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Inhibition of drug binding to human serum albumin by cholecystographic agents

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Abstract

The binding of two cholecystographic agents to human serum albumin (HSA) was evaluated by means of two different complementary methodologies. In particular, the inhibition of drug HSA binding caused by iopanoic- and iophenoxic-acid was investigated by circular dichroism (CD) and resonant mirror (RM) optical biosensor techniques. The CD study allowed to obtain information both on the cholecystographic agent binding site and on the effect of the binding on the protein conformation. Iopanoic acid (IOP), a drug potentially useful for thyrotoxic disorders, resulted a direct competitor for ligands that selectively bind to site II, in agreement to literature data. No definite evidence was obtained for the highest affinity binding site of iophenoxic acid (IOPH), however, this diagnostic tool markedly affected the binding of ligands to the most characterized high affinity sites on HSA, namely sites I, II and III. Binding parameters were obtained by optical biosensor analysis: K_D values were 3.6×10^{-7} and 2.8×10^{-8} M for IOP and IOPH, respectively.

Keywords: Human serum albumin; Drug binding; Cholecystographic agents; Optical biosensor; Circular dichroism

1. Introduction

As the most abundant soluble protein in plasma, human serum albumin (HSA) plays a fundamental physiological role in the transport of drugs, metabolites and endogenous factors [1,2]. In this regard, the investigation of drug–plasma protein interactions and the consequent determination of binding parameters are of fundamental importance to understand the overall drug activity. HSA interacts with a large spectrum of compounds: this interaction is essential since it determines the free, active concentration of the drug, provides a reservoir for a long duration of action, and ultimately affects drug absorption, metabolism, distribution and excretion. Thus it is evident that binding to HSA affects the pharmacokinetic properties of a drug and contributes to determining its toxic side effects.

Binding parameters can be evaluated by various methods, including equilibrium dialysis, ultrafiltration, ultracentrifugation, spectroscopic measurements, and biochromatography [1–5]. An interesting approach comes from circular dichroism (CD), a spectroscopic technique that allows to monitor directly in solution the binding interaction: the changes of the signal due to the drug to protein complexation (induced CD) is followed by selective measurements [3,6]. The induced CD is detected, either for achiral or chiral compounds, at wavelength where the electronic transitions of the drug chromophore occur.

Optical biosensor is a relatively new methodology that allows a real-time investigation of biorecognition phenomena by mean of a bioselective sensor chip. Unlike other techniques (i.e. those involving labelling of the species into examination), optical biosensor technique has a combined ability to monitor the amount of adsorbed complex both continuously and with high sensitivity. All optical biosensors share as their sensing principle a common phenomenology that is the total internal reflection (TIR) of light at an interfacial region. BIAcore and IAsys biosensors are two commercial instruments that at the moment are mostly employed within the biochemistry field. Both devices provide a

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direct measure of the refractive index change of the surface-adsorbed layer. The BIAcore utilizes the optical phenomenon of surface plasmon resonance (SPR) [7,8] whilst the IAsys biosensor is reliant upon another form of resonance known as the resonant mirror (RM) principle [9–11]. As complementary work of a previous study on small molecules–HSA interaction [12], we here accomplished a full characterization of the HSA binding of Iopanoic acid (IOP), still marketed, and iophenoxic acid (IOPH), two oral cholecystographic agents (Fig. 1), using CD and optical biosensor techniques. These two compounds have been widely used in the past for radiological visualization of the gallbladder. The concern about oral cholecystographic agents is raising because they represent nowadays an useful alternative for treating thyrotoxicosis in special situations [13]. This makes important the characterization of their binding to serum protein carriers and the evaluation of their potential to displace bound drugs and metabolites.

2. Experimental

2.1. Materials

All solutions were made using ultra high purity water. All reagents were analytical grade and were used without further purification. HSA, essentially fatty acids free, was supplied by Sigma–Aldrich (Milan, Italy) and it was used without further purification. IOP, IOPH, phenylbutazone (PBU), *rac*-ketoprofen (rKPF), bromophenol blue and bilirubin (BIL) were purchased from Sigma–Aldrich (Milan, Italy). Diazepam (DZP) was kindly provided by Professor A. Lucacchini, Dipartimento di Psichiatria, Neurobiologia, Farmacologia e Biotecnologie, Facoltà di Farmacia, Università di Pisa, Italy.

