



## Tocainide analogues binding to human serum albumin: A HPLAC and circular dichroism study

Marco Pistolozzi<sup>a</sup>, Carlo Franchini<sup>b</sup>, Filomena Corbo<sup>b</sup>, Marilena Muraglia<sup>b</sup>, Marcella De Giorgi<sup>b</sup>, Guy Felix<sup>c</sup>, Carlo Bertucci<sup>a,\*</sup>

<sup>a</sup> Department of Pharmaceutical Sciences, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy

<sup>b</sup> Department of Pharmaceutical Chemistry, University of Bari, Via E. Orabona n.4, 70125 Bari, Italy

<sup>c</sup> CNRS, Aix-Marseille Université, CINaM UPR 3118, Marseille, France

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### ABSTRACT

A series of synthesised tocainide analogues were characterized for their human serum albumin (HSA) binding, using high-performance liquid affinity chromatography (HPLAC) and circular dichroism (CD). The synthesis and physico-chemical characterization of compounds **7a–7d** is reported here. For the HPLAC investigation HSA was covalently immobilized to the silica matrix of the HPLC column, using an anchoring procedure, which allows the binding properties of the protein to be maintained. The HSA-based column was used for getting information on the high affinity binding sites of the tocainide analogues to HSA. According to the displacement chromatography approach, the retentions of the analytes were determined in the absence and in the presence of increasing concentrations of competitors known to bind to specific binding sites on the protein. The same system, drug/protein, was investigated in solution by CD.

The analysed compounds, proved active as sodium channel blockers, showed a much higher affinity to the serum carrier with respect to the parent compound, tocainide. Further, a non-cooperative interaction at sites I and II, and an almost independent binding at the bilirubin binding site on HSA were hypothesised on the bases of the competition experiments.

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### 1. Introduction

The monitoring of drug binding to serum proteins is essential to characterize the drug distribution, one of the ADMET (absorption, distribution, metabolism, excretion, toxicity) parameters, whose early determination is nowadays considered fundamental in drug discovery and development [1]. A quantitative and reliable characterization of the interaction of molecules with plasma proteins in an early stage of drug development is becoming essential in the field of drug discovery.

In particular, quantitative determination of drug binding to plasma proteins is important in clinical drug development, because the unbound drug fraction affects many critical pharmacokinetic parameters, and the steady-state distribution volume. Among serum carriers, human serum albumin (HSA) is the most abundant protein, and it has a fundamental role as carrier of drugs and metabolites, acting as a depot for hydrophobic compounds [2]. As the actual volume of distribution and concentration of a drug at the receptor site are strictly related to the amount of unbound drug fraction, the study of the binding mechanism to HSA has become

essential to the early characterization of the pharmacokinetic profile of new potential leads.

The potential application of tocainide as voltage gated sodium channel blocker has been diffusely investigated to symptomatically solve the myotonic syndromes, hereditary disorders caused by mis-sense mutations of the skeletal muscle sodium channel isoform Nav1.4. Unfortunately, clinical use of Tocainide was limited due to high general side effects [3]. For these reasons, tocainide was chosen as structural framework to create new potent and selective sodium channel blockers [4–7]. Recent QSAR studies have provided substantial improvement in the understanding of the key molecular requirements associated with the sodium channel blocking activity. The resulting pharmacophore hypothesis suggested that the introduction of a benzyl group on the amine nitrogen so as a certain distance between two aromatic rings are essential to enhance the sodium channel blocking action [8]. In particular, compound **7** (Fig. 1) has shown the highest increment of potency among all synthesised compounds [6]. Encouraged by these findings we optimised our investigation by focusing the attention on the xylydic moiety to prepare compounds **7a–7d** (Scheme 1). Preliminary *in vitro* data were registered by testing this short series on sodium currents of adult skeletal muscle fibres by using the voltage clamp method [9]. However, no substantial difference in potency was observed between the new molecules and the most

\* Corresponding author. Tel.: +39 0512099731; fax: +39 0512099734.  
E-mail address: [carlo.bertucci@unibo.it](mailto:carlo.bertucci@unibo.it) (C. Bertucci).

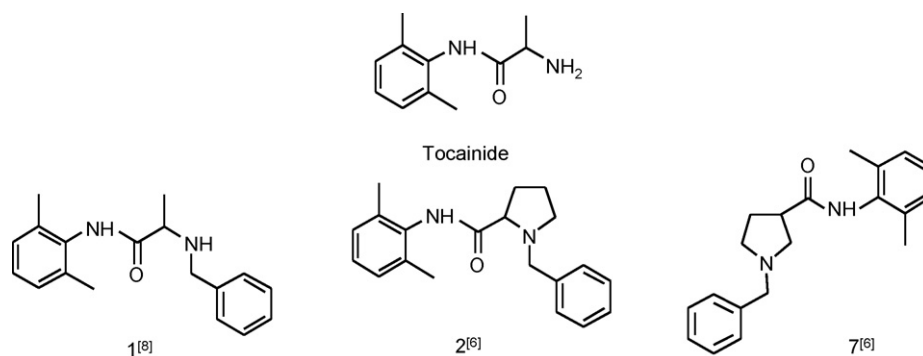


Fig. 1. Structures of tocaïnide and its analogues.

