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Studies on the interaction of diflunisal ion with cyclodextrins using ion-selective electrode potentiometry

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Abstract

The interaction of diflunisal ion (DF) with β-cyclodextrin (βCD), γ-cyclodextrin (γCD), and hydroxypropyl-β-cyclodextrin (HPβCD) was studied in phosphate buffer, pH 7.4, at 5–37°C and various CD concentrations using a home-made diflunisal ion-selective electrode. Typical direct binding plots and Scatchard plots were obtained with HPβCD. The Scatchard model for one class of binding sites was used for the estimation of binding parameters for the DF/HPβCD interaction. The estimates for n (number of binding sites per CD molecule) were in all cases very close to unity, indicating 1:1 complexation. The association constant (K) estimates decrease with increasing temperature. Sigmoidal direct binding plots and concave-downwards Scatchard plots were obtained with various βCD or γCD concentrations. The Hill model was used for the estimation of the binding parameters for the DF/βCD and DF/γCD interactions. Both the Hill coefficients and the binding constants were markedly dependent on the CD concentration. These findings indicate the cooperative character of DF/βCD and DF/γCD interactions. The free energy change, ΔG, and the thermodynamic parameters, ΔH and ΔS, were estimated for each of the interactions studied using the Van't Hoff equation.

Keywords: Binding; Cooperative binding; β-Cyclodextrin; γ-Cyclodextrin; Diflunisal; Hill model; Hydroxypropyl-β-cyclodextrin; Ion-selective electrodes; Scatchard model

1. Introduction

The mathematical treatment of drug–protein interactions has been based on the site-oriented model (Scatchard, 1949; Connors, 1987). According to this model, m classes of binding sites exist on the protein molecule, each with n_i independent, non-interacting and equivalent in the attraction for the ligand binding sites. In this model, the ratio $r = B/P_t$ is given by the equation:

$$r = \sum_{i=1}^{m} \frac{n_i K_i F}{1 + K_i F} \quad (1)$$

where F and B are the molar concentrations of the free and bound drug, respectively, P_t is the molar concentration of protein and K_i is the intrinsic binding constant for the ith class of binding sites. The non-interacting character of the binding sites leads to Scatchard plots with negative slopes (Connors, 1987) and the binding is termed classical. When the binding sites do not act independently of one another, the binding exhibits either positive or negative cooperativity and the empirical Hill equation (Hill, 1913; Connors, 1987) is used to describe the binding phenomenon:

$$r = \frac{nKF^h}{1 + KF^h} \quad (2)$$

where h is the Hill coefficient denoting positive (if $h>1$) or negative (if $h<1$) cooperativity. When cooperativity is operating, concave-downwards ($h>1$) and concave-upwards ($h<1$) Scatchard plots are obtained.

Cyclodextrins (CDs) form inclusion complexes with a large number of molecules. In most cases reported, the interaction is classical. However, there have been rare reports of cooperative interactions in the field of CDs, concerning either natural cyclodextrins (Harada and Nozakura, 1982; Cromwell et al., 1985; Eftink et al., 1989) or

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synthetic cyclodextrin derivatives (Harada et al., 1980; Petter and Salek, 1987; Petter et al., 1990; Kikuchi et al., 1992; Wang et al., 1994). In the case of natural cyclodextrins, cooperative binding with certain guest molecules has been mostly attributed to intermolecular hydrogen bonding between the CD molecules, while intermolecular interactions between both the host and guest molecules (hydrogen bonds, hydrophobic interactions and Van der Waals forces) contribute to cooperative binding processes when synthetic CDs are used.

In the course of our studies on drug–cyclodextrin interactions (Valsami et al., 1990, 1992; Sideris et al., 1992) we have studied the binding of diflunisal [5-(2,4-difluorophenyl)salicylic acid] ion with HPβCD and found no evidence of cooperative binding (Sideris et al., 1994). As a part of our efforts to elucidate the interaction of diflunisal with CDs, we present in this work additional studies on the diflunisal interaction with HPβCD and we extend our studies to the interaction of diflunisal with βCD and γCD. A diflunisal ion-selective electrode (ISE) was constructed and used for the study, which was performed in the mode of a potentiometric titration (additions of DF ion to a CD solution). The same electrode was used as a flow detector in a flow injection system for quality control of diflunisal formulations (Solich et al., 1995). Both the Scatchard (1949) and the Hill (1913) models were used for the estimation of the binding parameters. The analytical technique of ISE potentiometry has the advantage of the direct measurement of the concentration of the free small molecule in a wide concentration range in the presence of the CD and their complexes thus providing a complete profile of the interaction.