2.2. Methods

2.2.1. Circular dichroism

CD spectra were recorded using a JASCO J-810 spectropolarimeter (JASCO, Tokyo, Japan). The instrument was interfaced to a personal computer to acquire and elaborate data. All measurements were carried out at room temperature using a 1 cm pathlength cell and with the same instrumental parameters in order to minimize errors (time constant 4 s, scan speed 20 nm/min, resolution 0.2 nm, sensitivity 20 mdeg, spectral band width 1). Solutions of the protein were prepared in phosphate buffer (pH 7.4, 50 mM). Protein and ligand solutions were prepared immediately prior to use. Competition experiments were carried out on samples prepared as a 1/1 [marker]/[protein] complexes. HSA concentration was kept constant (15 μM), while the competitor concentration (iopanoic and iophenoxic acids) was varied according to the required ratio. In the case of rKPF, the HSA concentration was 7.5 μM.

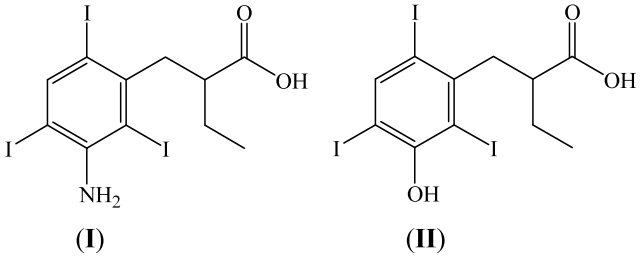

Fig. 1. Iopanoic acid (**I**) and iophenoxic acid (**II**) structures.

2.2.2. Biosensor technology

All analyses were performed using IAsys Plus optical biosensor (Labsystems Affinity Sensors, Cambridge, England). This instrument employs an open dual-well cuvette format, where the sample is added in a single step to deliver the material under investigation to the immobilized ligand. The device is supplied as a cuvette integrated with a glass prism through which the monochromatic light is directed. The instrument, when operating, permits a rapid stirring of the sample solutions by mean of a patent stirring system which leads to high solution homogeneity and to minimization mass transport to the surface. Optical biosensor was operated at 25 °C. Data were collected at 0.3 s per data point, the fastest possible data collection rate, and at 100% stirring in order to minimize mass transport effects. Biosensor data were analysed using Graph Fit v. 5.0.0.36 software and Graph Pad Prism 3.0 Software from Graph Pad Prism.

2.2.3. Immobilization of HSA and sample preparation

The protein was immobilized through its surface amine groups via amide bonds with the CM Dextran. The running buffer was PBS/T: 10 mM phosphate buffered saline pH 7.4, 0.05% Tween 20, (NaCl 136 mM, KCl 2.7 mM, 0.05% w/v Tween 20). CMD hydrogel in the cells was activated with a 1:1 EDC/NHS mixture (100 mM NHS and 400 mM EDC) for 7 min. HSA was added at a 1 mg/ml concentration in sodium acetate 10 mM, pH 5, diluted 1/10 into cell and let reacted for 7 min. Unreacted NHS-esters were blocked by washing with 1 M ethanolamine pH 8.5 for 3 min. PBS/T was added to stabilize the baseline.

Iopanoic acid stock solution was dissolved in 10 mM PBS/T and 10% of 0.01 M NaOH; IOPH stock solution was dissolved in 10 mM PBS/T. The same buffer in which the solutions were prepared was used as running buffer for the experiments. All compounds were freshly prepared as a 1 mg/ml stock solution and immediately prior to analysis were diluted with running buffer: a suitable range of concentrations was diluted into the cuvette. Addition of samples by dilution into the cuvette (usually 1–10) avoids disturbance of the baseline.

