Effect of Cyclodextrins on Protein Binding of Drugs: The Diflunisal/Hydroxypropyl-β-Cyclodextrin Model Case

Evangelos E. Sideris,[1] Michael A. Koupparis,[2] and Panayotis E. Macheras[1,3]

Received March 25, 1993; accepted June 8, 1993

The binding of diflunisal to hydroxypropyl-β-cyclodextrin (HPβCD), bovine serum albumin (BSA), human serum albumin (HSA), normal human plasma, and mixed solutions of HPβCD/protein was studied at 25°C, pH 7.4, by potentiometry using an electrode selective to diflunisal. The experimental data for diflunisal/HPβCD fit well to the 1:1 binding model. The binding of diflunisal with each of the studied proteins was compatible with a model having two independent classes of binding sites. The binding of diflunisal in mixed solutions HPβCD/BSA, HPβCD/HSA, and HPβCD/plasma increased considerably when the HPβCD concentration was increased. The binding behavior of the two biomolecules in the mixed solutions of HPβCD/BSA or HPβCD/HSA was described with an "additive" model formulated on the basis of the estimates of the binding parameters of diflunisal derived from the separate experiments with each one of the binders tested. The lower than theoretical binding observed in HPβCD/plasma solutions was ascribed to the competitive displacement of diflunisal from the HPβCD cavity by plasma cholesterol.

KEY WORDS: hydroxypropyl-β-cyclodextrin; diflunisal; protein binding; ion-selective electrodes.

INTRODUCTION

Cyclodextrins have been studied as complexing agents of many drug substances. Over the last several years, hydroxypropyl-β-cyclodextrin (HPβCD) has been studied as a useful cyclodextrin derivative for the solubilization and stabilization of various drugs (1–3). More recently, there has been interest in the use of HPβCD as a drug carrier in parenteral formulations (3–5), since it presents lower toxicity than natural cyclodextrins (3). In this context, the effects of HPβCD on the dissolution and transport of lipids and the plasma levels of cholesterol have been examined (6–8).

Concerns have been raised with respect to the potential effects of the parenterally used cyclodextrins on the disposition and pharmacokinetics of drugs (6,9–11). Under *in vivo* conditions cyclodextrins should not be regarded as simple inert carriers for drugs (11). Thus, the interaction of cyclodextrins with plasma proteins and protein drugs was the subject in recent studies (2,5,12,13). Consequently, studies are needed to clarify the interaction of drugs with the plasma proteins in the presence of cyclodextrins.

Only two previous studies (6,11) described the effect of cyclodextrins on the protein binding of spironolactone, naproxen, and flurbiprofen. These studies, utilizing ultrafiltration, were based on single-point measurements for the estimation of the extent of binding, and they do not provide the entire binding profile of drug in the presence of both binders, i.e., protein and cyclodextrin. Moreover, the estimates of the free fraction of drug contained also the fraction complexed to cyclodextrin (6,11).

The present study analyzes the effect of HPβCD on the binding of the nonsteroidal antiinflammatory drug diflunisal [5-(2,4-difluorophenyl)salicylic acid] to bovine serum albumin (BSA), human serum albumin (HSA), and plasma. Diflunisal was used as a model drug because of its high plasma protein binding (14). The binding of diflunisal to HPβCD, BSA, HSA, plasma, and mixed solutions of HPβCD/protein was continuously monitored potentiometrically (15–19) by means of a diflunisal ion-selective electrode (ISE). In the experiments with mixed solutions, the overall binding of diflunisal ion to both binders (HPβCD/protein) was estimated. The diflunisal ISE of the PVC type was constructed in our laboratory for the purposes of the present study.

MATERIALS AND METHODS

Reagents

All solutions were prepared in deionized water. Diflunisal was obtained from Sigma (St. Louis, MO) and used without any further purification. 2-Hydroxypropyl-β-cyclodextrin (average molar degree of substitution, 6.3) was obtained from Aldrich (Steinheim, Germany). Bovine serum albumin fraction V, human serum albumin fraction V, 2-nitrophenyl octyl ether, and tetraheptylammonium bromide were obtained from Fluka (Buchs, Switzerland). Polyvinyl chloride (PVC) of high molecular weight was obtained from Janssen Chimica (Beerse, Belgium). All other chemicals used were of analytical grade.

