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Fluorometric Determination of Stability Constants of Inclusion Complexes of Naproxen and Charged Cyclodextrins in Aqueous Solutions. Nonlinear *vs* Linear Data Processing

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Stability constants of complexes of naproxen with monoamino-β-cyclodextrin and carboxymethyl-β-cyclodextrin in both acidic and basic solution have been determined by fluorometry and compared with those of parent β-cyclodextrin. It appeared that a charged group linked to the cyclodextrin rim decreased its complexing ability. For determination of the stability constants, nonlinear fitting has been used as well as three methods, which consist in linearization of the original hyperbolic equation. These methods of data processing have been compared.

Key words: naproxen, β-cyclodextrin, amino-β-cyclodextrin, carboxymethylated-β-cyclodextrin, complexation, stability constants, fluorescence, nonlinear fitting

Cyclodextrins (CDs) are cyclic polysaccharides that form host-guest complexes with different compounds. They act as hosts for many drugs of different structures, modifying their physical and chemical properties, *e.g.* their solubility. The molar absorption coefficient in the optical absorption spectra and the fluorescence quantum yield belong to those physico-chemical properties of guest compounds that can be affected by complex formation. Changes in absorption coefficients [1–23] and fluorescence intensity [12,14,16,23–46] have been used for the determination of the stability constants of CD complexes.

Relationships between the change of the guest absorption (ΔA) or fluorescence intensity increments (ΔF) on the concentration of free ligand, [L], are hyperbolic. Expression for the dependence of ΔA on [L] was derived in [47]. For 1:1 complex formation, a similar derivation leads to the following dependence of ΔF on [L]:

$$\frac{1}{\Delta F} = I_o(\varepsilon\eta - \varepsilon_o\eta_o)\frac{KS[L]}{1+K[L]} \tag{1}$$

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where ε_o and ε – molar absorption coefficients of the uncomplexed and complexed guest, respectively; η_o and η – respective fluorescence quantum yields at the excitation wavelength; I_o – intensity of the incident light; S – total guest concentration; K – 1:1 stability constant.

Stability constants have been estimated either by a nonlinear fitting of the experimental data to the hyperbolic equations or by the linear transformations, which facilitate the extraction of the parameters from the linear plots. Three linear transformations of the original hyperbolic equation are described for light absorption measurements [47]. These transformations of (1) lead to the following equations:

$$\frac{1}{\Delta F} = \frac{1}{I_o(\varepsilon\eta - \varepsilon_o\eta_o)KS[L]} + \frac{L}{I_o(\varepsilon\eta - \varepsilon_o\eta_o)S} \tag{2}$$

Equation (2) is known by name of Benesi-Hildebrand [48]. By plotting $1/\Delta F = f(1/[L])$, one can estimate K as the intercept-to-slope ratio.

The second linear equation is known by name of Scott [49]:

$$\frac{L}{\Delta F} = \frac{1}{I_o(\varepsilon\eta - \varepsilon_o\eta_o)KS} + \frac{L}{I_o(\varepsilon\eta - \varepsilon_o\eta_o)S} \tag{3}$$

By using the plot $\Delta[L]/\Delta F = f(\Delta L)$, one can calculate K as the slope-to-intercept ratio.

Third linearization is known as a Scatchard one [50]:

$$\frac{\Delta F}{[L]} = -K\Delta F + I_o(\varepsilon\eta - \varepsilon_o\eta_o)KS \tag{4}$$

Here, K equals to slope of the $\Delta F/[L]$ *vs*. ΔF line taken with the opposite sign.

In the studies of binding of different substrates to a CD by spectrophotometry, K was mostly determined by non-linear fitting [3,5,10,11,15–18,23,25,28–32,34,40,44–46] and by the Benesi-Hildebrand method [1,2,4,6,8,9,12–14,21,24,26,27,33,35,36,38,39,41–43]. The other two linearizations have been used sparingly: that of Scott [7,19,22] and Scatchard [20,37]. However, Scatchard analysis is commonly used in studies of binding of small molecules to macromolecules (*e.g.* receptors). It can be shown that the linear Benesi Hildebrand dependence of $1/\Delta F$ *vs* $1/[L]^2$ holds when complex of different stoichiometry (1:2) is formed [14].

It has been claimed that the linear regression analysis of a linear transformation weights data points improperly, particularly in the case of the Benesi-Hildebrand data presentation, and, therefore, can lead to large errors. Hence, the plots should be only used for initial estimation of the parameters [25,27,28,51]. These estimates can be used to calculate more accurate values of the parameters, *e.g.* by non-linear curve-fitting model or by iteration.

