Journal of Chromatography, 444 (1988) 165–175
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1383

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND PHOTODIODE-ARRAY DETECTION OF THE HUMAN PROTEIN HC (HUMAN COMPLEX-FORMING GLYCOPROTEIN HETEROGENEOUS IN CHARGE), A CHROMOPHORE-ASSOCIATED PROTEIN

JULIO ESCRIBANO
Servicio de Endocrinologia, Centro Ramón y Cajal, 28034 Madrid (Spain)
RICARDO MATAS
Millipore Iberica SA., Waters Chromatography Division, Barcelona (Spain)
and
ENRIQUE MÉNDEZ*
Servicio de Endocrinologia, Centro Ramón y Cajal, 28034 Madrid (Spain)

SUMMARY

Photodiode-array ultraviolet–visible detection has been adapted to our high-performance liquid chromatographic system for the analysis and the characterization of the unknown yellow-brown chromophore associated with the human complex-forming glycoprotein, heterogeneous in charge (Protein HC). By using several post-experiment data processing modes, such as multichromatograms, automatic spectrum analyses or three-dimensional plots, the technique allows a direct verification of purity, quantification, as well as the identification of Protein HC without the necessity for further analytical systems. At least thirteen different shoulders in the absorption spectrum in the visible region between 300 and 480 nm have been identified for urinary Protein HC. However, no chromophore was found to be associated with Protein HC complexed with immunoglobulin A (HC–IgA complex). Comparison of spectra between plasma or urinary protein HC allows one to distinguish spectral differences in its chromophore, at least in the range from 300 to 480 nm. The technique was useful for easy identification of chromophore-containing peptides from the digested Protein HC.

INTRODUCTION

Photodiode-array (PDA) UV detection was recently introduced in high-performance liquid chromatography (HPLC) of aromatic amino acids, their metabolites[1] and aromatic-containing peptides[2]. Since then, PDA UV–VIS detectors with a wide range (from low ultraviolet to the visible region) have also been used for dinitrophenylhydrazone derivatives[3], iodine derivatives of Tyr[4] and more recently for several chromophores[5,6] including mycotoxins and other fungal metabolites[7]. This PDA detection is becoming increasingly popular for HPLC due to the wealth

0021-9673/88/$03.50

of information that now can be obtained about a sample to suplement data derived from the conventional single-wavelength UV–VIS detectors. It also provides data in areas such as multiwavelength absorbances, absorbance ratio monitoring and spectral routines for peak identification and purity.

In this paper we describe the use of a PDA UV–VIS detector in our HPLC system to initiate a study of the unknown yellow-brown chromophore associated with human Protein HC (human complex-forming glycoprotein, heterogeneous in charge). Also called α_1-microglobulin, this compound is a low-molecular-weight (LMW) glycoprotein, originally isolated from normal human urine[8]. The protein is widely distributed in physiological fluids as a LMW or a high-molecular-weight (HMW) component. The HMW component dislays, in addition to its Protein HC reactivity, immunoglobulin A (IgA) immunoreactivity[9]; it is also called HC–IgA complex.

Both protein HC and HC–IgA complex act as an "*in vitro*" inhibitor of neutrophil chemotaxis against endotoxin-activated serum at physiological roles in the regulation of the inflammatory response[10].

One of the peculiar features of the LMW Protein HC is that it displays considerable charge heterogeneity and includes an unidentified yellow-brown chromophore[8]. Preliminary studies in our laboratory have shown that the chromophore naturally associated with the protein was resolved in three fluorescent units, centred around 290, 360 and 410 nm, which do not correspond to any previously described chromophores[11].

The present work reports the use of a rapid PDA UV–VIS detection system in HPLC for initiating the characterization of this enigmatic chromophore(s) associated with Protein HC isolated from different physiological fluids. Preliminary studies of the direct location of chromophore-containing peptides, in HPLC fractions from the digested Protein HC, have also been made.

MATERIALS AND METHODS

Materials

Acetonitrile was from Scharlau (Barcelona, Spain), guanidinium chloride, iodoacetic acid and dithiotreitol from Sigma (St. Louis, MO, U.S.A.), tetrahydrofuran, 1-tosylamino-2-phenylethyl choromethyl ketone (TPCK)– trypsin trifluoroacetic acid and other compounds not specified from Merck (Darmstadt, F.R.G.). Ultra-pure water for HPLC, generated by a Milli-PO4, coupled with a Milli-Q water-purification system (Millipore, Bedford, MA, U.S.A.), was used in the preparation of all buffers.