Phosphate Buffer, 0.100 M, *pH 7.4.* This was prepared using sodium dihydrogen phosphate and adjusting the pH with an 18 *M* NaOH stock solution.

Diflunisal, 0.0100 M *Stock Solution.* This was prepared in phosphate buffer.

HPβCD Stock Solution. This solution was 0.0080 *M* with respect to HPβCD in phosphate buffer.

Diflunisal/HPβCD Mixed Working Solution. This solution was 0.0100 *M* with respect to diflunisal in the HPβCD stock solution.

BSA and HSA Solutions. These solutions were 22.5 g/L with respect to BSA or HSA in phosphate buffer and were prepared daily.

Diflunisal/BSA and Diflunisal/HSA Mixed Working Solutions. These solutions were 0.0100 *M* with respect to diflunisal in the BSA or HSA solutions.

Plasma Preparation. Human plasma from six drug-free healthy volunteers was collected and divided into 15-mL-volume glass vials, which were frozen at −25°C. Prepared plasma was used on the same day after dilution 1:1 with

[1] Department of Pharmacy, University of Athens, Panepistimiopolis, Athens 15771, Greece.
[2] Department of Chemistry, University of Athens, Panepistimiopolis, Athens 15771, Greece.
[3] To whom correspondence should be addressed.

0724-8741/94/0100-0090$07.00/0

phosphate buffer. The total plasma protein concentration was determined by the biuret reaction and was found to be 72 g/L.

Diflunisal/Plasma Mixed Working Solution. This solution was 0.0100 *M* with respect to diflunisal in plasma diluted 1:1 with phosphate buffer, 0.100 *M*, pH 7.4.

HPβCD/BSA, HPβCD/HSA, and HPβCD/Plasma Mixed Solutions. These solutions contained 1.00, 5.00, 8.00, and 10.00 m*M* HPβCD in 22.5 g/L BSA or HSA or 1:1 diluted plasma.

Diflunisal/HPβCD/BSA, Diflunisal/HPβCD/HSA, and Diflunisal/HPβCD/Plasma Mixed Working Solutions. These solutions were 0.0100 *M* with respect to diflunisal in the HPβCD/BSA, HPβCD/HSA, or HPβCD/plasma mixed solutions.

Electrode Construction

The diflunisal ISE was of the PVC membrane type (20). It consists of an electrode body [a conventional pH glass electrode (Cambridge Instrument Co.), the end of which has been cut off] and an electroactive PVC membrane attached to the end of the electrode body through a small silicone tube-cap filled with the internal reference solution. The electroactive PVC membrane was constructed by entrapping the corresponding liquid ion exchanger in a PVC matrix according to the method of Craggs *et al.* (20). The liquid ion exchanger for the diflunisal ISE was its ion pair with tetraheptylammonium cation in 2-nitrophenyl octyl ether (2-NPOE) at a concentration of ≈0.01 *M* and was prepared as follows: 5 mL of a 0.010 *M* tetraheptylammonium bromide solution in 2-NPOE was shaken six times with the 0.0100 *M* diflunisal solution in order to exchange Br^- with diflunisal ion, and every time the aqueous phase was separated by centrifugation and decanted. The organic phase was then dried with anhydrous sodium sulfate to remove any water traces. A small amount of this ion exchanger was used for the preparation of the PVC membrane. The internal reference solution was 0.0100 *M* with respect to diflunisal in 0.0100 *M* sodium chloride, saturated with silver chloride. After its construction, the indicator electrode was conditioned for 24 hr before use by immersing in a stirred solution of 0.0100 *M* diflunisal solution.

Apparatuses

The system used for the potentiometric measurements consists of an Orion Model SA720 pH/ISE meter, with a readability of ±0.1 mV, connected to an LKB Bromma Single Chart Recorder Model 2210. The emf 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 a temperature of 25 ± 0.5°C with an Edmund Buhler 7400 Tubingen Type UKT30 water bath, with constant magnetic stirring of solutions.

Procedures

Calibration Curve of the Diflunisal ISE. Five milliliters of the phosphate buffer was pipetted into the measurement cell, the pair of electrodes was immersed in it, and after the potential was stabilized (±0.1 mV), various aliquots of the 0.0100 *M* diflunisal stock solution were added (concentration range, 1×10^{-6}–6×10^{-3} *M*). The emf values were recorded and measured after stabilization (±0.1 mV), following each addition. The potential values *E* were plotted against $-\log C$ (*pC*) to obtain the calibration curve (response curve) using a least-squares fitting program. Corrections for the changes in volume after addition were performed by this program.

