have previously used solubility analysis, ultraviolet spectroscopy (UV), fluorescence and high performance liquid chromatography in the reversed phase mode (HPLC) for the determination of the complex formation constant K_f. In the present paper, the complex formation constant K_f for the 1 : 1 inclusion complex of Me-

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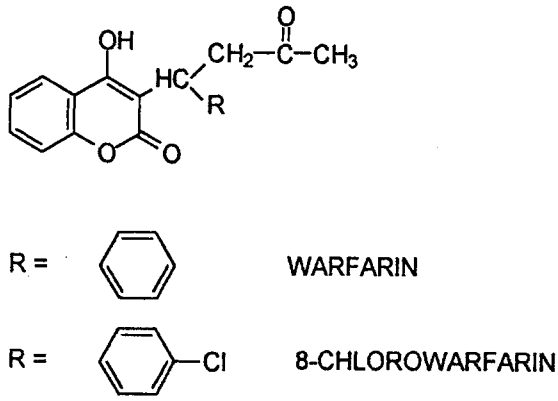

Figure 1. Warfarin.

β-CD with warfarin and 8-chlorowarfarin was determined by UV spectroscopy, fluorescence and HPLC.

2. Experimental

2.1. Reagents and Standards

Methanol (HPLC grade) was a Fluka reagent. All the other reagents were of analytical grade (Prolabo). Warfarin and 8-chlorowarfarin were obtained from Aldrich Chemical Co. Methyl-β-cyclodextrin, $MW_{ave.} = 1310$, $\alpha = 159°$, degree of substitution (DS) = 1.8, was purchased from Wacker-Chemie (Munich, Germany).

2.2. Apparatus and Procedure

UV-Visible absorption spectra data were obtained using a Philips PU 8740-UV/Vis spectrophotometer and a Philips PU 4900/20 printer-plotter. UV absorption changes of warfarin and 8-chlorowarfarin (10^{-4} mol L^{-1}) in the presence of methyl-β-cyclodextrin (varied from 0.9 to 17 $\times$ 10^{-3} mol L^{-1}) were measured at the appropriate UV absorption wavelength; the baseline was established for each measurement, using the solution of methyl-β-cyclodextrin at the same concentration. Each data point was evaluated in triplicate.

Fluorescence measurements were made using a Jobin Yvon JY3D spectrofluorimeter. The drug solutions were excited at a wavelength of 310 nm at pH = 7.4; the intensity of fluorescence of 3.5 $\times$ 10^{-5} mol L^{-1} drug solutions in the presence of methyl-β-cyclodextrin (0.9 to 12 $\times$ 10^{-3} mol L^{-1}) was measured at the emission wavelength of 388 nm. All spectra were measured at 25 °C. Each determination was evaluated in triplicate.

The chromatographic experiments were performed using a Spectra Physics 8800 ternary pump, connected to a Rheodyne model 7125 injection valve provided with

a 20 μl sample loop. Detection was accomplished using a Spectra Physics 100 variable wavelength UV detector set at 310 nm with a sensitivity of 0.05 a.u.f.s. The chromatographic column (C_{18}) was 150 × 4.6 mm i.d. stainless steel packed with 5 μm Nucleosil. The mobile phase consisted of a binary mixture methanol-phosphate buffer 0.01 mol L^{-1}, pH = 2.5; the flow rate was 1 mL min^{-1}. Before use, mobile phases are filtered under vacuum, with a glass filter Wathman GF/F 0.5 μm porosity filter and sonicated for 5 min. The column void volume was determined using potassium nitrite.