2. Experimental procedures

2.1. Reagents

Diflunisal was obtained from Sigma (St. Louis, MO, USA) and used without further purification. γCD was kindly donated by Prof. J. Szejtli (Cyclolab, Hungary). βCD, HPβCD, 2-nitrophenyl-octyl ether (2-NPOE) and tetraheptylammonium bromide were obtained from Fluka (Buchs, Switzerland). Polyvinyl chloride (PVC) of high molecular weight ($d=1.385$) was obtained from Janssen Chimica (Beerse, Belgium). All other chemicals used were of analytical grade.

All solutions were prepared in phosphate buffer, 0.10 M, pH 7.4. The diflunisal stock solution was 0.0100 M. βCD solutions of 0.0010, 0.0050, 0.0080 and 0.010 M, γCD solutions of 0.0050, 0.0080, 0.010, 0.020 and 0.030 M and an HPβCD solution of 0.0080 M were prepared. Mixed working solutions of 0.0100 M with respect to diflunisal were prepared in the aforementioned γCD, βCD and HPβCD solutions.

2.2. Electrode construction

The diflunisal ISE was of the PVC membrane type (Craggs et al., 1974). The electroactive PVC membrane was constructed by entrapping diflunisal liquid ion exchanger in a PVC matrix. The liquid ion exchanger was the ion pair of the diflunisal anion with the tetraheptylammonium cation in 2-nitrophenyl-octyl ether at a concentration of ≈0.01 M. The details of the preparation of the liquid ion exchanger and the PVC membrane have been described previously (Sideris et al., 1994). The membrane obtained was conditioned for 24 h before use in a 0.0100 M diflunisal solution. The diflunisal ISE (indicator electrode) was assembled by attaching the conditioned PVC membrane to a small silicone tube-cap filled with internal reference solution, and this was fitted to the end of an electrode body [a conventional pH glass electrode (Cambridge Instrument Co.), the end of which had been cut off]. The internal reference solution was 0.0100 M with respect to diflunisal in 0.0100 M sodium chloride, saturated with silver chloride.

2.3. Apparatus

The system used for the potentiometric measurements consisted of an Orion Model SA 720 pH/ISE meter, with a readability of ±0.1 mV, connected to an LKB Bromma Single Chart Recorder Model 2210. The emf (potential) values were measured against a Ag/AgCl reference electrode (Orion single junction, Model 900100, filled with Orion reference electrode filling solution 90-00-01) and recorded on a Brother M-1109 printer. All measurements were carried out in a 30-ml double-walled glass cell, thermostated at temperatures of 5, 15, 25 and 37°C (±0.5°C) by an Edmund Buhler 7400 Tubingen Type UKT30 water bath, under constant magnetic stirring of the solutions.

2.4. Construction of calibration curve

The pair of electrodes were immersed in 5 ml phosphate buffer, 0.10 M, pH 7.4. After the potential was stabilized (±0.1 mV), small volumes of the 0.0100 M diflunisal stock solution were added (concentration range achieved, 1×10^{-6} to 6×10^{-3} M). The potential values (mV) were recorded and measured after stabilization (±0.1 mV), following each addition. These were plotted against $-\log C_{drug}$ according to the Nernst equation, and the calibration curve was obtained by least-squares fitting of the Nernst equation to the experimental data.

2.5. Binding experiments

The pair of electrodes were immersed in 5 ml CD solution in phosphate buffer. After the potential was stabilized (±0.1 mV), small volumes of the corresponding

mixed DF/CD working solution were added. The emf values were recorded to check stabilization and measured after each addition.

2.6. Study of the effect of the pH on the response of the ISE

The pair of electrodes were immersed together with a combination glass electrode connected to a pH meter in 30 ml of diflunisal solution (5×10^{-4} M) in 0.10 M NaOH (initial pH≈12) in the presence of Na_2SO_4 0.10 M for adjustment of the ionic strength. After the potential was stabilized (±0.1 mV), small volumes of H_2SO_4 solution were added and the potential was continuously monitored in the region of pH 12–2.

2.7. Data analysis

The site-oriented (Scatchard, 1949) model for a single class of binding sites was applied for the estimation of the binding parameters for the DF/HPβCD interaction:

$$r = \frac{nKF}{1 + KF} \tag{3}$$

where n is the number of binding sites per CD molecule, K is the binding (association) constant (M^{-1}), r is the molar concentration of the bound drug (B) divided by the molar concentration of CD and F is the molar concentration of the free drug. For each addition, F was calculated from the calibration curve and B from the equation $B = T - F$, where T is the total molar concentration of diflunisal.

