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Journal of Chromatography A, 736 (1996) 115–123

JOURNAL OF CHROMATOGRAPHY A

Isolation by gel-permeation chromatography of a non-covalent complex of Cibacron Blue F3G-A with human serum albumin

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Received 15 September 1995; revised 12 December 1995; accepted 18 December 1995

Abstract

The isolation by gel-permeation chromatography on Sephadex G-100 of a non-covalent complex of Cibacron Blue F3G-A (CB) with human serum albumin (HSA) is described. The complex presents a molar ratio of 3:1 CB–HSA and can be re-chromatographed under the same conditions without modification of its composition. However, complete dissociation occurs when the complex is chromatographed in the presence of denaturing agents. The effect of pH on the molar composition of the complex was also investigated by gel-permeation chromatography. Analogous complexes between CB and A and C cyanogen bromide fragments of unreduced HSA were also isolated by gel-permeation chromatography on Sephadex G-50. They present a molar ratio of 0.8:1 and 1.3:1 CB–protein for fragments A and C, respectively. These results suggest that two of the three molecules of CB bound to HSA may be located in the hydrophobic pocket corresponding to subdomain IIA, with the other molecule in the hydrophobic site corresponding to subdomain IIIA. The UV–Vis and dichroic circular spectra of the isolated complexes are reported.

Keywords: Cibacron Blue F36-A; Albumin; Proteins; Dyes

1. Introduction

Human serum albumin (HSA) is the most abundant protein in the circulatory system. It consists of a single, non-glycosylated, polypeptide chain containing 585 amino acid residues and has many physiological functions. However, the most peculiar property of this protein is its unique ability to bind many endogenous and exogenous ligands, which range from inorganic cationic species to a large variety of organic molecules [1].

Among others, HSA interacts specifically and reversibly with Cibacron Blue F3G-A (CB), a textile dye commonly used in dye–protein affinity chromatography because of its ability to bind with apparent specificity to several proteins [2,3]. This property has been largely used for isolating HSA from human plasma, but the interaction mechanism is not fully understood. Elucidation of the interaction mechanism of HSA with ligands, and in particular with CB, is of interest because this knowledge may provide the basis for the design of better ligands for affinity chromatography.

We have been interested in the investigation of the HSA–CB interaction mechanism and have previously shown that, among three large cyanogen bromide

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0021-9673/96/$15.00
SSDI 0021-9673(95)01362-8

fragments of HSA, fragment B (sequence 1–123) does not interact with the dye immobilised on Sepharose CL-6B, whereas the binding properties of fragments A (sequence 299–585) and C (sequence 124–298) are similar to those of HSA [4].

We report here the isolation, by gel-permeation chromatography, of stable, non-covalent, complexes formed by HSA and CB and by cyanogen bromide fragments A and C of unreduced HSA with CB.

2. Experimental

HSA (essentially fatty acid and globulin free) was purchased from Sigma (Milan, Italy). Sephadex G-100 and G-50 were provided by Pharmacia LKB (Uppsala, Sweden). CB was obtained from Fluka (Buchs, Switzerland). All other chemicals were of the highest purity commercially available and were used without further purification unless otherwise specified.

Samples were filtered on Millex-HV (Millipore, Milan, Italy). All column chromatography steps were performed using Amicon glass columns. Eluents were monitored by an HP 1050 UV detector equipped with a preparative cell and coupled with an HP 3396A integrator (Hewlett-Packard) and an automated fraction collector (Gilson 201). Circular dichroic (CD) spectra of albumin solution, isolated fragments and respective complexes with CB, were measured on a Jasco J-600 instrument, in the wavelength region 296–700 nm, at a protein or protein–dye complex concentration of $8.2 \cdot 10^{-5}$ *M* in 0.025 *M* phosphate buffer, 0.1 *M* NaCl, pH 7.0, using a path length of 1 cm.

The UV spectra were measured using a Perkin-Elmer Lambda 2S spectrophotometer.