Binding Experiments. Five milliliters of the HPβCD, BSA, HSA, plasma, HPβCD/BSA, HPβCD/HSA, or HPβCD/plasma solutions were pipetted into the measurement cell, and the pair of electrodes was immersed in it. After the potential was stabilized (±0.1 mV), small amounts of the corresponding mixed working solutions were added. The emf values were recorded to check stabilization and measured after each addition. All experiments were run in triplicate.

Data Analysis. The binding parameters for the interaction of diflunisal with HPβCD, BSA, HSA, and plasma were calculated using the Scatchard model (21), which describes the binding of a substrate having *m* independent classes of binding sites on a ligand. The *i*th class contains n_i equal binding sites, which are characterized by the same binding constant $K_i(M^{-1})$. The mathematical expression of the generalized Scatchard model is described by the equation

$$B = \sum_{i=1}^{m} \frac{n_i K_i F}{1 + K_i F} L_t \qquad (1)$$

where *B* and *F* are the molar concentrations of the bound and free species of the substrate (diflunisal), respectively, and L_t is the total molar ligand (HPβCD or protein) concentration. 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 the substrate. The binding parameters were estimated by a computer program (15) performing nonlinear least-squares fitting of the Scatchard model to the experimental data (electrode potential *E* and *T* values were the dependent and independent variables, respectively).

Diflunisal binding to plasma required the use of a modified version of the generalized Scatchard model, as the molar concentration of the protein is not known (17,22). In this version, *B* and *F* are expressed as micrograms per milliliters, and L_t as grams per liter:

$$B = \sum_{i=1}^{m} \frac{N_i K_i F}{1 + (K_i F/1000\ \mathrm{MW})} L_t \qquad (2)$$

where N_i is the binding capacity of the *i*th class of binding sites expressed as moles per gram, and MW is the molecular weight of diflunisal.

RESULTS AND DISCUSSION

Electrode Characteristics

The diflunisal ISE shows a near-Nernstian response in

the concentration range of 2×10^{-5}–6×10^{-3} M. The slope at 25°C was 58–61 mV/decade ($r > 0.9997$) and the detection limit was 5×10^{-6} M.

The attached electrode membrane was found to have an operative life of about 2 months, after which a new one was attached to the silicone tube-cap. The slope of the electrode remained relatively constant and the potential for the same aqueous solution varied ±2 mV during its operative life. The response time was very short (the potential was stabilized in about 1–2 sec after each addition). The electrode presented no drift in the HPβCD and protein solutions or the 1:1 diluted plasma and was therefore adequate for precise binding measurements. The electrode potential was practically stable in the pH range 7–12 (pK_a of diflunisal = 3.0).

Binding Studies

A typical Scatchard plot for the binding of diflunisal ion to HPβCD at 25°C is shown in Fig. 1. The linearity of this plot is indicative of 1:1 complexation. This is also substantiated by the ideal x intercept found, 1.002 ± 0.003, which corresponds to the value of n in Eq. (1) for one class of binding sites, i.e., the cyclodextrin cavity. The binding constant obtained from the slope of the regression line in Fig. 1 was found to be 5564 ± 36 M^{-1} (for three titration experiments, the between-run standard deviation was 4%). This value is within the range of previously reported (11) complex stability constants of the structurally similar naproxen and flurbiprofen with HPβCD.

The nonlinear character of the Scatchard plots in Fig. 2 for the binding of diflunisal to BSA, HSA, and 1:1 diluted plasma at 25°C indicates the presence of more than one class of binding sites for diflunisal on the proteins studied. In all cases, computer analysis of data revealed two classes of binding sites. The estimates of the binding parameters are listed in Table I. It is interesting to note the similarity in the values of the diflunisal binding parameters to all binders examined. In all cases a high-affinity binding constant in the range 10–12 × 10^4 M^{-1} and a low association constant ≈2–3 × 10^3 M^{-1} were observed.

