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Spectroscopic characterization by photodiode array detection of human urinary and amniotic protein HC subpopulations fractionated by anion-exchange and size-exclusion high-performance liquid chromatography

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Abstract

A procedure for spectroscopic characterization and partial fractionation of human protein HC populations by high-performance liquid chromatography–photodiode array ultraviolet–visible detection is reported. Human protein HC from urine or amniotic fluid fractionated by anion-exchange HPLC in a protein Pak DEAE 5PW appeared to be heterogeneous as judged by the asymmetric elution pattern, consisting of a continuous irregular broad peak with several shoulders distributed along the whole chromatogram. Selected fractions containing shoulders were rechromatographed and finally six symmetrical homogeneous peaks with different retention times were obtained from each protein HC preparation. The direct automatic absorption spectra analyses at each peak maximum, indicated that all of the homogeneous peaks seemed to be protein HC, all of them associated to the same chromophore although with different stoichiometry ratios. Isoelectric focusing showed that each peak was composed of a limited number of subpopulations of protein HC with different isoelectric points. Size microheterogeneity has been also demonstrated in both urinary and amniotic protein HC preparations by a combination of size-exclusion HPLC on a TSK 3000 SW6 column and photodiode array detection. Partial fractionation of human albumin on an analytical anion-exchange Mono-Q PC 1.6/5 column, has allowed the identification of heterogeneous chromophore-containing populations displaying significant absorption in the visible region in resemblance to that of protein HC.

1. Introduction

Photodiode array ultraviolet–visible (DAD-UV–Vis) detection is becoming increasingly popular for the direct detection and characterization of molecules in high-performance liquid chromatography (HPLC) due to the vast amount of information that can be obtained from a sample by using several post-experimental data processing modes. The DAD-UV–Vis HPLC system has been used for the detection and characterization of a large number of compounds, including aromatic amino acids [1], aromatic containing peptides [2,3], iodine derived [4], as well as many chromophore containing molecules [5,6] including mycotoxins and

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other fungal metabolites [7]. In the last few years, the DAD-UV–Vis HPLC detection system was also employed for the characterization of the heterogeneous yellow–brown fluorescent chromophore associated to human protein HC [3,8].

Protein HC (human complex-forming glycoprotein, heterogeneous in charge), or α1-microglobulin, is a member of the recently defined lipocalin superfamily of hydrophobic ligand-binding proteins [9,10]. Protein HC is widely distributed in body fluids in at least two molecular forms, as a free monomer and as a HC–IgA complex [11-12]. Since these two forms of protein HC are always associated to an extremely heterogeneous yellow–brown fluorescent chromophore [13,14], the DAD-UV–Vis HPLC system was also successfully used for further studies of the protein HC chromophore. In fact, the system was essential for (i) the precise localization of the chromophore in the polypeptide chain of free protein HC, being covalently linked to the cysteine residue at position 34 by a reduction-resistant bond [13], and (ii) for the implication of the chromophore in the covalent linkage between the protein HC and the IgA in the HC–IgA complex in which are also involved the cysteine residue 34 of protein HC and cysteine at the penultimate residue of the carboxyl-terminal part of one of the IgA α-chains [14].

It has been shown that the remarkable charge heterogeneity of protein HC could probably be due to the variations in structure and/or amount of its chromophore, since no evidence of sequence variability in the polypeptide chain [15] or in the carbohydrate prosthetic groups after desialylation [11] was found. Protein HC seemed to consist of a mixture of heterogeneous populations of chromophore-containing molecules as judged by the diffuse band in agarose gel [11] or the high number of bands with different isoelectric points observed in isoelectric focusing [11,16,17]. At the present, the characterization of such populations has not yet been achieved.

The present work extends earlier investigation on the protein HC chromophore to determine spectroscopic characteristics and partial fractionation of reduced groups of protein HC components by a procedure combining anion-exchange and size-exclusion HPLC on line with DAD-UV–Vis detection system.

2. Experimental

2.1. Reagents

Acetonitrile was from Scharlau (Barcelona, Spain); other compounds not specified were from Merck (Darmstadt, Germany); ultra-pure water for HPLC, generated by a Milli-RO4, coupled with a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used in the preparation of all buffers.

