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Binding studies of taxanes to human serum albumin by bioaffinity chromatography and circular dichroism

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Abstract

The binding to human serum albumin (HSA) of the antitumoural drug paclitaxel and of several structural analogues has been characterized by bioaffinity chromatography and circular dichroism. A ranking of the taxanes was obtained for their affinity to the protein by measuring their retention times on an albumin chromatographic column. This also allowed the calculation of the drug bound percentage. Affinity resulted significantly affected by the nature of the isoserinic side chain, the presence of the 1,14-carbonate moiety and the substituent at C-7, showing that the hydrophobicity of the drug is fundamental in the binding process. The analysis demonstrated that the organic solvent highly alters the interaction mechanism of taxanes to the protein and so the affinity results. Circular dichroism experiments supported this hypothesis. Furthermore, taxanes binding to the serum carrier was characterized by displacement chromatography, by adding into the mobile phase selected competitors, (*S*)-ibuprofen and valproic acid, that are known to bind to specific binding sites on HSA. These experiments established a non-cooperative binding mechanism.

Keywords: Paclitaxel; Drug delivery; HSA binding; Circular dichroism; Bioaffinity chromatography

1. Introduction

Reversible binding to serum proteins modulates the distribution of drugs, and then affects the pharmacokinetics and pharmacodynamic properties [1]. Once administered, the free concentration of a drug can change due to its interaction with other drugs and endogenous factors, to its binding to plasma proteins, or to significant changes of the serum carrier concentration.

Therefore, investigation studies on the plasma protein drug binding site aimed to the determination of the binding parameters are relevant when studying a drug profile. This is particularly true when the drugs have as low aqueous solubility as paclitaxel. In such cases, the serum proteins act as selective drug carriers, because of their accumulation in tumour tissues.

HSA has a flexible structure with multiple domains, providing a variety of sites a broad spectrum of compounds can interact to Refs. [1–4]. For many drugs and endogenous factors this binding is selective and the affinity is relatively high (K_A 10^5 to 10^8 M^{-1}).

The binding of paclitaxel to HSA has been a topic of several scientific papers, the issue being very recently also associated to the potential clinical application of a novel HSA–paclitaxel cremophor free formulation [5]. A major problem related to the therapeutic use of paclitaxel, is a very low and also variable bioavailability. When given orally, it shows a complete lack of efficacy against hypersensitive human tumours, preventing oral administration for its therapeutic use [6]. The poor bioavailability of paclitaxel is partially due to the elimination of the drug from the cell by P-glycoprotein, Pgp, an efflux-pump which is abundant in the gastrointestinal tract [7]. Consequently, the research on paclitaxel analogues has been focused onto new generation taxanes, active compounds that are not substrates for PgP and so that exhibit a better bioavailability such as IDN5109 (ortataxel, otx, Fig. 1) [8]. Other new taxanes have been claimed to have peculiar mechanisms of action overcoming paclitaxel resistance due to tubulin overexpression [9] and to be active after prolonged treatment. In fact, IDN5390, a C-seco derivative (seco, Fig. 1), showed to be effective and well-tolerated when administered orally. This new drug has good potential to