Fragments A, B and C were obtained as described elsewhere [4].

2.1. Purification of CB

Commercial CB (150 mg) was dissolved in 50 ml of methanol. After addition of activated charcoal, the solution was stirred for 30 min and then filtered. 150 ml of diethyl ether were added to the filtrate and the resulting mixture was kept in the dark for 4 h. Pure CB which precipitated was filtered and dried in the air. The purity of the dye was checked by TLC on silica gel, using a mixture of *n*-butyl alcohol–ethyl acetate–*sec.*-propyl alcohol–water (20:10:40:30, v/v).

2.2. Isolation of the HSA–CB complex by gel-permeation chromatography on Sephadex G-100

In a typical procedure, 30 mg of HSA ($4.5 \cdot 10^{-7}$ mol) were dissolved in 10 ml of 0.025 *M* phosphate buffer, 0.1 *M* NaCl, pH 7.0. CB (5.1 mg, $6.07 \cdot 10^{-6}$ mol, corresponding to a dye–protein molar ratio of 13.5:1) was added to the solution. The solution was filtered and applied to a Sephadex G-100 column (50 × 2.2 cm I.D.) previously equilibrated with the same buffer used to prepare the sample solution. The column was eluted at room temperature using the equilibration buffer at a flow-rate of 1 ml/min and the eluate was monitored at 612 nm. Two absorbing fractions, the first corresponding to the albumin–dye complex and the second to the excess dye, were collected separately and their absorbance at 612 and 280 nm measured. The dye concentration was calculated using for the complexed dye an ε of 12 000 ± 150 at 612 nm.

The same procedure was repeated using starting solutions in which the molar excess of dye with respect to HSA was 12, 9, 4.5 and 3, respectively.

2.3. Isolation of the A- and C–CB complexes by gel-permeation chromatography on Sephadex G-50 and G-200

In order to verify the presence of aggregates of fragments A, B, C and also their possible involvement in the formation of complexes with CB, 10 mg of each fragment, corresponding to $3 \cdot 10^{-7}$ mol of A, $7.1 \cdot 10^{-7}$ mol of B and $5 \cdot 10^{-7}$ mol of C were dissolved in 5 ml of 0.025 *M* phosphate buffer, 0.1 *M* NaCl, pH 7.0. CB (1.5 mg, $1.8 \cdot 10^{-6}$ mol for A; 3.6 mg, $4.26 \cdot 10^{-6}$ mol for B; and 2.5 mg, $3 \cdot 10^{-6}$ mol for C, corresponding in each case to a dye–protein molar ratio of 6:1) was added to the solution. The solution was filtered and applied to a Sephadex G-200 column (625×3.2 cm I.D.) previously equilibrated with the same buffer used to prepare the sample solution. The column was eluted at room temperature, using the equilibration buffer at a flow-

rate of 1 ml/min and the eluent was monitored at 612 and 280 nm.

To determine the dye–protein molar ratio in the complex of fragments A and C with CB, the separation procedure was also performed on a Sephadex G-50 column (25 × 2.2 cm I.D.) under the same condition as described above, using starting solutions with different dye–protein molar ratios (see Table 2). Two absorbing fractions due to the fragment–dye complex and to the excess dye, respectively, were collected separately in each run and their absorbance at 612 and 280 nm measured. The dye concentration was calculated using for the complexed dye an ε of 12 000±150 at 612 nm.