The binding parameters for the interaction of diflunisal with βCD and γCD were estimated using the Hill equation (Eq. (2)) (Hill, 1913). For each addition, F was calculated as described above.

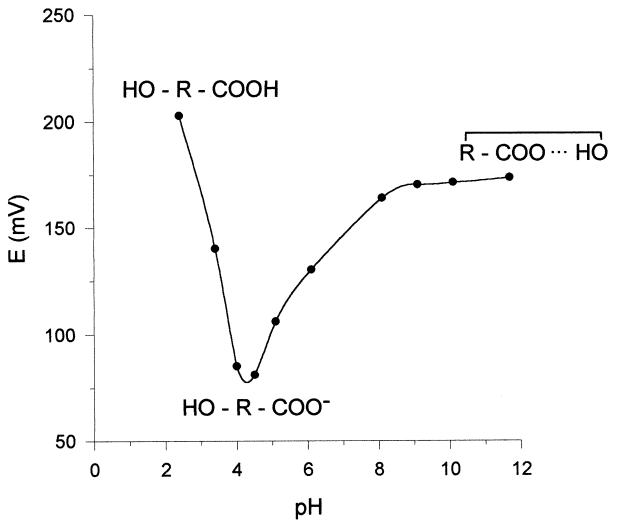

Fig. 1. Effect of pH on the potential, E, of the diflunisal ion-selective electrode at 25°C and diflunisal concentration of 5×10^{-4} M. The predominant diflunisal species are indicated at the critical pH regions.