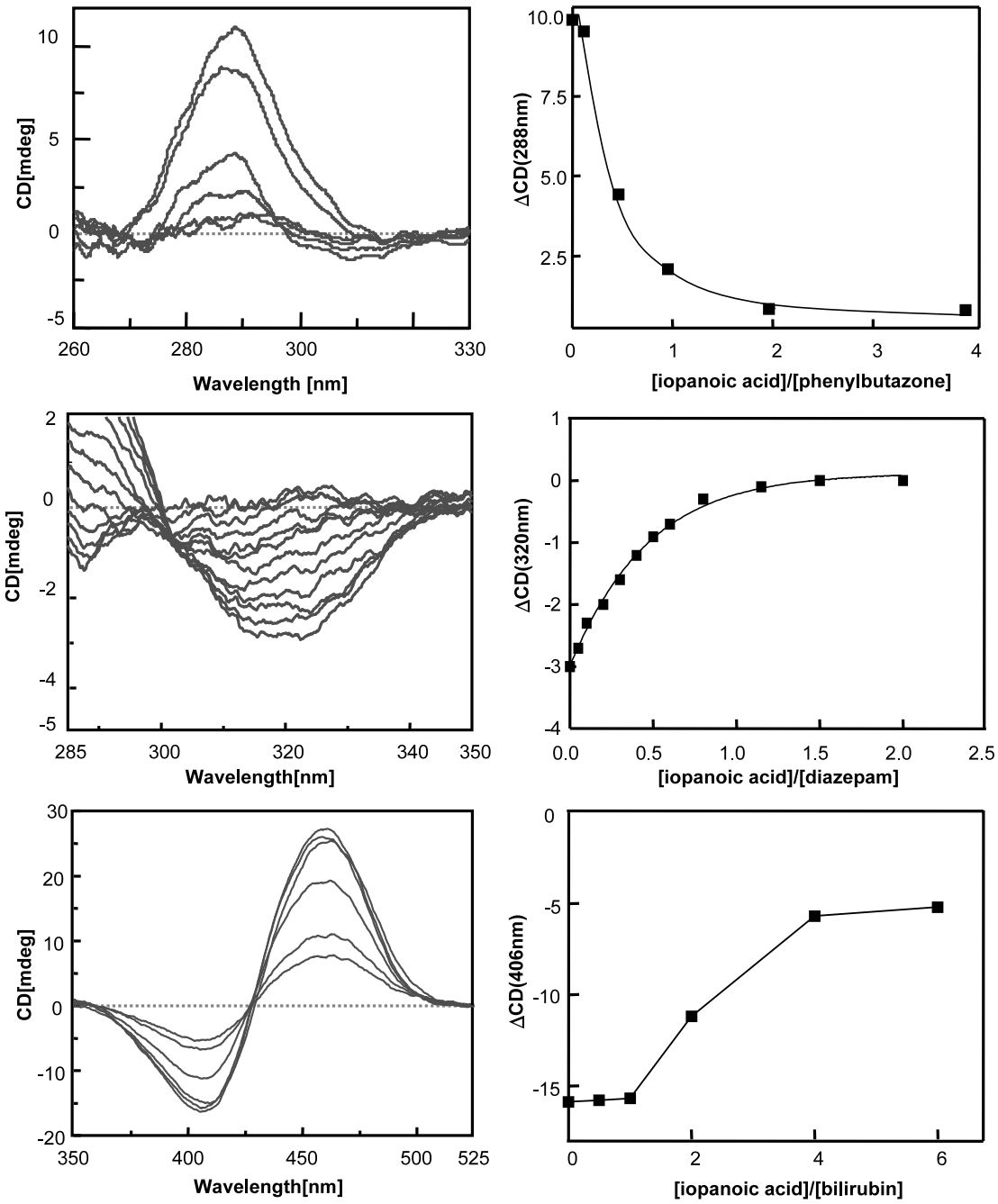

Fig. 2. Induced CD spectra of the [HSA]/[marker] 1/1 complexes in the presence of increasing concentrations of iopanoic acid as competitor ([HSA] = 15 μM in 50 mM phosphate buffer, pH 7.4; cell pathlength: 1 cm). [IOP]/[PBU] 0/1, 0.1/1, 0.5/1, 1/1, 2/1, 4/1 (upper); [IOP]/[DZP] 0/1, 0.05/1, 0.1/1, 0.2/1, 0.3/1, 0.4/1, 0.5/1, 0.6/1, 0.8/1, 1/1, 1.5/1, 2/1 (middle); [IOP]/[BIL] 0/1, 0.5/1, 1/1, 2/1, 4/1, 6/1 (lower).