active sodium channel blocker **7**. So, in the current work a considerable attention has been focused on the characterization of the pharmacokinetic properties both of tocaïnide analogues (**1** [8], **2** [6], Fig. 1) and **7** derivatives (**7a–7d**, Scheme 1). The synthetic procedure of compounds **7a–7d** is here in reported. Furthermore, although compounds **9** and **3c** are commercially available they have been included in our synthetic program due their prohibitive price.

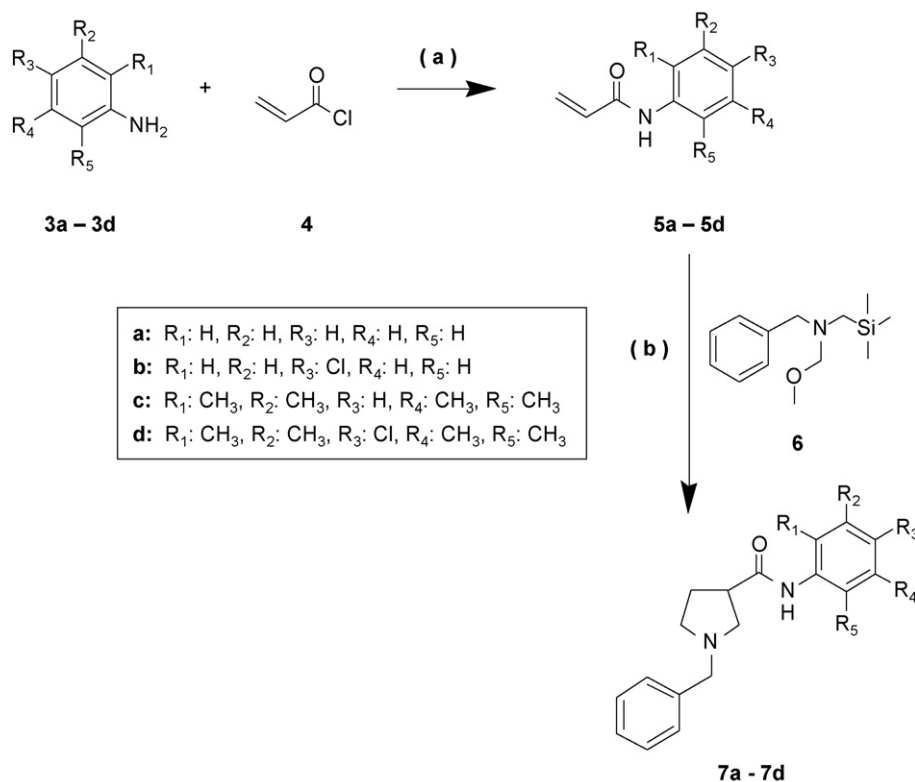
Here the distribution behaviour of this small series of tocaïnide analogues was studied by high-performance liquid affinity chromatography (HPLAC). A HSA-based column was used for ranking the compounds under investigation for their binding to the serum carrier, and for getting information on the involved binding areas on the protein. The bound fraction of the tocaïnide analogues was determined by measuring their retention on column, using the zonal chromatographic approach. Pivotal displacement experiments [10,11] by adding selected competitors [11–13] into the mobile phase were carried out to individuate the possible binding sites. The results were confirmed by circular dichroism (CD) competition studies. In this case, the compounds under investigation

were used as displacers of selected markers bound to specific binding sites on the protein, and the changing of the induced CD signal of the bound marker was monitored as measure of the competition mechanism [11].