Our results indicate that diflunisal is highly bound to plasma, and HSA binding accounts for the overall binding. Besides, the binding data with the diluted plasma show that 99% of diflunisal is bound to plasma proteins in the range of 70–300 μg/mL. For this concentration range, a similar extent of binding has been reported in the literature (14). The mea-

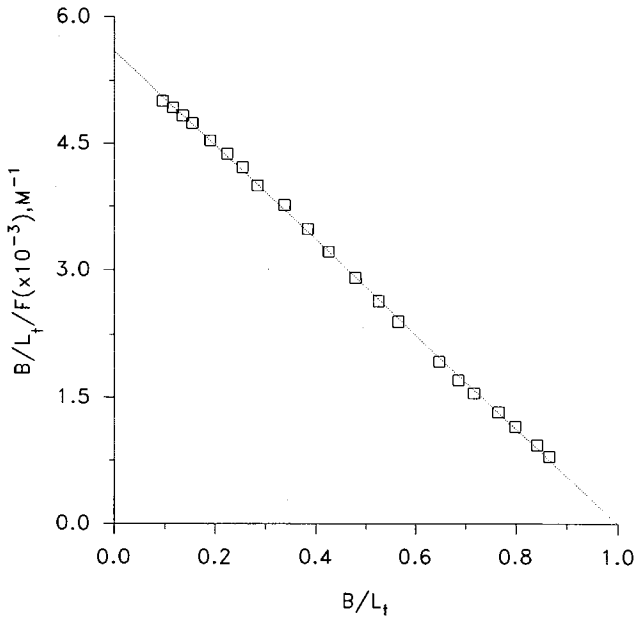

Fig. 1. Typical Scatchard plot for the diflunisal/HPβCD association at 25°C in phosphate buffer, pH 7.4. The theoretical Scatchard curve is drawn over the experimental points.

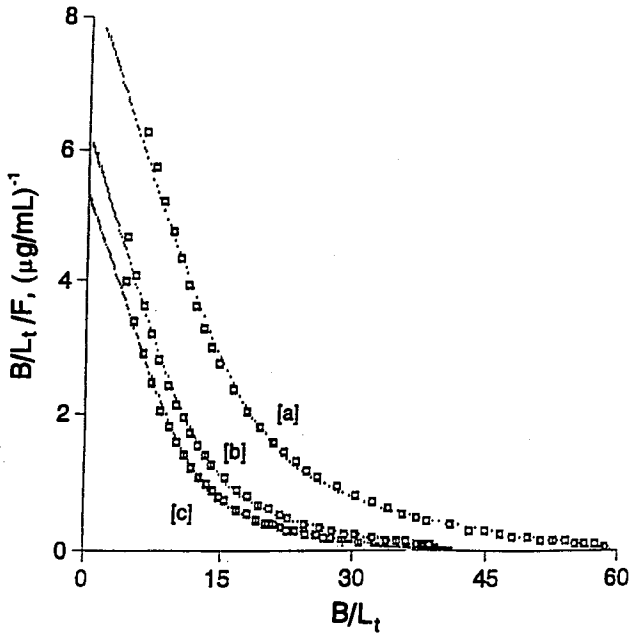

Fig. 2. Typical Scatchard plots for the binding of diflunisal to [a] BSA, [b] HSA, and [c] human plasma at 25°C. BSA and HSA concentration, 22.5 g/L, in phosphate buffer, pH 7.4. Plasma diluted 1:1 with phosphate buffer pH 7.4. The theoretical Scatchard curves are drawn over the experimental points.

Table I. Estimates[a] for the Binding Parameters of Diflunisal to BSA,[b] HSA,[b] and Plasma[c]

Binding parameter	BSA	HSA	Plasma
n_1	4.30 (0.12)	3.44 (0.08)	—
n_2	12.31 (0.15)	7.92 (0.11)	—
10^5N_1 (mol/g)	6.54 (0.19)	5.15 (0.12)	5.23 (0.11)
10^5N_2 (mol/g)	18.35 (0.22)	12.03 (0.17)	10.99 (0.22)
$10^{-4}K_1$ (M^{-1})	12.21 (0.59)	12.63 (0.58)	9.68 (0.43)
$10^{-4}K_2$ (M^{-1})	0.30 (0.01)	0.30 (0.02)	0.18 (0.01)
SD_{re}[d]	1.0	0.8	0.9

