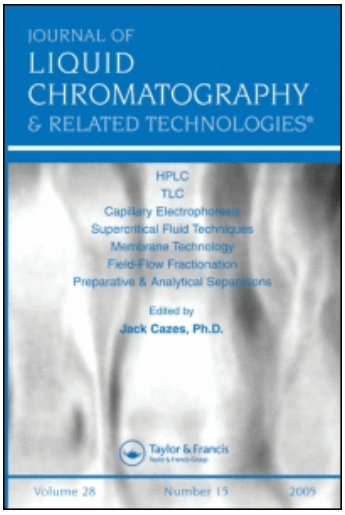

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Direct Combination of a High Performance Liquid Chromatograph and a Circular Dichroism Spectrometer for Separation and Structural Analysis of Proteins

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To cite this Article Takakuwa, Takashi , Kurosu, Yasuyuki , Sakayanagi, Nobuyuki , Kaneuchi, Fumiko , Takeuchi, Norismasa , Wada, Akio and Senda, Masaaki(1987) 'Direct Combination of a High Performance Liquid Chromatograph and a Circular Dichroism Spectrometer for Separation and Structural Analysis of Proteins', Journal of Liquid Chromatography & Related Technologies, 10: 12, 2759 — 2769

To link to this Article: DOI: 10.1080/01483918708066824

URL: http://dx.doi.org/10.1080/01483918708066824

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(12), 2759-2769 (1987)

DIRECT COMBINATION OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPH AND A CIRCULAR DICHROISM SPECTROMETER FOR SEPARATION AND STRUCTURAL ANALYSIS OF PROTEINS

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ABSTRACT

Basic studies of the combined system of a high performance liquid chromatograph (HPLC) and a circular dichroism (CD) spectrometer for separation and analysis of proteins are described. The HPLC-CD measurement of standard protein mixture was easily carried out by using a micro flow-cell device with a beam condenser and with a thin cell of a 1 mm-optical path. The effluent was firstly monitored at 280 nm by using an UV detector and subsequently monitored at 220 nm by using a CD spectrometer. The CD spectrum at each chromatographic peak by CD was measured in the wavelength region of 250 - 195 nm by a stopped flow method.

INTRODUCTION

The applications of high performance liquid chromatography (HPLC) to the separation of proteins have been remarkably utilized because this technique is one of the most common and convenient approach with simple operation, considerable resolution and high recovery of separated proteins. In particular, the maintenance of biological activity have been recently noticed in the HPLC of proteins (1-3). Since, as well known, the biological activity is related to the conformation of the protein, the detector which can sensitively reflect these properties will be strongly desired. However, the usual detectors, including UV one, are powerless in this purpose.

Circular dichroism (CD) is a useful tool for the conformational analysis of protein in relation to enzymatic activity, etc. (4-7). During recent several years, the direct combination of a HPLC instrument and a CD spectrometer have been examined to apply to low molecular weight chiral substances (8-12). If the HPLC-CD system can be easily utilized for proteins, it will be very useful for the conformational analysis or identification in the chromatographic separation. However, the extrema (220 - 190 nm) of the important CD bands, which reflect a backbone conformation of protein, exist in the vicinity of the short wavelength-limit (180 nm) of a CD spectrometer. Therefore, the shorter wavelength, the more difficult even the ordinary CD measurement becomes in this wavelength region. The HPLC-CD measurement by using a usual micro flow-cell is very difficult until now, for only part of the light-flux energy of a CD spectrometer has been used.

In this paper, we wish to demonstrate that our improved HPLC-CD system can sensitively detect a CD signal of protein and can offer the on-line CD spectrum by using a stopped flow method.

EXPERIMENTAL

Flow-cell device; To improve the efficiency of the light-flux energy of usual HPLC-CD system, a micro flow-cell was set into a cell holder with a beam condenser (fused silica lenses), which was modified from a JASCO micro CD attachment (10). The flow-cell of a 1 mm-optical path (a 19 μ l-volume) was used to reduce the far-UV absorption of a buffer itself.

Apparatus; JASCO HPLC system consisted of an intelligent pump (Model 880-PU), an injector (Model VL-614), an UV detector (Model 875-UV spectrophotometer) and a CD spectrometer (Model J-500C Spectropolarimeter attached with a flow-cell device and with a Model DP-500N Data Processor). Both an UV detector and a CD spectrometer were used connecting in that order.

