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Use of an immobilised human serum albumin HPLC column as a probe of drug–protein interactions: the reversible binding of valproate

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Abstract

The reversible binding of valproate to human serum albumin determines a decrease of the binding of ligands that selectively bind to site I, site II, and bilirubin binding site. The binding inhibition was followed by displacement chromatography methodology using increasing concentrations of the competitor, i.e. valproate, in the mobile phase. Significant binding inhibition was observed for drugs binding at site I and site II. The greater displacement was observed for the more retained enantiomer of benzodiazepines and profens. A reduction of the affinity was observed also in the case of phenol red, this compound being selected as representative of bilirubin binding site. Difference circular dichroism spectroscopy was also used to characterise the binding of valproate to human serum albumin. This antiepilectic drug was proved to affect the binding at site I, II, and bilirubin binding site. The data have physiological relevance because significant inhibition of the binding resulted at clinic concentrations of valproate.

Keywords: Enantiomer separation; Drug–protein binding; Human serum albumin; Valproate

1. Introduction

The fundamental role of human serum albumin [HSA] is as carrier for a variety of endogenous and exogenous compounds in serum [1]. Most drugs undergo some degree of reversible binding to HSA, a process that may often have significant effects on the overall activity profile of the compounds. In particular, the binding of drugs to the carrier controls the free concentrations and then their pharmacological activity and toxicity [1,2]. The free fraction concentration of a drug can change significantly because of its interaction with other drugs, which are co-administered. Furthermore the binding properties of HSA can change in pathological conditions either because of its concentration or because of the concentration of endogenous compounds that selectively bind to the carrier. Thus it is essential to determine the actual concentration of drugs and of their metabolites in plasma, but it is also essential to determine the mechanism(s) of their binding to the plasma proteins.

Many methodologies have been successfully employed to this purpose, but affinity chromatography resulted particularly suited to determining the binding parameters of the drugs. This is true, as in the case of immobilised HSA, if the chromatographic retention and enantioselectivity are related to the binding properties of the non-immobilised protein [3]. Drug–drug interactions at the protein binding level can be also observed and quantified, since the

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effect of one drug on the affinity of the other is reflected in the chromatographic retention of the test solute [4–6]. The simultaneous binding of drugs or endogenous ligands to HSA determines various potential interactions. Following the notation by Honorè [7] the following situations can occur: (a) an independent binding, i.e. the ligands bind independently and their affinities are not influenced; (b) a co-operative binding, i.e. the binding of one ligand increases the affinity of the other; (c) an anti-co-operative binding, i.e. the binding of one ligand decreases the affinity of the other; and (d) a competitive binding, i.e. the two ligands bind to the same binding site, this competition obviously determining a decrease of the affinity. In this last case usually a complete displacement of one of the ligands occurs, by using high concentration of the other. The drug under investigation can be used either as the analyte or as the displacer, i.e. added to the mobile phase [4–6].

In the present study the effect of valproate, an antiepilectic drug, on the chromatographic parameters of a series of drugs is reported with the aim to characterise the binding sites of valproate and then to underline interactions with potentially co-administered drugs, which can be of physiological relevance.

The therapeutic dose of valproate is relatively high (up to 60 mg/kg as daily dose). Valproate is reported to inhibit the binding of fatty acids [8], since valproate treated epileptics showed to have significantly higher levels of fatty acids in the plasma. This effect is likely to cause obesity. Valproate was also reported to displace site I [9–11] and site II [10–12] binding drugs, according to the notation by Sudlow et al. [13], and bilirubin [14]. The displacement of bilirubin can occur at clinical concentrations of valproate, this process being of physiological relevance because of the toxicity of bilirubin.

In addition to displacement chromatography experiments, difference circular dichroism (CD) spectroscopy proved that valproate affects the affinity binding at sites I, II and bilirubin binding site. Ligands that selectively bind to the three specific binding areas were selected, and the induced CD spectra of the single ligands were measured in the absence and in the presence of increasing concentrations of valproate.

