Journal of Chromatography, 515 (1990) 659–666
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1746

Apparatus for coupled high-performance liquid chromatography and capillary electrophoresis in the analysis of complex protein mixtures

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ABSTRACT

Apparatus for coupled high-performance gel permeation chromatography (HPLC) and capillary electrophoresis was constructed and applied to the analysis of complex protein mixtures. To avoid electric leakage to the HPLC system, an electromagnetic pinch valve was inserted in the line from the column outlet to the injection port of the capillary electrophoresis apparatus. All the procedures for chromatography and electrophoresis were automated with the aid of a system controller. The apparatus was successfully used for the automatic two-step separation of human serum proteins and water-soluble proteins in bovine brain. Proteins separated by gel permeation HPLC were further analysed by capillary electrophoresis according to their characteristic electrophoretic mobilities.

INTRODUCTION

The techniques of high-performance liquid chromatography (HPLC) and electrophoresis are widely used for the analysis of proteins. However, the resolution of each technique is low with respect to the number of protein species present in tissues or cells. In order to improve the resolution, instruments have been developed that combine two techniques having different separation principles. For example, gel permeation HPLC and reversed-phase HPLC were coupled for the analysis of glycosides in plant extracts[1], ion-exchange HPLC and reversed-phase HPLC for the analysis of polypeptides[2] and reversed-phase HPLC and sodium dodecyl sulphate polyacrylamide gel electrophoreis for the analysis of water-soluble proteins in *E. coli*[3]. Two-dimensional polyacrylamide gel electrophoresis, which offers the highest resolution of proteins at present, is a combination of gel isoelectric focusing and pore-gradient gel electrophoresis[4].

In a preceding paper, we reported on the construction of an apparatus combining low-pressure gel permeation chromatography (GPC) with capillary

0021-9673/90/$03.50

electrophoresis for the analysis of proteins with high sensitivity and simple operation[5]. The apparatus was used for the automatic two-step separation of purified proteins. Proteins were separated according to their molecular size in the first step, then separated according to their electrophoretic mobility in the second step.

To improve the resolution of proteins in the GPC step, we have now employed an HPLC system instead of the low-pressure chromatographic system. This apparatus was applied to the analysis of human serum proteins and water-soluble proteins in bovine brain.