[a] Calculated at 25°C; within-run standard deviations of estimates in parentheses; between-run relative standard deviations (n = 3) range from 3 to 10% for all parameters estimated.
[b] BSA and HSA concentration was 2.25% in 0.1 M phosphate buffer, pH 7.4, and was kept constant during the experiment.
[c] Plasma was diluted 1:1 with 0.1 M phosphate buffer, pH 7.4.
[d] Standard deviation of the sum of squared residuals utilizing either Eq. (1) (BSA, HSA) or Eq. (2) (BSA, HSA, plasma).

surements under the detection limit of the ion-selective electrode in the presence of proteins were based on the expansion of the least linear concentration limit, which is caused by the proteins (23,24). Thus, the minimum free drug concentration measured in the binding experiments with BSA, HSA, and plasma was 5×10^{-7} *M*. However, the use of the diflunisal ion-selective electrode for binding experiments in pharmacokinetic studies is not recommended, since (i) its detection limit is very close to the free concentrations encountered *in vivo* and (ii) the expanded detection limit presupposes knowledge of the binding parameters of diflunisal–plasma interaction.

The binding data for the binding of diflunisal to BSA, HSA, and plasma in the presence of various HPβCD concentrations are shown in Fig. 3. In all cases the free concentration of diflunisal ion decreased as the concentration of HPβCD increased. The effect is very significant at the higher HPβCD concentrations utilized. It is noteworthy that in a previous study (6) the binding of spironolactone was decreased when the β-cyclodextrin concentration was increased in mixed β-cyclodextrin/BSA solutions. Similar results, i.e., an increase in the free fraction with an increased HPβCD concentration, were also reported (11) by the same research group for the effect of HPβCD on the binding of naproxen and flurbiprofen to plasma. These results (6,11) are not in contrast with our observations if one takes into account that ultrafiltration was employed in the binding studies. This means that the fraction of drug bound to cyclodextrins that was filterable through the Amicon membranes was accounted for as free drug (6).

To analyze further the binding behavior of the two binders in the mixed solutions of HPβCD/protein, theoretical free (*F*) versus total (*T*) diflunisal ion concentration profiles were constructed using the estimates of the binding parameters derived from the experiments with each individual binder. HPβCD and each of the studied proteins were assumed to act independently and an "additive" model was developed on the basis of Eq. (3):

$$B = B_c + B_p \tag{3}$$

i.e., the total bound concentration, *B*, is the sum of the concentrations bound to HPβCD (B_c) and the protein (B_p). Pre-

