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The binding of benoxaprofen to human serum albumin. Classification of the primary binding site

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Summary

Benoxaprofen, a new non-steroidal anti-inflammatory drug, is shown by dialysis and microcalorimetry to have a very high affinity for human serum albumin. At low drug-to-protein ratios it does not share the same binding site as warfarin on albumin but does share a site with flufenamic acid and clofibrate. Data are presented which show that spectroscopic evidence can be unreliable in assigning binding sites and predicting drug interactions.

Introduction

It is important to not only determine the affinity of drugs for albumin but also to classify their binding sites so that drug interaction can be predicted. It has been documented that drugs bind to one of two discrete binding sites on human serum albumin (HSA). Site 1 is the warfarin site and is shared by the coumarins, carbon acids like phenylbutazone, sulfonylureas, and sulfonamides. Site 2 is the diazepam site and is shared with clofibrate, flufenamic acid and a range of acidic non-steroidal anti-inflammatories (Sudlow et al., 1975; Sjoholm et al., 1979). There is under certain conditions certain overlap and probably interaction between the sites.

In this paper investigations into the interaction of a new non-steroidal anti-inflammatory drug, benoxaprofen, 2-(4-chlorophenyl)-α-methyl-5-benzoxazolacetic acid, with human serum albumin (HSA) is investigated by dialysis, circular dichroism and microcalorimetry. Unfortunately benoxaprofen has been found to be unstable in the spectrofluorimeter. The intense light source causes photodegradation to occur, whereas in aqueous solutions a white precipitate forms instantaneously. This precludes the use of fluorescence spectroscopy in studying the binding properties of benoxaprofen.

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Materials and methods

Human serum albumin, fraction V (lot no. 30F-02271), was obtained from Sigma Chemicals (St. Louis, MO). Monobasic and dibasic sodium phosphate and sodium chloride, analytical reagent grade, were obtained from Mallinckrodt (St. Louis, MO). Dowex 50W-X8 cation exchange resins and 1-X8 anion exchange resins were supplied by J.T. Baker Chemicals (Phillipsburg, NJ). Deionized water was used in all experiments.

Prior to all experiments, solutions of HSA were deionized by running a solution through a column containing cationic and anionic exchange resins. The resins were prepared by adjusting the pH of the cationic and anionic forms to 1 and 12, respectively. The pH was then adjusted to 4.5 for the cationic form and 7.5 for the anionic form by rinsing with deionized water. Anionic and cationic resins were then added to a column and again rinsed with water. An albumin solution was run through the column and the final pH adjusted to 7.4 in 0.1 M phosphate buffer. This deionization procedure removed inorganic impurities and reduced the fatty acid content to a constant value of about 0.2 mol/mol of albumin (Wilting et al., 1980).

The concentration of HSA is determined gravimetrically and spectroscopically (mol. wt. = 66,500). The following drugs were used as supplied by the manufacturers: sodium benoxaprofen (903.5 mg free acid/g), Eli Lilly (Indianapolis, IN); fenbufen, Lederle Laboratories (Pearl River, NY); flufenamic acid, Aldrich Chemicals (Milwaukee, WI); sodium clofibrate, Imperial Chemical Industries (Alderley Park, England); oxyphenylbutazone and phenylbutazone, Ciba Pharmaceuticals (Summitt, NJ); ibuprofen, Upjohn (Kalamazoo, MI); sodium warfarin, Endo Labs (Garden City, NY) and phenprocoumon, Organon (W. Orange, NJ).

All experiments were performed in 0.1 M phosphate buffer at pH 7.4. Equilibrium dialysis was performed at 25°C for 12 h using the Dianorm system (Diachema A.G. Ruschlikon, Switzerland). Preliminary experiments showed there to be some adsorption of benoxaprofen onto the hydrated cellulose membranes. The free and bound concentrations of benoxaprofen were determined by the HPLC technique previously described (Fleitman et al., 1980). For the generation of a Scatchard plot 2.0×10^{-4} M HSA was used and total drug concentrations of $0.20–8.0 \times 10^{-4}$ M HSA were used. In the competitive displacement experiments only the free concentrations of benoxaprofen or warfarin were determined by HPLC.

