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Research paper

Spectrofluorimetric study of eflucimibe-γ-cyclodextrin inclusion complex

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Abstract

Eflucimibe, a novel and highly potent acyl-coenzyme A cholesterol *O*-acyl-transferase (ACAT) inhibitor, is sparingly soluble in aqueous media and exhibits a very weak natural fluorescence. However, when increasing concentrations of γ-cyclodextrin (γ-CD) are added, an increase in the fluorescence signal is observed, attesting the formation of a non-covalent inclusion complex between eflucimibe and the γ-CD. In this work, the stoichiometry of the complex and the corresponding association constant have been determined from fluorescence data by Benesi–Hildebrand's method (double reciprocal plots). As a result, a 1:1 stoichiometric ratio and a 20 M^{-1} formation constant were obtained. This apparent formation constant was determined in water containing 10% methanol, which was needed to improve 'aqueous' solubility of the drug in a CD-free medium. Owing to the extreme hydrophobicity of eflucimibe, these results provide valuable information for pharmaceutical formulation studies.

Keywords: Eflucimibe; Cyclodextrin; Spectrofluorimetry; Inclusion complex; Stoichiometry; Association constant

1. Introduction

Atherosclerosis, or the hardening of the arteries, is the cause of more than half of all mortality in developed countries. This disease, favoured by increased cholesterol amounts (especially LDL-cholesterol), is characterised by the clogging of the arteries, due to the build-up over time of fat and other substances. Among the novel therapeutic strategies, the inhibition of acyl-coenzyme A cholesterol *O*-acyl-transferase (ACAT) seems to be a promising approach to induce both hypolipidemic and antiatherosclerotic effects. Indeed, ACAT, an intracellular enzyme which catalyses esterification of cholesterol to cholesteryl esters, plays a crucial role in cholesterol absorption in intestine, assembly of very low density lipoprotein in liver, accumulation of cholesteryl esters in steroidogenic tissues and foam cell formation in atherosclerotic lesions [1,2]. Eflucimibe [(*S*)-2′,3′,5′-trimethyl-4′-hydroxy-α-dodecylthio-phenylacetanilide] (Fig. 1), a new ACAT inhibitor of great potency [3–5], has been developed in Pierre Fabre Research Center (Castres, France).

The poor solubility of eflucimibe in aqueous media can be ascribed to its extreme hydrophobicity; indeed, the estimated partition coefficient (log $P = 9.68 \pm 0.47$) was calculated by using the ACD/log *P* software 8.00 release (Advanced Chemistry Development, Toronto, Canada). The formation of a water-soluble inclusion complex would, therefore, improve its solubilization.

Cyclodextrins (CDs, α, β, γ) are cyclic oligosaccharides consisting of six to eight D-glucose units. They are able to form inclusion complexes with many compounds [6,7] since the inner conical hydrophobic cavity of the CD behaves as a host in which the guest molecule may fit selectively. Besides, the internal cavity being less polar than the surrounding water molecules, spectral and chemical properties of the guest may be dramatically affected, once included [8,9]. Indeed, CDs are known to improve certain properties of drugs (such as solubility,

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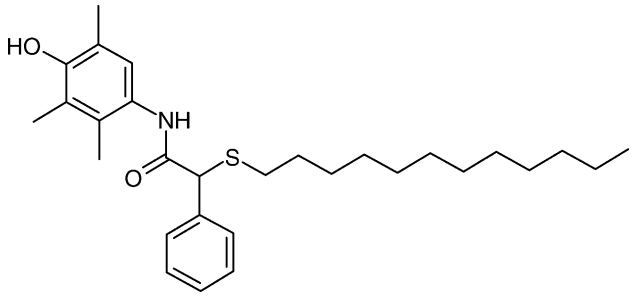

Fig. 1. Structure of eflucimibe.

stability and/or bioavailability) and enhance drug activity by encapsulation of the active molecule [10]. Therefore, the study of CD–drug complexation provides valuable information for the development of pharmaceutical formulations [11].

Different analytical methods may be used for such a purpose. In this work, the possible formation of an inclusion complex between eflucimibe and γ-CD is investigated by measurement of the enhancement of the fluorescence signal when adding increasing γ-CD concentrations. After determination of the stoichiometry of the complex, Benesi–Hildebrand's method (double reciprocal plots) was applied to calculate the corresponding association constant (K_f).

2. Experimental

2.1. Apparatus

Fluorescence measurements were performed at 25 °C on a Varian (Victoria, Australia) Cary Eclipse fluorescence spectrophotometer, equipped with a Xenon lamp and 10 mm quartz cells. Excitation and emission slits were both set at 5 nm. Data were acquired at a 600 nm min^{-1} scan rate.

2.2. Reagents

Eflucimibe was synthesised by the Medicinal Chemistry Division of Pierre Fabre Research Center (Castres, France). Methanol of HPLC quality was obtained from J.T. Baker (Noisy le Sec, France); 18 MΩ deionised water was prepared using Elgastat UHQ II system (Elga, Antony, France). Native gamma-cyclodextrin (γ-CD) was provided by Wacker Chemie (Munich, Germany).