Reagents and Proteins; Tris(hydroxymethyl)aminomethane (Tris) and hydrochloric acid (HCl) were both guaranteed grade purchased from Wako Pure Chemicals (Tokyo, Japan). Water purified by a Milli R/Q (Millipore, USA), was used for HPLC. Proteins were obtained from sources indicated in parentheses; horse spleen ferritin [MW 480,000], bovine pancreas ribonuclease A [MW 14,000](Pharmacia Fine Chemicals, Sweden), sperm whale skeletal muscle myoglobin [MW 17,000] and human transferrin [MW 80,000](Sigma Chemicals, St. Louis). These proteins were used without further purification. A Mixed protein solution was prepared in 50 mM Tris-HCl (pH 7.2) (each protein, 0.02 % (W/V)).

Column; A polyvinyl alcohol column, Asahipak GS-510 (0.76 ID x 50 cm) for gel permeation chromatography (GPC) was obtained from Asahi chemical Ind. Co.,Ltd. (Tokyo, Japan). A 100-cm length (i.e., two 50-cm columns coupled in series) was employed.

Chromatographic procedure and CD measurement; A mixed protein solution was introduced onto the column and eluted by the isocratic elution of 50 mM Tris-HCl (pH 7.2) at a flow rate of 1.0 ml/min at room temperature. The effluent was firstly

monitored at 280 nm by using an UV detector, and subsequently monitored at 220 nm by using a CD spectrometer (spectral band width (SBW) = 2 nm, time constant (TC) = 4 sec). The on-line CD spectrum was measured with the following procedures. The pump delivery was stopped at each chromatographic peak by CD and the pass was closed at the injector valve (stopped flow method) and then the each CD spectrum under the operating conditions of SBW = 1 nm, TC = 4 sec, scan speed = 20 nm/min, a computer of average transient (CAT) = 2 scans, was measured within 10 minutes. After completion of one CD measurement, the pump delivery was restarted to proceed the chromatography.

RESULTS AND DISCUSSION

Basic test of the CD spectrometer attached a micro flow-cell device

To estimate the efficiency of the light-flux energy of a CD spectrometer attached the improved micro flow-cell device, the high-tension voltage of the photomultiplier detector was measured by filling distilled water into the cell. As compared with ordinary measurement in the vicinity of 220 - 200 nm, the efficiency was estimated to about 50 % and the extent of an increase of the CD noise was within slight 1.4 fold. The short wavelength limit of a 50 mM Tris-HCl buffer solution was 195 - 200 nm with this system.

The CD blank of this system was measured by filling distilled water into the cell over the wavelength region of 185 - 700 nm. When SBW was set to 1 nm (standard condition), the maximum amplitude of CD blank was less than 10 mdeg. (ellipticity, θ) over the wavelength region of 185 - 700 nm, and this result dose not remarkably disturb a HPLC-CD measurement. However, when SBW was set to 2 nm, the maximum amplitude increased to more than twice. The enhanced baseline-amplitude at SBW = 2 nm is not suitable for CD spectral

measurement but did not disturb to monitor the CD signal at 220 nm.

The wavelength dependency of this CD system was calibrated by using a 0.06 % (W/V) water solution of ammonium d-10-camphorsulfonate (ACS; JASCO CD standard, obtainable as CD reagent from Katayama Chemicals; $[\theta]_{290.5}$ + 7910, $[\theta]_{192}/[\theta]_{290.5}$ - 2.03) (14) and the optical path of the flow-cell was estimated to 1.04 mm based on the CD maginitude of the ACS solution at 290.5 nm. The CD spectrum of the ACS solution measured by this system showed the low noise spectral feature in the wavelength region of 185 - 340 nm.

Application of the HPLC-CD system for proteins

To demonstrate the usefulness of the system, the HPLC-CD measurement for the mixed protein solution was carried out in the GPC system after the chromatographic separation was well established by an UV detection. A quantity of each protein charged was 80 μg (total injection volume, 400 μl), which was suitable for both the analytical separation and the CD detection.

Figure 1 shows a comparison of the chromatogram by UV (UV chromatogram) at 280 nm and the chromatogram by CD (CD chromatogram) at 220 nm. The CD chromatogram gives a low noise trace (0.4 mdeg. at SBW = 2 nm, TC = 4 sec). The CD detection limits (0.8 mdeg. at S/N ▫ 2) were estimated as follows; ferritin (80 μg), transferrin (13 μg), myoglobin (4.6 μg) and ribonuclease A (21 μg) as the injection quantity in this GPC. In Figure 1, the CD chromatogram strongly reflects the conformation of each protein component, while the UV chromatogram roughly reflects the each quantity. The CD-monitoring at 220 nm is suitable for the detection of helix and β-form in protein.