Te urinary Protein HC preparations were provided by Dr. A. O. Grubb, Malmö General Hospital, Sweden. Protein HC was purified by ion-exchange chromatography followed by gel chromatography and immunoabsorption, as described previously[5].

Reduction and alkylation

Three milligrams of native urinary Protein HC dissolved in 200 μl of 2 *M* Tris–HCl buffer (pH 8.6)–0.002 *M* EDTA–6 *M* guanidinium chloride were incubated with 35 m*M* dithiothreitol for 120 min at 37°C. Alkylation was achieved by adding 5.0 mg of iodoacetic acid and incubating for 15 min at room temperature in the

absence of light. The excess of reagents was removed by gel chromatography on a 200 mm × 200 mm column of Sephadex G-25.

Trypsin digestion

A 500-μg amount of reduced and carboxymethylated Protein HC was digested with 5 μg of TPCK-trypsin in 100 μl of 0.2 *M* *N*-methylmorpholine acetate buffer (pH 8.2) for 1 h at 37°C. After digestion, the material was freeze-dried, lyophilized and finally redissolved in 0.1% aqueous trifluoroacetic acid.

HPLC equipment

The chromatograph consisted of two Waters M6000A pumps, a Waters 680 automated gradient controller and a Waters 990 photodiode array detector with a dynamic range from ultraviolet to the visible (UV–VIS) region (190 to 600 nm), based on a NEC APC III personal computer. Sample injections were performed with a Waters U6K universal injector.

Size-exclusion HPLC was performed on a TSK 3000 SW6 (300 mm × 21.5 mm) column (Tokyo Soda, Tokyo, Japan) fitted with a TSK 3000 SW6 guard column, by isocratic elution with 0.1 *M* ammonium acetate buffer (pH 5.0). The column was operated at room temperature at a flow-rate of 1 ml/min. Reversed-phase HPLC was performed with a Nova-Pak column (Waters) (150 mm × 3.9 mm) protected by a guard column packed with μBondapak C_{18}/Corasil (Waters). The column was eluted with acetonitrile gradients containing 0.1% trifluoroacetic acid and operated at room temperature at a flow-rate of 0.5 ml/min.

RESULTS AND DISCUSSION

Since Protein HC was first described several years ago, every Protein HC preparation from either urine or plasma always presented a different coloration from almost white to a very dark brown. Conversely, no colour has been observed in the isolated Protein HC complexed with IgA (HC–IgA complex).

Taking account of the multiple possibilities the PDA UV–VIS detector offers, such as the wide dynamic range from the low ultraviolet to the visible region (190 to 600 nm), large data-storage capacity during an experiment, stored post-experiment analyses, etc., we consider this detector to be the most appropriate instrument with which to begin a study of the colour of the human protein HC.

PDA UV–VIS analysis by gel HPLC of a purified Protein HC preparation

Due to the fact that Protein HC has a tendency to aggregate, it is very common to observe a main strong band in the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified Protein HC preparations, corresponding to the Protein HC (30 000 daltons), as well as a few additional faint bands which may correspond either to Protein HC aggregates or small contaminants (data not shown). However, due to the small amount of such bands, the nature of these components has never been investigated.

One of these purified protein HC preparations (1.0 mg) was chromatographed by size-exclusion HPLC with a PDA UV–VIS detector. The experiment was recorded from 190 to 660 nm. Fig. 1 shows the post-experiment analysis in the spectrum index