In the present work, association of naproxen, (+)-6-methoxy-α-methyl-2-naphthalene acetic acid with two different cyclodextrins, *i.e.* a cationic and an anionic one, has been investigated. Naproxen is a non-steroidal anti-inflammatory drug. Solubility of this drug can be largely enhanced by complexation with β-cyclodextrin (β-CD) [52–54]. Stability constants of complexes of naproxen and uncharged cyclodextrins, such as α-CD, β-CD, γ-CD, 2-hydroxypropyl-β-CD and methyl-β-CD were previously determined by fluorometry [31]. In aqueous solution naproxen shows a strong fluorescence and its fluorescence intensity appeared to be enhanced by addition of cyclodextrins. In this way two conditions necessary for determination of stability constants by the fluorometric method are fulfilled.

In the present paper, stability constants of the naproxen-cyclodextrin complexes have also been determined by this method. The first objective of this work was to determine the role of charge in interaction of the charged and uncharged drug (naproxen) with negatively charged (carboxymethylated β-cyclodextrin, COOH-β-CD) and positively charged (6-monodeoxy-6-monoamino-β-cyclodextrin, NH_2-β-CD) modified β-cyclodextrins. The second objective was to compare the linear and nonlinear procedures of determination of stability constants.

EXPERIMENTAL

Reagents: Carboxymethylated-β-cyclodextrin, COOH-β-CD, and amino-β-cyclodextrin, NH_2-β-CD (as hydrochloride) were purchased from CYCLOLAB R&D Lab. Ltd. (Budapest, Hungary). COOH-β-CD (catalogue no. CY-2006) contained 3-3.5 carboxymethyl groups per CD ring. Degree of substitution of NH_2-β-CD (CY-E-2024) was one amino group per one CD ring. Naproxen is a weak acid ($pK_a = 4.2$). In aqueous unbuffered solutions it forms a mixture of dissociated (anionic) and undissociated (neutral) form and the solution pH is about 4.2 (for 5×10^{-5} M naproxen). After addition of a CD, four species are present in the solution, *i.e.* dissociated and undissociated naproxen and CD complexes of either form. In order to simplify the system, we have determined stability constants and carried out fluorescence measurements on solutions of naproxen in 0.1 M HCl (pH = 1) or in solutions of the naproxen sodium salt in 10^{-4} M NaOH, pH $\approx$ 10. In these solutions, naproxen was in its neutral and anionic form, respectively.

Fluorescence measurements: Fluorescence spectra were recorded with a Shimadzu RF-5000 spectrofluorometer equipped with a thermostatically controlled cell compartment. Spectra were measured at 35℃. The excitation wavelength was 329.6 nm. At this wavelength, molar absorption coefficient of naproxen and its anion was about 1520 M^{-1}, and 1600 M^{-1}, respectively. Fluorescence intensity was taken at maximum at 356 nm.

For fluorescence titration, 2 ml sample of about 5×10^{-5} M naproxen in diluted HCl or NaOH solution was titrated in a fluorescence cell, equipped with a Teflon stopper and a magnetic stirrer by successive addition 10 mM CD in an identical naproxen solution. The portions added were in the range 10–100 μl. Final concentrations of CDs ranged from 0 to 2.7 mM. After each titration, the fluorescence spectrum and the intensity at the maximum wavelength was recorded as a function of ligand concentration.

RESULTS AND DISCUSSION

Stability constants of complexes of neutral and anionic naproxen with charged CDs. Stability constants of neutral and anionic naproxen with the two CDs are summarized in Tables 1 and 2. Comparison of data given in Table 1 points out that

substitution of one hydroxyl group in β-CD by one amino group or three hydroxy groups by three carboxymethyl groups resulted in lower stability constant values as compared to those for unsubstituted β-CD. The origin of this effect has not been interpreted in terms of intramolecular interactions so far. An unexpected result is the large influence of the amino group substitution. In the acidic solution the amino group is protonized. Investigation of complexation of aryl phosphates by cationic cyclodextrins, such as guanidine-β-CD and NH_2-β-CD, revealed that phosphotyrosine was more strongly complexed than by unsubstituted β-CD at pH $\approx$ 7; for n = 2 the effect was even better, than for n = 1, where n is the degree of substitution [55]. Similarly, binding of nucleotides and nucleosides by aminocyclodextrins (n = 7) was stronger than that by the same ligand but at n = 2 [56]. Stability constants of complexes of arylphosphates with the same cationic cyclodextrin was independent of the substituent and degree of substitution (n = 1 or 2) [57]. Thus, formation of weaker complexes of naproxen with NH_2-β-CD has not been confirmed by the binding results with other substrates. The possible reason of the effect found here can be the interaction of the naproxen molecule with the ionized, $-NH_3^+$ group.