3. Results and Discussion

3.1. UV Absorption

Figure 2 shows the absorption spectra of warfarin in aqueous methanol solutions containing various amounts of Me-β-CD, (0.9×10^{-3}; 3.7×10^{-3}; 5.6×10^{-3}, 16.8×10^{-3} mol L^{-1}). The absorption band around 310 nm shifts towards longer wavelengths with increasing concentration of Me-β-CD. The isosbestic point appearing at 250 nm indicates an equilibrium:

$$W_f + \text{Me-}\beta\text{-CD} \leftrightarrows [W_f - \text{Me-}\beta\text{-CD}]$$

where W_f and Me-β-CD define warfarin and methyl-β-cyclodextrin respectively.

$$K_f = \frac{[W_f - \text{Me-}\beta\text{-CD}]}{[W_f] \cdot [\text{Me-}\beta\text{-CD}]}$$

$$[W_f]_t = [W_f] + [W_f - \text{Me-}\beta\text{-CD}]$$

$$[\text{Me-}\beta\text{-CD}]_t = [\text{Me-}\beta\text{-CD}] + [W_f - \text{Me-}\beta\text{-CD}]$$

where $[W]_t$ and $[\text{Me-}\beta\text{-CD}]_t$ describe the total concentration of warfarin and methyl-β-cyclodextrin, respectively.

The complex formation constant, K_f, for the inclusion complex W_f – Me-β-CD was determined according to the conventional Scott equation [7], assuming the formation of a 1 : 1 complex:

$$\frac{a \cdot c}{d} = \frac{1}{K_f \cdot \epsilon_c} + \frac{c}{\epsilon_c}$$

where a is the total concentration of warfarin, c is the total concentration of Me-β-CD, ϵ_c is the difference of the molar absorptivities for free and complexed warfarin and d is the change in absorbance of warfarin by the addition of Me-β-CD [8].

The plot of $(a \cdot c)/d$ versus c at 310 nm gives good linearity of slope of 5.02×10^{-4} and intercept of 15.40×10^{-7}, K_f can then be determined. For example, K_f = 310 ± 15 mol^{-1} L for warfarin at pH = 7.4 (methanol volume fraction: 10%).

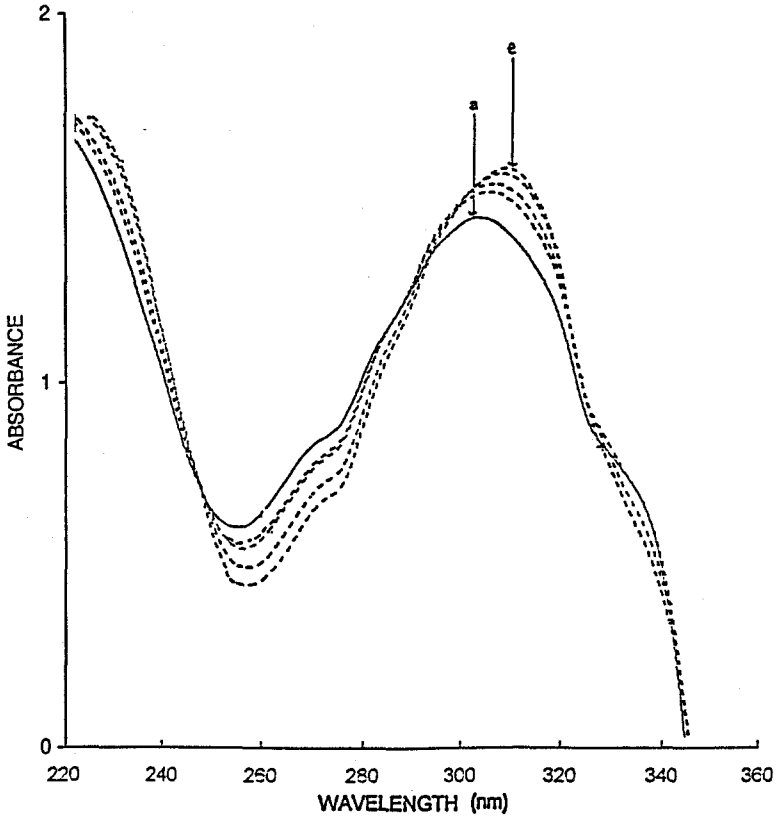

Figure 2. Absorption spectra of warfarin (10^{-4} mol L^{-1}) in methanol–phosphate buffer (0.01 mol L^{-1}, pH = 7.4, methanol volume fraction: 10%) at various concentrations of Me-β-CD. (Me-β-CD): (a) = without; (b) = 0.9×10^{-3} mol L^{-1}; (c) = 3.7×10^{-3} mol L^{-1}; (d) = 5.6×10^{-3} mol L^{-1}; (e) = 16.8×10^{-3} mol L^{-1}.