Eflucimibe (1.065 mM) and γ-CD (100 mM) stock solutions were prepared in methanol and pure water, respectively. Standard working solutions were obtained by appropriate dilution so that eflucimibe concentration (0.0213 mM) was kept constant in a water–methanol (9/1) mixture containing 0–18 mM of γ-CD. Indeed, the organic fraction was necessary to prevent precipitation of the free drug (e.g. in the absence of γ-CD) and allow the measurement of the corresponding fluorescence signal. All solutions were vigorously shaken for 6 h at room temperature, using a Bellco Glass, Inc. (Vineland, NJ, USA) mini-orbital shaker.

3. Results and discussion

A preliminary experiment was dedicated to the acquisition of the total fluorescence spectrum of eflucimibe–γ-cyclodextrin (Fig. 2): the resulting contour map was obtained by recording the emission spectrum (in a 200–500 nm range) acquired for each excitation wavelength scanned (in a 190–400 nm range). In this spectrum, excitation wavelengths (λ_{ex}, X) are plotted versus emission wavelengths (λ_{em}, Y), and level curves of fluorescence intensities correspond to lines of joint points of equal luminescence.

As a result, 232 and 346 nm were selected as excitation and emission wavelengths, respectively, for the study of the inclusion complex.

3.1. Stoichiometry of the inclusion complex

Experiments were performed on solutions that contained 0–18 mM of γ-cyclodextrin (six concentration levels), while the concentration of eflucimibe was kept constant. Fluorescence measurements were performed in triplicate to ensure repeatability. As expected when complexation occurs [12], the enhancement of the fluorescence intensity was observed with increasing γ-cyclodextrin concentration (Fig. 3).

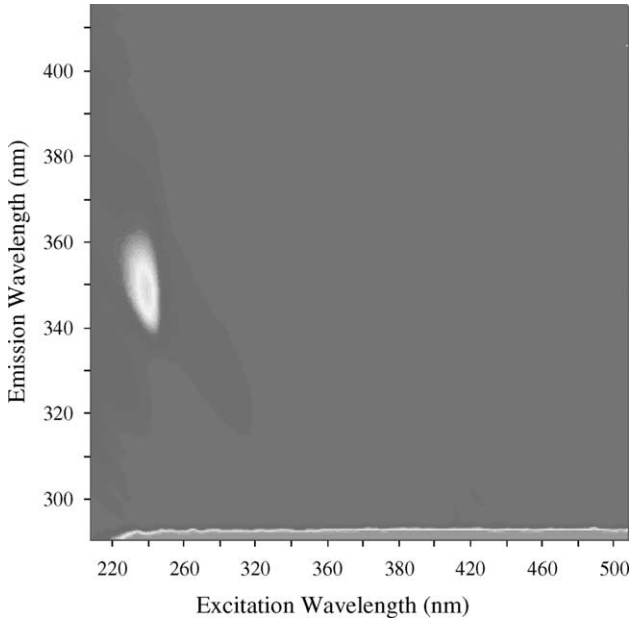

Fig. 2. Total fluorescence spectrum of eflucimibe–γ-cyclodextrin inclusion complex.

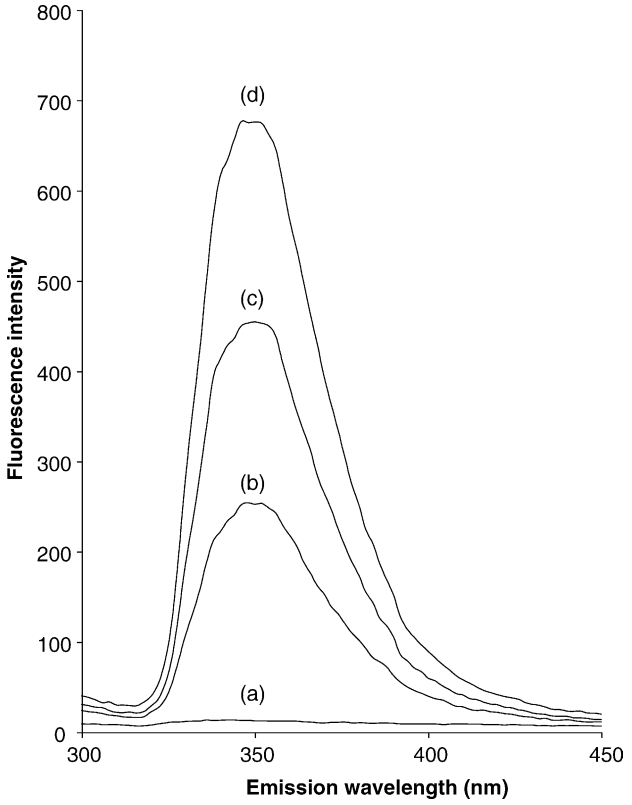

Fig. 3. Enhancement of fluorescence intensity with increasing γ-cyclodextrin concentration: (a) 0, (b) 6, (c) 10, and (d) 15 mM γ-CD in water/methanol (9/1). Eflucimibe: 0.0213 mM.