2.2. Proteins

The urinary protein HC was purified by ion-exchange chromatography followed by gel chromatography and immunoabsorption, as previously described [8]. Amniotic protein HC was purified from human amniotic fluid by two steps of 30% and 80% ammonium sulfate precipitation, a Concanavalin A-Sepharose column eluted with a gradient from 0–0.5 *M* methyl α-D-mannopyranoside, followed by a size-exclusion chromatography on Sephadex G 200 in 0.1 *M* ammonium bicarbonate pH 8.5. Human plasma serum albumin was purified as described [13] followed by Sepharose CL-4B chromatography.

2.3. HPLC equipment

The chromatograph consisted of two Waters M6000A pumps, a Waters 680 automated gradient controller and a Waters 990 photodiode array detector. Sample injections were performed with a Waters U6K Universal injector. Alternatively, a Smart HPLC system connected to the same photodiode array detection system was also used for micro-analyses.

Size-exclusion HPLC was performed on a TSK 3000 SW6 (300 × 21.5 mm) column (Toyo Soda, Tokyo, Japan) fitted with a TSK 3000 SW6 guard column, by isocratic elution with 0.1 *M* ammonium acetate buffer (pH 7.0). The column

was operated at room temperature at a flow-rate of 1 ml/min.

Anion-exchange was carried out with a Waters protein Pak DEAE 5PW (70 × 7.5 mm) column equilibrated with 20 m*M* Tris at pH 8.2, containing 17 m*M* NaCl and eluted with a gradient of NaCl from 17 m*M* to 35 m*M* in 100 min, from 35 m*M* to 59 m*M* in 20 min and from 59 m*M* to 85 m*M* in 1 min at room temperature and at flow-rate of 0.3 ml/min. Alternatively, human protein HC from urine and human albumin were also chromatographed on a Mono-Q PC 1.6/5 (50 × 1.6 mm) anion-exchange column, equilibrated with 20 m*M* piperazine, containing 50 m*M* NaCl (pH 5.3) and eluted with a linear gradient from 50 m*M* to 500m*M* NaCl over 65 min. The columns was operated at room temperature and at a flow-rate of 0.05 ml/min.

2.4. Photodiode array monitoring

A Waters 990 photodiode array detector with a dynamic range from the ultraviolet to the visible region (190–800 nm) connected on-line with the above Waters HPLC system was used. A NEC APC III personal computer was used to control the chromatographic system and analyze the resulting chromatograms. All the photodiode array spectra were run from 200 to 600 nm during ion-exchange or size-exclusion HPLC, and were analyzed using several different post-run data programs. The "spectrum index" plot was used to determine automatically all absorption spectra at the peak maximum. The "spectrum analysis" plot was used to provide another view of the spectra at any selected point or to easily discern any similarities or differences (see Figs. 1, 3, 4 and 6). Chromatograms produced by monitoring a separation for absorption at 230, 280, 330 and 400 nm were compared by using the "multichromatogram" mode of analysis. Peak areas were calculated by use of the "integrator" program.

2.5. Analytical gel electrophoresis

Isoelectric focusing [5 × 5 cm gels containing carrier ampholytes, Pharmalyte in 5% acrylamide (pH range 4–6.5)] was carried out with the Phast System (Pharmacia) apparatus. Gels were stained in 0.05% Coomassie blue R by using an automatic program recommended by the Phast System manufacturer. Densitometric scans were taken of the isoelectric focusing bands and integrated using an Image Quanter from Molecular Dynamics.

One-dimensional SDS-PAGE containing 18% acrylamide and 0.25% bisacrylamide was performed as described previously [18].