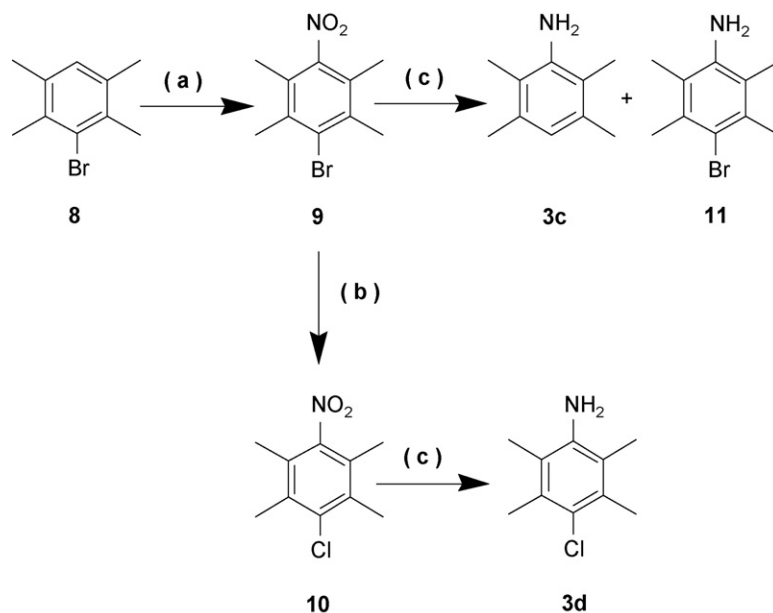
## 2. Experimental

### 2.1. Chemicals and instruments

Human serum albumin (HSA) (Cohn fraction V powder essentially fatty acid free, A1887), *L*-tryptophan (*L*-Trp), glycine, *N*-benzoyl-*D,L*-leucine, *rac*-warfarin, sodium valproate, *rac*-ibuprofen, sodium salicylate, bromophenol blue, and bilirubin were purchased from Sigma–Aldrich (Milan, Italy). Diazepam, flunitrazepam and nitrazepam were kindly provided by Prof. Regina H. Costa Queiroz, Faculty of Pharmacy, University of San Paolo, at Ribeirao Preto (Brazil). Other chemicals were reagent grade from commercial suppliers and were used without further purification. Potassium phosphate buffers (PB) were prepared by adjusting the pH of solu-



Scheme 1. Reagents and experimental conditions: (a) Et<sub>3</sub>N, anhyd. CH<sub>2</sub>Cl<sub>2</sub>, N<sub>2</sub>, from 0 °C (2 h) to rt (12 h); (b) CF<sub>3</sub>COOH, CH<sub>2</sub>Cl<sub>2</sub>, 3 h, rt.



**Scheme 2.** Reagents and experimental conditions: (a)  $\text{NaNO}_2$ ,  $\text{CF}_3\text{COOH}$ , 6 h, rt; (b)  $\text{CuCl}$ , anhyd. DMSO,  $\text{N}_2$ , 3 h,  $180^\circ\text{C}$ ; (c)  $\text{LiAlH}_4$ , anhyd. THF,  $\text{N}_2$ , 24 h, rt.

tions of  $\text{K}_2\text{HPO}_4$  with equimolar solutions of  $\text{KH}_2\text{PO}_4$ .  $\text{K}_2\text{HPO}_4$ ,  $\text{KH}_2\text{PO}_4$ , and  $(\text{NH}_4)_2\text{SO}_4$  powders were purchased from Carlo Erba Reagenti (Milan, Italy).

The HPLC apparatus consisted of a Jasco PU-2089 plus (Jasco, Tokyo) HPLC pump, a Rheodyne injector system with a  $20\ \mu\text{L}$  loop and a Jasco MD-2010 plus (Jasco, Tokyo) diode array detector. The column was thermostated at  $28^\circ\text{C}$  with a Column Chiller Model 7955 (Jones Chromatography Ltd., UK).

The chromatographic retention was reported as the capacity factor ( $k$ ), where  $k$  is defined as  $(t_{\text{drug}} - t_0)/t_0$  ( $t_{\text{drug}}$  = retention of the solute;  $t_0$  = retention of a non-retained solute).

Buffers were filtered with  $0.22\ \mu\text{m}$  mixed cellulose esters filter membranes (Millipore, Milan, Italy). The mobile phases were degassed prior to use by ultrasonic waves for at least 15 min.

All chemicals were purchased from Sigma–Aldrich in the highest quality commercially available. The structures of the compounds were confirmed by routine spectrometric and spectroscopic analyses. Spectroscopic data are given only for compounds not previously described.

Melting points were recorded on Gallenkamp melting point apparatus in open glass capillary tubes. The IR spectra were recorded on a PerkinElmer Spectrum One FT spectrophotometer and band positions were given in reciprocal centimetres ( $\text{cm}^{-1}$ ).  $^1\text{H}$  NMR spectra (300 MHz) were recorded on a FT Bruker Aspect 3000 spectrometer using  $\text{CDCl}_3$  as the solvent, unless otherwise indicated. Chemical shifts were reported in part per million (ppm) relative to solvent resonance:  $\text{CDCl}_3$ ,  $\delta$  7.26 ( $^1\text{H}$  NMR). Amino proton assignments were confirmed by  $\text{D}_2\text{O}$  exchange.  $J$  values are given in Hz. EIMS spectra were recorded with a Hewlett–Packard 6890-5973 MSD gas chromatograph/mass spectrometer at low resolution. Elemental analyses were performed on a Eurovector Euro EA 3000 elemental analyser, were indicated, C, H and N were within  $\pm 0.4$  of the theoretical values. Silica gel chromatographic separations were performed by chromatography with silica gel (Kieselgel 60, 40–63  $\mu\text{m}$ , Merck) packed in glass columns, using the technique described by Still et al. [14]. The eluting solvent indicated in parentheses, for each purification was determined by TLC, that was performed on precoated silica gel on aluminium sheets (Kieselgel 60 F<sub>254</sub>, Merck). TLC plates were visualized with UV light and/or in an iodine chamber.