Table 1. Stability constants of complexes of naproxen with three cyclodextrins in aqueous solutions (pH $\approx$ 1) at 35°C (σ is the standard deviation). Values in parentheses were obtained after iterative corrections (see text).

Cyclodextrin	K, M^{-1}			
	Non-linear fitting	Linear fitting method		
		Benesi-Hildebrand	Scott	Scatchard
β-CD	3000 σ = 114 n = 6	2400 (2880) σ = 100 (150) n = 6	2600 (2980) σ = 110 (140) n = 6	2460 (2900) σ = 60 (80) n = 6
COOH-β-CD	2500 σ = 55 n = 5	2000 (2300) σ = 240 (340) n = 6	2200 (2470) σ = 70 (100) n = 6	2070 (2380) σ = 150 (210) n = 6
NH_2-β-CD	930 σ = 90 n = 5	780 (840) σ = 120 (150) n = 4	800 (850) σ = 145 (160) n = 5	750 (800) σ = 160 (180) n = 5

Substitution of the β-CD rim with different groups can influence the complex formation due to steric effects. In the case of neutral hydroxy-β-cyclodextrin, OH-β-CD, it was found that stability constant of its complex with phenolphthalein systematically decreased with the degree of substitution n, *i.e.* number of OH groups [5,58]. In the case of charged CDs, these steric effects add to electrostatic effects. Here, the observed lower stability constant values for COOH-β-CD as compared to those for β-CD, presumably could be accounted for by some steric interactions of the guest with three

-COOH groups at the cyclodextrin cavity rim. Similarly, the stability constants of neutral indomethacin complexes are several times smaller for such negatively charged CDs as COOH-β-CD or sulfobutylether-β-CD (SBE-β-CD) than for neutral methylated cyclodextrin or hydroxypropyl cyclodextrin [59].

Table 2. Stability constants of complexes of naproxen anion with three cyclodextrins in aqueous solutions (pH ≈ 10) at 35°C (σ is the standard deviation). Values in parentheses were obtained after the iterative corrections.

Cyclodextrin	K, M^{-1}			
	Non-linear fitting	Linear fitting method		
		Benesi-Hildebrand	Scott	Scatchard
β-CD	620 σ = 34 n = 5	500 (530) σ = 230 (260) n = 5	540 (560) σ = 70 (70) n = 5	520 (550) σ = 80 (90) n = 5
COOH-β-CD	10 σ = 1 n = 4	too small	too small	too small
NH_2-β-CD	400 σ = 50 n = 8	510 (540) σ = 60 (70) n = 4	340 (350) σ = 110 (120) n = 8	330 (350) σ = 140 (150) n = 8

Out of anionic CD hosts, sulfoalkyl ether derivatives were the most frequently investigated. Investigation of the effect of the alkyl chain length and degree of substitution on complexation ability of sulfoalkyl β-cyclodextrin with respect to steroids showed that the binding ability increased with the increase of the distance of the sulfonate group from the cavity entrance (sulfobutyl *vs* sulfopropyl derivative). Direct sulfonation of β-CD inhibited the complexation [60]. The SBE-β-CD binding ability with respect to testosterone and progesterone was comparable to that of β-CD or was even higher. Increasing degree of substitution to a given optimum value assisted in complex formation. However, placement of more than an optimum number of sulfoalkyl groups at a rim of the CD torus resulted in inhibition of the complexation [60].

The complex stability constants for the neutral forms of the drugs (including naproxen) are larger for SBE7-β-CD than for OH-β-CD [20,61]. These data also point to the enhancing of the complexing ability of anionic CDs as compared to their neutral counterparts. The present results for naproxen and previously mentioned indomethacin do not fit in with the scheme. Presumably, the discrepancy is due to the proximity of the negatively charged groups in COOH- β-CD to the CD rim. Therefore, properties of the latter ligand should not be compared to the sulfopropyl or sulfobutyl CD derivatives but to CD directly sulfonated at the rim.