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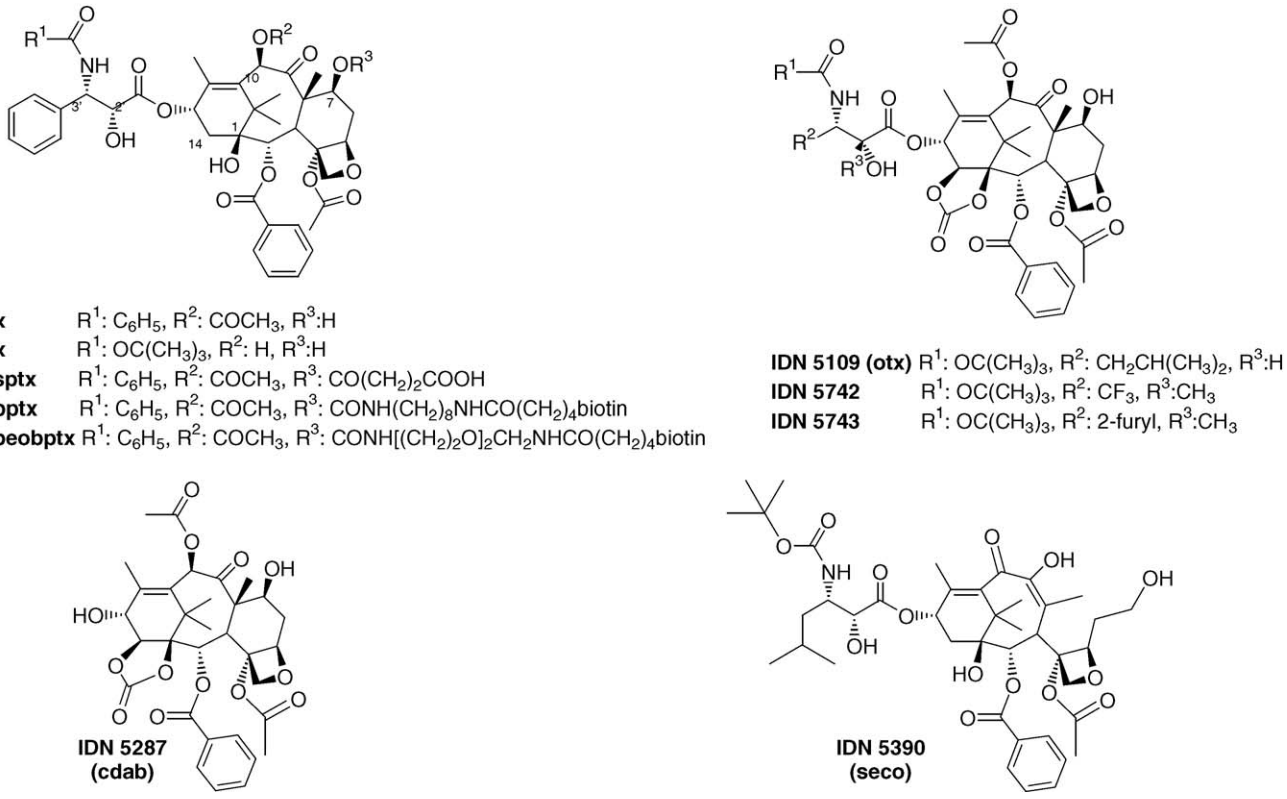

Fig. 1. Structures of taxanes.

become an oral drug candidate for a protracted chemotherapeutic treatment schedule [10].

The binding of paclitaxel to HSA has been so far studied through various methodologies. Optical biosensor [11], ultrafiltration [12], Fourier transform infrared (FT-IR) [13] and fluorescence and circular dichroism spectroscopy [14] allowed to get information on the binding sites on HSA, and to determine the binding parameters. Nevertheless, the comparison of the reported data shows significant differences in the affinity constant values. The adopted experimental conditions in carrying out binding experiments were indeed different: the assay always demands the use of a percentage of organic solvent in order to overcome the very low solubility of paclitaxel in aqueous solution. The use of ethanol [12] or methanol [13,14] as co-solvents for preparing paclitaxel solutions in truth modifies the interaction process being the nature of paclitaxel binding to HSA mainly hydrophobic. Actually, in the published papers, the co-solvent in use has never been given crucial importance when coming to the interpretation of paclitaxel–HSA binding mechanism.

Moreover, different values of the buffer pH were used, this affecting the conformation, and then the binding properties of the protein [1,15,16]. All this contributed to various interpretation of the binding mechanism of paclitaxel to HSA. Some authors showed that paclitaxel interacts to a high affinity binding site with HSA [12,14] while other authors described the binding process as a substantially non-specific drug/protein complexation [13].

Purcell et al. hypothesized that paclitaxel induces a conformational change on the protein, this based on a decreasing of the α-helix related CD signal once increasing the [paclitaxel]/[HSA] molar ratio. Although this behaviour might be reasonable, the change of the protein conformation could be likewise related to the action of increasing concentration of organic solvent in the aqueous solution [17]. On the other hand, the binding of paclitaxel has been found to affect the binding in the protein area where $Thryp_{214}$ is located [14]. This residue forms part of the wall in one of the two main drug binding cavities on HSA, i.e. site I, subdomain IIA. But circular dichroism (CD) displacement experiments carried out with the more water soluble, 7-hemisuccinyl-paclitaxel (7-sptx, Fig. 1) suggested that HSA does not have a high-affinity binding site for this taxane, and that the relatively low affinity of 7-sptx to the protein is a result of interactions with several low affinity binding sites [18]. This result was in accordance with the optical biosensor investigations, where 7-sptx and few other taxanes demonstrated that the interaction strength with the protein was low (K_A, ca. 10^3 to 10^4 M^{-1}) [11].