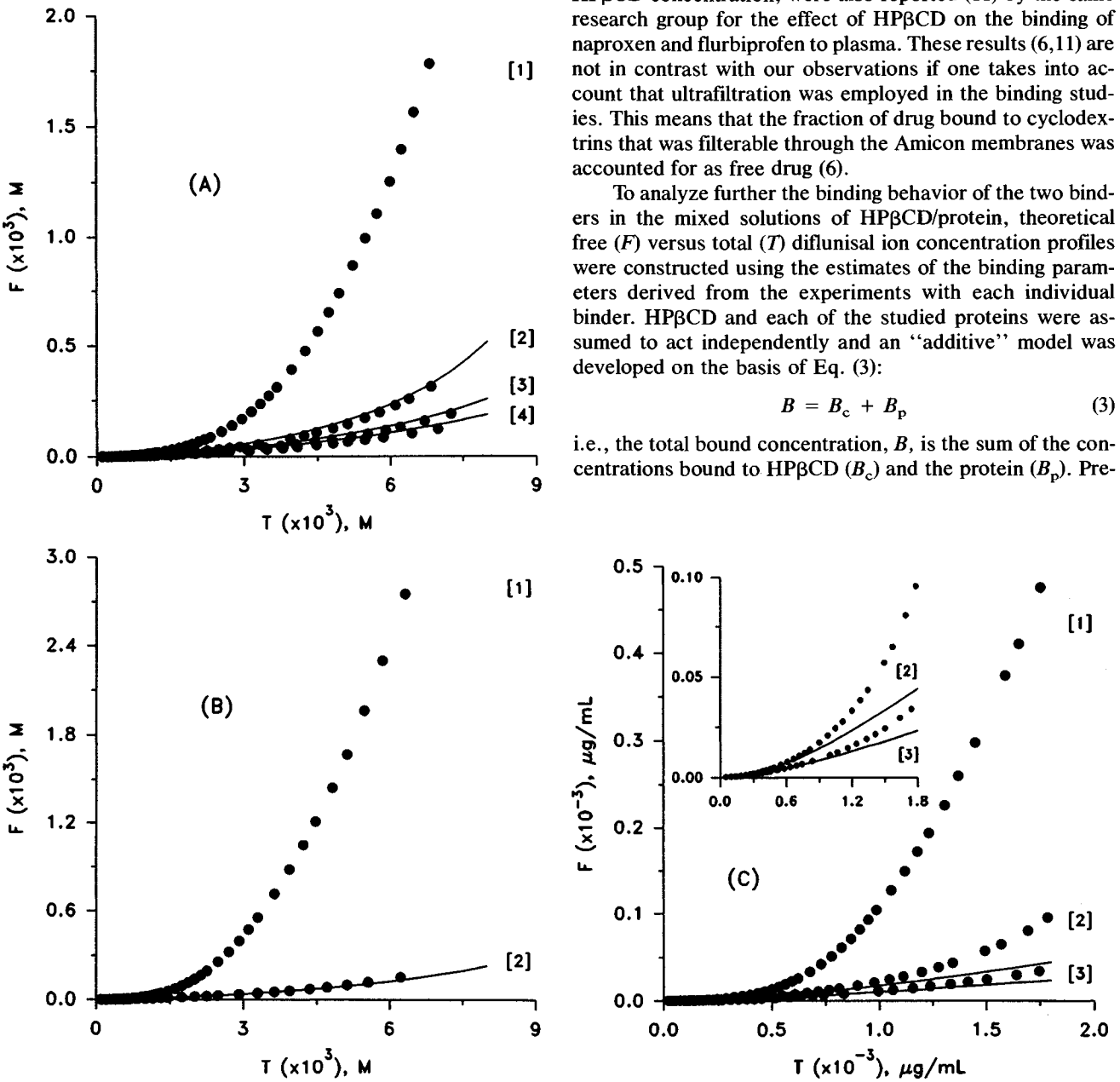

Fig. 3. Binding of diflunisal to BSA, HSA, and plasma in the absence and presence of various concentrations of HPβCD. Plots of free (*F*) vs total (*T*) drug concentration: (A) in 2.25% BSA in [1] the absence and the presence of [2] 0.005 *M*, [3] 0.008 *M*, and [4] 0.010 *M* HPβCD; (B) in 2.25% HSA in [1] the absence and [2] the presence of 0.01 *M* HPβCD; (C) in plasma diluted 1:1 with phosphate buffer, pH 7.4, in [1] the absence and the presence of [2] 0.005 *M* and [3] 0.010 *M* HPβCD. Solid lines represent the predicted free-total drug profiles for the corresponding HPβCD concentration. The inset magnifies the region of the *y*-axis origin.

dicted values for B_c and B_p in experiments with BSA or HSA were calculated from Eq. (1); theoretical predictions for B_p in experiments with plasma were based on Eq. (2). The estimates listed in Table I were assigned to the parameters n_i, N_i, and K_i in Eqs. (1) and (2). The bound drug concentration, B, was replaced by $T - F$ in Eq. (3); the solution of Eq. (3) in terms of F was accomplished iteratively, utilizing the MINSQ computer program.

The binding of diflunisal to the mixed HPβCD/BSA and HPβCD/HSA solutions in all cases studied is very nicely predicted by the theoretical model (Figs. 3A and B). Therefore, the hypothesis of the model considered, i.e., additivity in the binding behavior of the two binders, HPβCD/BSA and HPβCD/HSA, is verified. This means that there is no significant interaction between the two biomolecules in solution. Thus, any significant conformational change for HPβCD and protein affecting their binding properties should be ruled out. Similar results have recently been reported for the system HPβCD/interleukin-2 (2). Based on derivative spectroscopy data, no conformational changes of interleukin-2 in the presence of HPβCD were observed (2).