Table 2 shows similar results as Table 1, but for solution of anionic naproxen. Stability constants are markedly lower than those for acidic solutions (Table 1) for the three CDs investigated here; for parent β-CD, OH-β-CD, and CH_3-β-CD the effect has been already found previously [31]. Presently, it is also shown for the other two β-CD derivatives. The complex stability constant for neutral NH_2-β-CD and ionized naproxen is still lower than for ionized NH_2-β-CD and neutral naproxen (Table 1). In other words, ionization of the guest hindered the complex formation.

The effect has been also found for other guest molecules; for example, neutral OH-β-CD/charged substrate complexes exhibited a 2 to 31 times decrease in complexation strength as compared to the neutral forms of the same substrates [20]. Moreover, the effect was found for many β-CD complexes of charged substrates. However, the opposite tendency was also reported, *e.g.* for the anionic agents, the binding constants between SBE7-β-CD and OH-β-CD were similar [61]. Please, address also to discussion and references in [31].

In the case of COOH-β-CD, a very low, practically negligible stability constant of a complex with naproxen in alkaline solution can be accounted for by repulsion of the host and guest negatively charged molecules. Some other effects of changing the neutral to charged cyclodextrin on complexation of various drugs can be easily explained by the interaction of charges: compared to drug complexes with neutral CDs, the K values were 20 to 1600% larger when the drug and CD molecules carried opposite charges, but 50 to 80% smaller when the molecules carried the same type of charge [59]. The effect was also reported in [20] for the same charges. However, other interactions can reverse the binding ability ordering. β-CD-sulfate binding ability of positively charged chlorpromazine was inferior to that of parent β-CD [19]. The results of comparison of binding ability of drugs by neutral or charged CDs depend upon nature of the substrates used. However, the investigations of the influence of the CD charge on the substrate binding are relatively scarce.

Substitution of only one -OH group by one charged $-NH_3^+$ group lowered the CD complexation ability with respect to naproxen. Binding of naproxen anion with CD, bearing a neutral -NH_2 group, is still weaker. Complexation of naproxen by COOH-β-CD is also weaker than by parent β-CD, but the effect is not so distinct as in the case of NH_2-β-CD.

Solubility of carboxymethylated-β-CD and amino-β-CD is larger than 100 g/100 ml, whereas that of unsubstituted β-CD is 1.85 g/100 ml (25°C, water, according to producer specification). Due to such solubility enhancement (by a factor greater than fifty), it could be possible to solubilize much more naproxen in solutions of carboxymethylated-β-CD and amino-β-CD than in solution of unsubstituted β-CD.

Determination of the complex stability constants by different methods. The stability constants were determined directly by the non-linear curve-fitting model, the underlying hyperbolic curve being presented in (1), as well as according to three linearizations (Tables 1 and 2). Differences in K values, determined by different methods, are displayed by comparing the three linearizations (2–4) used for the processing of the same experimental data for NH_2-β-CD and naproxen anion (Figs. 1–3).

The results of the determination with these particular plots were 360, 325, and 310 M^{-1}, respectively. When one uses the Benesi-Hildebrand regression, quite often very high or very low (or even negative K values) are obtained. That is why, after such extreme values were neglected, the number of experiments, n, is sometimes lower for that regression than for the other. The data have been interpreted assuming a 1:1 complex stoichiometry. The justification for the assumption arises from linearity of the three plots (Figs. 1–3). They were plotted according to linear equations (2–4), which are valid only in the case of the 1:1 stoichiometry. This procedure has been used previously for naproxen complexes [31] and for other substrates [6,11,20,27,28,62]. In the case of nonlinear plots [14,21,27], it was assumed that complexes of higher stoichiometry, *e.g.* 1:2, were also formed. In the special case of linearity of the plot $1/\Delta F$ *vs* $1/[L]^2$, this linearity was taken as an evidence of 1:2 complex formation [14].

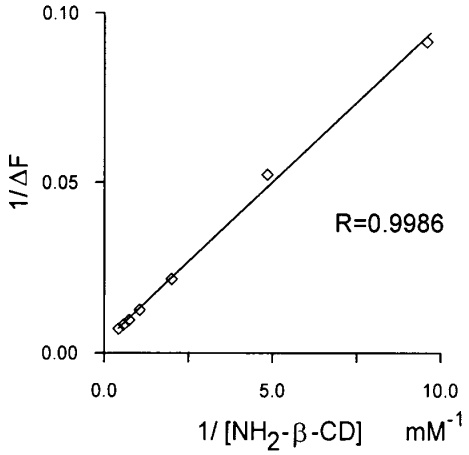

Figure 1. Benesi-Hildebrand plot for interaction between naproxen and NH_2-β-CD. Solvent NaOH, pH ≈ 10.