Microcalorimetry

Heat fluxes for the interaction between HSA and benoxaprofen were measured at 25°C in a flow microcalorimeter (Model 2107-121 L.K.B. Bromma, Sweden). For the titration, 2.56×10^{-4} M HSA was mixed with drug concentrations of $0 - 5.12 \times 10^{-4}$ M. For the Job's plot (Job, 1928) a final total concentration of 2.5×10^{-4} M was used. For the experiments involving competition for the binding sites, final concentrations of 2.5×10^{-4} M HSA and drugs were used.

Circular dichroism

Circular dichroic (C.D.) measurements were made using a Jasco model J-500

spectropolarimeter (Jasco International, Tokyo, Japan). The extrinsic benoxaprofen HSA signal was measured at a slit width of 1 nm, wavelength expansion of 5 $nm \cdot cm^{-1}$, chart speed 1 $cm \cdot min^{-1}$, time constant 32 s and a sensitivity of 0.5 or 1.0 $m^0 \cdot cm^{-1}$. Concentrations of benoxaprofen and HSA were as stated in the figures. Solutions were scanned from 290–320 nm in 10 mm cells. Under these experimental conditions, HSA and its complexes with the competing drug made no contribution to the C.D. spectrum. The observed spectra are entirely due to benoxaprofen–HSA complexes. The dynode voltage was kept below 0.5 in all experiments.

Results and discussion

The deionization step in the preparation of HSA solutions removes inorganic impurities and allows reproducible heats of reaction to be obtained (Hardee et al., 1979). Microcalorimetry was used in early experiments to obtain a value for the binding constant at the highest affinity site on HSA and also to ascertain whether or not more than one binding site contributed to the heat evolved following interaction with benoxaprofen. Several sites contributed to this heat as shown by the Job's plot in Fig. 1. Had only one site contributed to the heat flux then the limiting slopes would be equal in magnitude but opposite in sign and meet at 0.5 mole fraction, clearly this is not so. In spite of this, some insight into the primary binding constant can be obtained from calorimetery. In Fig. 2 the heat flux, in terms of μV as a

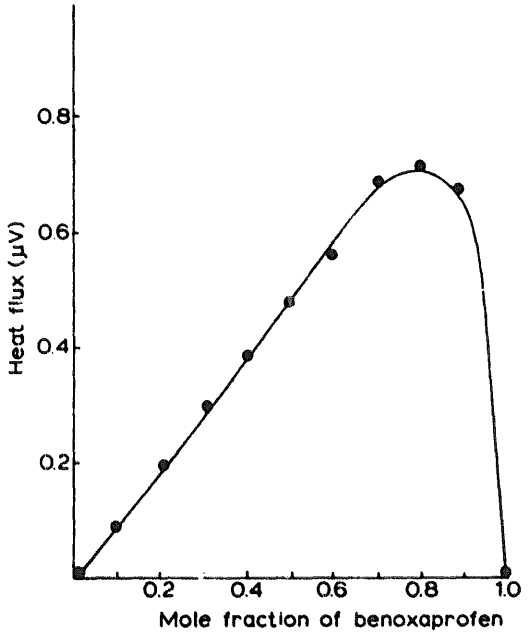

Fig. 1. Job's plot for the benoxaprofen–HSA interaction. Microcalorimetric data at 25°C at pH 7.4 in a 0.1 M phosphate buffer.