3. Results and discussion

3.1. Characterization of the cholecystographic agents binding to HSA by CD study

CD can be employed to characterize the binding of drugs and of small organic ligands to serum proteins, by analysing the contribution that can be generated upon their binding [3,6,14–17]. The induced CD signal arises only from the complexed ligand and it selectively reflects a stereoselective binding phenomenon. Alternatively, the study can be carried out looking at the interaction of the ligand with markers that are known to bind to specific binding areas on the protein. This can be easily obtained by measuring the change of the induced CD signal of the HSA/marker complex in the presence of an increasing amount of the compound under investigation, which acts as an interacting or competing drug. The induced CD can be observed at the wavelengths where the electronic transitions of the ligand chromophores occur.

Experimentally this contribution is obtained by subtracting the CD of the components from those of the HSA/ligand complexes.

3.1.1. Iopanoic acid displacement experiments

Phenylbutazone, DZP and BIL were chosen as selective markers for the three most characterized binding sites on HSA, i.e. sites I, II and III, according to the definition reported in literature [1,2,18]. The changes of the induced CD signal due to the bound marker were monitored in the presence of increasing concentrations of IOP as the competitor. Iopanoic acid displays an intense absorption at 300 nm, i.e. in the spectral region where induced CD signal is observed for the HSA/marker complex (260–350 nm), when PBU and DZP are the markers. However, by conducting binding experiments with IOP as the ligand, we observed that there was no induced CD signal even at high IOP excesses ([HSA]/[IOP] up to 1/10 as ratio value). Values higher than 10 were not used, due to experimental limits as consequence of the compound isotropic absorption. Assuming the CD contribution of IOP negligible, we then proceeded with displacement experiments. The induced signal due to the [HSA]/[PBU] 1/1 complex (site I) decreased markedly in its intensity upon an increase of the IOP concentration (Fig. 2). The CD signal at 288 nm was almost negligible for a [PBU]/[IOP] 1/4 molar ratio. This behaviour suggested an anticooperative binding, following the definition by Honoré [19]. The induced signal due to the [HSA]/[DZP] interaction (site II) was completely brought to zero by a 1.5 excess of IOP suggesting a direct competition with DZP for the binding to site II, in agreement to literature data [20]. As a matter of fact, a K_{aff} of 4.8×10^6 M^{-1} was determined for IOP when acting as competitor, a value that reflects its affinity to the primary binding site. Peculiar behaviour was registered for binding to site III, using BIL as marker. The induced CD signal did not change significantly until a 1/2 [BIL]/[IOP] molar ratio, whilst it decreased (in absolute value) markedly for higher ratios (Fig. 2). This suggested that site III is not the highest affinity site for IOP, but high concentrations of IOP can displace efficiently BIL. Thus an anticooperative binding can occur for the IOP/BIL interaction, but an almost independent binding resulted at low [IOP]/[BIL] molar ratios. Alternatively, at high ratio values it could be possible a conformational change of the protein leading to a deep modification of the binding site properties. To verify this hypothesis, the ordinate structure of HSA was studied with high energy CD experiments (180–260 nm). However, the CD spectrum of the protein did not change for a [IOP]/[HSA] molar ratio up to 10 (Fig. 3).

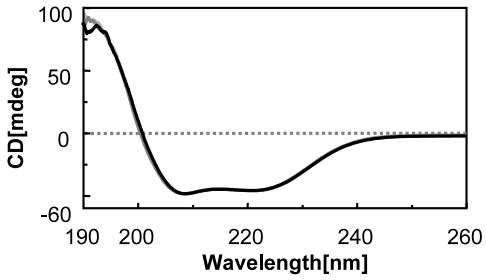

Fig. 3. Superimposed CD spectra of HSA in the absence and in the presence of iopanoic acid: [IOP]/[HSA] 0/1, 5/1, 10/1.

3.1.2. Iophenoxic acid displacement experiments

Iophenoxic acid was first characterized for its spectroscopic properties. Electronic absorption spectrum of IOPH showed a band about 320 nm and, in the same spectral region, a negative CD band was observed in the presence of HSA (Fig. 4). For a fixed concentration of protein, the induced CD contribution increased reaching a constant value for [IOPH]/[HSA] molar ratio higher than 1. Such a signal was obviously taken into consideration when performing CD analysis with IOPH as the competitor compound. In order to get a neat CD signal variation, markers were accurately selected having an induced CD at lower energy with respect to that observable with IOPH. In particular, bromophneol blue