3. Results and discussion

Since the early purifications of protein HC, it had been noticed that all preparations displayed considerable charge heterogeneity and included a yellow–brown chromophore [11]. Besides, a protein HC preparation is considered pure and homogeneous when it presents a single N-terminal amino acid sequence and gives one unique band of ca. 30 000 u in SDS-PAGE. However, the same protein HC preparation has an extensive charge heterogeneity, migrating either as a heterogeneous wide band on agarose gel electrophoresis [11], or as a mixture of many defined bands distributed between pH 4.0–5.3 on isoelectric focusing [11,16,17]. The heterogeneous chromophore showed a typical absorption band without defined peaks in the visible region between 300 and 600 nm and an inflection point at about 330–345 nm (see Fig. 1, insert, top). Whether the heterogeneous absorption spectrum of protein HC is due to the contribution of a mixture of protein HC populations associated with either distinct ligands, or with different amounts of the same ligand, has not been investigated. Due to the high heterogeneity of protein HC, separation of single components is extremely difficult, and thus the precise characterization of such heterogeneous protein HC containing populations has not yet been established. To fractionate and characterize populations of protein HC, two different preparations from human urine and amniotic fluid were fractionated by a chromatographic system based on

separations by charge, such as anion-exchange HPLC.

3.1. Fractionation by anion-exchange HPLC of urinary protein HC and spectroscopic characterization of subpopulations with different charges

A urinary protein HC preparation was chromatographed by an anion-exchange HPLC system on a protein Pak DEAE 5PW column. The column was eluted with a gradient of NaCl from 17 to 85 m*M*. Fig. 1 shows the profile at 280 nm. Protein HC appeared to be heterogeneous in charge as judged by the asymmetric elution pattern, consisting in a continuous irregular broad peak with several shoulders distributed along the whole chromatogram. The material of six selected shoulder-containing fractions was pooled, dialyzed against 20 m*M* Tris-HCl, and then rechromatographed twice under the same chromatographic conditions. Finally, six symmetrical peaks A–F with different retention times were obtained.

The absorption spectra of each of the fractionated peaks, at the peak maxima from 240 to 600 nm, were automatically determined by the spectrum index mode (Fig. 1, insert, bottom). All the spectra were found to be nearly identical, displaying significant absorption in the visible region between 300 and 600 nm, in resemblance to the unfractionated urinary protein HC spectrum, but with different 330/280 nm absorbance ratios, ranging from 0.17 for peak A to 0.34 for peak F.

These data demonstrated that urinary protein HC represents a complex mixture of identical polypeptide chains associated to the same heterogeneous chromophore, but with different stoichiometry. They also indicated a direct relationship between the amount of chromophore and charge. In fact, the above spectroscopic characteristics indicated that peaks A and F seem to be populations containing less or more chromophores, respectively (Fig. 1, inserts). Moreover, the chromatographic elution behavior on the anion-exchange column also indicated that peak F corresponds to the most negatively charged population.