2. Experimental

2.1. Chemicals and reagents

Phenylbutazone, valproic acid sodium salt, *rac*-ketoprofen, *rac*-fenoprofen, *rac*-suprofen, phenol red, *rac*-warfarin, and *rac*-naproxen were obtained by Sigma Aldrich (Milan, Italy). Bilirubin was supplied from Fluka Chemika (Buchs, Switzerland). Human serum albumin (HSA) (essentially fatty acids free) was supplied by Sigma (Milan, Italy) and it was used without further purification. Diazepam, *rac*-oxazepam and *rac*-temazepam were kindly provided by Professor A. Lucacchini, Dipartimento di Psichiatria, Neurobiologia, Farmacologia e Biotecnologie, Facoltà di Farmacia, Università di Pisa, Italy. The hemisuccinic ester of *rac*-oxazepam, *rac*-oxazepam hemisuccinate, was prepared by acylation with succinic anhydride in the presence of pyridine, following a reported procedure [15]. All reagents were analytical grade and were used without further purification. The buffer solutions were filtered through a 0.45 μm membrane filter and degassed before their use for high-performance liquid chromatography (HPLC). The solutions of the protein and of the ligands were prepared immediately before their use.

2.2. Instrumentation and chromatographic conditions

The chromatographic system consisted of a Jasco PU-980 solvent delivery system, and a Jasco MD-910 Multiwavelength Detector connected to a computer station. A Rheodyne model 7125 injector with a 20 μl loop was used. The column was thermostated at 25°C with a Column Chiller Model 7955 (Jones Chromatography Ltd., UK).

The HSA column was from Shandon (Pittsburgh, PA; 15×0.4 cm I.D.). The mobile phase was phosphate buffer 100 m*M* (pH 7.4)/1-propanol, (95/5 or 90/10) (v/v), 1 ml min^{-1}. The chromatographic elution of the solutes [injected solutions $c=0.02$–0.1 mg ml^{-1} in phosphate buffer 100 m*M* (pH 7.4)/1-propanol, (95/5) (v/v)] was followed at 220, 260 and 300 nm.

The chromatographic retention of the solutes was

expressed as capacity factors (k) defined as $(t_{drug} - t_0)/t_0$ (t_{drug} = retention time of the drug; t_0 = retention time of a non retained solute). The enantioselectivity ($\alpha = k_2/k_1$), was also calculated (k_2 and k_1 are the capacity factors of the second and first eluted enantiomers).

2.3. Reversible binding of valproate to HSA

A series of compounds known to bind to site I, site II, and bilirubin binding site was first injected on the HSA column in order to characterise the binding of each solute to the protein (k values), as well as the enantioselective binding (α values).

Then, increasing concentrations of valproate (up to 5 m*M*) were added to the mobile phase [phosphate buffer 100 m*M* (pH 7.4)/1-propanol, 95/5, v/v)]. Each modified mobile phase was allowed to equilibrate for 3 h and the chromatographic parameters determined when the injected samples reached a stable retention. The column was finally checked with all the analysed compounds in order to verify the change of the binding properties of the protein at the specific binding site I, site II, and bilirubin binding site. The pH of the mobile phase did not significantly change upon addition of the modifier, even for the highest concentration of valproic acid used, i.e. 5 m*M*.

2.4. Determination of the competitor affinity constant

The relationship between the k of the solute and the mobile phase concentration of the displacer is expressed by Eq. (1):

$$\frac{1}{(k' - X - Y)} = \frac{V_M K_2 [D]}{K_3 m_L} + \frac{V_M}{K_3 m_L} \quad (1)$$

where V_M is the void volume of the column; K_2 and K_3 the equilibrium constants for binding of the displacer and solute, respectively; m_L the moles of the solute bound to the stationary phase; [D] the concentration of the displacer in the mobile phase; X, the residual k' resulting from binding at sites on the protein unaffected by the displacer; and Y the non-specific chromatographic interactions.