Here we present a study on the HSA binding of paclitaxel and of several structural analogues (Fig. 1) conducted by bioaffinity chromatography where a HSA-based column was used as affinity phase (high performance affinity liquid chromatography, HPALC). The tested taxanes were ranked based on their affinity to the protein, by measuring their retention times on column, using a zonal chromatography approach. The binding of taxanes was monitored at different percentage of organic solvent (here

Table 1
Capacity factor (k) values of taxanes determined on HSA-based column

PB/1-propanol: 90/10	7-bptx	otx	7-peobptx	IDN5742	IDN5743
$k_{[90:10]}$	11.2	7.8	6.5	6.2	5.3
PB/1-propanol: 97.5/2.5	ptx	7-sptx	dtx	IDN5287	IDN5390
$k_{[97.5:2.5]}$	11.4	10.9	4.5	3.4	2.8

1-propanol) and it was shown that the co-solvent does interfere with the binding process, which might give rise to misleading assumptions. Circular dichroism (CD) experiments proved that 1-propanol does influence the binding mechanism of taxanes to the protein. In order to remark a possible selective binding area for these compounds on HSA, we carried out pivotal displacement chromatography experiments by adding into the mobile phase selected competitors, (*S*)-ibuprofen and valproate.

2. Experimental

2.1. Materials

Water was doubly distilled. HPLC grade 1-propanol was from Sigma–Aldrich, Milan, Italy. The buffer solutions were filtered through a 0.45 μm membrane filter and degassed before their use.

2.1.1. Samples

Docetaxel (dtx), paclitaxel (ptx), IDN5390 (seco), 14β-hydroxy-baccatin III 1,14-carbonate (IDN5287, cdab), and IDN5109 (ortataxel, otx) were from Indena Spa, Italy. 7-Hemisuccinyl paclitaxel (7-sptx), 7-polyethoxy-biotinylpaclitaxel (7-peobptx), 7-biotinyl paclitaxel (7-bptx), 2′-Metil-3′-deisobutil-3′-trifluorometil IDN5109 (IDN5742) and 2′-Metil-3′-deisobutil-3′-(2-furil) IDN5109 (IDN5743) were prepared by Andrea Guerrini (ISOF, CNR, Bologna, Italy).

Bilirubin (bil), sodium salicylate (sal), (*S*)-ibuprofen [(*S*)-ibu], and valproic acid (val) were purchased by Sigma–Aldrich (Milan, Italy).

All samples were dissolved in 1-propanol (1 mg/ml) and stored at −20 °C. Dilutions of the compounds were made in appropriate buffers just before use.

Buffer: PB (potassium phosphate buffer) 50 mM, pH 7.4.

2.1.2. Apparatus

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 cm × 0.4 cm i.d.).

2.1.3. Chromatographic conditions

Mobile phase: PB/1-propanol, 100/0–80/20 (v/v); flow rate: 0.8 mL min^{-1}. Solutions were injected at a $c = 0.1$ mg mL^{-1} and the chromatographic runs were followed at 230 nm wavelength.

2.1.4. Binding parameters determination

The solute chromatographic retentions were expressed as capacity factors (k):

$$k = \frac{t_{\text{drug}} - t_0}{t_0} \quad (1)$$

where t_{drug} is the drug retention time and t_0 is the non-retained solute retention time (Table 1).

The bound drug percentage (%b) was calculated from the k-values, obtained by zonal elution chromatography, according to the following equation (2) [19]:

$$\%b = \frac{k}{k+1} \times 100 \quad (2)$$

The bound drug parameter calculation in only aqueous phase was extrapolated by linearly plotting the $\log k$ values (averages of triplicate measurements) against the percentage (v/v) of 1-propanol in the eluent mixtures (Table 2).