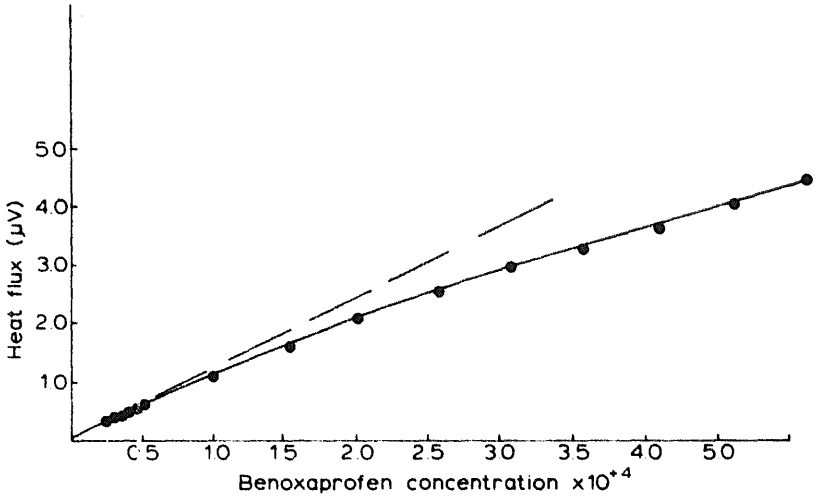

Fig. 2. Heat flux as a function of benoxaprofen concentration in 0.1 M phosphate at pH 7.4 at 25°C [HSA] = 2.52×10^{-4} M. A primary binding constant of 5.05×10^{6} M^{-1} can be estimated from the data.

function of benoxaprofen concentrations at a fixed concentration of HSA is shown. A plateau value for the heat flux was never reached because of the number of binding sites contributing to the heat and the limited solubility of the benoxaprofen. A value can be estimated for the first binding constant if data at drug-to-protein ratios of less than 0.5 are used in the estimation as outlined below.

If it is assumed that at drug-to-protein ratios below 0.5 all of the benoxaprofen is bound to the first site, the slope of a line drawn through points and the origin is represented by the dashed line in Fig. 2 and will give the value of the maximum heat flux produced by the formation of one mole of bound complex (μV_{max}). The heat is equivalent to the plateau value if one could be obtained for a 1 : 1 complex and its units are $\mu V \cdot mol^{-1} \cdot$ liter. A linear regression program is used to find the value of μV_{max} and K_1 is calculated from Eqn. 1 where a and b are the initial concentrations of reactants.

$$K_1 = \frac{C}{(a - c)(b - c)} \tag{1}$$

and C is given by

$$C = \frac{\mu V}{\mu V_{max}} \tag{2}$$

representing the concentration of the 1 : 1 complex formed.

Using this method a value of 5.05×10^{6} l/mol for K_1 is obtained. This allows the heat of the reaction in J/mol to be calculated from the maximum heat flux.

$$\Delta H \text{ (J/mol)} = \mu V_{max} \times \frac{1}{0.057} \times \frac{1}{\text{total flow rate}} \tag{3}$$

where 0.057 is the calibration constant for this microcalorimeter in $\mu V/\mu W$. A value of $-21{,}000$ J/mol is obtained for the heat of formation of one mole of complex at 25°C. From Eqns. 4 and 5, ΔG and ΔS are found to be -38.242 J/mol and 57.9 J/mol °K, respectively.

$$\Delta G = -RT \ln K \tag{4}$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \tag{5}$$

A more reliable determination of the binding parameters can be obtained from equilibrium dialysis data obtained at very low drug-to-protein ratios, which necessitates the use of very sensitive analytical techniques.

Fig. 3 illustrates a Scatchard plot (Scatchard, 1949) for the benoxaprofen-HSA interaction. Each data point represents the average of two determinations from an equilibrium dialysis experiment. Both the bound and free sides were analyzed by high performance liquid chromatography since preliminary studies have shown that 5–6% of the drug is bound to the membrane. Computer fitting the data to a 3-site