If both the solute and displacer bind at only one identical site on the immobilized protein, then $X = 0$ and a plot of $1/(k' - Y)$ vs. [D] will produce a linear relationship with a slope of $(V_M K_2/K_3 m_L)$ and an intercept of $(V_M/K_3 m_L)$. The value of K_2, the binding affinity constant for the displacer, can be determined directly by calculating the ratio of the slope to intercept for this plot [4–6].

2.5. Circular dichroism measurements

Circular dichroism (CD) and absorption spectra were recorded using a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan) and a Jasco V-530 spectrophotometer (Jasco, Tokyo, Japan). Both instruments were interfaced to personal computers to acquire and elaborate data. All measurements were carried out at room temperature using 1 cm pathlength cell. The same instrumental parameters were employed to reduce the errors: time constant 4 s, scan speed 20 nm/min, resolution 0.2 nm, sensitivity 20 mdeg, sbw 1. Solutions of the protein were prepared in phosphate buffer (pH 7.4, 0.1 *M*) and actual concentrations were determined from the absorbance at 280 nm (ε_{280} 32180 calculated as optical density per mol of protein) [16]. Competition experiments were carried out on [marker]/[HSA] 1/1 complexes. HSA concentration was kept constant (15 μM), while the concentration of the competitor was varied according to the required ratio. Difference CD methodology were carried out selecting achiral markers, thus the interaction with the protein was responsible for the observed induced CD signal, which arose only from the complexed ligand and it should selectively reflect the steroselective binding [17].

3. Results and discussion

3.1. Displacement chromatography

The retention of one drug, the solute, onto the column containing the immobilized protein was measured before and after the addition of valproate, the competitor. The magnitude and the direction of the resulting changes in k' allowed to determine the

binding site of the ligand and to indicate the type of the occurring interaction. Phenyl butazone and *rac*-warfarin were selected as representative drugs for investigating the influence of valproate to the binding site I. Both drugs were displaced by valproate, a quite impressive reduction of the retention occurring for concentrations of the competitor up to 5 m*M* (Fig. 1). The displacement phenomenon was concentration dependent, the changes in the retention being significant for concentrations of valproate higher than 0.1 m*M*. In the case of *rac*-warfarin the binding inhibition upon the addition of valproate took place for both the enantiomers, the process being slightly enantioselective. Indeed a reduction of the enantioselectivity of about 11% was determined in the presence of 5 m*M* valproate. These results are in agreement with the reported interaction of valproate with site I binding site [9–11]. Quantitative analysis of the displacement experiment data for

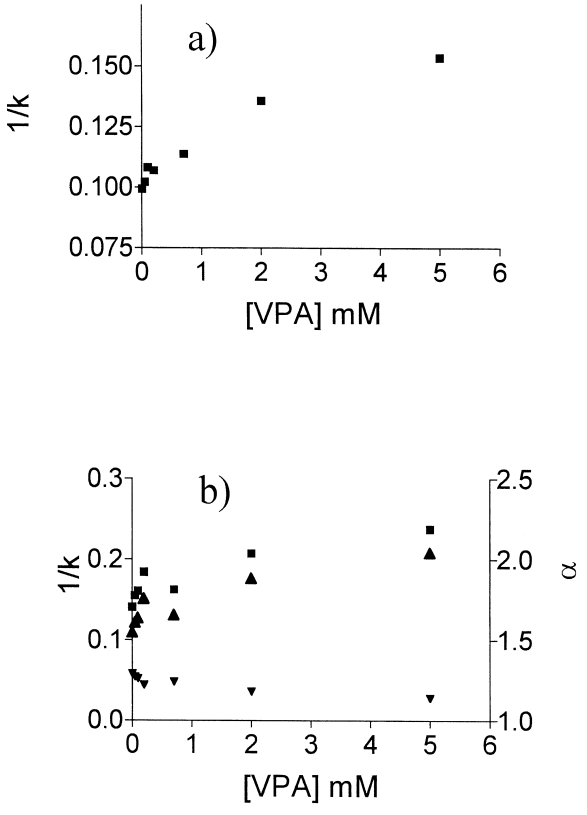

Fig. 1. The effect of valproate (VPA) on the chromatographic parameters [k_1 (■); k_2 (▲); α(▼)] of phenylbutazone (a) and *rac*-warfarin (b) on a HSA-based column.