Benesi–Hildebrand's method [13] was used to determine the stoichiometry of the inclusion complex; two possible stoichiometries, 1:1 and 1:2, were considered in this study.

Assuming that eflucimibe forms a 1:1 inclusion complex with gamma cyclodextrin (γ-CD), the following equilibrium can be written

$$\text{Eflucimibe} + \gamma - \text{CD} \rightleftharpoons \text{Eflucimibe} - \gamma - \text{CD} \quad (1)$$

and the formation constant (K_f) of the complex is given by

$$K_f = [\text{Eflucimibe} - \gamma - \text{CD}]/[\text{Eflucimibe}][\gamma - \text{CD}] \quad (2)$$

where [Eflucimibe−γ-CD], [Eflucimibe] and [γ-CD] are equilibrium concentrations.

Then, according to [13], the relationship between the observed fluorescence and the γ-CD concentration tested is given by

$$\frac{1}{F - F_0} = \frac{1}{(F_\infty - F_0)K_f[\gamma - \text{CD}]} + \frac{1}{F_\infty - F_0} \quad (3)$$

where F denotes the observed fluorescence signal at the [γ-CD] concentration tested, F_0 the fluorescence intensity in the absence of the γ-CD, and F_∞ the fluorescence intensity when all of the eflucimibe molecules are effectively complexed. Six concentration levels appear in Fig. 4.

When $1/(F-F_0)$ is plotted versus 1/[γ-CD], a linear relationship is observed ($r=0.996$), reflecting a 1:1 inclusion complex (Fig. 4a). The linear relationship was also confirmed by ANOVA and examination of the residuals, eliminating thus a possible lack-of-fit.

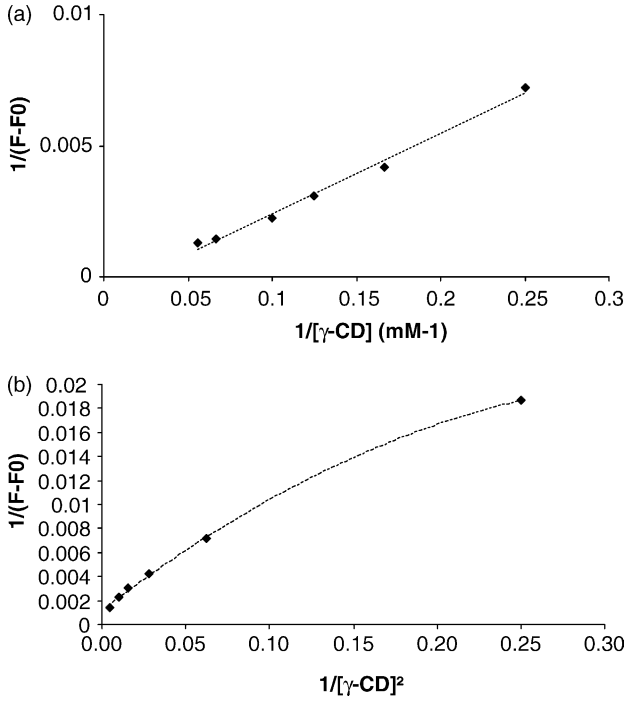

Fig. 4. Double reciprocal plots: determination the stoichiometry of eflucimibe–γ-cyclodextrin inclusion complex: (a) $1/(F-F_0)$ versus 1/[γ-CD] (assumption of 1:1 complex); (b) $1/(F-F_0)$ versus $1/[\gamma\text{-CD}]^2$ (hypothesis of 1:2 complex).

In order to check this 1:1 stoichiometric ratio, the hypothesis of a 1:2 complex was also investigated by plotting $1/(F-F_0)$ as a function of $1/[\gamma\text{-CD}]^2$ [14]. A non-linear correlation was obtained (second order polynomial relationship), confirming thereby the previous result of a 1:1 stoichiometric ratio (Fig. 4b).

3.2. Determination of the association constant

Once the stoichiometric ratio is determined, the former double reciprocal plot method ($1/(F-F_0)$ versus 1/[γ-CD]) can also be used to calculate the association constant (K_f) of the inclusion complex. Indeed, least squares regression results in a linear relationship, where the association constant (K_f) is given by the ratio of the intercept to the slope. Thereby, a 20 M^{-1} value was obtained.

4. Conclusion

The formation of an inclusion complex between eflucimibe and γ-CD has been demonstrated; indeed, the enhancement of the fluorescence signal has been observed upon addition of increasing concentrations of γ-CD. The double reciprocal plot method enabled determination of

both the (1:1) stoichiometry of the complex and its association constant (20 M^{-1}).

These results are important for pharmaceutical formulation considerations since γ-CD allows 'aqueous' solubilization of the extreme hydrophobic drug eflucimibe, a new and very promising ACAT inhibitor in atherosclerosis research.

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