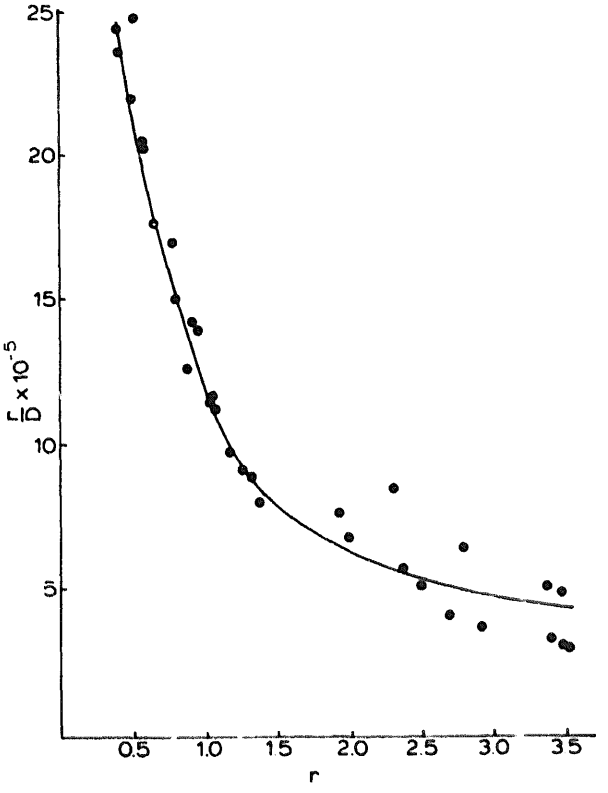

Fig. 3. Scatchard plot using data from equilibrium dialysis at 25°C in 0.1 M phosphate buffer. Binding parameters of $K_1 = 6.02 \times 10^6$ M^{-1} $n_1 = 1.0$; $K_2 = 6.93 \times 10^4$ $n_2 = 2.0$; $K_3 = 1.08 \times 10^4$ $n_3 = 3.0$ can be computed from the data.

model utilized Eqn. 6.

$$r = \frac{n_1 K_1[d]}{1 + K_1[D]} + \frac{n_2 K_2[D]}{1 + K_2[D]} + \frac{n_3 K_3[D]}{1 + K_3[D]} \quad (6)$$

Where r is the number of moles of benoxaprofen bound per mole of albumin, [D] is the free benoxaprofen concentration and K_i and n_i are the binding constants and number of sites of the i^{th} class. The binding constant obtained for the first site ($K_1 = 6.02 \times 10^6$ 1/mol) agrees well with the calorimetry data. A good graphical estimate of n_2 and n_3 is not obtained since solubility problems with benoxaprofen preclude getting higher values of r. Using lower HSA concentrations could solve this problem, but data near physiological concentrations of HSA were preferred and were necessary to compare with the calorimetric data.

This primary binding constant is high, but a value of 2.73×10^{-6} 1/mol has been reported for ibuprofen a drug which is also a propionic acid derivative.

To determine whether or not benoxaprofen competes with warfarin for its primary binding site a series of dialysis experiments were performed. In these laboratories the coumarins have been found to be more reliable markers for determining whether a drug is a site 1 or a site 2 drug and so warfarin was chosen as the marker in the data of Table 1. The drugs in Table 1 are ranked in order of the ability to displace warfarin by a significance test on the difference between two means ($\Delta\bar{X}$). Mean values of free warfarin concentration are compared with and without competitor and the 90% confidence limit of the difference between these two means is calculated. If $\Delta\bar{X} >$ C.L., the difference between the means is due to drug displacement and not random scatter. If $\Delta\bar{X} \leq$ C.L., the samples are not significantly

TABLE 1

EFFECT OF COMPETITORS ON THE FREE WARFARIN CONCENTRATION IN 0.1 M PHOSPHATE BUFFER AT pH 7.4 AND 25°C. [HSA] = 2.5×10^{-4} M TOTAL WARFARIN = TOTAL COMPETITOR = 1.25×10^{-4} M

Competitor	Free warfarin		
	($\bar{X} \pm$ S.D.; μg/ml)	C.L. ($\alpha = 0.05$) *	$\Delta\bar{X}$ - C.L. **
Buffer	1.00 ± 0.03		
Phenylbutazone	1.77 ± 0.01	0.036	0.734
Oxyphenylbutazone	1.66 ± 0.21	0.069	0.491
Flufenamic acid	1.27 ± 0.05	0.050	0.220
Ethacrynic acid	1.26 ± 0.08	0.069	0.191
Clofibrate	1.18 ± 0.03	0.028	0.152
Fenbufen	1.13 ± 0.04	0.039	0.091
Benoxaprofen	1.04 ± 0.02	0.025	0.015
Ibuprofen	1.01 ± 0.08 (n = 5)	0.068	−0.058

* 90% confidence limit for the difference between two means.