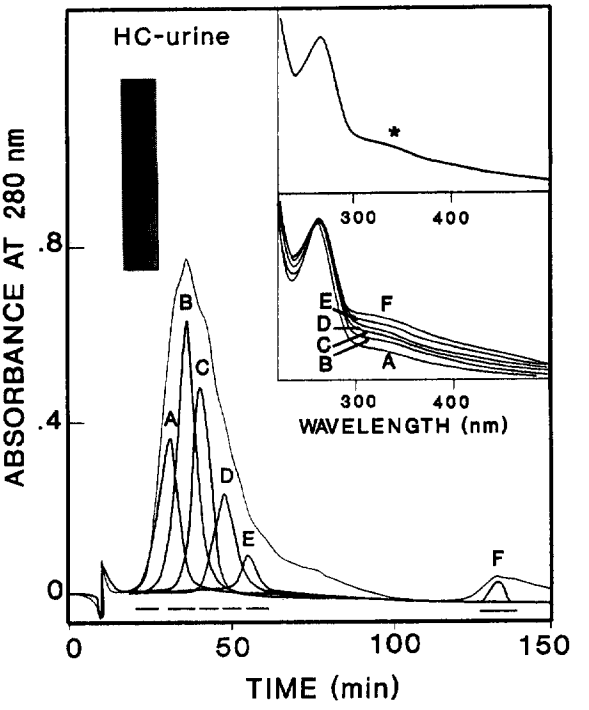

Fig. 1. Chromatography of heterogeneous protein HC from urine on a Protein Pak DEAE 5PW anion-exchange column. Separation of protein HC from urine on a column (70 × 7.5 mm) of Protein Pak DEAE 5PW, with mobile phase of (A) 20 m*M* Tris-HCl (pH 8.2) and (B) 20 m*M* Tris-HCl (pH 8.2) containing 100 m*M* NaCl, at a flow-rate of 0.3 ml/min. The proteins were eluted with a gradient from 17 to 35% in 100 min, from 35 to 59% in 20 min and from 59 to 85% in 1 min. Peaks indicated by bars were dialyzed against 20 m*M* Tris-HCl (pH 8.2) and rechromatographed as above. The elution profiles of the corresponding rechromatographed peaks are indicated by letters (A–F). The inserts show the absorption spectra from 240 to 500 nm of unfractionated urinary protein HC (top) and the rechromatographed peaks recorded automatically from the peak maximum of each fraction (normalized at 280 nm), by using the spectrum index mode. SDS-PAGE of unfractionated protein HC is inserted.

In addition, the existence of a limited number of protein HC subpopulations in each of the symmetrical peaks was investigated by isoelectric focusing. The remarkable charge heterogeneity of this urinary protein HC preparation seemed to be caused by a limited number of subpopulations corresponding to multiple bands of unequal intensity within the pH range 4–5.3 (Fig. 2, top,

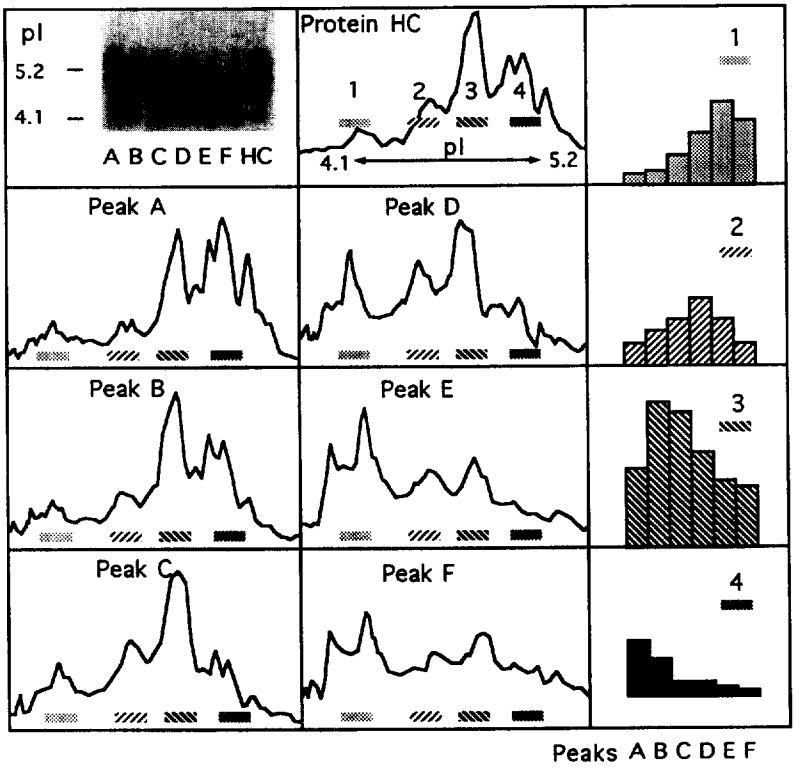

Fig. 2. Densitometry and isoelectric focusing of the urinary protein HC and the isolated populations thereof by anion-exchange HPLC. Isoelectric focusing of peaks A–F from Fig. 1 and urinary protein HC (top, left). Densitometric scan of the corresponding lanes A–F and protein HC (left down and middle). Relative percentages of lines 1–4 in the different densitometric analyses (right).

left, HC). These isoelectric points were lower than the calculated 6.1 based on the amino acid sequence, and supported the negative charge of the chromophore.

On the other hand, isoelectric focusing of the protein HC containing peaks A–F, showed almost the same characteristic pattern, but with clear differences in the population ratio of the focused bands along the pH range (Fig. 2, top, left A–F). Fig. 2 also shows the densitometric analysis of the corresponding isoelectric focusing bands in each of the protein HC containing peaks A–F (Fig. 2, protein HC and peaks A–F). The distribution of some of the protein HC subpopulations in four selected focused bands 1–4, in peaks A–F is also indicated (Fig. 2 right, 1–4).