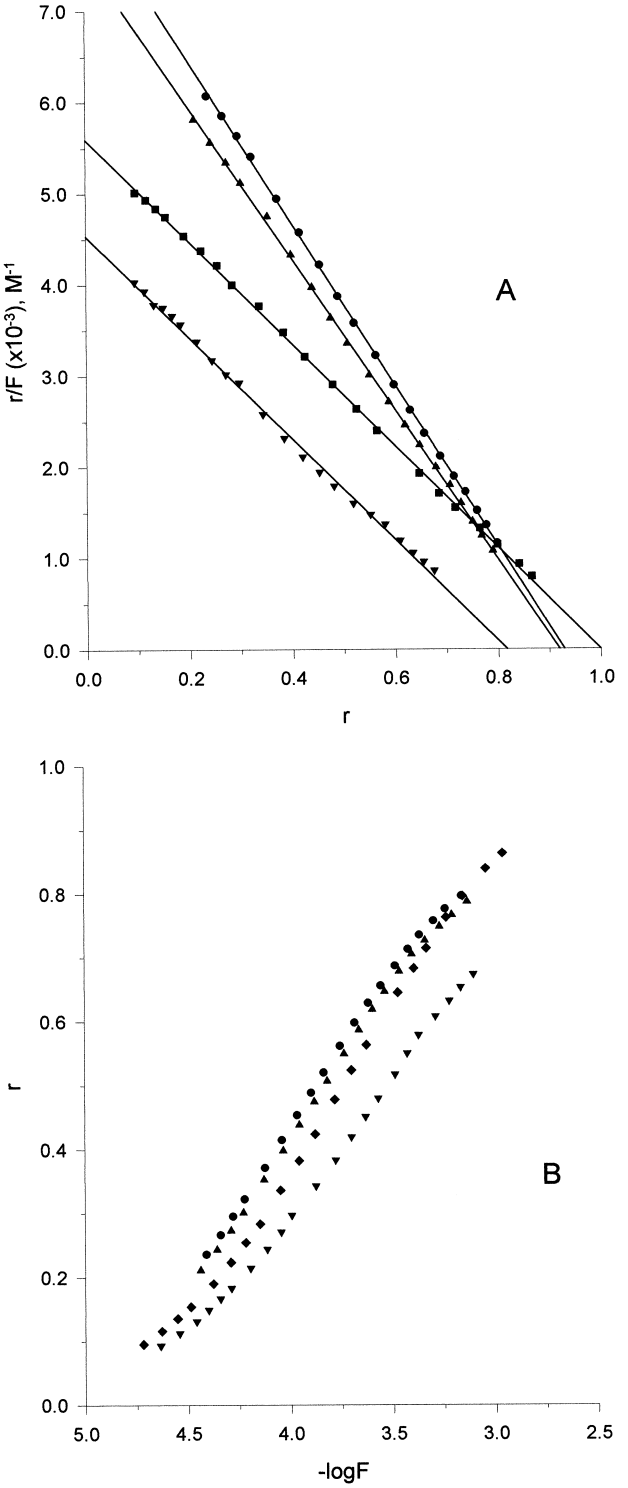

Fig. 2. (A) Scatchard plots of the diflunisal/HPβCD interaction at temperatures 5 (●), 15 (▲), 25 (♦) and 37°C (▼); total HPβCD concentration 0.008 M. The fitted lines of the linear form of Eq. (3) are drawn over the experimental data. (B) Saturation plots (r vs. $-\log F$) of the interaction of diflunisal with HPβCD at temperatures 5 (●), 15 (▲), 25 (♦) and 37°C (▼).

The parameters of both the Scatchard model (n, K) and the Hill model (n, K and h) were estimated by non-linear least-squares fitting of Eqs. (3) and (2), respectively, to the experimental data.

3. Results and discussion

3.1. Electrode characteristics

The diflunisal ISE showed a near-Nernstian response (slope 58–61 mV/decade) in the concentration range 2×10^{-5} to 6×10^{-3} M at all temperatures utilized in the present study. The detection limit was 5×10^{-6} M. The electrode presented no drift in the presence of CDs and was therefore adequate for the binding experiments performed. Fig. 1 shows the dependence of the response of the diflunisal ISE on the pH of the solution. For pH values lower than 8 the potential decreases (corresponding to an increase of the diflunisal anion activity to which only the ISE responds). This would not normally be expected, since a decrease of pH below pK_a+2 results in the formation of the diflunisal acid species leading to an increase of the ISE potential. Similar results have been reported previously for salicylic acid (Mitsana-Papazoglou et al., 1984), which presents structural similarities with diflunisal. This behaviour is due to the formation of intra- and/or intermolecular hydrogen bonds between the diflunisal molecules at alkaline pH (R–COO$\cdots$HO), which progressively break as the pH decreases and measurable anions of the drug (HO–R–COO$^-$) are released (Fig. 1). The diflunisal molecule possesses three groups (–OH, –COOH, –F) which would participate in intra- or inter-hydrogen bonding. At pH below 4 the difflunisal anion is protonated ($pK_a\approx3$), resulting in the normal increase of the ISE potential. However, the response curve of the DF ISE obtained using pH-buffered solutions (pH 7.4 in this application) is linear up to the concentration of 6×10^{-3} M.

3.2. Binding studies

The Scatchard plots obtained for the interaction of DF with HPβCD at temperatures ranging from 5 to 37°C are shown in Fig. 2A. The estimates of the binding parameters in the temperature range studied are presented in Table 1. The linearity of the Scatchard plots at all temperatures studied is indicative of the 1:1 complexation of diflunisal with HPβCD. In addition, the estimates of parameter n (number of binding sites per HPβCD molecule) were found to be, in all cases, almost equal to unity (Table 1). It is interesting to note that the estimate for n at 25°C was found to be equal to the theoretical value, calculated from Eq. (3) for one binding site, i.e. the CD cavity. Although the value of n does not change with temperature, the value of K (binding constant) decreases with increasing temperature (Table 1). This kind of behavior is characteristic for exothermic interactions. In Fig. 2B, the saturation plots of HPβCD with DF at all temperatures studied are shown. The covered range of free diflunisal concentration is about two decades of the molarity scale and corresponds to 16.7–79.8, 21.1–86.0, 7.4–86.55 and 9.3–67.5% saturation of HPβCD at 5, 15, 25 and 37°C, respectively.

The Scatchard plots (r/F vs. r) obtained for the interactions of DF with βCD and γCD at various CD concentrations (Fig. 3A,B) and at various temperatures (Fig. 4A,B) are concave-downwards. Curvilinear Scatchard plots with downwards concavity are generally considered to indicate positive cooperativity at the binding site (Werthen and Nygren, 1993). In addition, all direct plots (r

Table 1
Estimates of the binding parameters for the DF/HPβCD and DF/γCD interactions at various temperatures[a,b]