concentrations of the competitor up to 150 μ*M* allowed to determine the affinity constant of the competitor, the values obtained being related to the binding site where the competition occurs [4–6]. A value of affinity constant of the order of 10^3 M^{-1} was obtained for the two enantiomers of *rac*-warfarin, that one related to the more retained enantiomer being a little higher (1.5×10^3 and 1.9×10^3). The decreasing of the enantioselectivity in the *rac*-warfarin binding in the presence of valproate, suggests that the interaction is not strictly competitive. In this case the competition between valproate and both the enantiomers of warfarin at that site should determine an increase of the enantioselectivity because of the larger displacement of the less retained enantiomer.

The binding inhibition effect of valproate was even stronger for site II binding drugs, like benzodiazepines and profens. As an example in Fig. 2, the behaviour of the capacity factor and of the enantioselectivity upon increasing concentration of valproate in the mobile phase, are reported for *rac*-oxazepam hemisuccinate, and *rac*-ketoprofen. Reduction up to almost 70% was observed for the retention in the presence of 5 m*M* valproate. Also in the case of site II binding drugs, the binding inhibition process resulted enantioselective. A reduction of the enantioselectivity of about 28 and 24% was observed for *rac*-oxazepam hemisuccinate, and for *rac*-ketoprofen, at a 5 m*M* concentration of the displacer. These results clearly demonstrate an interaction of valproate in the binding of benzodiazepines and profens. Furthermore the reduction of the enantioselectivity upon increasing the concentration of the competitor suggests that the binding to the highest affinity binding site, i.e. site II, is significantly affected by valproate. Quantitative analysis of the displacement experiment data up a competitor concentration of 150 μ*M*, allowed to determine the affinity constant of the valproate, the values obtained being related to the highest affinity binding site II. A value of affinity constant of the order of 10^3 M^{-1} was obtained for both the enantiomers of *rac*-oxazepam hemisuccinate (1.2×10^3 and 1.8×10^3) and *rac*-ketoprofen (2.5×10^3 and 2.7×10^3), that one related to the more retained enantiomers being higher. Also in the case of site II binding drugs a pure competitive interaction can not be invoked to

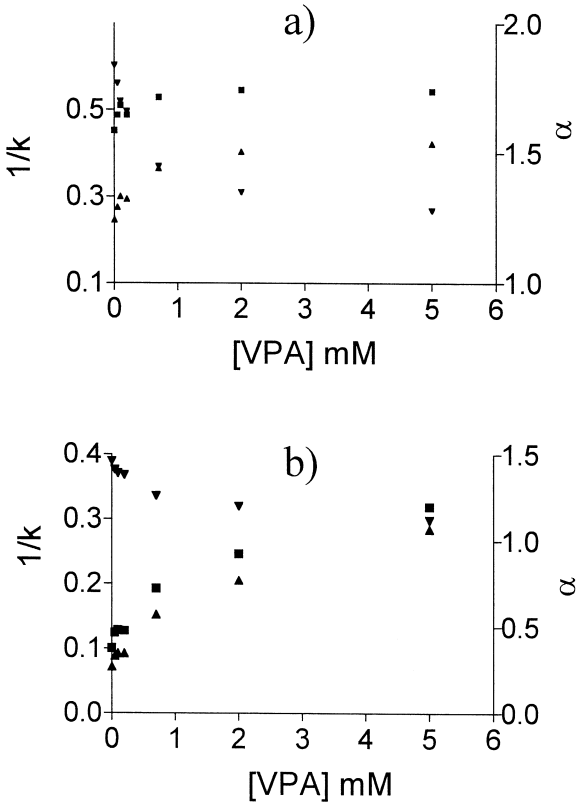

Fig. 2. The effect of valproate (VPA) on the chromatographic parameters [k_1 (■); k_2 (▲); α(▼) of *rac*-oxazepam hemisuccinate (a) and of *rac*-ketoprofen (b) on a HSA-based column.