EXPERIMENTAL

Apparatus

A schematic diagram of our apparatus is shown in Fig. 1. The HPLC system was

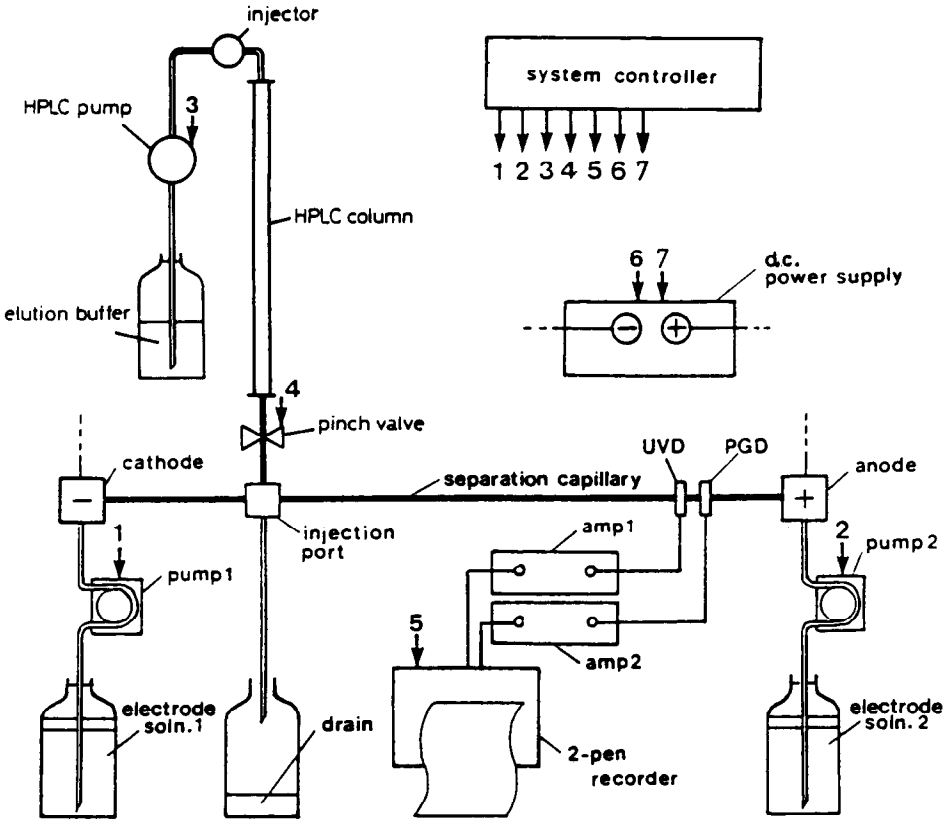

Fig. 1. Apparatus combining HPLC and capillary electrophoresis. The terminating electrolyte (solution 1) and leading electrolyte (solution 2) are pumped by peristaltic pumps (pumps 1 and 2) to wash the electrodes and separation capillary. Elution buffer for GPC is pumped through the HPLC column by a high-pressure pump (HPLC pump) and the effluent from the column is loaded through the injection port. An electromagnetic pinch valve is closed to avoid electric leakage to the HPLC system and then a d.c. high voltage is applied between the electrodes. Protein zones are detected with UV and potential gradient detectors (UVD and PGD) during the analysis. The numbered arrows indicate the output lines connecting the system controller to the equipment.

combined with a capillary electrophoresis apparatus. The HPLC system consisted of a glass column (500 mm × 4.0 mm I.D.), packed with the gel permeation packing Asahipak GS-520 (polyvinyl alcohol-type gel, particle diameter 9 μm, exclusion limit for pullulan 300 000) (Asahi Chemical Industry, Kawasaki, Japan), a Model NP-DX-8 high-pressure pump (Nihon Seimitsu Kagaku, Tokyo, Japan) and a Model KLS-3T line sample injector (Kyowa Seimitsu, Tokyo, Japan).

In order to avoid electric leakage, the outlet of the HPLC column was connected to the sample injection port of the capillary electrophoresis apparatus in the following manner. A perfluorinate ethylene–propylene (PFEP) copolymer tube (50 mm × 0.25 mm I.D.) from the column outlet was connected to a silicone-rubber tube (10 mm × 0.5 mm I.D. × 2.3 mm O.D.), equipped with a Model PM-02 electromagnetic pinch valve (Takasago Electric, Tokyo, Japan) in the middle. The other end of the silicone-rubber tube was connected to a PFEP tube (50 mm × 0.25 mm I.D.). A glass capillary tube (50 mm × 0.24 mm I.D. × 0.35 mm O.D.) was connected to the end of the PFEP tube and the other end was introduced into the injection port through a septum. The column effluent was introduced into the separation capillary through the port and the excess effluent was drained off. A constant volume (*ca.* 5 μl) of the effluent was retained in the separation capillary and was subjected to electrophoresis. The part of the apparatus for capillary electrophoresis is basically the same as that reported previously[5,6].

System controller

For the automatic operation of the apparatus, all the components (the HPLC pump, the electromagnetic pinch valve, peristaltic pumps, a recorder and a high-voltage d.c. power supply) were controlled by a system controller. Construction details have been described fully elsewhere[6].

Computer programs and time schedule for automated operation

After equilibration and sampling, the cycle of HPLC elution and electrophoresis of the effluent was repeated automatically. The microcomputer programs for the automatic operation were written in BASIC. The time schedule of the program is as follows: (1) pump the leading electrolyte solution to rinse the anode and the separation capillary (2 ml in 2 min); (2) pump the termination electrolyte solution to rinse the cathode (0.7 ml in 3 min); (3) open the electromagnetic pinch valve; (4) pump the chromatographic elution buffer into the column to introduce the column effluent into the injection port (70 μl in 30 s); (5) close the pinch valve; (6) turn on the d.c. power supply to start electrophoresis (150 μA constant current); (7) turn on the two-pen recorder and reduce the current to 50 μA; (8) turn off the d.c power supply and the recorder. These procedures are repeated from step 1. The time needed for one cycle of analysis is 23 min.

Preparation of protein samples

Sera from normal human adults were freshly obtained and stored at −20°C. Soluble proteins from bovine brain were prepared in basically the same way as described previously[7]: fresh bovine brain tissue was washed thoroughly with distilled, deionized water to remove clotted blood, then combined with an equal weight of distilled deionized water and homogenized with a Potter–Elvehjem homogenizer in an

ice-bath. The homogenate was centrifuged at 35 000 *g* for 1 h and the supernatant was stored at −20°C.