** Largest value = greatest displacement.

different or there is an enhancement on binding in the presence of a competitor. The magnitude of $\Delta\bar{X}$ − C.L. is used to rank the samples in terms of warfarin displacement

Phenylbutazone and oxyphenylbutazone share the same site as warfarin whereas ibuprofen, benoxaprofen, fenbufen and clofibrate do not. This is consistent with the literature. Flufenamic acid and ethacrynic acid slightly displace warfarin, but not to the extent expected from values of their affinity constants. The increased concentration of free warfarin may be due to the effect of the competitors on the N → B conformational change in HSA rather than by direct displacement. Ibuprofen gives a negative value for $\Delta\bar{X}$ − C.L. and this may signify an enhancement of warfarin binding at this low drug-to-protein ratio. This observation is supported by spectroscopic evidence presented by other authors (Sudlow et al., 1976; Otagiri et al., 1979, 1980). Ibuprofen probably pushes HSA into the B-conformation, which has a greater affinity for warfarin (Wilting et al., 1980).

Table 2 contains more dialysis data in an attempt to confirm that benoxaprofen is a site 2 drug. The data for flufenamic acid, clofibrate, and phenylbutazone conform to the literature and support the hypothesis that benoxaprofen is a site 2 drug. If direct competition at a binding site was the only factor involved, then ibuprofen and fenbufen should also displace benoxaprofen (unpublished observations from dialysis data suggest that fenbufen and ibuprofen push HSA to the B form, a form in which drugs usually have an increased affinity for HSA). The net result of the experimental conditions of Table 2 on the N → B conformational change is not always a decrease in affinity as would be expected from conditions of direct displacement. Oxyphenylbutazone may cause displacement by affecting the N → B equilibrium. Oxyphenylbutazone and phenylbutazone are the only drugs so far reported to favor the N-form of HSA (Elbory et al., 1981). In the presence of drugs which have large effects on the N → B change, clear classification of drugs into sites 1 or site 2 types may not be possible. Microcalorimetric data and circular dichroic spectra support

TABLE 2

EFFECT OF COMPETITIORS ON THE FREE BENOXAPROFEN CONCENTRATION IN 0.1 M PHOSPHATE AT pH 7.4 AND 25°C. [HSA] = 2.5×10^{-4} M

Competitor	Free benoxaprofen		
	($\bar{X} \pm$ S.D.; μg/ml)	C.L. ($\alpha = 0.05$) *	$\Delta\bar{X}$ − C.L. **
Buffer	0.207 ± 0.006	-	-
Flufenamic acid	0.243 ± 0.004	0.00819	0.02781
Clofibrate	0.240 ± 0.003	0.00790	0.02510
Oxyphenylbutazone	0.230 ± 0.010	0.01453	0.00847
Ibuprofen	0.213 ± 0.003	0.00795	−0.00195
Phenylbutazone	0.214 ± 0.006	0.01028	−0.00328
Fenbufen	0.213 ± 0.016 (n = 5)	0.01309	−0.00709

* 90% confidence limit for the difference between two means.** Largest value = greatest displacement.

the above hypotheses. The heat flux of binding for the mixture of a drug and competitor (ΔH_{AB}) is compared to the sum of the values obtained with each drug acting alone with HSA ($\Delta H_A + \Delta H_B$). If ΔH_{AB} is equal to $\Delta H_A + \Delta H_B$ no interaction between drugs A and B has occurred. If the value of ΔH_{AB} is less than the sum of $\Delta H_A + \Delta H_B$ then a mutual displacement is probable. If ΔH_{AB} is greater than the sum, an interaction causing enhancement of the binding has taken place. These interpretations can be complicated by major conformational changes occurring following a drug–HSA interaction.