The theoretical curves for the binding of diflunisal ion to the mixed HPβCD/plasma solutions differ considerably from the experimental data (Fig. 3C). The extent of binding predicted was higher than that found experimentally. The theoretical model overestimates the extent of binding and this overestimation becomes more patent at the lower HPβCD concentration used. A plausible explanation for these results can be based on the competitive displacement of diflunisal from the HPβCD cavity by plasma constituents. Frijlink *et al.* (6,11) have provided evidence for the displacement of naproxen and flurbiprofen from HPβCD by plasma cholesterol. The same mechanism can be postulated for diflunisal, which is structurally relevant to flurbiprofen. Both compounds are substituted biphenyls, while the association constant for diflunisal/HPβCD is much lower than that for flurbiprofen/HPβCD (11). Two important experimental observations derived from Fig. 3C should also be pointed out. First, the theoretical overestimation of binding is manifested mainly at the higher total drug concentrations utilized. Obviously, at the lower drug concentrations examined, the effect of HPβCD cannot be detected due to the extensive binding (>98%) to the plasma proteins (see inset of Fig. 3C). Second, the theoretical overestimation of binding becomes negligible at the higher HPβCD concentration (Fig. 3C). It is reasonable to argue that at this high HPβCD concentration (0.01 *M*), the effect of the competitive displacement of cholesterol is minimized since the HPβCD molecules are in sufficient excess and therefore capable of binding both diflunisal and cholesterol.

Proper interpretation of the binding data obtained with the two methodologies employed, namely, ultrafiltration (6,11) and potentiometry, can be useful in understanding the effect of cyclodextrins on the drug action as well as the disposition of drugs. It is known that the pharmacological effect at the receptor sites in extravascular tissues is dependent on the unbound concentration of drug in plasma. HPβCD is able to cross the vascular endothelium, and therefore the fraction of drug bound to HPβCD can reach the interstitial spaces. In other words, HPβCD should be viewed as an efficient carrier of the drug to membranes, and therefore ultrafiltration studies are appropriate to provide binding estimates when disposition to interstitial fluid is considered. Potentiometry, unlike ultrafiltration, where complexed drug is measured as free drug, measures unbound and uncomplexed drug. Accordingly, potentiometry is the method of choice for providing binding estimates when disposition to the intracellular fluid and/or pharmacological effect is examined, since the cyclodextrin molecules are not able to permeate the cell membranes. The overall binding, i.e., to cyclodextrin and protein, will facilitate interpretation of the effect of cyclodextrins on the disposition of drugs. It should be noted that discrepancies between the bound fraction of flurbiprofen estimated with ultrafiltration and tissue concentrations were observed (11).

In summary, the technique of ion-selective electrodes provides a powerful tool for studying the binding of drugs to proteins in the presence of cyclodextrins. In addition, the use of potentiometry can facilitate evaluation of the beneficial effect of HPβCD as a solubilizing and stabilizing excipient for protein drugs (2,13). Finally, protein instability in aqueous solutions in the presence or absence of cyclodextrin(s) can be assessed with direct potentiometry since the binding profile of a drug or a probe is a true reflection of the three-dimensional protein structure.

REFERENCES

1. J. Pitha, J. Milecki, H. Fales, L. Pannell, and K. Uekama. Hydroxypropyl-β-cyclodextrin: Preparation and characterization; Effects on solubility of drugs. *Int. J. Pharm.* 29:73–82 (1986).
2. M. E. Brewster, M. S. Hora, J. W. Simpkins, and N. Bodor. Use of 2-hydroxypropyl-β-cyclodextrin as a solubilizing and stabilizing excipient for protein drugs. *Pharm. Res.* 8:792–796 (1991).
3. D. Duchene (ed.), *New Trends in Cyclodextrins and Derivatives,* Editions de Sante, Paris, 1991.
4. K. S. Estes, M. E. Brewster, A. I. Webb, and N. Bodor. A non-surfactant formulation for alfaloxone based on amorphous cyclodextrin: Activity studies in rats and dogs. *Int. J. Pharm.* 65:101–107 (1990).
5. M. E. Brewster, J. W. Simpkins, M. S. Hora, W. C. Stern, and N. Bodor. The potential use of cyclodextrins in parenteral applications. *J. Parent. Sci. Technol.* 43:231–240 (1991).
6. H. W. Frijlink, A. C. Eissens, N. R. Hefting, K. Poelstra, C. F. Lerk, and D. K. F. Meijer. The effect of parenterally administered cyclodextrins on the cholesterol levels in the rat. *Pharm. Res.* 8:9–16 (1991).
7. T. Irie, K. Fukunaga, and J. Pitha. Hydroxypropyl-cyclodextrins in parenteral use. I. Lipid dissolution and effects on lipid transfers in vitro. *J. Pharm. Sci.* 81:521–523 (1992).
8. T. Irie, K. Fukunaga, M. K. Garwood, T. O. Carpenter, J. Pitha, and J. Pitha. Hydroxypropylcyclodextrins in parenteral use. II. Effects on transport and disposition of lipids in rabbit and humans. *J. Pharm. Sci.* 81:524–528 (1992).
9. T. Nagai, O. Shirakura, and N. Nambu. Hypnotic potency, and the plasma and brain concentration of hexobarbital in the presence of cyclodextrins. In *3rd Int. Conf. Pharm. Technol., Paris, 31 May/2 June 1983, Vol. V,* pp. 253–262.
10. O. Shirakura, N. Nambu, and T. Nagai. Effect of β-cyclodextrin on disposition of hexobarbital and phenobarbital in mice. *J. Incl. Phenom.* 2:613–621 (1984).
11. H. W. Frijlink, E. J. F. Franssen, A. C. Eissens, R. Oosting, C. F. Lerk, and D. K. F. Meijer. The effects of cyclodextrins on the disposition of intravenously injected drugs in the rat. *Pharm. Res.* 8:380–384 (1991).
12. A. Sharma, K. Anderson, and J. W. Baker. Flocculation of serum lipoproteins with cyclodextrins: Application to assay of hyperlipidemic serum. *Clin. Chem.* 36:529–532 (1990).