justify the enantioselective inhibition of the binding in the presence of valproate. A multiple site binding should occur or the competition could be allosteric in nature. Actually different binding sites are reported for benzodiazepines depending on their stereochemistry [18] and the stereoselective binding of profens is still an open question [19].

A valproate concentration dependent binding inhibition was observed also in the case of phenol red. The behaviour of the retention of this ligand is reported in Fig. 3 for concentration of valproate up to 5 m*M*. The effect of valproate on the retention of phenol red is particularly efficient for concentrations of valproate up to 1.5 m*M* (Fig. 3). These results are in agreement with the reported interaction of valproate with the binding of bilirubin [10], which is expected to bind at the same site where the phenol red does [1]. Quantitative analysis of the displacement experiment data allowed to determine the affinity constant of the valproate, the values obtained being related to the phenol red binding site. A value of affinity constant of 6×10^2 M^{-1} was obtained.

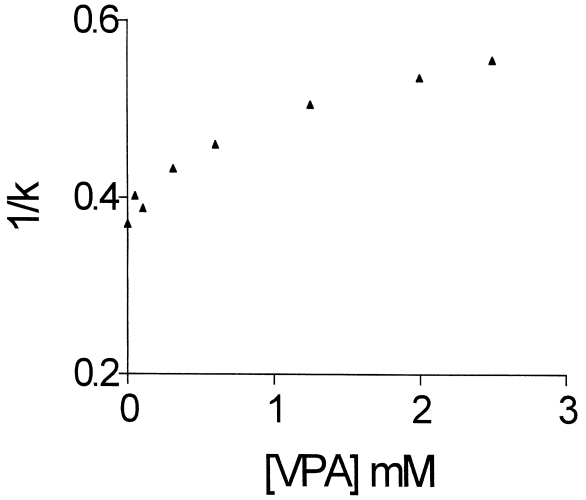

Fig. 3. The effect of valproate (VPA) on the chromatographic parameter ($1/k$) of phenol red on a HSA-based column.

The effect of valproate on the binding of drugs was studied also for other ligands, in order to investigate how this modifier can be used to modulate the drug binding. HSA-based columns are indeed currently used to determine the enantiomeric excess of chiral drugs, and the performances of the column can be improved either changing the experimental condition adopted (temperature, buffer concentration, pH, organic modifier) and adding a selective modifier to the mobile phase that reversibly binds to specific binding areas [20–22]. As far as the valproate is concerned, it was proved to significantly reduce the affinity, and then the retention, of a series of drugs binding to site I and II. In Table 1 the values of the capacity factors and of the enantioselectivity are reported for the analysed drugs, in the absence and in the presence of 2 and 5 m*M* valproate.

This change of the binding properties of HSA resulted in an improvement of the chromatographic performances of the HSA based column, and this improvement was particularly useful for the analysis of profens. Some of these drugs, like naproxen and suprofen, show very high affinity to the protein, and

Table 1
Chromatographic parameters in the absence and in the presence of 2 or 5 m*M* valproate in the mobile phase