2.4. Gel-permeation chromatography of HSA–CB on Sephadex G-100 at different pHs

A 30-mg amount of HSA and 5.1 mg of CB, corresponding to a dye–protein molar ratio of 13.5:1, were dissolved in 10 ml of one of the following buffers: 0.025 *M* sodium acetate, 0.1 *M* NaCl, pH 4.5; 0.025 *M* NaH_2PO_4, 0.1 *M* NaCl, pH 4.5; 6; 6.5; 7.0; 8.5 or 12; 0.025 *M* Tris–HCl, 0.1 *M* NaCl, pH 8.5; 0.025 *M* Na_2CO_3, 0.1 *M* NaCl, pH 10 or 10.5. The solution was filtered and applied to a Sephadex G-100 column (50 × 2.2 cm I.D.) previously equilibrated with the same buffer used to prepare the sample solution. The column was eluted at room temperature using the equilibration buffer at a flow-rate of 1 ml/min and the eluent was monitored at 612 nm. Two absorbing fractions, the first corresponding to the albumin–dye complex and the second to the excess dye were collected separately and their absorbance at 612 and 280 nm was measured. The dye concentration was calculated using an ϵ of 12 000±150 at 612 nm for the complexed dye.

2.5. Gel-permeation chromatography of the HSA–CB 1:3 complex in the presence of denaturing agents

The 1:3 HSA–CB complex, isolated as described in Section 2.2, was re-dissolved in 10 ml of 0.025 *M* phosphate buffer, pH 7, containing one of the following denaturing agents: 1 *M* sodium thiocyanate; 0.035 *M* sodium dodecyl sulfate (SDS) or 8 *M* urea. The solution was filtered and applied to a Sephadex G-100 column (50 × 2.2 cm I.D.) previously equilibrated with the same buffer used to prepare the sample solution. The column was eluted at room temperature using the equilibration buffer at a flow-rate of 1 ml/min and the eluent was monitored at 612 and 280 nm. Absorbing fractions were collected separately and their absorbance at 612 and 280 nm was measured.

3. Results and discussion

3.1. Isolation by gel-permeation chromatography of the complex between HSA and CB and the effect of pH

Investigations concerning the interaction of HSA and CB in solution have been based mainly on dialysis techniques, CD spectra, NMR spectra and, mainly, on UV–Vis spectroscopy [5,6]. A gel-permeation method for the determination of the number of dye molecules linked to the protein has also been reported, but it does not allow the isolation of the complex [7]. Isolation of protein–dye complexes has not been reported and therefore the direct characterisation of these species has not been feasible. Based on the previously described work [7], we have attempted to isolate possible complexes formed between HSA and CB using gel-permeation chromatography.

Dye–protein solutions (10 ml), containing a molar excess of dye from 3 to 13.5 with respect to the HSA content, were prepared in 0.025 *M* phosphate buffer containing 0.1 *M* NaCl, pH 7.0. The solutions were applied to a G-100 column, previously equilibrated with the same buffer and the column was eluted using the equilibration buffer, at a flow-rate of 1 ml/min. The eluent was monitored at 612 nm.

The chromatographic profile for the 9:1 dye–protein starting solution is reported in Fig. 1. For solutions in which the excess of dye with respect to the protein content was greater than 3:1, the chromatograms showed two peaks. The first eluting peak presented a retention volume identical to that of HSA, whereas the retention volume of the second eluting peak was identical to that of CB. Therefore

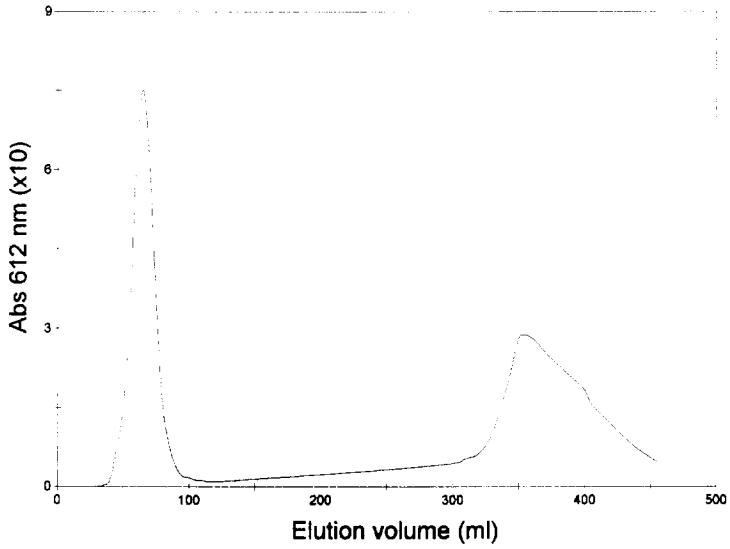

Fig. 1. Gel-permeation chromatography of CB–HSA (9:1, mol/mol) on Sephadex G-100.

the two fractions could be attributed to the dye–protein complex and excess dye, respectively.