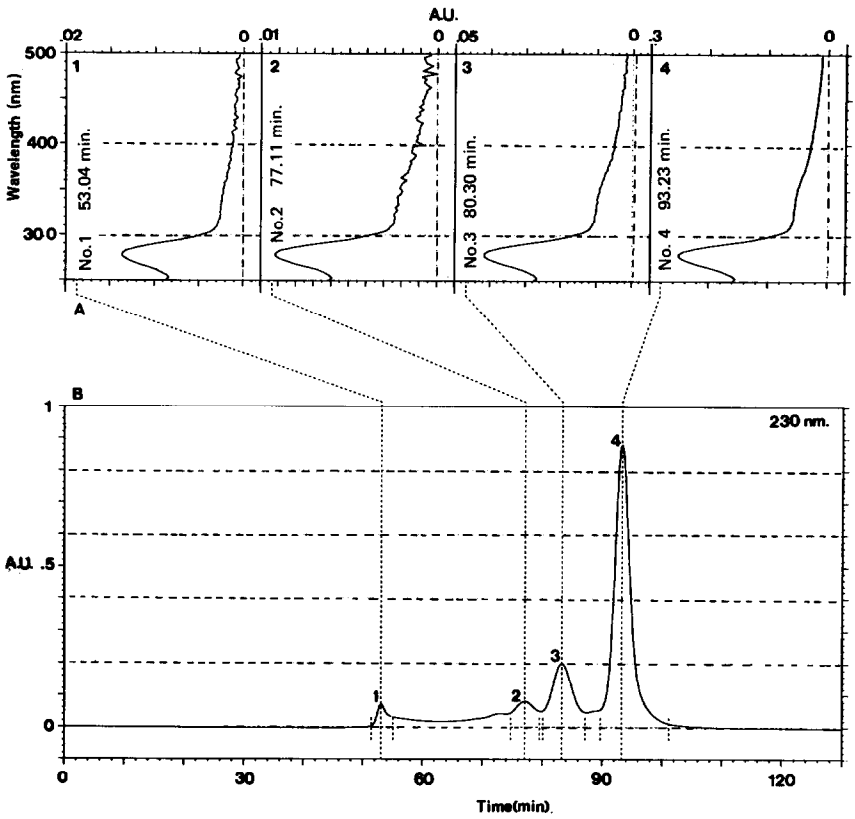

Fig. 1. Fractionation of an urinary Protein HC preparation by gel HPLC. Column: TSK 3000 SW6 (300 mm × 21.5 mm). Flow-rate: 1.0 ml/min. Proteins were eluted at room temperature with an isocratic mobile phase, consisting of 0.1 *M* ammonium acetate buffer (pH 5.0). The chromatogram was analyzed by monitoring the absorbance at 230 bm (lower). Automatic spectra were acquired from the peak maxima from 250 to 500 nm (upper).

mode. The lower portion represents the pattern distribution selected at 230 nm (single-wavelength chromatogram), the upper is an automatic profile of the respective absorption spectra at each peak maximum from 250 to 500 nm.

Direct observation of these spectra shows that the Protein HC (peak 4) can be automatically identified, owing to the typical broad band present in its spectrum in the visible region. Furthermore, the same broad band was also present in the spectra of the other three peaks (1, 2 and 3) of the chromatogram. Since peaks 1–3 displayed the same amino acid composition as Protein HC (data not shown), these peaks may be related to the faint bands observed in SDS-PAGE and not to contaminants.

Fig. 1 also permits the calculation of the protein concentration of each peak, by using the extinction coefficient of Protein HC[11] and the absorbance value at the peak maximum (278 nm) of each component. A total of 95% was found to be distributed in all Protein HC-containing molecules in the chromatogram (Fig. 1). The band observed in the visible region in the absorption spectra of the four peaks (Fig.