3.2. Fractionation by anion-exchange HPLC and spectroscopic characterization of populations of amniotic protein HC

Until the present time, all preparations of human protein HC were isolated from plasma or urine. In this report we have also studied the charge heterogeneity and spectroscopic characteristics of a new protein HC preparation isolated from human amniotic fluid.

The amniotic protein HC preparation presented (i) a single band on SDS-PAGE with an apparent molecular mass of 30 000 (data not shown), (ii) an N-terminal identical to that of the urinary protein HC, and (iii) displayed an absorption spectrum, with a rather broad absorption band between 300 and 600 nm nearly

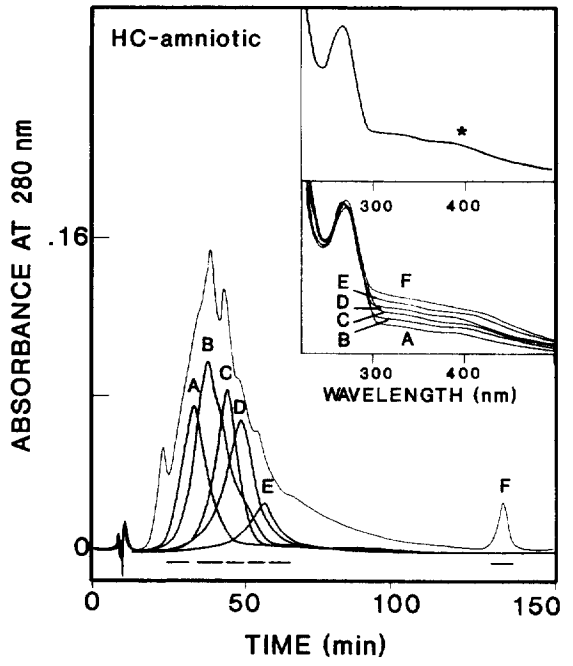

Fig. 3. Chromatography of heterogeneous protein HC from amniotic liquid on a Protein Pak DEAE 5PW anion-exchange column. Separation was carried out as described in Fig. 1. Peaks indicated by bars were dialyzed against 20 m*M* Tris-HCl (pH 8.2) and rechromatographed as above. The elution profiles of the corresponding rechromatographed peaks are indicated by letters (A–F). The absorption spectra from 240 to 500 nm of unfractionated amniotic protein HC (top) and the rechromatographed peaks recorded automatically from the peak maximum of each fraction (normalized at 280 nm) are inserted. SDS-PAGE of unfractionated protein HC is inserted.

identical to that of the urinary protein HC but with an inflection point at about 425 nm, and a 330/280 nm absorbance ratio of 0.27 (Fig. 3, insert, top).

Also the amniotic protein HC seemed to be heterogeneous by the asymmetric elution pattern consisting of an irregular broad peak distributed along the whole chromatogram with well defined shoulders on the above anion-exchange protein Pak DEAE 5PW column (Fig. 3).

The fraction of the wide peak was divided in six pools A–F, dialyzed again 20 m*M* Tris-HCl, rechromatographed twice, and finally, six symmetrical peaks A–F with different retention times were obtained. The automatic absorption spectra at the peak maximum of each individual peak (A–F) from 240 to 600 nm (Fig. 3, insert, top), indicated that all spectra showed a similar broad band and displayed significant absorption in the visible region between 300 and 600 nm with different 330/280 nm absorbance ratios, ranging from 0.21 for peak A to 0.33 for peak F. Amniotic protein HC also migrated as multiple bands of unequal intensity and with different isoelectric point in isoelectric focusing (data not shown). Thus, these data indicated that the amniotic protein HC seems to be very similar to urinary protein HC.

3.3. Verification of size microheterogeneity in protein HC by size-exclusion HPLC with DAD-UV–Vis detection

The fact that both urinary and amniotic protein HC displayed charge heterogeneity due to the existence of mixtures of populations bound to different amounts of chromophore, seems to suggest the existence of size microheterogeneity as well.