Analyte	[valproate] = 0		[valproate] = 2 m*M*		[valproate] = 5 m*M*	
	k_1	α	k_1	α	k_1	α
phenylbutazone	10.1		7.4			
rac-warfarin	7.1	1.29	4.8	1.18		
rac-temazepam	2.8	1.84	2.5	1.62		
rac-oxazepam hemisuccinate	2.2	1.84	1.8	1.35		
rac-ketoprofen	9.9	1.46	4.1	1.20	3.1	1.12
rac-fenoprofen	n.d.	n.d.	n.d.	n.d.	6.9	1.36
rac-suprofen	n.d.	n.d.	n.d.	n.d.	12.0	2.53
rac-naproxen	n.d.	n.d.	n.d.	n.d.	15.9	1.57
phenol red	2.7		1.8			

then their analysis results usually very difficult or almost impossible because of the unusually high retention time on the HSA column [21]. On the contrary the presence of valproate in the mobile phase makes much easier the analysis of these important drugs, with a relatively shorter time of analysis. It is also important to observe that the enantioselectivity is still high also in the presence of high concentrations of the modifier (Table 1).

3.2. Difference circular dichroism (CD) spectroscopy

The binding of valproate to HSA was investigated also by difference CD spectroscopy. The change of the induced CD spectra of ligands complexed to HSA was measured in the presence on increasing concentrations of valproate, which was used as the competitor. Valproate is very likely used as the displacer because of its favourable spectroscopic characteristics. This drug does not show any electronic transitions at wavelengths longer than 250 nm and then it results transparent in the spectral region where induced CD spectra arising from the binding of the examined ligands to the protein occur.

Three non-chiral ligands were selected because of their selective binding at site I (phenylbutazone), at site II (diazepam), and at site III (bilirubin) [1,2,16,23,24]. The CD spectra were measured for the complexes of these three ligands with HSA ([HSA]/[DRUG] 1/1). The change of the CD spectra was then monitored upon the adding of increasing concentrations of valproate (Fig. 4). The analysis of these induced CD spectra allowed to get information on the type of interaction of valproate occurring at any of the studied binding areas. When phenylbutazone, known to bind to site I, was used as the marker, the intensity of the induced CD signal for the 1/1 [phenylbutazone]/[HSA] complex resulted significantly reduced in the presence of increasing concentrations of valproate (Fig. 4A). This result is in agreement to the reported binding inhibition of site I binding drugs in the presence of valproate [9–11], and with the observed reduction of the retention of phenylbutazone and *rac*-warfarin on HSA-based column, when the mobile phase was modified with valproate (Fig. 1 and Table 1). The change of the induced CD signal of phenylbutazone complexed to HSA strongly suggests that the higher affinity binding site for this drug is involved in the competition, being this site the only stereoselective one and then the only one that can be studied by CD. The decreasing of the CD signal was phenylbutazone concentration dependent and its intensity resulted very low when the [valproate]/[phenylbutazone] molar ratio was 40 or higher. The residual contribution to the CD arising from the phenylbutazone complexed to HSA can be razionalised by considering an anti-co-operative interaction in the co-binding of phenylbutazone and valproate.

A significant change of the induced CD spectra was observed also in the case of HSA bound diazepam, this drug being selected as marker of site II. A remarkable decreasing of the induced CD signal observed for the 1/1 [diazepam]/[HSA] complex occurred upon increasing the concentration of