Figure 2 shows the on-line CD spectra with a good S/N ratio in the region of 250 - 195 nm. In the CD spectrum of myoglobin (Figure 2), the large double negative peak (about 207 nm and 222

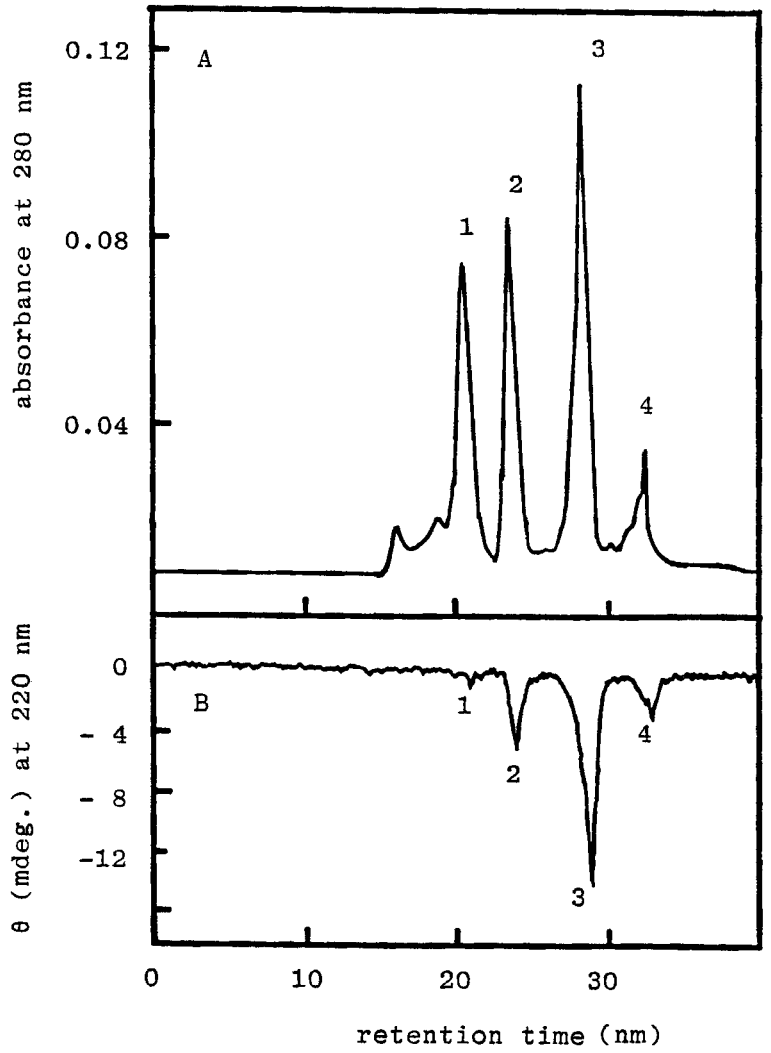

Figure 1 GPC of proteins. (A) UV chromatogram and (B) CD chromatogram. Sample: 1. ferritin (80 μg), 2. transferrin (80 μg), 3. myoglobin (80 μg), 4. ribonuclease A (80 μg). Chromatographic conditions: column; Asahipak GS-510 (0.76 ID x 50 + 50 cm), eluent; 50 mM Tris-HCl (pH 7.2), flow rate; 1.0 ml/min, column temperature; room temperature. CD conditions: TC; 4 sec, SBW; 2 nm.

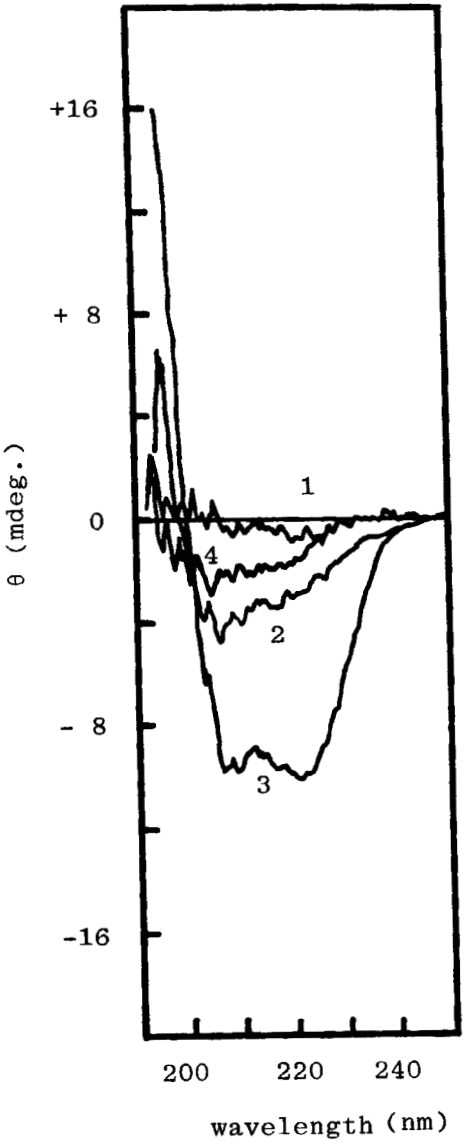

Figure 2 CD spectra of proteins by a stopped flow method. Conditions: TC; 4 sec, SBW; 1 nm, CAT; 2 scans, scan speed; 20 nm/min.