Temperature (°C)	K (M^{-1})	h	n	R^2 [c]
HPβCD				
5	$8.85(0.04)\times10^3$		0.929(0.002)	0.9999
15	$8.25(0.04)\times10^3$		0.920(0.002)	0.9999
25	$5.57(0.04)\times10^3$		1.002(0.003)	0.9999
37	$5.45(0.06)\times10^3$		0.820(0.005)	0.9999
βCD				
5	$9.66(4.54)\times10^6$	1.620(0.046)	0.810(0.006)	0.9992
15	$4.26(1.22)\times10^6$	1.640(0.029)	0.824(0.005)	0.9996
25	$7.83(0.38)\times10^4$	1.259(0.005)	0.620(0.001)	0.9999
37	$2.03(0.10)\times10^4$	1.189(0.005)	0.798(0.002)	0.9999
γCD				
5	$1.98(0.43)\times10^6$	1.88(0.02)	1.66(0.05)	0.9999
15	$6.13(1.26)\times10^5$	1.75(0.02)	1.73(0.05)	0.9999
25	$8.82(0.94)\times10^4$	1.62(0.01)	1.60(0.03)	0.9999
37	$5.93(1.64)\times10^4$	1.62(0.03)	1.47(0.06)	0.9998

[a]Standard deviations of the estimates in parentheses.
[b]βCD, γCD and HPβCD concentration 0.008 M.
[c]Multiple regression coefficient utilizing Eq. (2) or Eq. (3).

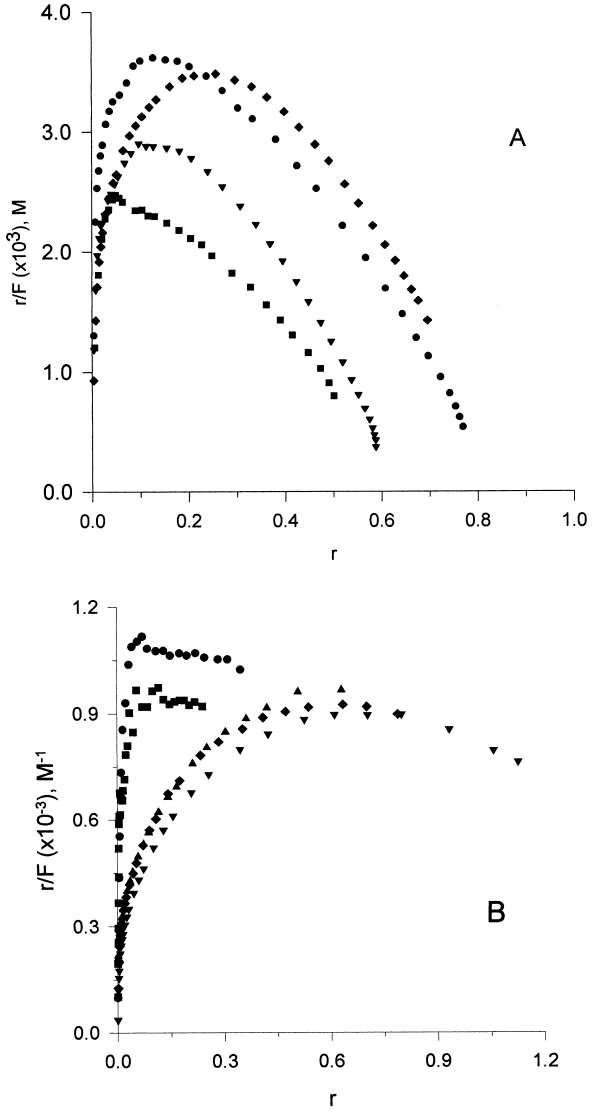

Fig. 3. Scatchard plots of (A) the diflunisal/βCD interaction at total CD concentrations of 0.0010 M (■), 0.0050 M (▼), 0.0080 M (♦) and 0.010 M (●), and (B) diflunisal/γCD at total CD concentrations of 0.0050 M (▼), 0.0080 M (♦), 0.010 M (▲), 0.020 M (●) and 0.030 M (■) (at 25°C and pH 7.4).