The chromatogram of the solution in which the dye–protein molar ratio was 3:1 showed only the first eluting peak. The peak was collected and its absorbance at 612 nm was measured. It gave a value of 1.60±0.02 (Table 1), identical to that of the starting solution, from which an ϵ of 12 000±150 for the bound dye could be inferred. Under the same conditions an ϵ of 12 250±200 was determined for the free dye.

The absorbance of the fractions corresponding to the dye–protein complex, collected in the chromatograms of samples at different dye–protein molar ratios, are reported in Table 1. All values are close to that determined for the sample with a 3:1 dye–protein molar ratio, indicating that the dye–protein complex isolated at pH 7 has a constant stoichiometry of 3:1 and that this stoichiometry is independent of the excess of dye in the starting solution.

In Table 1 the absorbance ratio A_{612}/A_{280} measured in all the collected fractions corresponding to the dye–protein complex is also reported. Since only the dye absorbs at 612 nm, whereas both dye and protein absorb at 280 nm, a constant A_{612}/A_{280} ratio would indicate a constant dye–protein molar ratio in the complex. Thus, the values reported in Table 1 constitute further evidence that a constant 3:1 dye–protein complex can be isolated under these conditions.

The molar composition of the isolated 3:1 complex remained unchanged when it was chromato-

Table 1
Absorbance at 612 nm and the absorbance at 612 nm/absorbance at 280 nm ratio of fractions corresponding to CB–HSA complex collected during the chromatography of samples with different CB–HSA molar ratios

CB–HSA (mol/mol) applied sample	Absorbance at 612 nm first eluting peak	Calculated[a] CB–HSA (mol/mol)	Absorbance 612 nm/absorbance 280 nm
13.5	1.66	3.1	0.28
12	1.64	3.0	0.28
9	1.61	2.9	0.28
4.5	1.55	2.8	0.29
3	1.62	3.0	0.27

[a]Calculated using an ϵ of 12 000 for bound dye. The values reported are the average of at least three measurements.

graphed again under the same conditions. However, when the isolated 3:1 complex was chromatographed in the presence of denaturing agents, such as 1 *M* sodium thiocyanate, 0.035 *M* SDS or 8 *M* urea, the chromatograms showed two peaks, the first detectable at 280 nm corresponding to the free HSA, and the second absorbing at 612 nm corresponding to the free CB. The complete dissociation that occurred clearly indicates the non-covalent nature of the complex.

The gel-permeation technique was also used to investigate the effect of pH on the dye–protein ratio of the complex. Solutions containing 9:1 mol/mol of CB–HSA were prepared in appropriate buffer at pH 4.5, 6.0, 6.5, 7.0, 8.5, 10, 10.5 and 12 and applied to a column previously equilibrated with the same buffer used to prepare the sample. The column was eluted with the equilibration buffer at a flow-rate of 1 ml/min. The peak corresponding to the HSA–CB complex was collected and the absorbance at 612 nm was measured. The concentration of CB was calculated using an ϵ of 12 000.

The results obtained are summarized in Fig. 2. They indicate that the CB–HSA ratio has a practically constant value of 3 in the pH range 6.5–10, whereas at pHs below 6.5, the CB–HSA ratio increases to reach a value of 7.5 at pH 4.5. At pH values higher than 10 the CB–HSA ratio decreased to reach a value of about 1.9 at pH 12.