Reagents

The following reagents were used for the preparation of the electrode solutions without further purification: 2-Amino-2-methyl-1-propanol (Nakarai Chemicals, Kyoto, Japan), tranexamic acid (Daiici Seiyaku, Tokyo, Japan), hydrochloric acid (1 *M*, special grade for amino acid sequence analysis), potassium hydroxide and hexane (Wako, Osaka, Japan), hydroxypropylmethylcellulose (HPMC) (Aldrich, Milwaukee, WI, U.S.A.), sodium azide (Kanto Pure Chemical, Tokyo, Japan) and ampholyte mixture (Ampholine, pH 3.5–10) (Pharmacia–LKB, Uppsala, Sweden).

Conditions for gel permeation HPLC and capillary electrophoresis

Protein samples (200–600 μg of protein in 30 μl) were applied to the HPLC column. The elution buffer was 0.25% Ampholine (pH 3.5–10)–0.00625% sodium azide. The conditions for capillary electrophoresis were as follows. The leading electrolyte solution was 5 m*M* HCl–9.3 m*M* 2-amino-2-methyl-1-propanol (pH 9.9) and the terminating electrolyte solution was 50 m*M* tranexamic acid–potassium hydroxide (pH 10.8). The solutions were kept in amber-glass bottles and overlayed with a 1-cm layer of hexane to minimize the dissolution of carbon dioxide. A PFEP capillary tube (230 mm × 0.5 mm I.D. × 1.0 mm O.D.), the inner surface of which had been coated with HPMC, was used as the separation tube. Electrophoresis was performed at a constant current of 150 μA for 4.8 min (initial voltage *ca.* 6 kV) and then at a constant current of 50 μA for about 12 min.

RESULTS AND DISCUSSION

Combination of HPLC system with capillary electrophoresis apparatus

In the preceding study[5] we used an open column packed with Sephadex G-50 and a micro-peristaltic pump for GPC. To improve the resolution of proteins by GPC, we experimented with connecting the HPLC system to the electrophoresis apparatus. When the outlet line (PFEP tubing) of the HPLC column was directly connected to the injection port, malfunction of the computer frequently occurred during electrophoresis and the automatic operation was interrupted. These effects were found to be due to the electric discharge of the high-voltage d.c. through the metallic parts of the HPLC system (the stainless-steel tubing, high-pressure pump and injector). In order to avoid electric leakage, an electromagnetic pinch valve was installed between the column outlet and the injection port, as described under Experimental. The time schedule for automatic operation was reprogrammed so that the pinch valve was closed during electrophoresis. With the aid of these devices, the two-step separation could be performed automatically.

Gel permeation HPLC of human serum proteins

Before the two-step separation, gel permeation HPLC was performed separately. Fig. 2 shows the elution profile of a human serum sample. Human serum (10 μl) was diluted 5-fold with the chromatographic elution buffer (40 μl) and a 30-μl portion was applied to the column and eluted at a flow-rate of 140 μl/min. As Fig. 2 shows, the

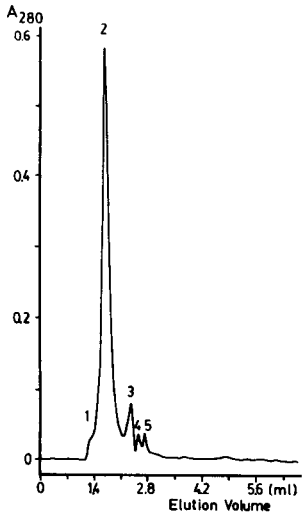

Fig. 2. Elution profile of human serum obtained by gel permeation HPLC. Human serum, diluted 5-fold (30 μl), was applied to the glass column (500 mm × 4 mm I.D.) and eluted with the elution buffer (0.25% Ampholine–0.00625% sodium azide) at a flow-rate of 140 μl/min.