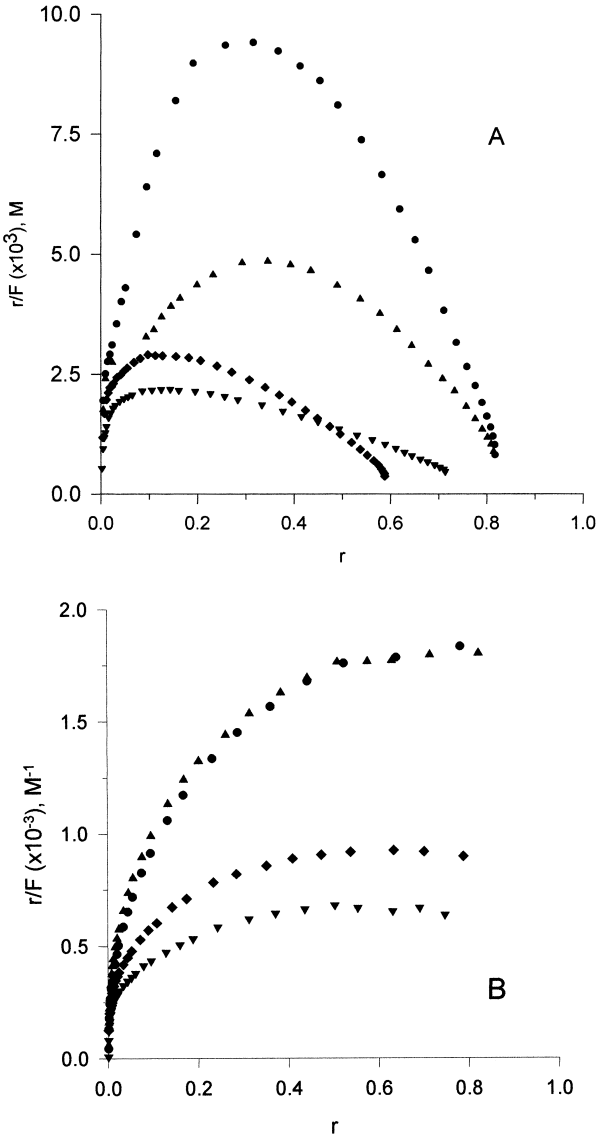

Fig. 4. Scatchard plots of (A) the diflunisal/βCD interaction and (B) the diflunisal/γCD interaction, at temperatures of 5 (●), 15 (▲), 25 (♦) and 37°C (▼); total concentration of CD, 0.0080 M (pH 7.4).

vs. F) obtained for the DF/βCD (Fig. 5A,B) and DF/γCD interactions (Fig. 6A,B) are sigmoidal. The fitted line of the Hill model (Eq. (2)) follows the experimental data very closely (Figs. 5 and 6) and this is reflected in the value of the multiple regression coefficient (R^2) which in all cases is very close to unity (Tables 1 and 2). In nearly all cases (Tables 1 and 2), the value of h was found to be greater than unity, which is indicative of the positive cooperativity of the binding process (Mazoit et al., 1996). The estimated values of the Hill coefficient are within the range of previously reported values for the interaction of aggregated βCD derivatives with various guests (Petter et al., 1990).

Both the Hill coefficient and the association constant increase on increasing the βCD concentration (Table 2). In contrast, these two parameters are shown to decrease with increased γCD concentration (Table 2). At the higher γCD concentrations (0.020 and 0.030 M), the value of h is practically constant and equal to unity (Table 2), while at the higher βCD concentrations (0.008 and 0.010 M) higher values of h were found. In parallel, the direct plots of the DF/γCD interaction at the higher γCD concentrations utilized are not sigmoidal, i.e. the interaction is not cooperative (Fig. 6A). The non-cooperative character of the interaction at the higher γCD concentrations is also confirmed by the estimated K values, which remain practically constant (Table 2). This kind of concentration dependency is common in cooperative binding phenomena (Bowmer and Lindup, 1978a,b).

Both DF/βCD and DF/γCD interactions behave similarly to temperature variations. Both h and K increase with decreasing temperature. In the case of the DF/βCD