To investigate such a possibility, both urinary and amniotic protein HC preparations were fractionated by a size-exclusion HPLC system using a TSK-3000 SWG column. Apparent size homogeneity was evident for urinary and amniotic protein HC since on gel filtration both gave symmetrical peaks, i.e. a main peak (monomer) and a minor peak (dimer) (Fig. 4).

To study size microheterogeneity, the absorption spectra from 240 to 600 nm were acquired automatically, at four selected points (1–4) of fractions eluted earlier, later, and of the main protein HC. The spectra clearly revealed the presence of molecules associated with different amounts of chromophore (Fig. 4A,B, inserts), the first and the last eluted fractions being populations of protein HC associated with the highest and lowest amount of chromophore, respectively. This data indicated a linear relationship between the amount of chromophore associated with the polypeptide chain of protein HC and their elution from the size-exclusion column. Moreover, it confirmed once more the existence of protein HC populations associated

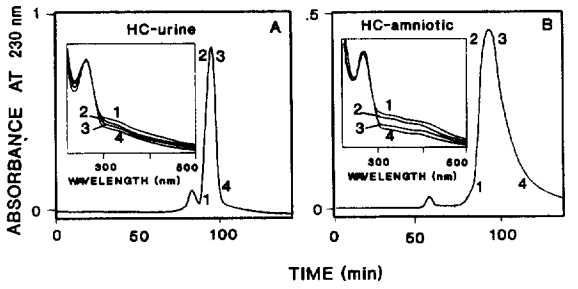

Fig. 4. Elution profile on a size-exclusion HPLC system of protein HC from urine and amniotic liquid. (A) Separation of heterogeneous protein HC from urine and (B) from amniotic liquid on a TSK-3000 SWG column (300 × 21.5 mm). The proteins were eluted at room temperature with an isocratic mobile phase, consisting of 0.1 *M* ammonium acetate buffer (pH 6.9). The insets show the absorption spectra from 240 to 500 nm (normalized at 280 nm) of different elution times (indicated by number) from the peak, recorded automatically by the spectrum index mode.

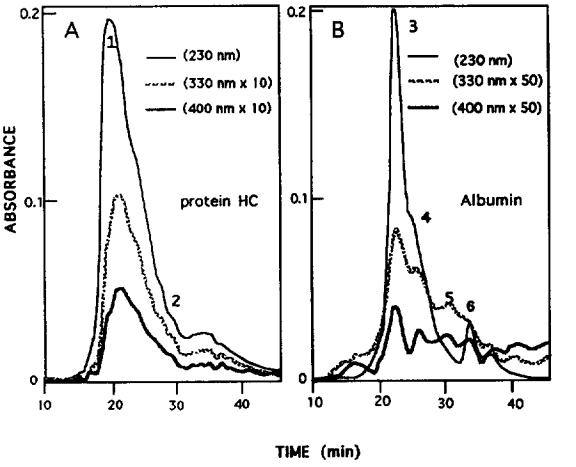

Fig. 5. Chromatography of human protein HC from urine and human albumin on a Mono-Q PC 1.6/5 (50 × 1.6 mm) anion-exchange column. (A) Separation of protein HC from urine and (B) from human albumin on a column (50 × 1.6 mm) of Mono-Q-PC 1.6/5 with mobile phase of (a) 20 m*M* piperazine, containing 50 m*M* NaCl (pH 5.3) and (b) 20 m*M* piperazine (pH 5.3) containing 500 m*M* NaCl, at a flow-rate of 0.05 ml/min. The proteins were eluted with a linear gradient from 50 to 500 m*M* NaCl in piperazine (pH 5.3) over 65 min. The absorbance of the effluent was monitored at 230, 330 and 400 nm, by using the multichromatogram mode analysis.

with different amounts of probably the same chromophore.