13. M. E. Ressing, W. Jiskoot, H. Talsma, C. W. van Ingen, E. C. Beuvery, and D. J. A. Crommelin. The influence of sucrose, dextran, and hydroxypropyl-β-cyclodextrin as lyoprotectants for a freeze-dried mouse IgG_{2a} monoclonal antibody (MN12). *Pharm. Res.* 9:266–270 (1992).
14. R. K. Verbeeck, A. Boel, A. Buntinx, and P. J. de Schepper. Plasma protein binding and interaction studies with diflunisal, a new salicylate analgesic. *Biochem. Pharmacol.* 29:571–576 (1980).
15. T. Christopoulos and E. Diamandis. Binding studies using ion-selective electrodes. Examination of the picrate-albumin interaction as a model system. *Anal. Chem.* 62:360–367 (1990).
16. G. N. Valsami, P. E. Macheras, and M. A. Koupparis. Binding studies of ions to cyclodextrins using ion-selective electrodes. *J. Pharm. Sci.* 79:1087–1094 (1990).
17. G. N. Valsami, P. E. Macheras, and M. A. Koupparis. Binding study of the fluorescence probe 1-anilino-8-naphthalene-sulfonate to human plasma and human and bovine serum albumin using potentiometric titration. *Pharm. Res.* 8:888–892 (1991).
18. G. N. Valsami, M. A. Koupparis, and P. E. Macheras. Complexation studies of cyclodextrins with tricyclic antidepressants using ion-selective electrodes. *Pharm. Res.* 9:94–100 (1992).
19. E. E. Sideris, G. N. Valsami, M. A. Koupparis, and P. E. Macheras. Determination of association constants in cyclodextrin/drug complexation using the Scatchard plot: Application to β-cyclodextrin-anilinonaphthalenesulfonates. *Pharm. Res.* 9:1568–1574 (1992).
20. A. Craggs, G. J. Moody, and J. D. Thomas. PVC matrix membrane ion-selective electrodes. *J. Chem. Educ.* 51:541–544 (1974).
21. G. Scatchard. The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* 51:660–672 (1949).
22. W. Loscher. A comparative study of the protein binding of anticonvulsant drugs in serum of dog and man. *J. Pharmacol. Exp. Ther.* 208:429–435 (1979).
23. M. Otto, P. May, K. Murray, and J. D. R. Thomas. Calibration of ionized calcium and magnesium with ligand mixtures for intracellular ion-selective electrode measurements. *Anal. Chem.* 57:1511–1517 (1985).
24. A. Angelakou, G. Valsami, M. Koupparis, and P. Macheras. Use of 1-anilino-8-naphthalene-sulfonate as an ion probe for the potentiometric study of the binding of sulphonamides to bovine serum albumin and plasma. *J. Pharm. Pharmacol.* 45:434–438 (1993).