Table 2
Bound percent (%b) values of taxanes determined for different 1-propanol concentrations

Samples	Bound percentage, %b										
	0[a]	2[a]	4[a]	6[a]	7[a]	8[a]	10[a]	13[a]	15[a]	17[a]	20[a]
IDN5390	77.7	64.1	56.3	50.0	–	44.4	40.2	–	–	–	–
IDN5287	75.0	66.7	60.0	53.3	–	46.2	41.7	–	–	–	–
dtx	–	70.8	63.1	56.3	–	50.0	46.2	–	–	–	–
7-sptx	–	81.1	74.1	67.6	62.1	60.0	53.3	–	–	–	–
ptx	–	86.3	82.0	76.7	72.0	69.6	–	–	–	–	–
IDN5743	–	–	89.2	86.0	82.9	80.5	74.6	–	–	–	–
IDN5742	–	–	90.5	87.7	85.1	82.5	77.4	–	–	–	–
7-peobptx	–	–	–	–	–	83.4	77.4	69.0	60.0	50.7	–
otx	–	–	–	90.0	88.3	86.0	80.0	–	–	–	–
7-bptx	–	–	–	–	–	–	–	91.9	86.8	79.4	66.7

[a] 1-Propanol %.

2.1.5. Displacement chromatography assay

Displacement chromatography experiments were carried out by the zonal elution approach. By this method, a known concentration of a competing agent is continuously applied in the mobile phase to the HSA-based column, while small amounts of the taxanes are injected. Taxanes were first injected on the HSA column in order to characterize the binding of each solute to the protein (k-values) when the injected samples reached a stable retention. Then, increasing concentrations of the competitors [sodium salicilate (sal), (*S*)-ibuprofen ((*S*)-ibu), and valproic acid (val)] were added to the mobile phase. The pH of the mobile phase upon the addition of the competitors was checked and did not change significantly, even for the highest concentration of valproic acid used, i.e. 3 mM. Each modified mobile phase was allowed to equilibrate for 3 h. For each competing agent concentration triplicate injections of the analytes were made.

Displacement chromatography allows the affinity constant of the competitors to be determined at their common binding sites. The relationship between the k of the taxane and the mobile phase concentration of the competitor is expressed by Eq. (3):

$$\frac{1}{k - X - Y} = \frac{V_M K_2 [D]}{K_3 m_L} + \frac{V_M}{K_3 m_L} \quad (3)$$

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

If both the taxane and competitor bind at only one identical site on the immobilized protein, then $X = 0$ and a plot of $1/(k - Y)$ versus [D] will produce a linear relationship with a slope given by $V_M K_2/K_3 m_L$ and an intercept given by $V_M/K_3 m_L$. The value of K_2, the binding affinity constant for the competitor, can be determined directly by calculating the ratio of the slope to intercept for this plot [20,21].

2.1.6. Circular dichroism (CD) measurements

CD spectra were recorded using a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan). 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, 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 ($\varepsilon_{280} = 32\,180$ calculated as optical density per mol of protein) [22]. Competition experiments were carried out adding increasing concentrations of paclitaxel to the 1/1 marker/protein complexes. HSA and marker concentrations were kept constant (15 μM), while the concentration of paclitaxel was varied according to the required ratio, [marker]/[HSA]/[paclitaxel] = 1/1/0, 1/1/3, 1/1/5. The same experiments were carried out in the presence of the same concentration of 1-propanol used for the solubilization of ptx in the various complexes.

3. Results and discussion

3.1. Determination of bound fraction of taxanes

Paclitaxel and its structural analogues (Fig. 1) were injected over the HSA column at a fixed concentration (0.1 mg/ml). The capacity factor values obtained for the examined taxanes are reported in Table 1. Note the different eluent phase composition. Affinity resulted significantly affected by the nature of the isoserinic side chain, the presence of the 1,14-carbonate moiety and the C-7 substituent, suggesting that the hydrophobicity of the drug is fundamental in the binding process. The new generation taxanes (otx, IDN5742, IDN5743) had significant increased binding to HSA, with respect to the parent paclitaxel. On the contrary IDN5390, the C-seco derivative, showed a much lower affinity. However, the k values were obtained by using different concentration of 1-propanol, because the investigated compounds could not be eluted in reasonable time from the HSA column without adding an organic modifier in the mobile phase. Thus, a direct comparison of the affinities was not possible on these bases. However, the $\log k$ values can be extrapolated to a zero percentage content of 1-propanol by linear plotting of $\log k$ against the percentage of 1-propanol in the mobile phase. The bound fraction of the drugs was then determined, by applying Eq. (2): [19] 7-sptx showed a very close affinity to that of the underivatized paclitaxel (Table 2). IDN5390 (seco) and IDN5287 (cdab) were also studied without 1-propanol addition, this pointing out a good agreement between the experimental (Table 2) and the 1-propanol bound drug extrapolated at zero percentage (Fig. 2).