## 2.2. Synthesis of tocinamide analogues

Compounds **7a–7d** (Scheme 1) were achieved according to the procedure previously reported for the preparation of compound **7** [6]. Briefly, the acrylamides **5a–5d** were obtained starting from substituted anilines under nitrogen atmosphere in the presence of acrylic chloride. The desired final products (**7a–7d**) were obtained to the dipolar cycloaddition reaction of the acrylamides intermediates and compound **6**. This latter was prepared by using a reported synthetic approach [15].

The synthesis of substituted anilines **3c** and **3d** (Scheme 2) started from the commercially available compound **8** that was converted in 1-bromo-2,3,5,6-tetramethyl-4-nitrobenzene (**9**) by using  $\text{NaNO}_2$  in presence of a catalytic amount of trifluoroacetic acid. The nitro group of **9** was reduced under nitrogen atmosphere by using  $\text{LiAlH}_4$  to generate the corresponding aniline **3c** in a mixture. Therefore, chromatographic purification was required to obtain the pure product **3c**.

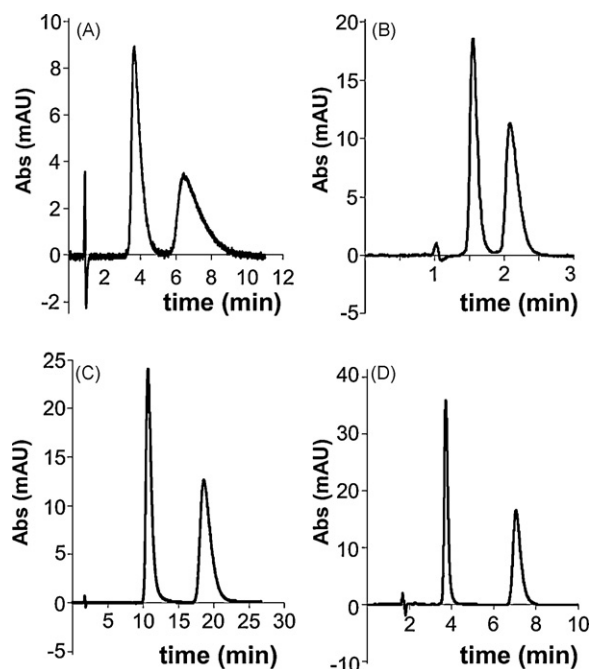
The key intermediate **9** was treated with  $\text{CuCl}$  at  $180^\circ\text{C}$  to give the corresponding chlorine derivative (**10**) that was reduced to produce the final product **3d** by following the conditions previously described to obtain compound **3d**.

Anilines **3a** and **3b** are commercially available.

Experimental details and physico-chemical properties of intermediates and final compounds here described, are reported in Supplemental Material.

## 2.3. Column preparation

The silica columns (50 mm  $\times$  4 mm i.d., and 150 mm  $\times$  4.6 mm i.d.) packed with Kromasil 200 Å (5  $\mu\text{m}$ ) were silanized by an *in situ* process as previously reported [16]. In brief, the packed silica gel column was dried at  $150^\circ\text{C}$  for 6 h under helium. After cooling, the column was flowed with dry toluene (30 mL) at 1 mL/min. The column was then heated to  $110^\circ\text{C}$ , and derivatized *in situ* by pumping through a toluene solution of 3-glycidypropyltrimethoxysilane (5 mL in 45 mL of toluene) at 0.5 mL/min to give an epoxide silica column. The column, after cooling, was then washed with anhydrous toluene (20 mL) and anhydrous dioxane (80 mL).



**Fig. 2.** Chromatographic enantioresolution on the HSA-based column 50 mm × 4 mm i.d. of *rac*-warfarin (0.01 mg/mL) (a) and N-benzoyl-DL-leucine (0.005 mg/mL) (b). Mobile phases were PB (pH 7.4; 67 mM)-1-propanol (97:3, v/v) in the case of *rac*-warfarin and PB (pH 7.4; 67 mM)-1-propanol (99:1, v/v) in the case of N-benzoyl-DL-leucine. Chromatographic enantioresolution on the HSA-based column 150 mm × 4.6 mm i.d. of *rac*-warfarin (0.05 mg/mL) (c) and N-benzoyl-DL-leucine (0.01 mg/mL) (d). Mobile phase was PB (pH 7.4; 67 mM)-1-propanol (90:10, v/v) for both samples. The runs were monitored at  $\lambda = 308$  nm (*rac*-warfarin) and at  $\lambda = 228$  nm (N-benzoyl-DL-leucine).