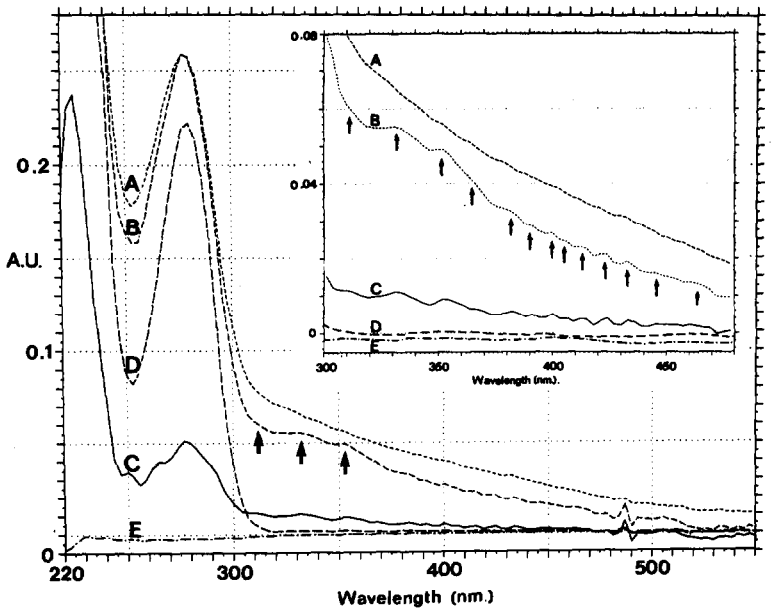

Fig. 2. Comparative spectral analyses from 220 to 550 nm. (A) Plasma HC (peak 4 from Fig. 4); (B) and (C) urinary Protein HC (peak 3, dimer and peak 4, monomer from Fig. 1); (D) HC–IgA complex (peak 2 from Fig. 4); (E) blank corresponding to retention time of 10 min from Fig. 1. Arrows indicate the positions of shoulders. For clarity, the spectral analyses from 300 to 480 nm at high sensitivity (0.08 a.u.f.s.) are shown in the insert.

1, top) is too broad and not sufficiently resolved to be assigned to only one chromophore. However, using another post-experiment analysis in the spectrum analysis mode, a more detailed view of the spectra around the visible region of some of the peaks, *e.g.*, peak 4 (monomeric Protein HC) and peak 3 (dimeric Protein HC), is seen (Fig. 2). Since spectra (A, B, C and D) are recorded directly from the chromatograms, quantitative differences are not significant, as they are not normalized for protein concentration. The spectrum analysis from 220 to 550 nm of the monomeric (B) and dimeric (C) Protein HC shows an absorption maximum at 280 nm and three shoulders at 310, 332 and 352 nm, as well as additional minor shoulders between 360 and 480 nm. Furthermore, a better view of the small shoulders can be observed in the same spectrum analysis mode, using a wavelength range between 300 and 480 nm and high sensitivity (0.08 a.u.f.s.) (Fig. 2, insert). At least nine additional shoulders between 365 and 462 nm were observed for both monomeric urinary Protein HC. Although the shoulders observed for the dimeric (C) Protein HC are less defined in comparison to those of the monomeric (B) Protein HC, they are due to the low concentration and not from the contribution from the noise. As is seen there are no shoulders present in the baseline (E) or for the HC–IgA complex (D), from which the chromophore is absent. The same type of absorption spectrum was observed for all Protein HC preparations from urine, analyzed by the same PDA UV–VIS detector (data not shown). By using another post-experiment analysis, the three-dimensional plot, additional information can be obtained from the same chromatogram (Fig. 1).