Table 3 lists the results for different displacement experiments at pH 7.4. As expected, the heat flux generated for a mixture of two site 1 drugs, warfarin and phenprocoumon, gave a heat which was less then the additive heat when each was measured separately. This indication of a displacement is in agreement with the dialysis data in Table 1. Within experimental error, there does not appear to be competition between benoxaprofen and warfarin or phenprocoumon. There is a displacement of benoxaprofen in the presence of flufenamic acid and clofibrate and this again is confirmed by the dialysis data in Table 1. In the presence of ibuprofen and fenbufen ΔH_{AB} was greater than ΔH_A and ΔH_B indicating some type of cooperative phenomenon or conformational change resulting in the enhanced binding of benoxaprofen may be taking place.

All drugs listed in Table 3 reacted exothermally with HSA. Phenylbutazone was to be included in this table but it was found that at drug-to-protein ratio less than

TABLE 3

MICROCALORIMETRIC DATA FOR BINDING SITE SPECIFICITY AND POSSIBLE COMPETITION. IN 0.1 M PHOSPHATE AT pH 7.4 AND 25°C. [HSA]=DRUG CONCENTRATIONS=2.5 $\times 10^{-4}$ M

Drug	Steady-state heat flux ($\mu J : s^{-1}$)	Expected value ($\Delta H_A + \Delta H_B$)	Interaction	Site
Benoxaprofen	16.31			?
Fenbufen	17.37			?
Ibuprofen	2.63			II
Flufenamic acid	23.15			II
Clofibrate	1.05			II
Warfarin	15.26			I
Phenprocoumon	6.32			I
Benoxaprofen and fenbufen	38.94	33.68	+	
Benoxaprofen and ibuprofen	21.58	18.94	+	
Benoxaprofen and flufenamic acid	38.57	39.46	−	
Benoxaprofen and clofibrate	16.31	17.36	−	
Benoxaprofen and warfarin	31.11	31.57	0	
Benoxaprofen and phenprocoumon	22.63	22.63	0	
Warfarin and phenprocoumon	16.84	21.58	−	

(+) = greater than additive heat.
(−) = less than additive heat.
(0) = additive heat is the same as observed heat.

1.0 an endothermic reaction occurred. The value obtained for ΔH at this ratio was approximately +4.0 kcal/mol which is close to the literature value (Maes et al., 1979). It was not clear whether adding heats of different signs would be applicable to the model described.

It would not be possible to assign a site to benoxaprofen from this microcalorimetric data. Some of the problems associated with the use of spectroscopic techniques for the assignment of binding sites are exemplified by the circular dichroic data presented below. As can be seen from Fig. 4 a small concentration of phenylbutazone increases the size of the C.D. signal of the benoxaprofen–HSA complexes but this cannot be due to the increased affinity of benoxaprofen for HSA in the presence of phenylbutazone because of the dialysis data. Phenylbutazone possibly increases the N-form of HSA and benoxaprofen–HSA complexes have a larger C.D. signal when HSA is in the N-form. The reverse is true for the situation when ibuprofen and fenbufen are used as competitors as is shown in Figs. 5 and 6. In both cases the spectra is greatly reduced in the presence of the competitors suggesting a strong displacement; however, only minimal displacement is seen from the dialysis data. The reason for this is apparently that although the 3 drugs share the same high affinity site (site 2) both fenbufen and ibuprofen increase the B-form of HSA at pH 7.4 and this increases the affinity of the primary site for benoxaprofen (Fleitman and Perrin, 1981) and probably also increase the affinity of the secondary site for the drug. When HSA is in the B-form, benxoprofen–HSA complexes have a decreased ellipticity which is consistent with the hypothesis that the decrease in ellipticity in Figs. 5 and 6 is due to the effect on the N → B equilibrium rather than a displacement mechanism.