Significant differences have been observed between the reported affinity values for the binding of paclitaxel to HSA [12,14]. These controversial results are most likely related to differences in the sample preparation. In addition, taxanes solutions tend to aggregate, which can result in precipitation during experiments.

3.2. HSA binding site investigation: displacement chromatography approach

Displacement chromatography experiments, carried out by zonal elution methodology, were conducted in order to examine the competition displayed by taxanes on the binding of selected agents with a known high affinity binding site on HSA. The first competitor used was salicylate, which binds to site I as high affinity binding site [1]. The adding of salicylate up to 500 μM concentration in the mobile phase did not change significantly the retention of the seco derivative. Experimental limitations, i.e. retention time of analytes close to the systemic peak of the competitor and its absorption, did not allow a reliable analysis of the other taxanes. However, the almost independent binding of seco and salicylate is in agreement with the non-competition at site I previously monitored by CD for the analogue 7-sptx [17].

However, the interaction of ptx with the warfarin/phenylbutazone binding site has been recently reported, as studied by fluorescence and CD spectroscopy. The observed

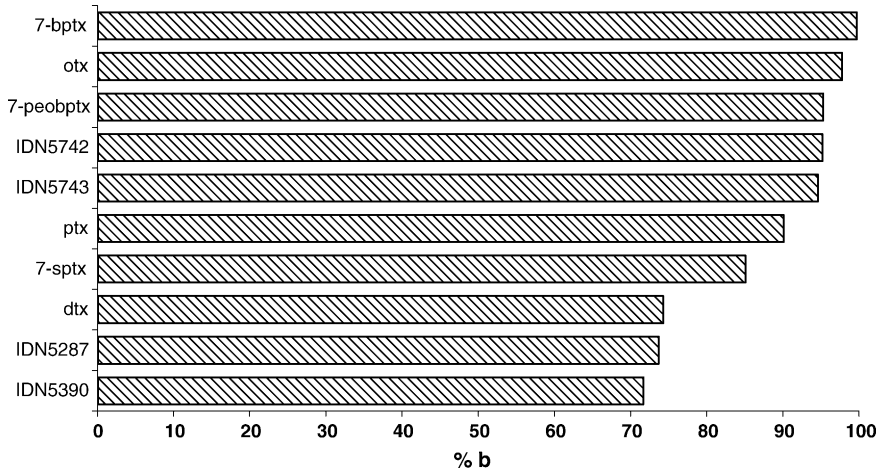

Fig. 2. Screening of taxanes: bound percent (%*b*) values extrapolated for 100% aqueous solution.

behaviour could be due to the use of methanol as organic modifier [14]. This hypothesis is strongly supported by the significant decreasing of the k values of taxanes on the HSA-based stationary phase by increasing the concentration of the organic modifier in the eluent (Table 2). Accordingly, the calculated percent of bound drug decreases upon the increasing of the organic solvent percent (Table 2). The influence of the 1-propanol concentration on the drug binding to HSA was demonstrated also by CD experiments (see next section). Furthermore, an increasing percent of the unbound fraction of paclitaxel in plasma has been recently monitored for drug formulated with a Cremophor EL and ethanol mixture [23]. A significant binding inhibition of taxanes was observed using (*S*)-ibuprofen, site II marker, as the competitor (Fig. 3). This anti-inflammatory drug, known to selectively bind to site II [1] was added to the mobile phase up to 10 μM, and resulted efficient in the displacement of all the investigated taxanes (Fig. 3). This interaction determined a lowering of the retention of the drugs; however evidence was not obtained for a direct competition mechanism. The analysis of the chromatographic data allowed the affinity constant of the competitor to be determined for the interacting site. Values in the range $5.7–1.1 \times 10^4\ M^{-1}$ were obtained, i.e. much lower with respect to the reported affinity constant for (*S*)-ibuprofen, this suggesting a non-cooperative binding of taxanes at site II. Also valproate (val) resulted efficient in displacing taxanes (Fig. 4), but again a non-cooperative mechanism has to be taken into account to describe the process. This anti-epileptic drug, known to bind to site II, is reported to interact also to site I and to the bilirubin binding site [24–27]. Concentrations up to 5 mM of val in the mobile phase determined a significant decreasing of the binding of taxanes to the protein. The analysis of this behaviour allowed again the determination of valproate affinity constant values for the taxanes sites. Values of $1–2 \times 10^2\ M^{-1}$ were obtained for the investigated taxanes, suggesting a non-cooperative binding with valproate.