The different interactions of HSA with CB at various pHs can be probably related to the various

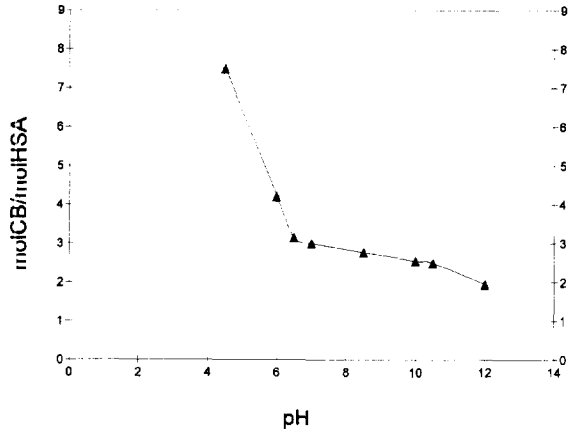

Fig. 2. Molar ratio CB–HSA as a function of pH.

transitions occurring in the HSA molecule at different pHs. It is in fact known [8] that at pH 4.5 the N form of HSA, which is present at pH 7, is converted to the F form. This transition is accompanied by a slight decrease in the helical content from 55 to 45%, but implies that about one-half of the carboxyl groups and one-quarter of the phenolic groups, which are normally masked in the N form, become exposed. This occurs as a consequence of the rupture of the ion pairs with protonated basic side chains in which these groups are involved in the N form. The resulting protonated basic residues may thus form new ion pairs with the sulfonated groups of CB. Hence, in addition of the three molecules bound to specific sites in the N form, further CB molecules may result which are linked through electrostatic forces. The slight decrease of the HSA–CB ratio observed at basic pH, instead, may be related to the deprotonation of basic residues occurring at high pH.

3.2. Isolation by gel-permeation chromatography of the complexes between cyanogen bromide fragments A and C of HSA and CB

The cleavage of unreduced HSA by cyanogen bromide gives rise to three large fragments which, according to the naming convention of McMenamy et al. [9], are called A (sequence 299–585), B (1–123) and C (124–298). Fragment C consists of a single polypeptide chain. Fragments A and B are composed of four and two subfragments, respectively, held together by disulphide bonds.

The above fragments were obtained and separated as previously described [4] and the formation of complexes between these fragments and CB was tested by gel-permeation chromatography. To detect whether the interaction of fragments A, B and C with CB involves monomeric species or aggregates, solutions of CB and fragments A, B and C in a molar ratio of 6:1 dye–protein were eluted on a Sephadex G-200 column, as previously described for HSA. The presence of aggregates is expected to be detectable under these conditions since aggregate species would be separated from the corresponding monomeric species. The chromatographic pattern of the three fragments is reported in Fig. 3. Peaks were detected at 612 nm and 280 nm.