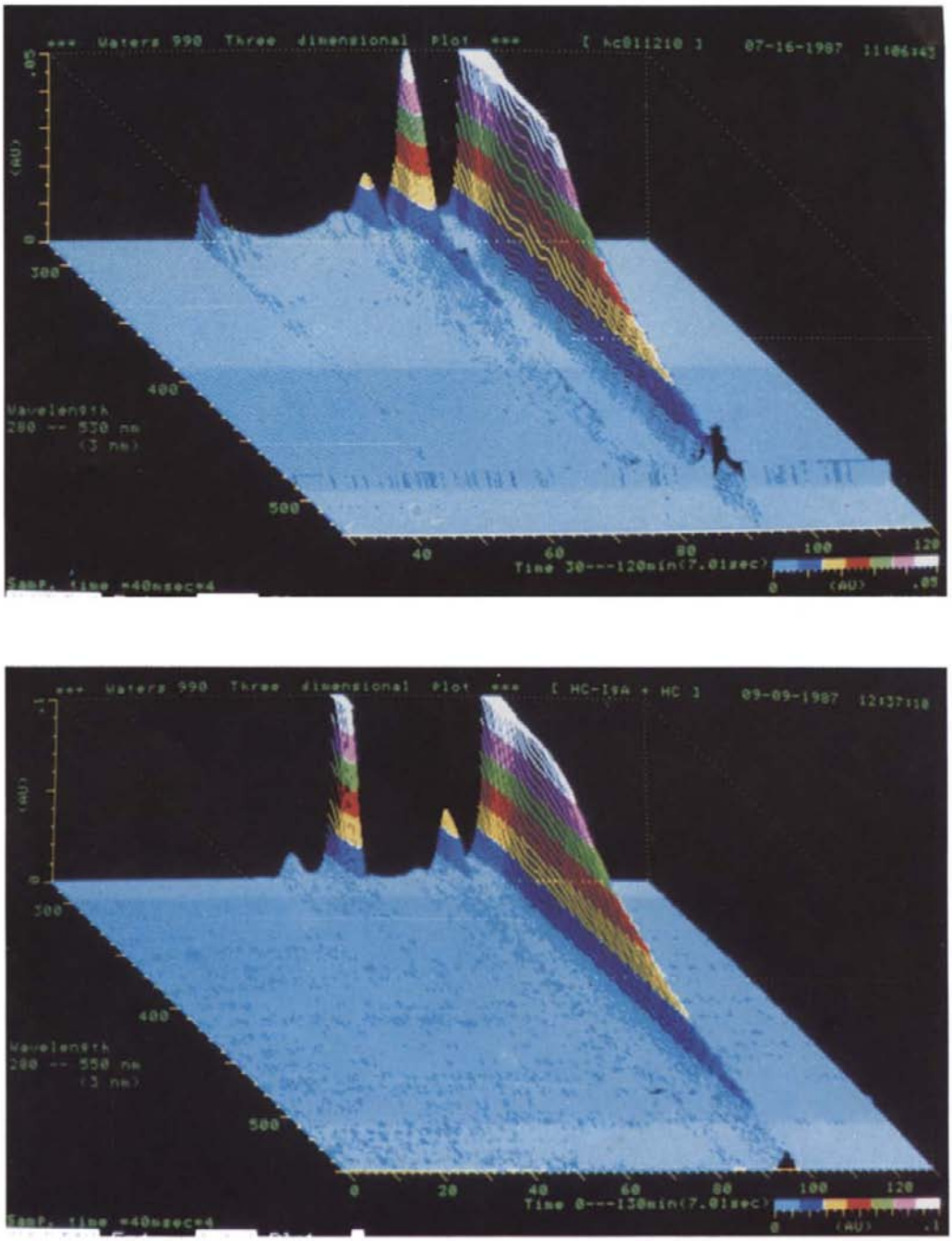

Fig. 3. Photodiode array detector three-dimensional plot from 280 to 550 nm of the chromatograms in Figs. 1 (top) and 5 (bottom). Views of Protein HC and HC–IgA complex from a 45° left angle are displayed.

Fig. 3 (top) shows the three-dimensional mode in the wavelength range of 280 to 550 nm and viewed from a 45° left angle. Direct visualization of the picture immediately shows that the chromophore is associated with the monomeric Protein HC (peak 4, Fig. 1). However, the exact range of the chromophore(s) is difficult to estimate since, as is shown in the picture and in Figs. 1 and 2, it is overlapped within the ultraviolet region, from which the chromophore apparently emerges, ending in the visible region of the spectrum around 500–550 nm. Traces of the chromophore, associated with the Protein HC-aggregated peaks 1–3 (Fig. 1) can also be seen.

Direct identification by PDA UV–VIS detection of plasma Protein HC and HC–IgA in HPLC

For the location of Protein HC and HC–IgA during purification, we have routinely used several immuno-techniques, such as immunoelectrophoresis[12], crossed-immunoelectrophoresis[13] and more recently enzyme-linked immunosorbent assay (ELISA)[14].

Fig. 4 shows a another post-experiment analysis in the multichromatogram mode on an HPLC size-exclusion column to identify protein HC and HC–IgA complex in a final step of purification from a mixture containing only these two components. A multichromatogram analysis of an experiment at three different absorbances, 280, 350 and 450 nm, is presented. It is evident that, plasma protein HC can be identified immediately, since the profile of Protein HC (peak 4) and peak 3 (dimer) can be visualized not only in the low ultraviolet but also in the visible region, while HC–IgA (Peak 2) and Peak 1 (HC–IgA aggregate) are only see in the ultraviolet region.

Fig. 3 (bottom) shows the three-dimensional plot of this chromatogram (Fig. 4), carried out in the wavelength range from 280 to 550 nm. The direct three-dimen-