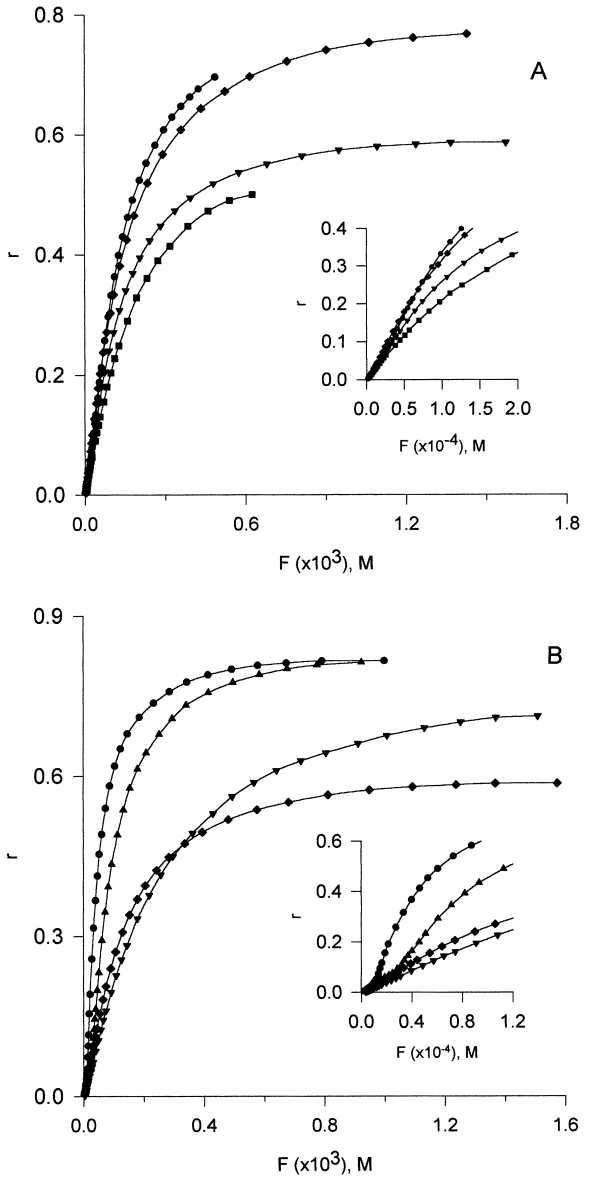

Fig. 5. Direct plots (r vs. F) of the diflunisal/βCD interaction (pH 7.4) (B) at temperatures of 5 (●), 15 (▲), 25 (♦) and 37°C (▼) (total concentration of βCD, 0.0080 M); (A) at 25°C and total βCD concentrations of 0.0010 M (f), 0.0050 M (▼), 0.0080 M (♦) and 0.010 M (●). The fitted lines of Eq. (2) are drawn over the experimental data (the inserts show the sigmoidal segments of the curves).

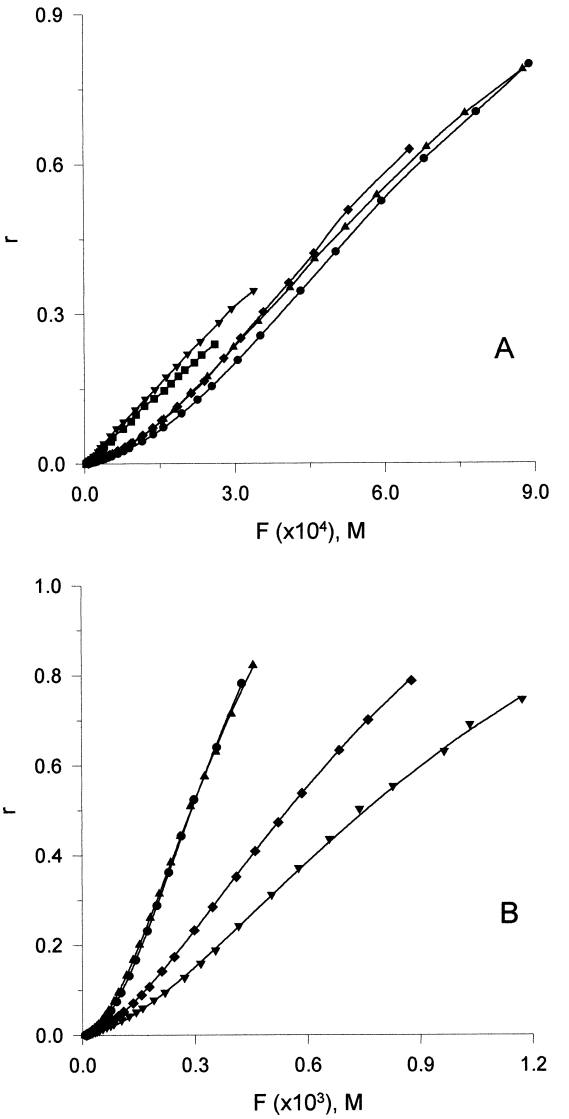

Fig. 6. Direct plots (r vs. F) of the diflunisal/γCD interaction (pH 7.4) (B) at temperatures of 5 (●), 15 (▲), 25 (♦) and 37°C (▼) (total concentration of γCD, 0.0080 M); (A) at 25°C and total γCD concentrations of 0.0050 (●), 0.0080 M (♦), 0.010 M (▲), 0.020 M (▽) and 0.030 M (■). The fitted lines of Eq. (2) are drawn over the experimental data.

interaction the value of K increased dramatically when the temperature decreased from 25 to 15°C and a larger value for h was also found (Table 1). This may be attributed to the large contribution of the specific non-covalent interactions involved in inclusion complex formation, as indicated by the high negative ΔH_{obs} value (Eftink et al., 1989) (Table 3) calculated after thermodynamic analysis of the experimental data (see below).