3.2. FLUORESCENCE

The fluorescence spectra were measured for warfarin and 8-chlorowarfarin in aqueous methanol Me-β-CD solutions in order to calculate the inclusion constants.

Figure 3 shows the fluorescence spectrum of warfarin in phosphate buffer (0.01 mol L^{-1}, pH = 7.4, methanol volumic fraction: 10%) solutions containing Me-β-CD at various concentrations. The spectra were obtained by irradiating the solutions with 310 nm light, and recording the emitted fluorescence intensity between 325 and 500 nm. The fluorescence intensity is markedly enhanced with increasing concentration of Me-β-CD. Inclusion probably gives rise to a decrease in intramolecular rotational freedom of these molecules by fixing them inside the cavity of Me-β-CD.

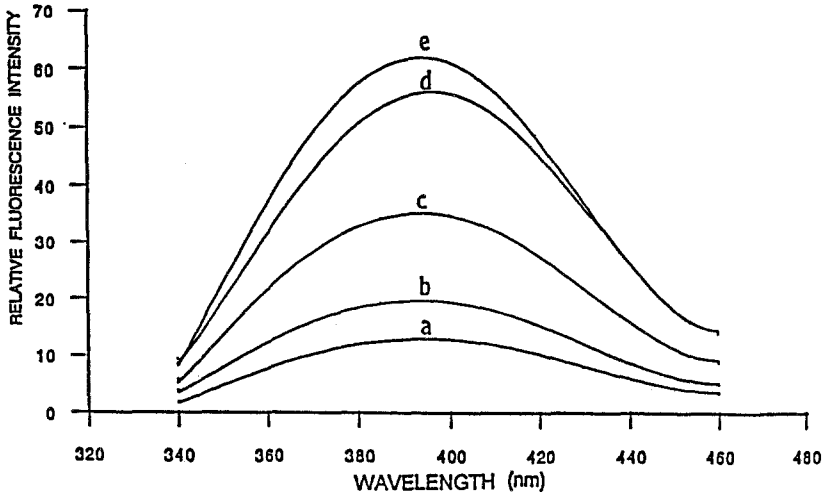

Figure 3. Effect of Me-β-CD on the fluorescence spectrum of warfarin (3.5×10^{-5} mol L^{-1}) in phosphate buffer (0.01 mol L^{-1}, pH = 7.4, methanol volume fraction: 10%). (Me-β-CD): (a) = without Me-β-CD; (b) = 0.9×10^{-3} mol L^{-1}; (c) = 1.9×10^{-3} mol L^{-1}; (d) = 7.5×10^{-3} mol L^{-1}; (e) = 11.2×10^{-3} mol L^{-1}.

The complex formation constants, K_f, were calculated by the method described by Mataga and Tsuno [9] for a 1 : 1 complex using the equation:

$$\frac{1 - f_o/f_m}{c} = -K_f + \frac{\phi_c}{\phi_a} \cdot \frac{\epsilon_c}{\epsilon_a} \cdot K_f \cdot (f_o/f_m)$$

ϵ_a and ϵ_c are the molar absorptivities of the drug and the complex at the wavelength of excitation, respectively; ϕ_a and ϕ_c are the quantum yields of the drug and the complex; f_o and f_m are the fluorescent intensities of the drug in the absence and presence of Me-β-CD at concentration c.

A good linear relationship between $(1 - f_o/f_m)/c$ versus f_o/f_m is obtained (slope: 1.24×10^3; correlation coefficient: 0.99). K_f is determined by the value of the intercept of the line (Table I). For example warfarin yields $K_f = 262 \pm 12$ mol^{-1} L, pH = 7.4 (methanol volume fraction: 10%).