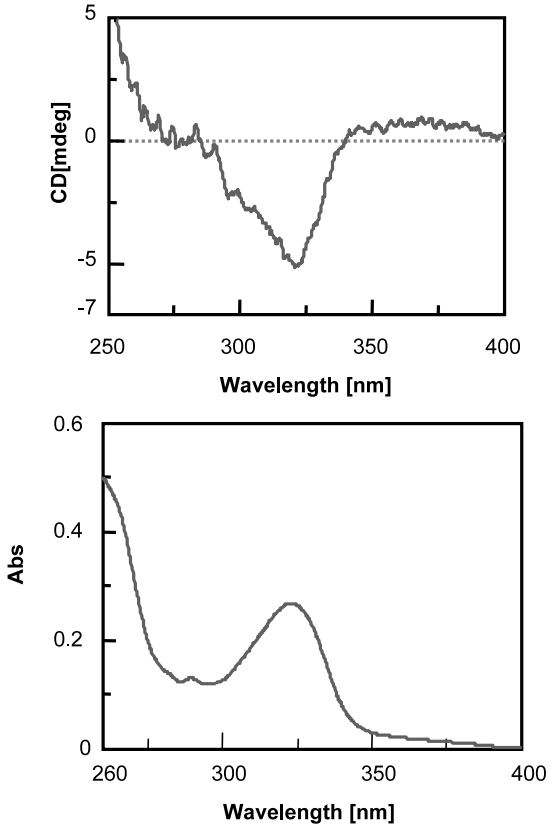

Fig. 4. Induced CD (upper curve) and UV (lower curve) of iophenoxic acid bound to HSA ([IOPH]/[HSA] 2/1, [HSA] 15 μM).

(BPB), rKPF and BIL were selected as markers for site I, II, and III, respectively. The induced CD spectrum for the [BPB]/[HSA] 1/1 complex (Fig. 5) decreased (absolute value) when IOPH concentration was increased. However, the displacement phenomenon was significant only for [IOPH]/[BPB] molar ratios higher than 1, as shown in Fig. 5 where a plot of CD signal variation at 606 nm versus [IOPH]/[BPB] ratio is represented. In contrast the induced CD spectrum due to the HSA bound IOPH was concentration dependent for [IOPH]/[HSA] molar ratio up to 1, being almost constant at higher ratios. This behaviour suggested a two sites interaction, being stereoselective the highest affinity site of IOPH, and not affecting to a large extent the affinity of BPB to HSA. At higher concentrations IOPH binds to a second site, affecting markedly the binding of