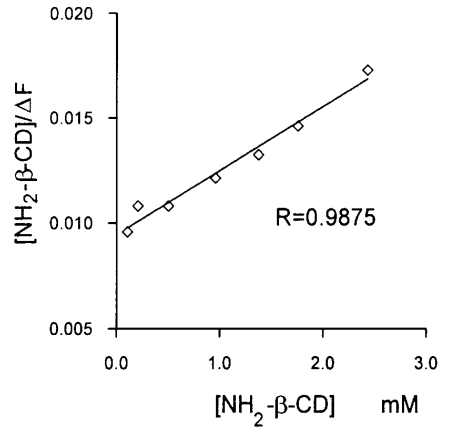

Figure 2. Scott plot for interaction between naproxen and NH_2-β-CD. Solvent: NaOH, pH ≈ 10.

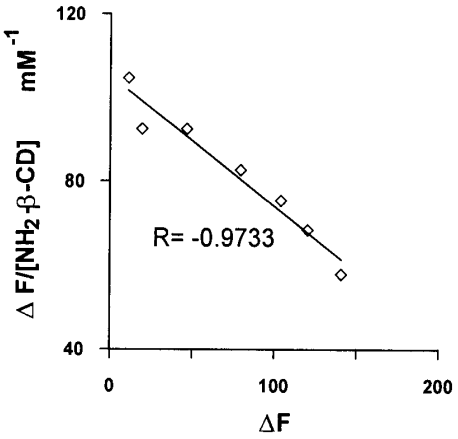

Figure 3. Scatchard plot for interaction between naproxen and NH_2-β-CD. Solvent: NaOH, pH ≈ 10.

The stability constants estimated by all four methods are not equal, despite processing the same experimental data in every case. The constants determined by non-linear fitting are mostly larger than those determined by linear regression fitting.

Comparison of non-linear and linear fits (values outside parentheses) shows that the difference between resulting K values amounts to 20%. One of the reasons of such discrepancy may be as follows: in (1–4), ΔF is expressed as a function of a free ligand concentration, which is not *a priori* known. In literature on determination of K it is usually assumed that a large ligand excess, relative to the substrate, is employed, so the total concentration of ligand can be substituted in the equations instead of the free ligand concentration (here CD). This assumption is not always valid. In our experiments, the concentration of a CD bound to substrate amounts to a few percent (assuming K = 2000 M^{-1}). In order to find the concentrations of free CD, when stability constants are not known, an iterative procedure was used [24]. The results obtained after three iteration steps are given in parentheses. They are always larger than the uncorrected values. Whereas the uncorrected K values, resulting from the three linearizations, were systematically lower than those after the nonlinear fit, after the iterative corrections all the methods produced results, which were in agreement within the experimental error. Still, the results remained not the same. In papers, dealing with the evaluation of stability constants, only one of the methods has been used at the same time; the most frequently used were the non-linear fitting or the Benesi-Hildebrand linearization, without iterative corrections. The difference of results generated with different methods of data processing are independent of the experiments' accuracy and repeatability and could not be reduced by more accurate experimental conditions. This is why the comparison of data on stability constants for different compounds or under different conditions (medium/temperature) should be made only, when data to be compared have been recorded and processed simultaneously by the same method. In the case when data are processed by one of the linearized plots, the iterative

procedure should be used [24]. The assumption that the free ligand concentration equals that of the total ligand added, causes a lowering of the stability constants by a dozen percent.

CONCLUSIONS

Of the complexes investigated, the complex of parent β-CD with neutral naproxen is of the largest stability. Substitution of one -OH group of β-CD by a neutral -COOH one results in the lowering of the stability constant by about 17%. On substitution of the -OH by a charged $-NH_3^+$ group, the stability constant of naproxen is lowered three times. Still lower is the stability of the negatively charged naproxen with the β-CD bearing a neutral NH_2 group. Analysis of the literature data on host-guest interaction in the case when one of the reagents is charged, did not help us to elucidate the reasons of the deterioration of binding.

Analysis of the experimental data by nonlinear fitting and by the linearizations of the original hyperbolic equation showed that the methods are not equivalent: stability constants obtained by a linear fitting were lower than those obtained by a nonlinear fit. After the iterative corrections, allowing to introduce free, instead of the known total ligand concentrations in the linearized plots, the constants resulting from the linear and nonlinear fits were, within experimental error, the same. These corrections are then required for the linear fits even in the case when concentrations of bound ligand amount to a few percent.

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