HSA was covalently immobilized on the packed epoxy-silica columns by the reported [17,18] online procedure, with slight modifications. In brief, a HSA solution (10 mg/mL of protein in 50 mM PB, pH 6.5, 1 M ammonium sulphate) was circulated through the epoxy-silica columns in closed circuit for 24 h. The columns were then washed with 120 mL of 50 mM PB, pH 7.4; and 100 mL of 1 M glycine dissolved in PB. The two HSA-based columns (50 mm × 4 mm i.d. and 150 mm × 4.6 mm i.d.) were employed for the determination of the HSA bound fraction and for the displacement studies, respectively.

#### 2.4. Method evaluation

To rule out any effects on the binding properties of HSA by the immobilization procedure, two of racemates, known to bind HSA enantioselectively, were injected into the column. *Rac*-warfarin, and N-Benzoyl-DL-leucine were selected. Stock solutions of each racemate were prepared as 1 mg/mL solutions in 1-propanol, and then diluted to the appropriate concentration with PB (pH 7.4; 67 mM). The chromatographic runs were performed at a flow rate of 1 mL/min, injecting 20  $\mu$ L of the analytes (see caption to Fig. 2 for more details).

#### 2.5. Affinity chromatography: bound fraction determination

Stock solutions of tocinide and its analogues were prepared at a concentration of 1 mg/mL in 1-propanol and were diluted 10-fold with PB (pH 7.4; 67 mM) just before injection into the HSA column. The mobile phase was composed of PB (pH 7.4; 67 mM), and the flow was set at 1 mL/min.

The bound drug fraction ( $B\%$ ) was determined as the measured capacity factor, according to a previously reported method [19]

$$B\% = 100 \left( \frac{k}{k+1} \right) \quad (1)$$

#### 2.6. Affinity chromatography: displacement experiments

A series of displacement experiments were carried out to have information on the binding site(s) of tocinide and of its analogues. Increasing amounts of the markers for the three major binding sites of HSA were added to a mobile phase containing PB (pH 7.4; 67 mM)-1-propanol (97:3, v/v). Sodium salicylate, *rac*-ibuprofen and sodium valproate were used as markers of Sudlow's site I, II and the bilirubin site, respectively. The concentration of the marker in the mobile phase ranged from 0 to 100  $\mu$ M for sodium salicylate, from 0 to 20  $\mu$ M for *rac*-ibuprofen and from 0 to 5 mM for sodium valproate.

All the compounds were injected at the same concentration used for the determination of the bound fraction described in Section 2.5. The solutions of tocinide and its analogues were injected and the capacity factors of the drugs were determined in the absence and in the presence of increasing concentrations of the competitors. The plotting of the reciprocal of the capacity factor ( $1/k$ ) versus the molar concentration of the marker gives information on the interaction mechanism. A direct competition at a single site gives a straight line with a positive slope in this plot, while a non-competitive relationship gives only random variations [10,11,20,21].

The following linear equation is satisfied in the case of direct competition, where the analyte binds to a single binding site:

$$\frac{1}{k_A} = \frac{V_M K_I [I]}{K_A m_L} + \frac{V_M}{K_A m_L} \quad (2a)$$

where  $k_A$  is the measured capacity factor of the analyte A,  $V_M$  is the column void volume,  $K_I$  is the affinity constant of the competitor for the single binding site of A,  $[I]$  is the molar concentration of the competitor,  $K_A$  is the association equilibrium constant of A for its binding site, and  $m_L$  is the total moles of analyte binding sites in the column. This last parameter,  $m_L$ , corresponds to the protein moles correctly anchored to the silica matrix.

The equilibrium dissociation constant ( $K_D$ ) of the displacer for the single binding site of the analyte can be then calculated by linear fitting of the data.

In particular,  $K_D$  can be obtained as

$$\frac{\text{intercept}}{\text{slope}} = \frac{1}{K_I} = K_D \quad (2b)$$

Multisite binding determines deviation from this linear behaviour.

#### 2.7. Circular dichroism: displacement experiments

Circular dichroism measurements were performed with a Jasco J-810 spectropolarimeter. The displacement experiments were carried out using tocinide and compound 7 as the competitors, and phenylbutazone, diazepam and bromophenyl blue as selected markers for Site I, Site II and bilirubin binding site, respectively. The change in the CD signal after addition of different amounts of tocinide and compound 7 to a solution containing equimolar concentrations of HSA and marker [15  $\mu$ M in PB (pH 7.4; 67 mM)] were monitored. Stock solutions of tocinide and compound 7 (1 mM in 1-propanol), of diazepam, phenylbutazone and bromophenyl blue (15 mM in 1-propanol), and of HSA (150  $\mu$ M in PB, pH 7.4, 67 mM) were prepared immediately before the experiments. All the measurements were carried out using a 10 mm path length cell. The working solutions were prepared mixing the HSA, marker and displacers at molar ratios of 1:1:0, 1:1:1, 1:1:2, 1:1:4, 1:1:6 and 1:1:8, respectively.