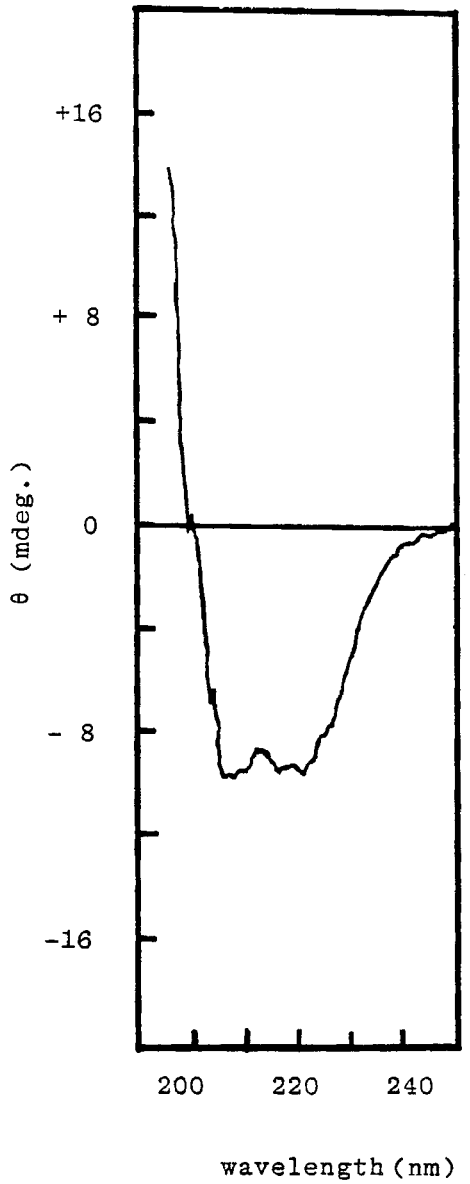

Figure 3 The reference CD spectrum of myoglobin. Concentration: 0.005 % (W/V) in 50 mM Tris-HCl (pH 7.2). Conditions are same as those in Figure 2.

nm) and the crossover at about 200 nm, which characterize the spectral feature of a protein of high helical content (79 %) (15,16), are clearly catched. In the CD spectra of transferrin and ribonuclease A as compared with it of myoglobin, the shorter wavelength shifts (199 - 197 nm) of the crossover, which reflect the increase of random structural contents as well known, can be also clearly catched by this HPLC-CD system.

For the calibration of the concentration of CD chromatographic peaks in Figure 1 and the on-line CD spectra in Figure 2, each reference CD spectrum of protein was measured by filling the solution of known concentration into the flow-cell. As an example, Figure 3 shows the reference CD spectrum of 0.005 % myoglobin in a 50 mM Tris-HCl (pH 7.2). Detection limit of only CD spectrometer with this flow-cell device independent of the GPC separation (corresponded to 0.8 mdeg. of CD signal at S/N =2) were estimated based on the CD magnitude at 220 nm of the reference spectrum as follows; ferritin (27 μg/ml), transferrin (6.4 μg/ml), myoglobin (4.3 μg/ml) and ribonuclease A (16 μg/ml).

In conclusion, our HPLC-CD system for protein analysis allows to be operated with a small quantity in this system, except for proteins having a very weak CD band such as ferritin. It is sufficiently expected that this HPLC-CD system is practically a useful tool for conformational analysis and identification of proteins in a chromatographic separation.

In future, improving the efficiency of the light energy, this HPLC-CD system will have a higher sensitivity.

ACKNOWLEDGEMENT

We are indebted to Drs.Takateru Uchida and Kohji Noguchi, Technology and Development 2nd Division of Asahi Chemical Industry Co.,Ltd. for the donation of Asahipak GS-510 column. We also wish to thank Dr.Muneo Saito, Manager of Liquid

Chromatography and Medical Instruments Division and Dr.Nobuhiko Kokubun, General Manager of Spectroscopic Instruments Division of JASCO Factory for thier encouragement and useful advices.

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