These results are in agreement to the CD experiments carried out on the 7-sPTX/HSA complex in solution, the data suggesting a relative low binding of taxanes to different binding sites [17].

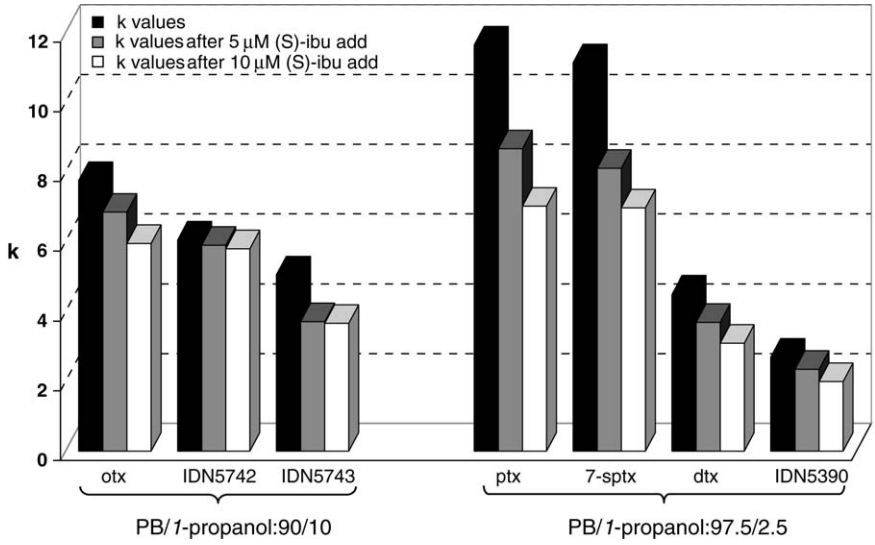

Fig. 3. Binding inhibition of taxanes upon addition of (*S*)-ibuprofen as competitor in the mobile phase.

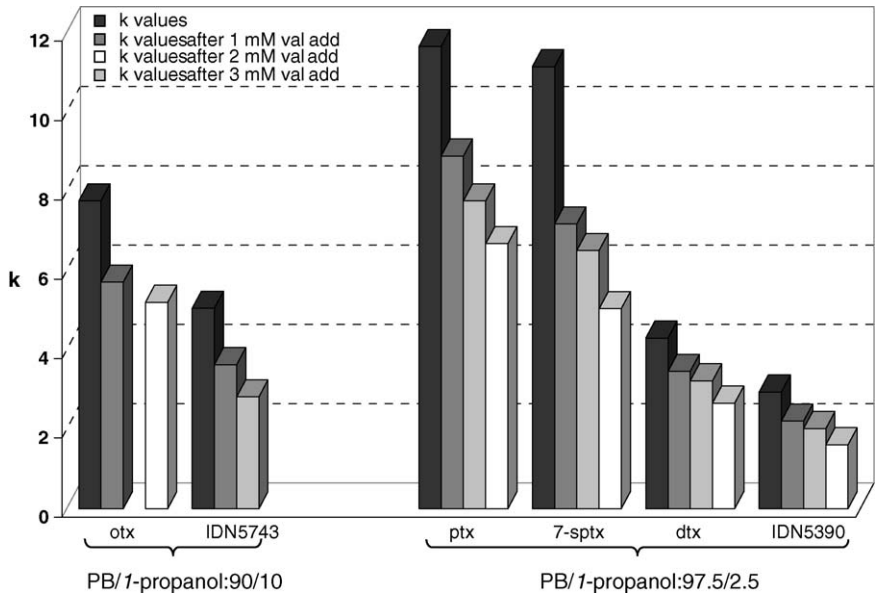

Fig. 4. Binding inhibition of taxanes upon addition of valproic acid as competitor in the mobile phase.