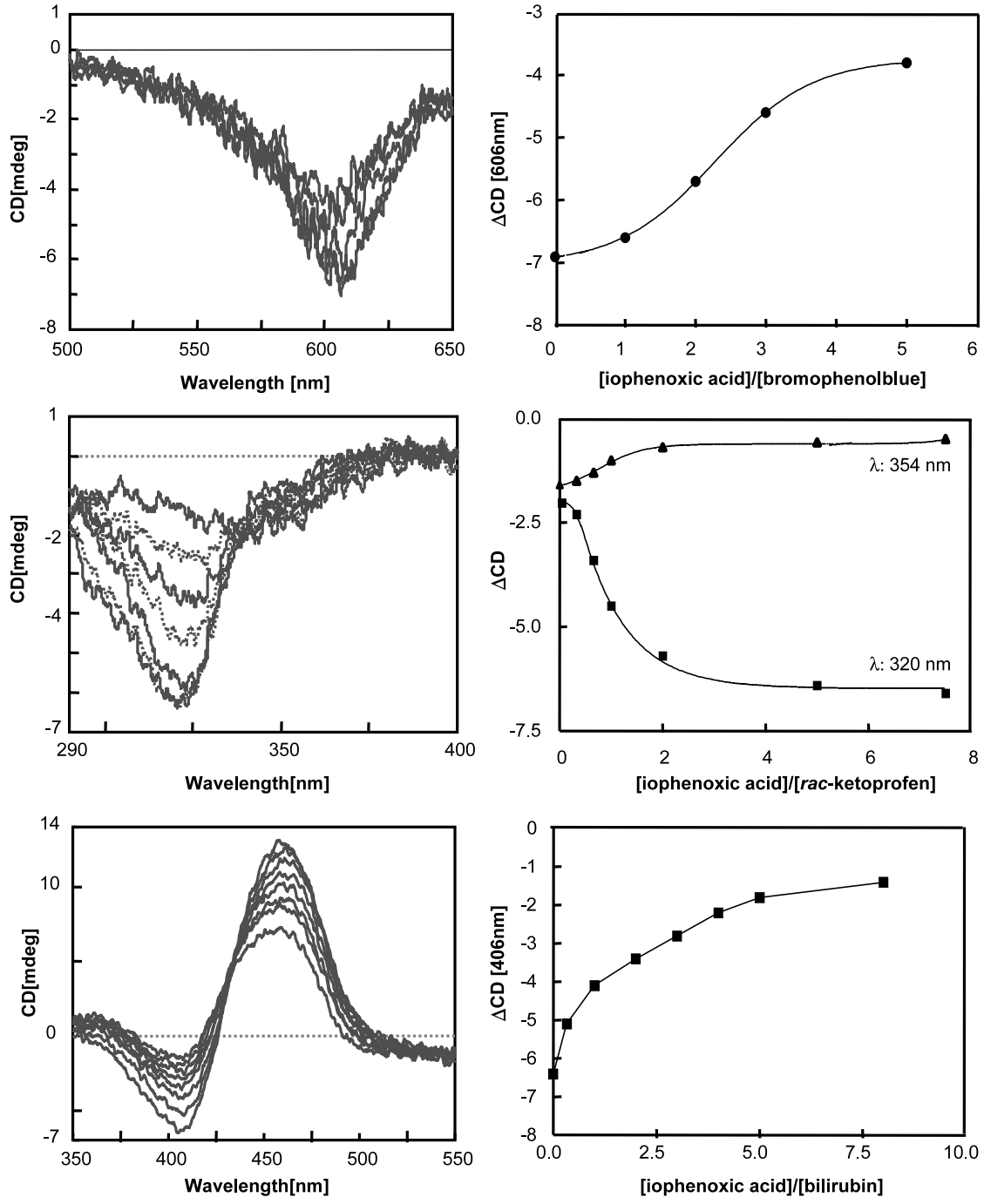

Fig. 5. Induced CD spectra of the [HSA]/[marker] 1/1 complexes in the presence of increasing concentrations of iophenoxic acid as competitor. ([HSA] = 15 μM in the cases of BPB and BIL complexes and 7.5 μM for the rKPF complex in 50 mM phosphate buffer, pH 7.4; cell pathlength: 1 cm). [IOPH]/[BPB] 0/1, 1/1, 2/1, 3/1, 4/1 (upper); [IOPH]/[rKPF] 0/1, 0.33/1, 0.66/1, 1/1, 2/1, 5/1, 7.5/1 (middle) [▲▲▲▲ induced CD of bound rKPF monitored at 354 nm, ■■■■ induced CD of bound IOPH monitored at 320 nm]; [IOPH]/[BIL] 0/1, 0.5/1, 1/1, 2/1, 3/1, 4/1, 5/1, 8/1 (lower).

BPB at site I. This behaviour is not in agreement to literature data [1,20], being IOPH expected to bind to site I as primary binding site. Thus the competition experiments were carried out also with a different site I marker, i.e. PBU, in order to support the obtained results. Also using PBU as marker, the displacement experiments exhibited two distinct phases, the first one being related to the binding of IOPH to its highest affinity binding site and the second one when IOPH inhibited the binding of PBU to site I. Indeed IOPH resulted efficient as competitor only when binding to a non-stereoselective binding site, i.e. when the induced CD contribution was constant upon increasing the [IOPH]/[HSA] molar ratio (Fig. 6). Thus the possible mechanism of binding was confirmed and, on the bases of these observations, we suggested an almost independent binding at low concentration of the competitor and an anticooperative binding for [IOPH]/[PBU] molar ratios higher than 1.

A similar behaviour was observed in the case of rKPF, used as marker for site II on HSA. Indeed the induced CD signal of the bound IOPH augmented by increasing its concentration, reaching a plateau for [IOPH]/[HSA] molar ratio higher than 1 (Fig. 5). In these conditions, the presence of IOPH did not affect markedly the affinity of rKPF, while a significant decrease of the induced CD due to HSA bound rKPF was observed at higher [competitor]/[marker] molar ratio values. However, a signal, even if very low in its intensity, was still observable at high [IOPH]/[rKPF] molar ratios. Once again we can suggest an independent binding and an anticooperative binding at low and high [competitor]/[marker] molar ratios, respectively. Finally, IOPH was proved as competitor with BIL, as marker for site III. A concentration dependence was observed with a decrease of the induced CD signal of the 1/1 [BIL]/[HSA] complex in the presence of increasing concentrations of IOPH (Fig. 5). This behaviour clearly suggests an anticooperative binding at all the [IOPH]/[BIL] molar ratios.