**Table 1**  
Bound fraction (B%) obtained by affinity chromatography compared with literature data, and predicted log *P* values.

Sample	B% <sup>a</sup>	miLog <i>P</i> <sup>b</sup>	B% from literature <sup>c</sup>
1	89.1	2.494	
2	94.5	2.799	
7	97.1	2.912	
7a	97.7	3.047	
7b	98.3	3.725	
7c	98.1	3.689	
7d	99.3	4.295	
Tocainide	25.4	0.187	10 ± 15
R-Warfarin	98.3		99 ± 1
S-Warfarin	98.7		
Flunitrazepam	85.3		77–79
Diazepam	93.5		98.7 ± 0.2
Nitrazepam	85.2		87 ± 1

<sup>a</sup> Calculated as the average of the results obtained by two or three injections. Standard deviations of all the calculated B% are always ≤0.11.

<sup>b</sup> Calculated using online [www.molinspiration.com/cgi-bin/properties](http://www.molinspiration.com/cgi-bin/properties) site (Molinspiration Property Engine v2009.01).

<sup>c</sup> In whole plasma [22].

### 3. Results and discussion

The reliability of the prepared HSA-based columns for affinity chromatography studies was checked by injecting racemates of molecules known to bind the protein in an enantioselective manner to verify if the protein, once anchored to the silica matrix, maintains its binding properties. As selective markers for the most important binding sites on HSA, *rac*-warfarin and *N*-benzoyl-DL-leucine, were analysed by zonal chromatography. Baseline resolution was obtained for both the markers on the two columns (Fig. 2). These results strongly support a correct anchoring of the HSA onto the columns.

#### 3.1. HSA bound fraction determination by affinity chromatography

Tocainide and its analogues were injected into the HSA covalently immobilized column. The ranking of the tocainide analogues for their HSA bound fraction was performed on the basis of the capacity factor, *k*, as determined in aqueous solution. Additional drugs, whose bound fraction to serum proteins was already known, were analysed in the same experimental conditions to check the dependability of the column for ranking the new synthesised compounds for their HSA binding (Table 1). A quite good agreement was obtained in the comparison of the bound fraction values experimentally obtained for the HSA binding and the literature data of these selected drugs.

All the tocainide analogues showed a higher affinity to HSA comparing to the parent compound (Table 1). In particular, both the constriction of the amino group into a rigid pyrrolidine moiety and the derivatization of the amino group with a benzyl group showed

to increase the binding percentage. The increasing of the lipophilicity of the xylylidic moiety also increases the affinity of this class of compounds to HSA (Table 1). This behaviour fits very well with the predicted log *P* values of the analysed compounds (Table 1). Tocainide shows a much lower predicted log *P* than the synthesised structural analogues, and the ranking of the compounds based on the log *P* parameter mirrors that one based on the bound fraction values. A quite close value for both log *P* and B% was obtained for compounds **7b** and **7c**. These data suggest that hydrophobicity is important in determining the interaction between tocainide analogues and HSA. A better insight into the HSA/drug provides an invaluable structural framework for the interpretation of drug binding data. This should facilitate efforts to modify new therapeutic compounds to optimise their distribution within the human body, by obtaining a better balance between distribution and half-life of the drug.

#### 3.2. HSA binding site investigation: displacement affinity chromatography and circular dichroism

Displacement affinity chromatography experiments were carried out by determining the capacity factor of each analyte upon increasing concentrations of selected competitors added to the mobile phase. The analysis of the chromatographic data allowed the affinity constant of the competitors (*K*<sub>1</sub>) to be determined (see Section 2). The competition studies were performed using the same system described for the bound fraction determination. The change of the capacity factor (*k*) was monitored upon addition of increasing concentrations of selected competitors into the mobile phase. The displacers were selected for their selective binding to the main binding sites on albumin, sodium salicylate (site I), *rac*-ibuprofen (site II) and sodium valproate (site III, known also as bilirubin site) (Table 2). As an example that illustrates competitive experiments, the binding characterization of compound **7** is reported in Fig. 3. Salicylate up to 100 μM and *rac*-ibuprofen up to 20 μM determined a decrease of the retention of tocainide and of its analogues. The *K*<sub>1</sub> values of the displacers were calculated for all the analysed compounds. The values obtained (Table 2) were significantly lower with respect to the reported affinity constants values for salicylate and *rac*-ibuprofen [2], then suggesting an allosteric competition, with a non-cooperative binding of tocainide and its analogues with salicylate and *rac*-ibuprofen. We can then hypothesise that both Site I and Site II are not primary binding sites for the analysed compounds. Further, very small changing of the retention was observed when valproate was used as the displacer up to 5 mM. Very low *K*<sub>1</sub> values were obtained for all the analytes with respect to the affinity constant of valproate reported in the literature [2] (Table 2). Thus, an almost independent binding should be suggested for tocainide and its analogues, and valproate, selective marker of the bilirubin binding site.