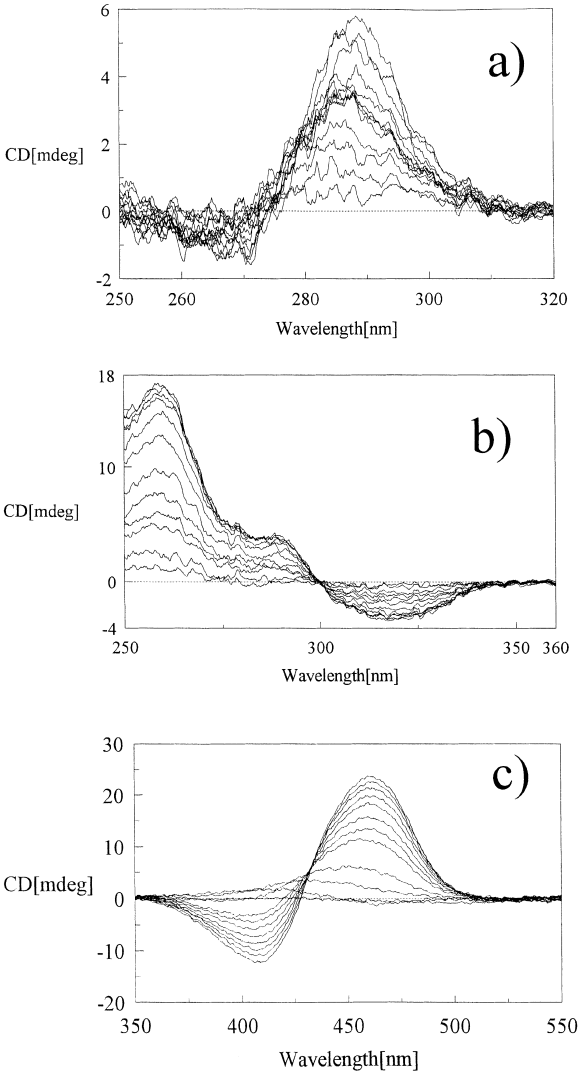

Fig. 4. Induced CD spectra of the [drug]/[HSA] 1/1 complexes in the presence of increasing concentrations of valproate [VPA]. [HSA]: 15 μ*M* in phosphate buffer 0.1 *M*, pH 7.4. [VPA]/[phenylbutazone] 0/1; 1/3; 2/3; 1/1; 2/1; 4/1; 8/1; 10/1; 15/1; 20/1; 40/1; 80/1 (A). [VPA]/[diazepam] 0/1; 1/3; 2/3; 1/1; 2/1; 4/1; 8/1; 12/1; 16/1; 20/1; 30/1; 40/1 (B). [VPA]/[bilirubin] 0/1; 1/1; 2/1; 4/1; 6/1; 10/1; 15/1; 20/1; 40/1; 60/1; 100/1; 200/1 (C). The induced CD signal decreases by increasing the competitor concentration. The inversion of the CD couplet (c) occurs for [VPA]/[bilirubin] 60/1 or higher.

valproate (Fig. 4B). This reduction of the induced CD signal was evident for relatively high concentrations of valproate, being the induced CD almost zero for a [valproate]/[diazepam] molar ratio of 30/1. On the contrary only a little reduction was monitored for low concentrations of the competitor ([valproate]/[diazepam] molar ratio up to 2/1). These results are in agreement with the displacement chromatography data, when a significant reduction of the affinity was observed only for relatively high concentrations of valproate in the mobile phase. Both the methodologies strongly suggest a multiple binding for valproate, in agreement with literature data [10–12], and site II could be a secondary binding site for valproate.

Finally the interaction between bilirubin and valproate in binding to HSA was investigated. Valproate has been reported to compete with bilirubin in the binding to HSA [14]. This interaction is important because a significant inhibition of the bilirubin binding can determine a dangerous concentration of free bilirubin in plasma. Actually a reduction of the induced CD spectrum of bilirubin bound to HSA was observed in the presence of valproate (Fig. 4c). The decreasing of the CD signal was valproate concentration dependent in between 1/3 and 20/1 [valproate]/[bilirubin] molar ratio. This behavior is in agreement with the observed HSA binding inhibition of phenol red in the presence of valproate, as observed by displacement chromatography experiments. Phenol red is known to bind to the bilirubin binding site [1]. This characteristic was confirmed by the observed decreasing of the induced CD spectrum of the [bilirubin]/[HSA] 1/1 complex in the presence of phenol red. A 29% reduction of the CD signal at 463 nm was measured for a 2/1 [phenol red]/[HSA] molar ratio. Higher concentrations of valproate added to the [bilirubin]/[HSA] complex solution, determined a dramatic change in the induced CD spectrum of bilirubin complexed to HSA, with a significant decreasing of the CD intensity and a final inversion of the sign of the CD couplet observed between 400 and 500 nm for a [valproate]/[bilirubin] molar ratio of 100 or higher. This phenomenon could be explained with a change of the conformation of the HSA bound bilirubin, as due to a local change at the bilirubin binding area because of the binding of valproate. In acqueous solution, bilirubin exists as an isoenergetic mixture of two conformational isomers of P- and M-helicity [25]. The apparence of a bisegnate CD spectrum when bilirubin binds to HSA has been interpreted as HSA