The chromatogram of fragment B recorded at 612

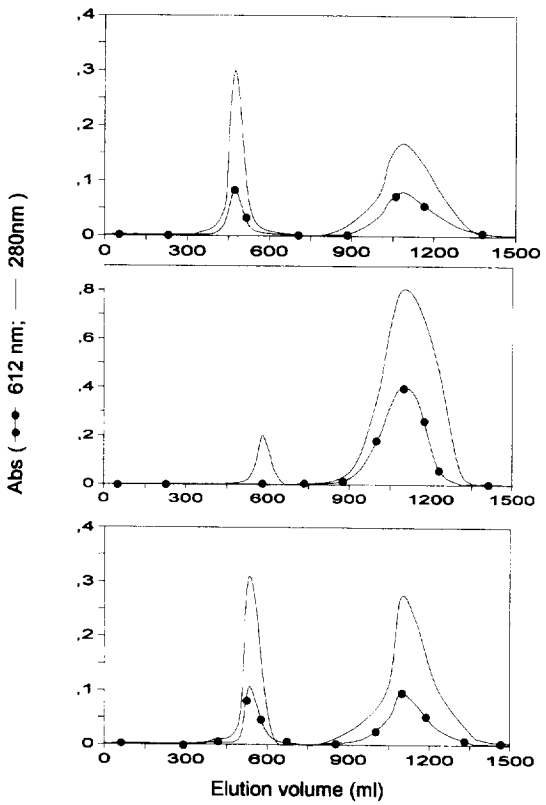

Fig. 3. Gel-permeation chromatography on Sephadex G-200 of (Top) CB–Fragment A (6:1, mol/mol); (Middle) CB–Fragment B (6:1, mol/mol) and (Bottom) CB–fragment C (6:1, mol/mol).

nm shows a single peak with a retention volume (1092 ml) identical to that of free CB. In contrast, the 280 nm trace shows two peaks, a faster eluting one having a retention volume analogous to that of fragment B (584 ml) and a second one coincident with the 612 nm peak. The first peak, which is only detectable at 280 nm, is attributable to fragment B, whereas the second one which is detectable both at 280 nm and 612 nm is due to the free dye.

The chromatogram of fragment A recorded at 612 nm and 280 nm shows, in both traces, two coincident peaks with maxima at 472 ml and 1092 ml, clearly corresponding to the CB–fragment A complex and free dye, respectively. The chromatogram of fragment C shows a similar profile with maxima for the two peaks at 536 ml and 1092 ml, respectively. From these results, it appears that fragment B does not interact with the dye, thus paralleling the behaviour already observed for this fragment with respect to CB immobilized on Sepharose CL-6B [4]. Fragments A and C are able to interact with the dye. Furthermore, the chromatographic profiles in Fig. 3 show that peaks corresponding to fragments A, B and C are single and sharp, thus indicating that aggregates are not present and that the interaction occurs between monomeric species and the dye.

In order to calculate the dye–protein molar ratio for the complexes of fragments A and C with the dye, the complexes were isolated using a Sephadex G-50 column, which permits the collection of the corresponding peak in a small volume. To this aim, solutions of CB and fragments A and C at different dye–protein molar ratios (see Table 2) were prepared and eluted on the Sephadex G-50 column, as described in Section 2.3.

From the absorbance at 612 nm of fractions corresponding to the complex of CB with fragments A and C, a dye–protein molar ratio of about 0.8:1 and 1.3:1 for fragments A and C, respectively, can be calculated. It appears that this value is independent of the dye–fragment molar ratio in the starting

Table 2
Absorbance at 612 nm and absorbance at 612 nm/absorbance at 280 nm ratio of fractions corresponding to the first eluting peak collected in the chromatograms of solutions of CB and fragments A and C on Sephadex G-50

CB–protein (mol/mol) applied sample	Absorbance at 612 nm first eluting peak		Calculated[a] CB–protein (mol/mol)		A_{612}/A_{280}	
	A	C	A	C	A	C
4.5:1	0.62	1.55	0.82	1.31	0.26	0.3
3:1	0.62	1.53	0.82	1.27	0.26	0.3
1.5:1	–	1.64	–	1.42	–	0.29
0.85:1	0.60	–	0.80	–	0.27	–

[a]Calculated using an ϵ of 12 000 for bound dye. The values reported are the average of at least two measurements.

solution. Moreover, it should be observed that chromatography of solutions with molar ratios of 0.85:1 and 1.5:1 resulted in the formation of a single 612 nm absorbing peak, corresponding to the dye–protein complex.

It is worth noting that recent X-ray investigations of the tertiary structure of HSA have concluded that the binding sites of this protein for small organic molecules are located within the subdomains IIA and IIIA [10]. Subdomain IIA is totally retained within fragment C, whereas subdomain IIIA is partially coincident with fragment A. Taking into account a partial disruption of the binding sites due to the cyanogen bromide cleavage, the finding that fragments A and C are able to bind 0.80 and 1.3 molecules of CB per molecule of fragment, respectively, suggests that two of the three CB molecules bound to HSA may interact with subdomain IIA and the third molecule with subdomain IIIA.