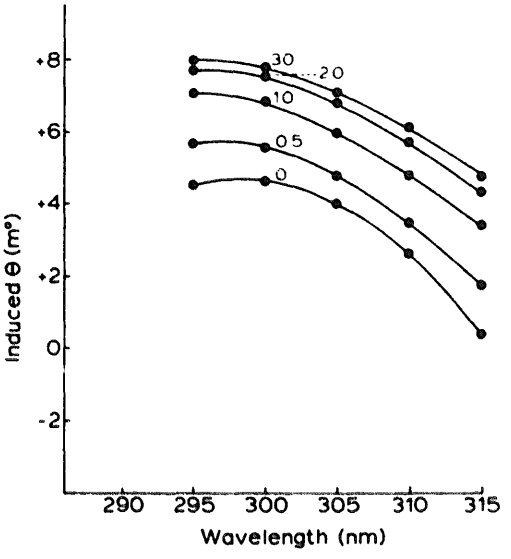

Fig. 4. Effect of phenylbutazone on the induced C.D. curves for the benoxaprofen–HSA interaction. [HSA] = benoxaprofen = 2.0×10^{-5} M. Ratios of phenylbutazone to benoxaprofen are stated on the curves.

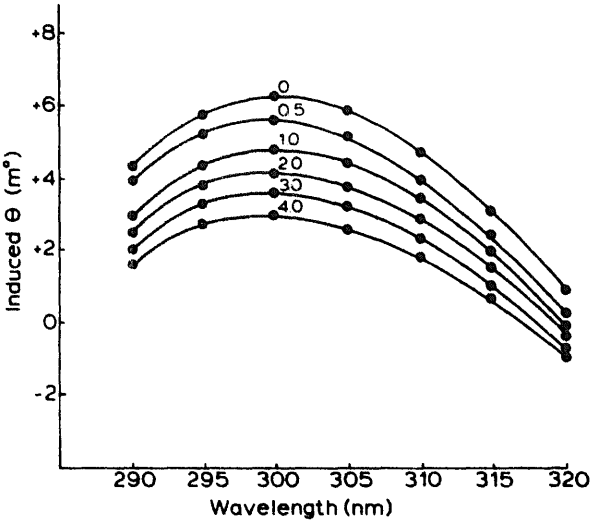

Fig. 5. Effect of ibuprofen on the induced C.D. curves for the benoxaprofen–HSA interaction. [HA]= benoxaprofen = 2.0×10^{-5} M. Ratios of ibuprofen to benoxaprofen are stated on the curves.

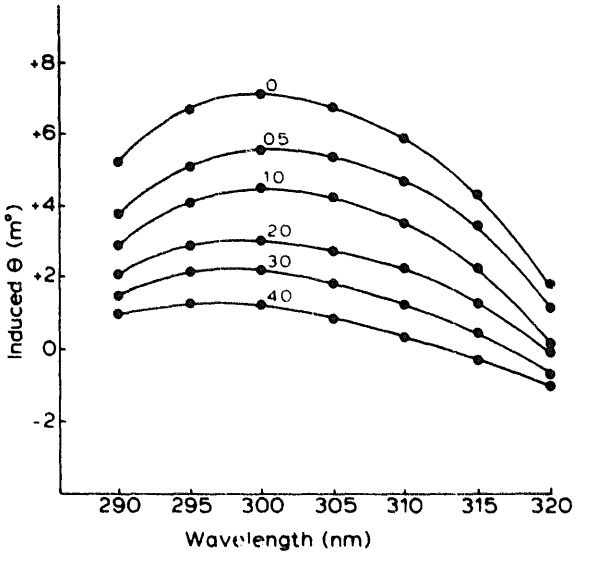

Fig. 6. Effect of fenbufen on the induced C.D. curves for the benoxaprofen–HSA interaction. [HSA]= benoxaprofen = 2.0×10^{-5} M. Ratios of fenbufen to benoxaprofen stated on the curves.

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