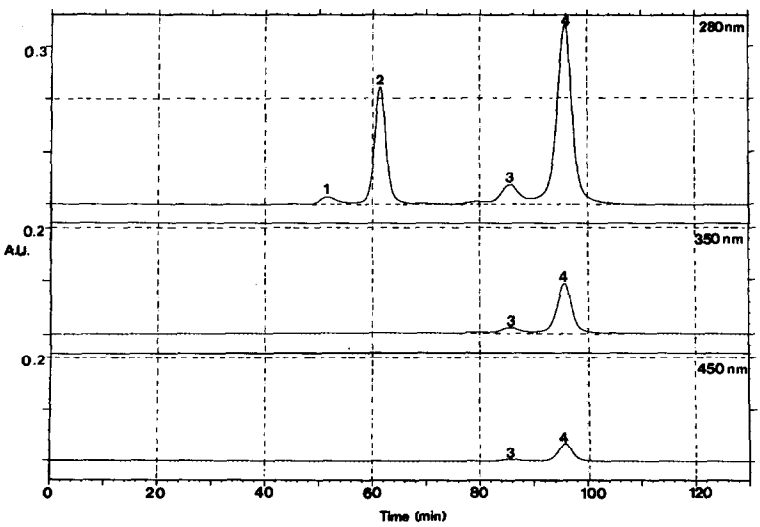

Fig. 4. Multichromatogram analysis of the absorbances at 280, 350 and 450 nm of a mixture containing plasma Protein HC and HC–IgA complex. Column, mobile phase and flow-rate as in Fig. 1.

sional shows the presence of a chromophore(s) associate with Protein HC (retention time 96 min), which clearly is not present in the HC–IgA complex (retention time 61 min). This demonstrates that by using the multichromatogram (Fig. 4), the three-dimensional (Fig. 3) or the spectrum index (Fig. 1) modes, Protein HC from plasma or urine can be directly identified without recourse to immuno-techniques. Furthermore, Figs. 3 and 4 also demonstrate the absence of chromophore from the HC complexed with the IgA (HC–IgA complex).

Using the spectrum analysis mode, we compared the difference in spectra be-

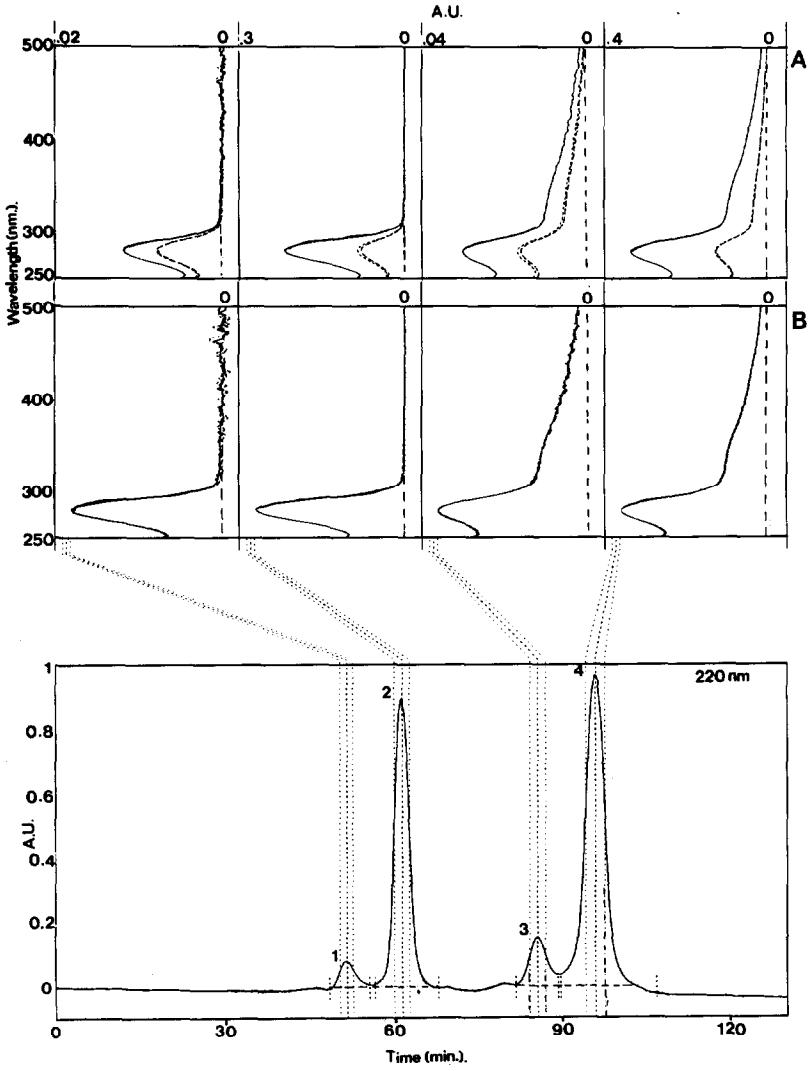

Fig. 5. Spectral comparison to confirm the peak purity of plasma Protein HC and HC–IgA complex separated by gel HPLC (Fig. 4). The chromatogram was analyzed to monitor the absorbance at 220 nm. Automatic overlay of spectra acquired from peak maxima and right and left slopes (top A), and automatic plotter spectra, normalized to 280 nm from peak maxima and right and left slopes (top B).