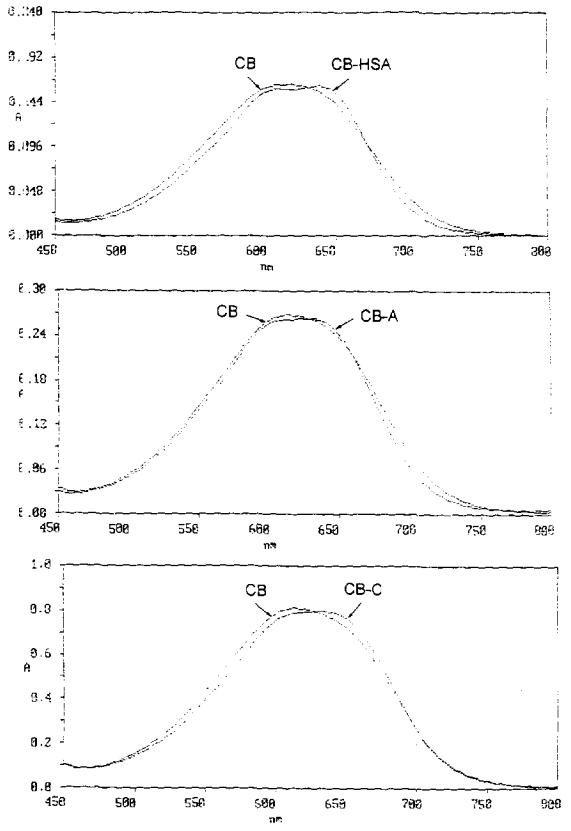

Fig. 4. Visible spectra in the region 450–800 nm of (top) CB–HSA complex; (middle) CB–A complex; and (bottom) CB–C complex in comparison with free CB.

3.3. UV–Vis and CD spectra of isolated complexes

The visible absorption spectrum of CB in phosphate buffer in the 450–800 nm range is constituted by a large band with a maximum between 610 and 615 nm [5]. The absorption spectra of the isolated complex of HSA, fragments A and C with CB are reported in Fig. 4, in comparison with that of the free dye. The spectra of the bound dye show a slight hypochromic effect and a red shift with respect to the free dye. The latter is more pronounced in the CB–HSA spectrum.

Neither HSA nor CB show a CD signal in the 300–700 nm region. However, the spectrum of the CB–HSA complex shows positive maxima at 315, 386 and 425 nm and a negative peak at 660 nm (Fig. 5). Maxima at 315 and 386 nm are also present in the CB–A spectrum, whereas the CB–C spectrum shows a negative peak at 310 nm and a positive peak at 425 nm. These data indicate that the anthraquinone chromophore interacts strongly with the proteins, giving rise to induced CD signals.

4. Conclusion

Gel-permeation chromatography allows the isolation of non-covalent complexes formed by CB with HSA and with A and C cyanogen bromide fragments of unreduced HSA. The complexes present a molar ratio of 3:1, 0.8:1 and 1.3:1 dye–protein for HSA, fragments A and C, respectively. The results also show that fragment B does not form any complex with CB. The stoichiometry of the CB–HSA complex remains practically constant in the pH range 6.5–10. Higher molar ratio values found at pHs lower than 6.5 and lower molar ratio values observed at pHs higher than 10 are probably related to structural transition occurring in the HSA molecule as a function of pH. Finally, these data suggest that two of the three molecules of CB bound to HSA interact with the binding site located on subdomain IIA, whereas the third is linked to the binding site on subdomain IIIA.