3.4. Comparative spectral analyses of human urinary protein HC and human albumin fractionated by anion-exchange chromatography

In order to obtain more information on the nature of the heterogeneous protein HC chromophore, we have investigated if other plasmatic human ligand-binding proteins could have common bound components. We first investigated plasmatic human albumin, which not only carries many different ligands, such as fatty acids, bilirubin, hematin, glycans etc., as well as unknown compounds [19–21], but which also seems to be structurally implicated in the formation of plasmatic complexes with protein HC [11].

Fig. 5 shows the chromatographic behavior of these two human proteins in an analytical anion-exchange system using a Mono-Q PC 1.6/5 column. The resulting chromatograms show that human albumin eluted as a broad and irregular peak distributed along all fractions in a pattern similar to that of protein HC (Fig. 5), and resembling the patterns obtained above for urinary and amniotic protein HC with the DEAE anion-exchange column (Figs. 1, 2). The absorption spectra at four selected points (3–6) of the albumin chromatogram showed similar broad bands and displayed significant absorbance in the visible region at 330 and 450 nm (Fig. 6) compared with the corresponding protein HC spectra of two selected points (1, 2) (Fig. 6). Moreover, the albumin-containing fractions 3 and 6 contained the populations associated with low and high amounts of chromophore respectively, similar to the equivalent fractions 1 and 2 of protein HC (Fig. 5A).

The similar spectroscopic characteristics of the two human proteins might suggest the existence of common ligands. The possibility that these are also involved in the linkage of the HC–albumin complex, in a manner similar to the way protein HC chromophore is implicated in the HC–IgA complex, can not be discarded.

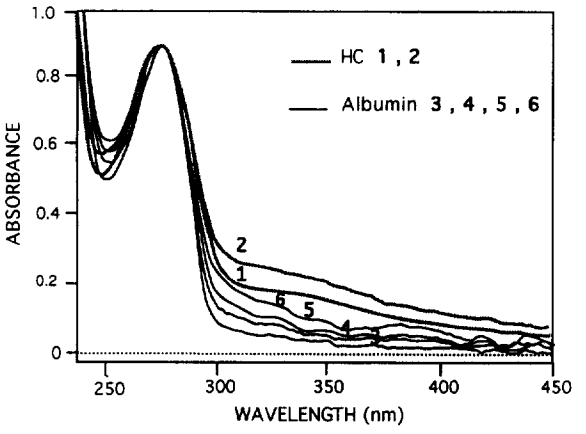

Fig. 6. Comparison of spectral analyses from 240 to 450 nm of different elution times from the main peak of Fig. 5A (protein HC, 1 and 2) and Fig. 5B (albumin, 3–6). For better comparison spectra are normalized at 280 nm by using the spectrum analysis mode.

4. Conclusion

The results presented here indicate that anion-exchange HPLC combined with DAD-UV–Vis detection is a suitable tool to study the nature of the colored unknown protein HC chromophore. The method allowed the partial fractionation of urinary or amniotic protein HC in populations of chromophore-containing molecules grouped in six symmetrical peaks with different retention time and similar spectroscopic characteristics. These subpopulations seemed to be simple mixtures of protein HC polypeptides bound apparently to different amounts of the same chromophore. Each one showed a different distribution pattern of bands of disproportionated intensity in isoelectric focusing. The isoelectric distribution of urinary protein HC (Fig. 2, top, left, HC) is not a characteristic pattern for all protein HC preparations. Most patterns corresponding to other protein HC preparations were similar, but not identical depending on the source of the human fluid (urine, plasma or amniotic fluid) and from batch to batch (data not shown). Additional systematic studies have to be performed to confirm spectral similarity in the key region of interest (300–600 nm). Moreover, the chromophore seemed to be negatively charged since the protein HC populations carrying the highest amount of chromophore were eluted at the end of the salt gradient in the anion-exchange column. Furthermore, a linear correlation between the chromatographic elution and the size of the chromophore-containing protein HC subpopulations was observed in size-exclusion chromatography, demonstrating the existence of size microheterogeneity in the two protein HC preparations. The chromatographic behavior in the anion-exchange system, as well as the absorption spectra data indicated that amniotic protein HC is nearly identical to the urinary protein HC and that both are very similar to human albumin. Whether or not these two types of human proteins share common ligands remains to be investigated.

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