It is also interesting to note that the value of parameter n (reflecting complex stoichiometry in the stoichiometric model; Connors, 1987) was found not to be dependent on temperature or CD concentration (Tables 1 and 2), being approximately 0.75 for βCD and 1.5 for γCD. These values reveal a complex stoichiometry of 3:4 and 3:2 (CD:DF) for the DF/βCD and DF/γCD interaction, respectively. Unfortunately, our experimental procedure does not provide an insight into the structural features of the complexes. However, the intermolecular hydrogen bonding of diflunisal molecules (see electrode characteristics, Fig. 1, and Solich et al., 1995) may play a key role in the cooperative binding phenomena. It is worth mentioning that the rare literature reports for the cooperative binding between cyclodextrins and guest molecules have been mostly attributed to intermolecular hydrogen bonding

Table 2
Estimates of the binding parameters of the DF/γCD and DF/βCD interactions at 25°C and various CD concentrations[a]

CD (M)	K (M^{-1})	h	n	R^{2} [b]
γCD				
5.0×10^{-3}	$2.31(0.24)\times10^{5}$	1.76(0.01)	1.59(0.01)	0.9999
8.0×10^{-3}	$8.82(0.94)\times10^{4}$	1.62(0.01)	1.60(0.03)	0.9999
1.0×10^{-2}	$7.11(2.10)\times10^{4}$	1.62(0.03)	1.90(0.14)	0.9998
2.0×10^{-2}	$1.47(0.72)\times10^{3}$	1.09(0.03)	1.79(0.38)	0.9998
3.0×10^{-2}	$1.51(1.06)\times10^{3}$	1.09(0.05)	1.51(0.64)	0.9998
βCD				
1.0×10^{-3}	$2.17(0.28)\times10^{4}$	1.158(0.012)	0.628(0.006)	0.9999
5.0×10^{-3}	$3.80(0.22)\times10^{4}$	1.195(0.006)	0.821(0.001)	0.9999
8.0×10^{-3}	$7.83(0.38)\times10^{4}$	1.259(0.005)	0.620(0.001)	0.9999
1.0×10^{-2}	$3.14(0.23)\times10^{5}$	1.410(0.007)	0.795(0.003)	0.9999

[a]Standard deviations of the estimates in parentheses.
[b]Multiple regression coefficient utilizing Eq. (2) or Eq. (3).

between CD molecules 'cooperating' to bind the guest molecules (Harada and Nozakura, 1982; Cromwell et al., 1985; Eftink et al., 1989). In addition, one should note that cyclodextrins undergo self-aggregation in solution (Eftink et al., 1989; Coleman and Nicolis, 1992).

3.3. Thermodynamic evaluation of the DF/βCD, DF/γCD and DF/HPβCD interactions

Despite the complexity of the binding of diflunisal with βCD, γCD and HPβCD, we attempted a thermodynamic analysis according to the Van't Hoff equation utilizing the estimates for the association constants:

$$\log K = \frac{\Delta S}{2.303R} - \frac{\Delta H}{2.303R}\frac{1}{T} \quad (4)$$

where ΔS and ΔH are the observed changes in entropy and enthalpy, respectively, T is the absolute temperature (K), R is the gas constant (1.985 cal/deg) and K is the binding (association) constant at temperature T. Linear relationships ($0.93 \leq r \leq 0.97$) were established in all cases and the associated observed thermodynamic parameters are listed in Table 3. The high negative ΔH_{obs} and ΔS_{obs} values for the DF/βCD and DF/γCD interactions can probably be attributed to the cooperative character of the interaction. However, due to the complexity of the binding process, the observed thermodynamic parameters are not interpreted in relation to the mechanism(s) involved.

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Table 3
Estimates of the thermodynamic parameters for the DF/HPβCD, DF/βCD and DF/γCD interactions[a,b]

Interaction	ΔH_{obs} (kcal/mol)	ΔS_{obs} (cal/mol/deg)	ΔG_{obs} (kcal/mol)	R [c]
DF/HPβCD	−2.7(0.8)	7.4(2.8)	−4.9(1.2)	0.93
DF/βCD	−47.5(8.6)	−137.0(29.3)	−6.7(12,2)	0.97
DF/γCD	−20.14(4.2)	−43.7(14.1)	−7.1(5.9)	0.97

[a]Standard deviations of the estimates in parentheses.
[b]βCD, γCD and HPβCD concentration, 0.008 M.
[c]Correlation coefficient utilizing Eq. (4).

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