tween the isolated plasma HC and HC–IgA complex with the urinary Protein HC (Fig. 2). The absorption spectrum of the HC–IgA complex (Fig. 2D) shows only a maximum around 278 nm while the plasma Protein HC exhibits the same peak and an additional band in the visible region, which was apparently similar to the urinary Protein HC (Fig. 2B). However, in the insert (Fig. 2A) the spectrum of the plasma Protein HC does not show defined shoulders in comparison with the ones observed for urinary Protein HC (Fig. 2B and C). These spectral comparisons indicate some differences in the composition of the chromophore(s) attached to Protein HC in urine or in plasma. Our results suggest that the chromophore(s) attached to Protein HC present in various human fluids could be different. This is at present being investigated in our laboratory using the PDA UV–VIS detector.

Verification of peak purity of Protein HC and HC–IgA complex by PDA UV–VIS detection

To verify the purity of peaks, the spectra of all peaks were automatically taken at the peak maxima and inflection points. Fig. 5 shows the post-experiment analysis in the spectrum index mode of the data from Fig. 4. The pattern distribution was selected at 220 nm and automatic absorption spectra from the peak maxima and inflection points were selected from 250 to 500 nm (top A). The plots were also automatically normalized at 280 nm (top B). It is evident from the results presented in Fig. 4 that the four peaks of the chromatogram are pure, confirmed by SDS-PAGE and amino acid composition analysis data (not shown).

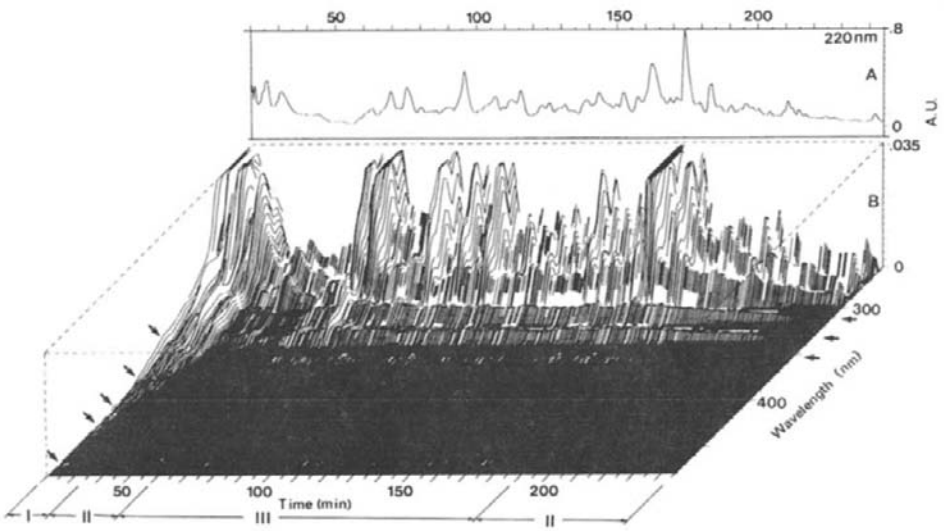

Fig. 6. Separation of a tryptic digest of reduced and carboxymethylated Protein HC. Sample: 3.0 nmol. Column; Nova-Pak C_{10} (300 mm × 21.5 mm). Flow-rate: 0.5 ml/min. Peptides were eluted at room temperature with acetonitrile gradients containing 0.1% trifluoroacetic acid. The chromatogram was analyzed to monitor the absorbance at 220 nm (0.8 a.u.f.s.) (top). Three-dimensional plot of the chromatogram from 255 to 480 nm. View from a 45° right angle (bottom). Groups of chromophore-containing peptides I, II and III are indicated on the time axis.