The K_f values obtained at pH = 7.4, although slightly lower, are in good agreement with those obtained by the UV method. At pH = 7.4, warfarin, the pK_a of which is 4.8 [10], is essentially fully ionized. It has previously been found that unionized warfarin is preferentially bound with β-CD [11]. The data of Table I show that warfarin has a significantly higher complex formation constant with Me-β-CD at pH = 2.5 (unionized form) than at pH 7.4 (fully ionized form).

The spectrophotometric methods, fluorescence in particular, are easy to perform, and the measurements can be made at different values of pH and percentage of methanol. The main advantage of fluorescence spectrometry over UV spectrometry is the difference of relative fluorescence intensity observed between free and com-

Table I. Formation constants of Me-β-CD complexes, K_f (mol^{-1} L), with warfarin and 8-chlorowarfarin obtained by several techniques at 25 °C.

		Fluorescence		UV		HPLC	
% MeOH	pH	log K_f	K_f	log K_f	K_f	log K_f	K_f
10	7.4	2.40	262 ± 12	2.50	310 ± 15		
2	7.4	2.55*	350 ± 20	2.70*	500 ± 30		
				2.65	440 ± 22		
10	2.5	2.80	650 ± 20				
60	2.5					1.78	60 ± 12

* 8-Chlorowarfarin.

plexed forms (Figure 3). This is of great interest in the case of warfarin where the difference of absorbance between free and complexed forms in UV spectrometry are small (Figure 2).

3.3. Chromatographic Studies

In conventional reversed phase HPLC, differences in the physicochemical interactions of the eluate with the mobile phase and the stationary phase determine their partition coefficients and, hence, their capacity factor k'. The utilisation of cyclodextrins in a mobile phase in chromatographic reversed-phase systems may form inclusion complexes, host-guest compounds, with a variety of organic molecules or ions in aqueous solution. Since the host-guest interaction depends on the fit of the structural features of the guest molecule to the cavity of cyclodextrin, the introduction of cyclodextrin in the chromatographic system can be expected to vary the retention time. When cyclodextrin was added to the aqueous methanolic mobile phase, the retention time of a solute that is less hindered for inclusion becomes usually shorter, reflecting the fact that the interaction between the solute and the stationary phase is weakened by complex formation. Since the change of the retention time caused by the formation of the inclusion complex is closely related to the stability of the inclusion complex, some attempts have been made to estimate the complex formation or dissociation constant from the relationship between the retention time and the concentration of CD in the mobile phase [12–15].

The present section examines the retention mechanism of warfarin and 8-chlorowarfarin in a reversed-phase system involving methyl-β-cyclodextrin (Me-β-CD) inclusion complex formation. We thereafter derive the associated formation constant.

In a reversed phase system containing Me-β-CD in the mobile phase, two phenomena are involved in the equilibrium between the two phases: adsorption of the compound, both free and bound to Me-β-CD, and complexation in the mobile phase.

It is assumed that only 1 : 1 stoichiometric complexes are formed [16, 17], when warfarin (W_f) is introduced into the column in the presence of Me-β-CD in the mobile phase. The following simplified scheme describes the equilibria:

$$W_{f_m} + \text{Me-}\beta\text{-CD} \leftrightarrows [W_f\text{-Me-}\beta\text{-CD}]_m$$

$$\left(\text{formation constant of } [W_f\text{-Me-}\beta\text{-CD}] \; : \; K_f = \frac{[W_f\text{-Me-}\beta\text{-CD}]_m}{[W_f]_m \cdot [\text{Me-}\beta\text{-CD}]_m}\right)$$

$$W_{f_m} \leftrightarrows W_{f_s}$$

$$\left(\text{distribution constant of } W_f : K_o = \frac{[W_f]_s}{[W_f]_m}\right)$$

$$[W_f\text{-Me-}\beta\text{-CD}]_m \leftrightarrows [W_f\text{-Me-}\beta\text{-CD}]_s$$

$$\left(\text{distribution constant of } [W_f\text{-Me-}\beta\text{-CD}] \; : \; K_1 = \frac{[W_f\text{-Me-}\beta\text{-CD}]_s}{[W_f - \text{Me-}\beta\text{-CD}]_m}\right)$$

where the subscripts s and m denote the stationary and mobile phase, respectively.