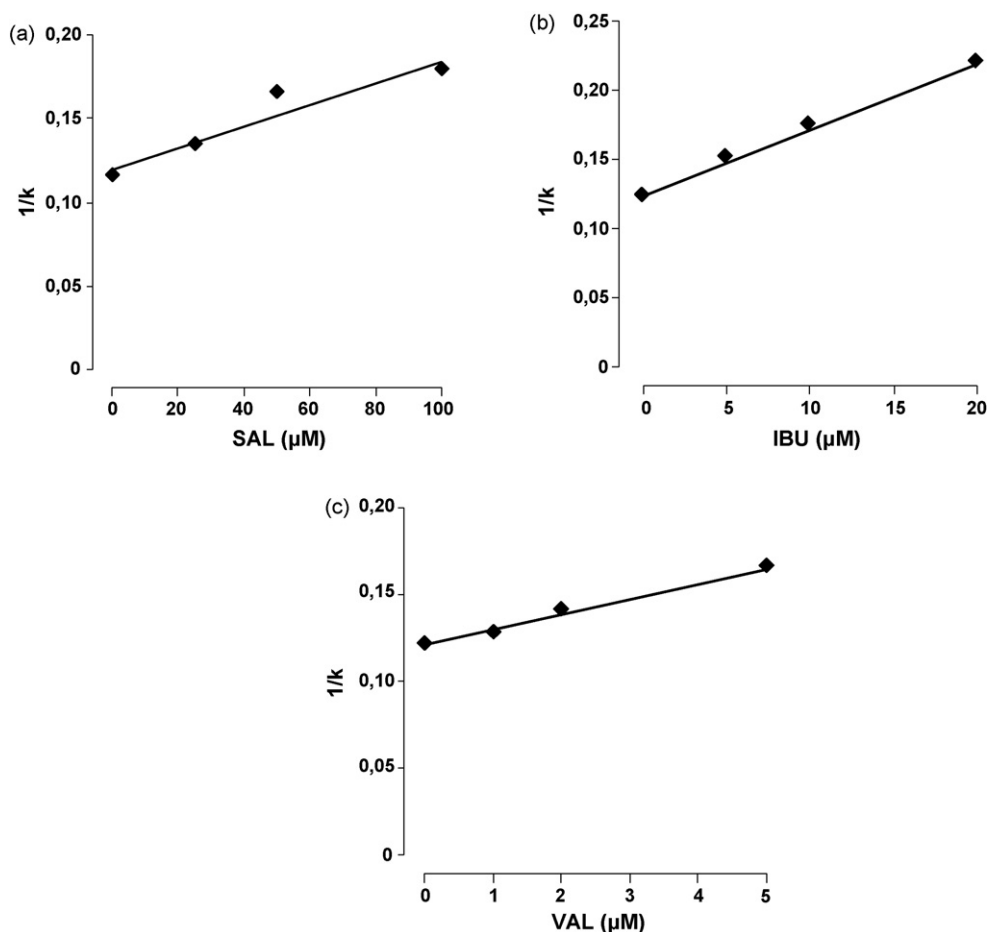
Competition experiments were also carried out by CD spectroscopy, by monitoring the change of the induced CD spectrum

**Table 2**  
*K*<sub>1</sub> values obtained by displacement chromatography analysis. The competitor affinity values were determined by slope and intercept of the 1/*k* plot of the analyte upon increasing the concentration of the competitor (see Eqs. (2a) and (2b), Section 2).

Sample	<i>K</i> <sub>1</sub> Salicylic Acid (M <sup>-1</sup> ) (Lit. 1.9 × 10 <sup>5</sup> M <sup>-1</sup> ) <sup>a</sup>	<i>K</i> <sub>1</sub> Ibuprofen (M <sup>-1</sup> ) (Lit. 2.7 × 10 <sup>6</sup> M <sup>-1</sup> ) <sup>a</sup>	<i>K</i> <sub>1</sub> Valproic Acid (M <sup>-1</sup> ) (Lit. 5.9 × 10 <sup>4</sup> M <sup>-1</sup> ) <sup>b</sup>
1	4.06 × 10 <sup>3</sup>	2.04 × 10 <sup>4</sup>	7.35 × 10 <sup>1</sup>
2	2.36 × 10 <sup>3</sup>	2.24 × 10 <sup>4</sup>	5.75 × 10 <sup>1</sup>
Tocainide	2.06 × 10 <sup>3</sup>	1.40 × 10 <sup>4</sup>	1.93 × 10 <sup>1</sup>
7	5.35 × 10 <sup>3</sup>	3.87 × 10 <sup>4</sup>	7.21 × 10 <sup>1</sup>
7c	4.62 × 10 <sup>3</sup>	3.35 × 10 <sup>4</sup>	5.93 × 10 <sup>1</sup>
7d	4.84 × 10 <sup>3</sup>	6.03 × 10 <sup>4</sup>	1.06 × 10 <sup>2</sup>

<sup>a</sup> [2].