3.3. HSA binding of taxanes by difference circular dichroism spectroscopy

Difference CD spectroscopy represents a useful tool to characterize the binding mechanism of drugs to HSA. To this end, the induced CD spectra of markers that selectively bind to sites I–III on HSA were measured. Than, the change of the induced spectra upon the addition of increasing concentrations of the investigated drug was monitored. The data reported in the literature for ptx [14] and for 7-sptx [17] are not in agreement. Indeed no significant changes were observed in the value of the induced CD spectra upon increasing the concentration of 7-sptx added to the marker/HSA complex solution [17] while significant binding inhibition was reported when ptx was used as competitor [14]. A possible explanation of this behaviour, beside the structural difference of the two taxanes, resides in the role of the organic solvent, used to solubilize paclitaxel, on the binding process. A decreasing of the induced CD signal for bilirubin (bil) was observed either in the presence of increasing concentration of ptx, up to [ptx]/[bil]/[HSA]: 10/1/1 molar ratio, and in the presence of the same concentration of 1-propanol used for the solubilization of ptx in the various complexes (Fig. 5). Thus, organic solvent did affect the binding process, and, most probably, the CD signal allied to the HSA bound bilirubin is not affected in a significant extent by the presence of increasing concentration of ptx. These results strongly support the view that none of the most important binding sites, i.e. sites I–III, represent high affinity binding sites for taxanes, as reported in the CD study of 7-sptx [17]. This behaviour is also in agreement with the relatively low affinity constant value previously determined [11,13] suggesting a non-selective binding at low affinity binding sites. Further support to this hypothesis arises from the comparison of the CD spectra of the free and the HSA bound 7-sptx [17]. No significant differences were observed, while the binding to sites I–III usually determines some extent of stereoselectivity [20].

On the contrary, other authors [12] reported much higher affinity constant value for the binding of ptx to HSA, but, again the experimental condition adopted likely heavily affected the results obtained. Unfortunately the low solubility of taxanes, and

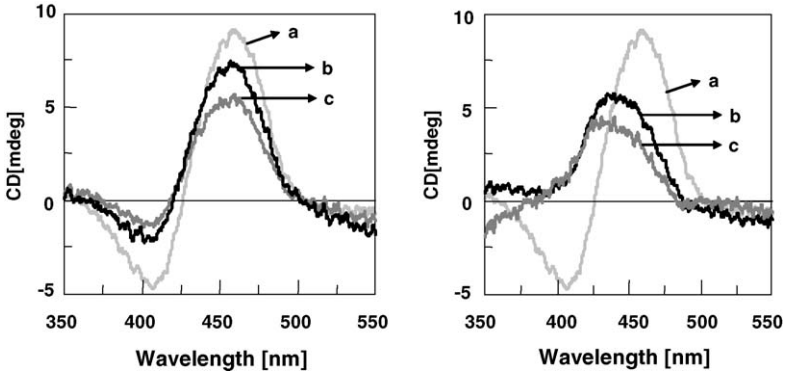

Fig. 5. Decreasing of the induced CD of bilirubin bound to HSA before (a) and after addition of 1-propanol (b) and after addition of paclitaxel (c): [HSA]/[bilirubin]/[PTX], 1/1/3 (left) and 1/1/5 (right); [HSA]/[bilirubin]/[1-propanol], 1/1/0.6% (left) and 1/1/2% (right).

the extremely low solubility in the case of ptx and otx, prevents a reliable investigation by means of spectroscopic studies of their binding to the serum proteins. Thus, even if a binding of ptx to sites I–III on HSA cannot be excluded, all the obtained data indicate a relatively low affinity binding process at several binding sites.

4. Conclusions

A method has been developed for rapidly characterizing the binding of taxanes to HSA. The bound percent of the drug can be determined by retention data on the HSA-based stationary phase. The affinity is strongly affected by the presence of even low concentration of organic solvents in the solution.

No evidence of a high affinity binding site for taxanes on HSA has been obtained, either by bioaffinity chromatography and CD. However, an inhibition of the taxanes binding to the carrier was monitored using (*S*)-ibuprofen and valproate as selective modifiers in the mobile phase. The observed behaviour suggests a non-cooperative binding for both the competitors.

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