3.2. Affinity constants determination by optical biosensor (IAsys) analysis

IAsys is based on the RM principle [9,10]: a RM is integrated with a micro-cuvette device and permits to follow the optical phenomenon of the evanescent wave. Changes in refractive index at the surface of the device, or the biological layer, modify the angle at which light can be made to propagate in the waveguide: conditions for propagation are met at only one discrete angle (resonant angle). The change in angle is linear with respect to the mass.

3.2.1. IAsys experiments

A typical IAsys experiment of drug binding requires the protein (ligand) immobilized on the biosensor cuvette surface followed by different analyte (ligate) concentration adds. For HSA immobilization and binding experiment cycles details refer to previous results [12]. Iopanoic acid and IOPH were investigated for their reversible binding to the serum carrier. A similar behaviour was observed for both contrast agents: a steady state was reached in a few seconds, after the drug was added to the cuvette over the HSA modified CMD surface. Both drugs dissociated very rapidly when the ligate solutions were replaced by running buffer: this behaviour is elucidated in Fig. 7 where overlaid sensorgrams are reported at different IOP concentration values (2.4×10^{-8}–2.4×10^{-6} M). As dissociation process was practically immediate after running buffer washes, we studied only the association

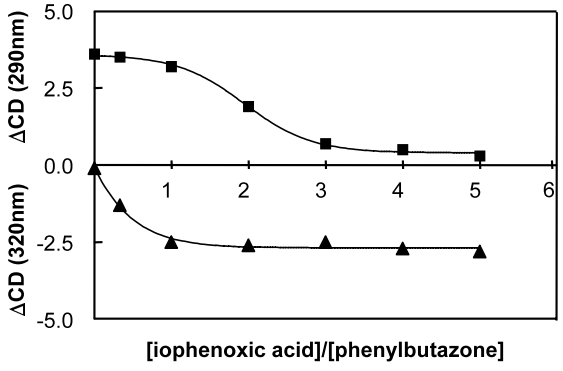

Fig. 6. Induced CD spectra of [HSA]/[PBU] 1/1 complexes in the presence of increasing concentrations of iophenoxic acid ([HSA] = 15 μM in 50 mM phosphate buffer, pH 7.4; cell pathlength: 1 cm). [IOPH]/[PBU] up to 5/1 [■■■■ induced CD of bound PBU measured at 290 nm; ▲▲▲▲ induced CD of bound IOPH measured at 320 nm].

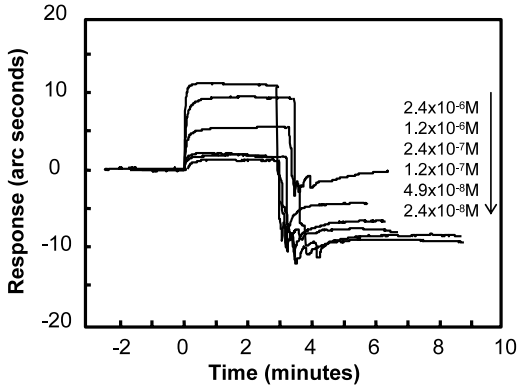

Fig. 7. Sensorgram overlays obtained at reported iopanoic acid concentration (from 2.4×10^{-8} to 2.4×10^{-6} M).

process: we considered the observed steady state value (at a selected time, i.e. always after 90 s of the binding process) at each concentration to be the R_{eq} (response at equilibrium) value. The values were corrected by subtracting the reference channel (simply activated CMD surface) response, in order to minimize the a specific binding contribution to the biosensor matrix, and subsequently plotted against concentrations (Fig. 8A). In order to estimate the two compound affinity for the plasma protein, we assumed that HSA binding sites were equally available and that at very low concentration values, as the ones tested, only the high affinity site was populated. With this assumption we analysed the binding events by mean of the binding isotherm equation in which, if the curve has a Lamgmuirian behaviour (1:1 interaction), K_D can be directly calculated as the ligate concentration at 50% of $R_{max.}$ value. Data were fitted by means of GraFit software and the highest affinity binding site equilibrium dissociation constants were evaluated to be: $K_{D(IOP)}$ 3.7×10^{-7} and $K_{D(IOPH)}$ 2.2×10^{-8} M. The same sets of data were analysed using Scatchard regression method (Fig. 8). In the Scatchard analysis, a plot of R_{eq}/[L] versus R_{eq} yields a slope of—K_A with an x axis intercept of $R_{max.}$. The y intercept is equivalent to $K_A R_{max}$ (Fig. 8B). By this approach we obtained the following parameters: $K_{A(IOP)}$ 2.8×10^6 M^{-1} (r^2: 0.914); $K_{A(IOPH)}$ 3.6×10^7 M^{-1} (r^2: 0.937), which are in accordance with those previously reported [12,20].