<sup>b</sup> [23].

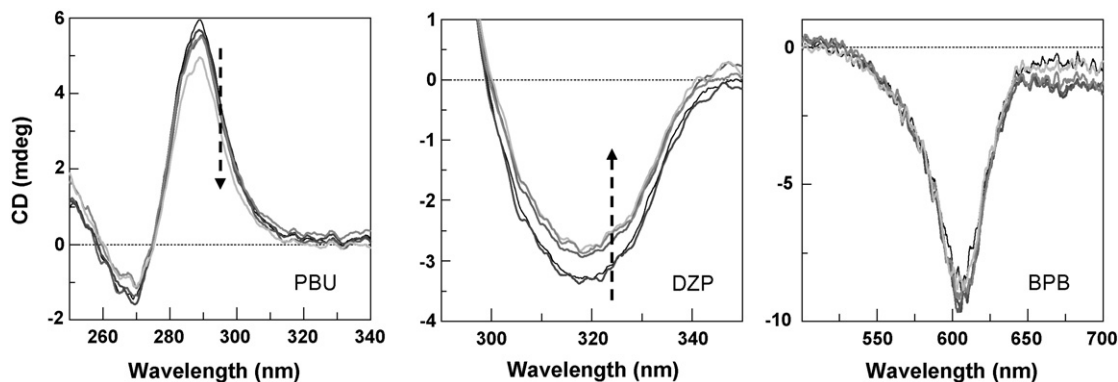


**Fig. 3.** Displacement experiments. Behaviour of  $1/k$  values of compound **7** in the absence and in the presence of increasing concentrations of salicylate (a), rac-ibuprofen (b) and valproate (c).

observed for a [HSA]/[marker] complex 1:1 (15  $\mu\text{M}$ ) in the presence of increasing concentrations of tocinide and of **7**. Compound **7** was selected as representative of the tocinide analogues. The markers employed were phenylbutazone (selective binding to site I), diazepam (selective binding to site II) and bromophenol blue (selective binding to site III). Competition experiments for the protein binding can be carried out using competitors known to bind to specific binding sites, as it was done for the affinity chromatography experiments. Alternatively the compound under investigation can be used as the competitor, and analysed against specific markers known to bind to specific binding areas. In the case of displacement

experiments using CD spectroscopy, the tocinide and its analogues were used as the competitors. The strategy of the experiment is determined by the spectroscopic characteristics of the involved compounds, being the measurements based on the monitoring of the induced CD signal in the absence and in the presence of the competitor [24]. In order to make easier the analysis of the data, the marker should present absorption and an induced CD signal at lower energy with respect to the absorption of the competitor and of the protein [24].

According to the results obtained by affinity chromatography, compound **7** resulted efficient in decreasing the induced CD spectra



**Fig. 4.** Effect on the induced CD of phenylbutazone (PBU), diazepam (DZP) and bromophenol blue (BPB), bound to HSA upon addition of increasing concentration of compound **7** to the [HSA]/[Marker] complexes. Stoichiometry of the [HSA]/[Marker]/[**7**] complexes: 1/1/0, 1/1/1, 1/1/2, 1/1/4, 1/1/6, and 1/1/8.

of both the markers at Site I and Site II (Fig. 4). No change at all was observed in the induced CD spectrum of bromophenol blue upon addition of compound 7 up to [HSA]/[marker]/[7] 1/1/8. These results support the hypothesis of a non-cooperative binding at Sites I and II, and an independent binding at Site III. Tocainide was also used as displacer. No significant change of the induced CD spectra of all the markers was observed upon adding increasing concentration of tocainide. This should be due to the much lower affinity to HSA of tocainide with respect to its structural analogues.

#### 4. Conclusions

The design, synthesis and distribution behaviour of a small series of Tocainide analogues potentially useful as sodium channel blockers were rationalised. In particular, all the compounds analysed showed a much higher affinity to HSA with respect to the parent compound tocainide. Thus the structural modifications of the drug determined an improvement of the pharmacokinetics of its analogues, giving fundamental information on the interaction protein/drug. This should facilitate the design of optimised structures showing a better balance between distribution and half-life of the drug. Both the constriction of the amino group into a rigid pyrrolidine moiety and the derivatization of the amino group with a benzyl group determined a significant increase of the bound fraction. Furthermore, the increasing of the lipophilicity of the xylididic moiety also increases the affinity of this class of compounds to the serum carrier, as supported by the good correlation between bound fraction and predicted  $\log P$  values. According to the results obtained by affinity chromatography and CD competition experiments, an anti-cooperative binding to sites I and II, and an almost independent binding for the bilirubin site can be hypothesised for the molecular recognition mechanism.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2010.03.005.

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