Three-dimensional visualization of the chromophore containing tryptic peptide by PDA UV–VIS detection

In order to determine the location(s) of the chromophore(s) in the polypeptide chain of Protein HC, we analyzed the tryptic peptides chromatographed on a reversed-phase (RP)-HPLC column by PDA UV–VIS detection. Fig. 6 shows the three-dimensional plot of the tryptic peptides, carried out in the wavelength range from 255 to 480 nm. Direct visualization of Fig. 6 shows that the chromophore(s) is apparently associated with nearly all peptides in the chromatogram. At least three groups of chromophore-containing tryptic peptides can be distinguished: the first corresponds to those with retention times from 20 to 35 min, presenting shoulders at 432, 412, 382 and 352 nm, as well as a few more around 300 nm; the second, with retention times from 35 to 60 and 190 to 245 min, presents mainly two shoulders at 310 and 332 nm; the third group with retention times from 60 to 190 min presents two major shoulders at 310 and 332 nm and a minor one at 352 nm.

These distributions of shoulders associated with the tryptic peptides are in agreement with those observed in the spectra of urinary Protein HC, around the visible region (Fig. 2). The presence of chromophore(s) associated apparently with the totality of the peptides is coincident with the fact that most of the peptides isolated during the elucidation of the primary structure of Protein HC[12] were coloured. The precise characterization of all chromophore-containing tryptic peptides from the urinary Protein HC will be investigated using this PDA UV–VIS detector. In conclusion, the results presented in Figs. 1–6 from tests performed with four different software routines (multichromatograms, spectrum analysis, spectrum index plot and three-dimensional plot) demonstrate the large amount of information that can eaily be obtained in a short time from an HPLC experiment by using a PDA UV–VIS detection system. The possibility of working in the ultraviolet and in the visible range simultaneously has allowed confirmation of the direct association of the chromophore with Protein HC, but not with the HC–IgA complex. It also provides detailed spectral characteristics of the chromophore in urinary and plasma Protein HC as well as verification of the purity and quantification of the protein. In addition the PDA UV–VIS detector is a very useful tool, not only for studying in more detail the nature of this enigmatic chromophore(s) associated with Protein HC, but also for localizing the precise attachment of the chromophore(s) to the polypeptide chain of this human protein.

ACKNOWLEDGEMENTS

This work was supported by grants from the Comisión Asesora para Desarrollo de la Investigación Cientifica y Técnica and Fondo de Investigaciones Sanitarias de la Seguridad Social. We thank Dr. J. Gavilanes for his critical evaluation.

REFERENCES

1 A F. Fell, B. J. Clark and H. P. Scott, *J. Chromatogr.*, 297 (1984) 203.
2 F. Nyberg, C. Pernow, U. Moberg and R. B. Eriksson, *J. Chromatogr.*, 359 (1986) 541.
3 E. Puputti and P. Lehtonen, *J. Chromatogr.*, 353 (1986) 163.
4 L A. Edwards, R. E. Huber and T. J. Carve, *Methods in Protein Sequence Analysis*, Humana Press, Clifton, NJ, 1986, p. 457.

5 W. A. Dark, *J. Chromatogr. Sci.*, 24 (1986) 495.
6 T. Alfredson and T. Sheelan, *J. Chromatogr. Sci.*, 24 (1986) 473.
7 J. C. Frisvad and U. Thrane, *J. Chromatogr.*, 404 (1987) 195.
8 L. Tejler and A. O. Grubb, *Biochim. Biophys. Acta*, 439 (1976) 82.
9 A. O. Grubb, C. López, L. Tejler and E. Méndez, *J. Biol. Chem.*, 258 (1983) 14698.
10 E. Méndez, J. L. Fernandez-Luna, A. O. Grubb and F. Leyva-Cobian, *Proc. Natl. Acad. Sci. U.S.A.*, 88 (1986) 1472.
11 J. Gavilanes, C. López, F. Gavilanes and E. Méndez, *Biochemistry*, 23 (1984) 1234.
12 A. O. Grubb, E. Méndez, J. L. Fernandez-Luna, C. López-Otin, E. Mihaesco and J. P. Vaerman, *J. Biol. Chem.*, 261 (1986) 14313.
13 J. L. Fernandez-Luna, J. L. Moneo, A. O. Grubb and E. Méndez, *J. Imunol. Methods*, 82 (1985) 101.
14 C. López-Otin, A. O. Grubb and E. Méndez, *Arch. Biochem. Biophys.*, 228 (1984) 544.