4. Conclusion

CD and optical biosensor methodologies allowed a better characterization of the binding properties of the two cholecystographic agents to HSA. CD allowed to identify the high affinity binding site on HSA for the cholecystographic agents under investigation. IOP has been proved to act as direct competitor for site II ligands, in agreement to literature data. On the contrary, IOPH inhibited the binding of site I, II and III ligands, but it did not act as a direct competitor for site I markers, as previously reported in the literature.

Biosensor resulted a rapid and efficient system for monitoring, in real-time, the binding of the two contrast agents to the plasma protein leading to informative results without need of labelling. The investigation of small molecule–macromolecule interactions by RM biosensor is a relatively new approach. The low refractive index increment observed with small ligands has to be considered a limit of the technique, however, the obtained value of K_D obtained for the two analytes are in agreement with those obtained by independent techniques, suggesting the biosensor as a suitable tool

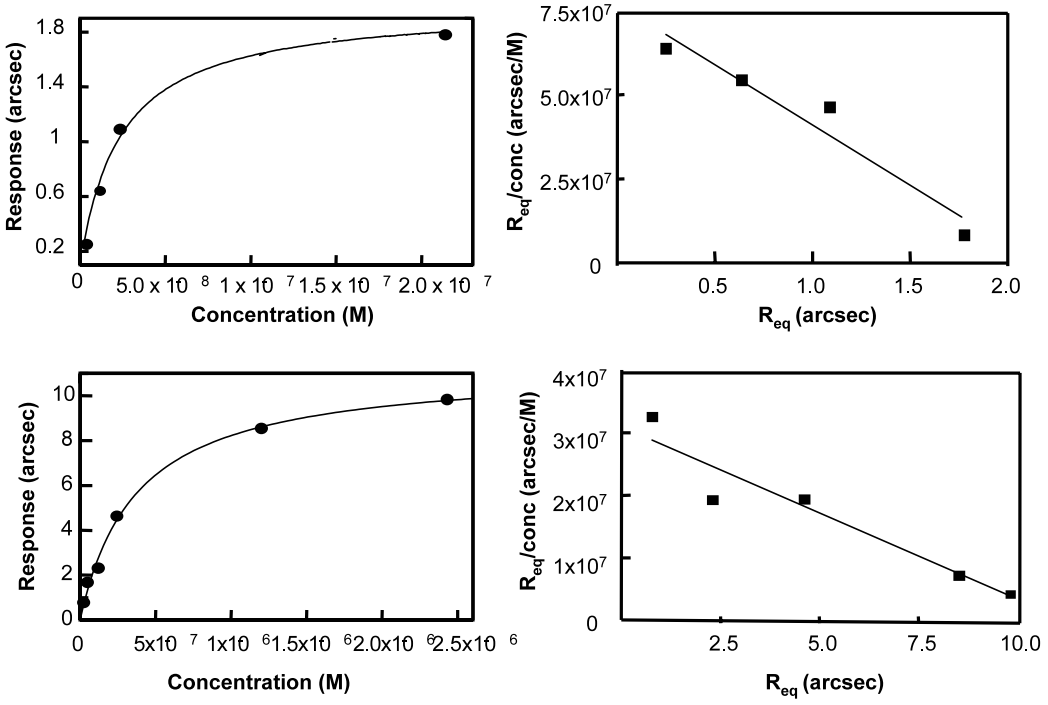

Fig. 8. (A) Iophenoxic acid (left up plot) and iopanoic acid (left down plot) binding isotherms; (B) Iophenoxic acid (right up plot) and iopanoic acid (right down plot) Scatchard plots.

for the screening of drugs based on their binding to target proteins.

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