bound bilirubin adopting a prevailing conformation of P-helicity [26]. The protein acts as a water soluble chiral complexation agent that fix the conformation of the bilirubin The induced spectrum allied to the bilirubin complexed to HSA decreased significantly upon increasing the concentration of valproate up to [valproate]/[bilirubin] 20/1 (Fig. 4C). Furthermore, higher concentrations of the competitor determined a dramatic change in the induced CD spectrum of bilirubin complexed to HSA, with a significant decreasing of the CD intensity and a final reverse of the sign of the CD couplet observed with maxima at about 460 and 410 nm (Fig. 4c). Thus a change in the intensity of the induced CD spectrum of the HSA bound bilirubin in the presence of valproate is not necessarily determined by a change of the bound fraction of bilirubin. The inversion of the bisegnate CD spectrum can be interpreted as a conversion of the P-helicity conformation, at lower [valproate]/[bilirubin] molar ratio, to the M-helicity conformation at higher [valproate]/[bilirubin] molar ratio concentration. This phenomenon has been previously evidenced upon saturation of an acqueous bilirubin/HSA mixture with chloroform and other volatile anesthetics [27]. Recently an inversion of the CD couplet was observed also in the case of chiral bilirubin analog after addition of amines and diamines [28]. Furthermore spectroscopic studies of mutant HSA/bilirubin complexes revealed that despite the similarity of the K_{aff} values, quite dramatic changes had occurred in the structure of the adducts [29].

4. Conclusions

Displacement chromatography methodology resulted efficient in monitoring pharmacologically relevant interactions between ligands in binding to HSA. Valproate affects the ligand binding at site I, site II and bilirubin site. The monitoring of the binding inhibition of bilirubin was particularly important taking into account the toxicity of this endogenous compound. The binding inhibition was valproate concentration dependent and it resulted particularly efficient for site II binding drugs, like the most retained enantiomers of benzodiazepines and profens. For these drugs the retention was markedly reduced in the presence of valproate, while an useful enantioselectivity was maintained for concentrations of valproate up to 2 m*M*.

Furthermore difference circular dichroism spectroscopy allowed to have a deeper insight to the binding properties of valproate on HSA. This drug was proved to affect the binding to the highest affinity binding sites, as shown by the significant decrease of the induced CD of phenylbutazone, diazepam, and bilirubin complexed to HSA. In the case of diazepam, the ligand was, in practice, completely removed from the carrier, this suggesting a direct competition of valproate for binding site II. On the contrary a significant reduction of the induced CD spectra of HSA complexed phenylbutazone and bilirubin was observed even if a residual contribution to CD remained for relatively high concentration of the competitor. This behaviour suggests an anti-cooperative interaction between these ligands and valproate.

In conclusion, displacement chromatography and difference circular dichroism data, demonstrate the active role of valproate in the binding inhibition of site I and site II binding drugs and of bilirubin.

Finally valproate can be used as selective modifier in the mobile phase when using the HSA based column for determining the enantiomeric excess of chiral drugs. The presence of valproate reduces the time of the analysis, while the enantioselectivity is still high enough to allow a reliable application of the chromatographic method for the enantioselective analysis of both drugs binding to site I and to site II. These data demonstrate that the study of binding mechanism can assist in developing enantioselective chromatographic methods.

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