The above schemes take no explicit account of dissociation and/or protonation. The formation constant involved should be regarded as the apparent formation constant at the pH of the mobile phase. Furthermore, the distribution equilibrium of Me-β-CD itself between the hydrophobic stationary phase and the hydrophilic phase is assumed to be negligible; the retention time of Me-β-CD was nearly the same as that of potassium nitrite used as a marker for measuring the column dead volume.

Neglecting the interaction of the Me-β-CD complexed solute with the stationary phase, the capacity factor k' of the sample solute can therefore be written as:

$$k' = \phi \frac{[S]_s}{[S]_m + [S - \text{Me-}\beta\text{-CD}]_m}$$

where ϕ denotes the phase mobile ratio of the column.

The complex formation constant, K_f, was calculated by the method described by Fujiwara and Ueada [18] using the equation:

$$\frac{1}{k'} = \frac{1}{k'_0} + \frac{K_f \cdot [\text{Me-}\beta\text{-CD}]_t}{k'_0} \tag{1}$$

where k'_0 is obtained in the absence of Me-β-CD.

The plot of $1/k'$ versus $(\text{Me-}\beta\text{-CD})_t$ will give a straight line, the slope of which is equal to K_f/k'_0, and the intercept to $1/k'_0$. The formation constant, K_f, of the inclusion complex can thus be calculated from the slope to intercept ratio of the regression straight line [19].

Table II. Effect of the concentration of Me-β-CD in the mobile phase on $1/k'$ for warfarin.

Me-β-CD	0 mmol L^{-1}	3 mmol L^{-1}	4 mmol L^{-1}	5 mmol L^{-1}	7.5 mmol L^{-1}
$1/k'$	0.150	0.182	0.185	0.200	0.214

The pH of the mobile phase used in this work (pH = 2.5) was much lower than the pK_a value (pK_a = 4.8), therefore only one species of solute (i.e. neutral molecule) should be taken into account. To optimize the chromatographic conditions with a view to the determination of the inclusion constant, we have studied the influence of the percentage of methanol on the capacity factor k' of warfarin. The values of the capacity factor k' increase with a decreasing percentage of methanol in the mobile phase, and a linear relationship exists between log k' and the percentage of methanol. The mobile phase of phosphate buffer (0.01 mol L^{-1}, pH = 2.5, methanol volume fraction: 40%) was retained for the determination of the complex formation constant.

The effect of addition of Me-β-CD in the mobile phase on the reversed-phase retention are shown in Table II.

As expected from Equation (1), k' values decreased with an increase in the concentration of Me-β-CD ([Me-β-CD]$_t$) in the mobile phase, and a linear relationship was observed between $1/k'$ and [Me-β-CD]$_t$; the slope is 16.88, with intercept 0.13.

The complex formation constant, K_f, calculated from the slope is, for example, $60 \pm 12\ mol^{-1}\ L$ (25 °C, pH = 2.5, methanol volume fraction: 60%). The correlation coefficient $R > 0.99$ indicate good linearity, showing that a 1 : 1 complex was formed.

As for the preceding methods, the complex formation constant obtained by the chromatographic method depends on the mobile phase compositions used: the retention time of the solute is influenced not only by the concentration of Me-β-CD but also by the type and the content of organic solvent in the mobile phase. In addition, it can be influenced by additional interaction mechanisms, such as a direct interaction of the complexed solute with the alkyl chains of the stationary phase or a modification of the contribution of residual silanol groups in the presence of Me-β-CD.

The results obtained do clearly indicate an interaction between warfarin, 8-chlorowarfarin and methyl-β-cyclodextrin; attempts will be made to investigate its interest in the biopharmaceutical field.

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