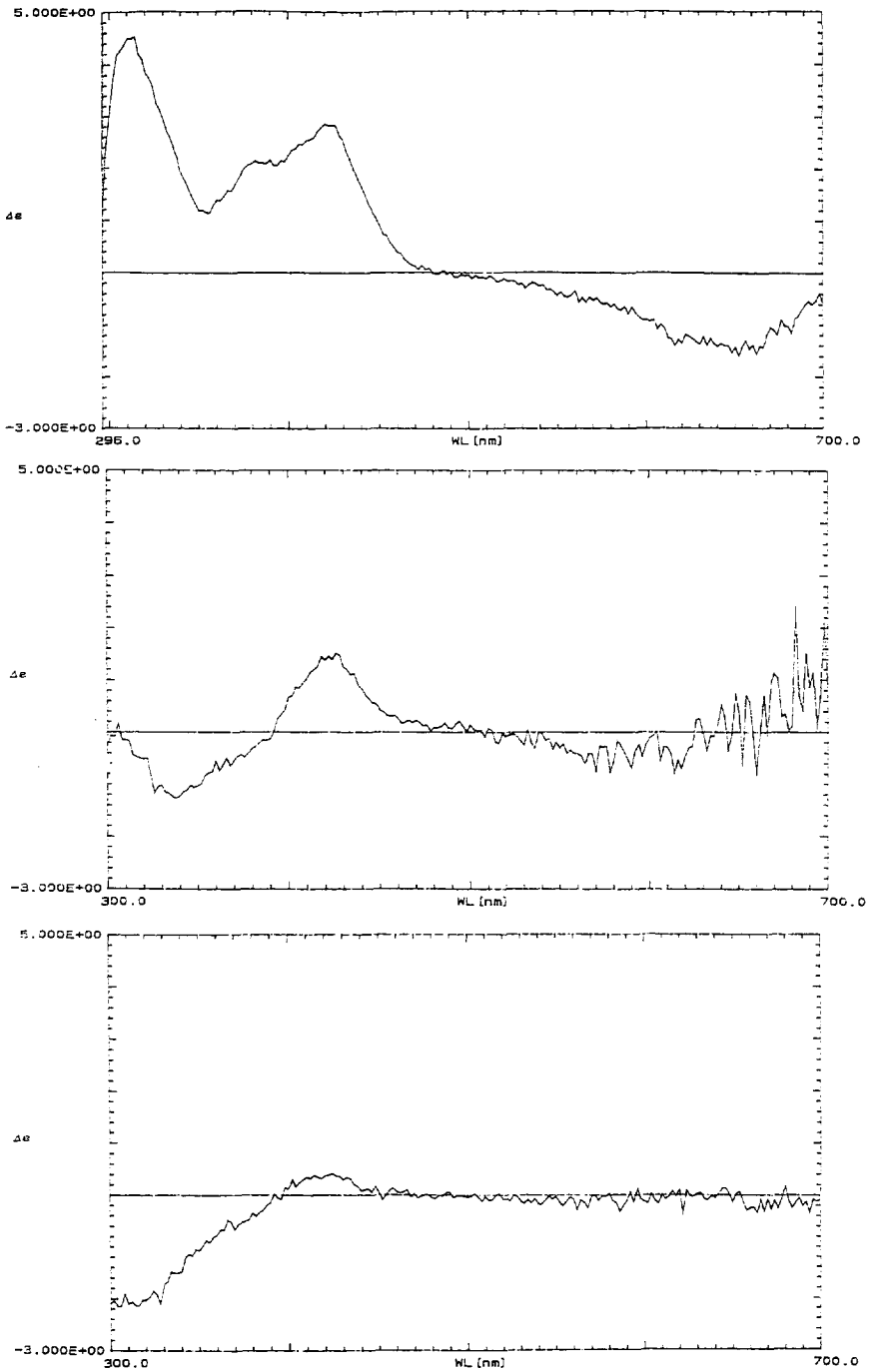

Fig. 5. Circular dichroic spectra in the region 300–700 nm of (top) CB–HSA complex; (middle) CB–A complex and (bottom) CB–C complex.

Acknowledgments

Financial support of Ministero dell' Università e della Ricerca Scientifica e Tecnologica (MURST) is gratefully acknowledged.

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