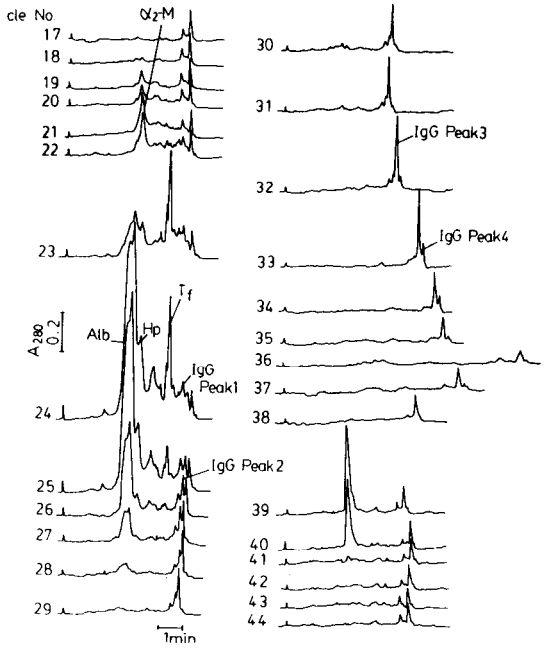

Fig. 3. Separation of serum proteins and other constituents with the combined apparatus. A serum sample solution (30 μl) was applied and the UV patterns of the electropherograms were traced. Serum proteins eluted from the gel permeation HPLC column were further separated with their characteristic electrophoretic mobility. α_2-M = α_2-Macroglobulin; Hp = haptoglobin (type 1-1); Tf = transferrin; Alb = albumin; IgG peak 1, etc. = separated peaks of IgG.

UV-absorbing constituents of human serum were separated into four peaks and one shoulder.

Gel permeation HPLC capillary electrophoresis of serum proteins

The serum sample solution (30 μl) was applied to the column. The column volume was 6.28 ml (500 mm × 4 mm I.D.), the line dead volume from the HPLC column outlet to the injection port was calculated to be 9 μl and the volume of the effluent pumped in one cycle was tentatively set at 70 μl. Then, the cycle of analysis described under Experimental was repeated 90 times without manual intervention.

Of the 90 electropherograms obtained, those from the 1st to the 17th cycle and from the 44th to the 90th cycle were fairly reproducible, showing the UV pattern of Ampholine. The UV- absorbing constituents in human serum appeared from the 18th to the 43th cycles, as shown in Fig. 3. From the elution volumes, the shoulder numbered 1 in Fig. 2 corresponded to cycles 18–20, peak 2 to cycles 21–30, peak 3 to cycles 31–34, peak 4 to cycles 35–38 and peak 5 to cycles 39–43.

Some of the UV peaks in the patterns were identified by analysing either purified serum proteins or the effluent from the HPLC column by micro two-dimensional gel

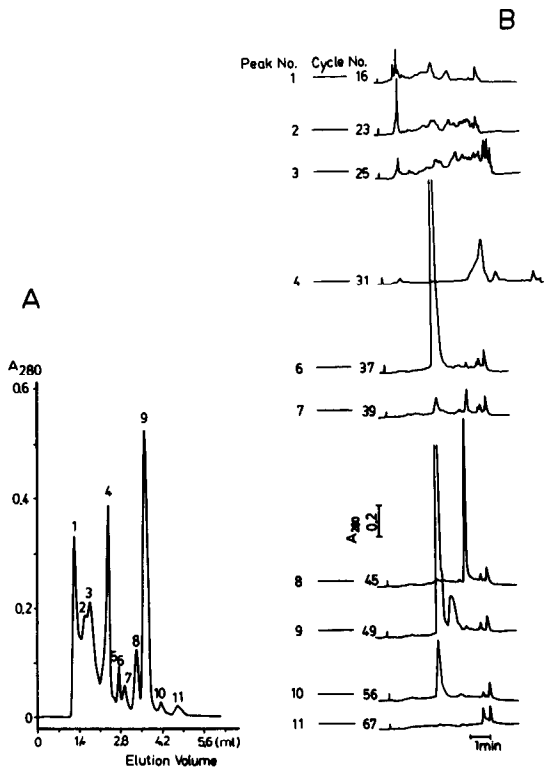

Fig. 4. Examples of the electropherograms obtained with the combined apparatus for a soluble brain fraction. Soluble fraction of bovine brain (30 μl) was applied to the combined apparatus. The electropherograms (UV patterns) corresponding to the UV peak positions of gel permeation HPLC (shown in A) are shown in B.

electrophoresis[8]. The peaks of α_2-macroglobulin (molecular weight estimated by pore-gradient fel electrophoresis, *ca.* 500 000), haptoglobin 1-1 (100 000), transferrin (90 000), albumin (69 000) and immunoglobulin G (IgG) (150 000) were identified as indicated in Fig. 3. The serum proteins were eluted from the HPLC column according to their molecular weight, except IgG, which seemed to be retarded by ionic interaction with the column material. IgG appeared as four peaks in the electropherograms and the peak ratio changed as shown in Fig. 3.

Each identified protein appeared in four or five cycles, corresponding to an effluent volume of *ca.* 300 μl. The plate number of the HPLC column under the conditions employed was calculated to be about 600–1000.

The UV peak appearing in cycles 39–40 (Fig. 3), which corresponded to peak 5 in Fig. 2, was identified as being due to uric acid. The component corresponding to peak 4 in Fig. 2 was not detected in the electropherograms, suggesting that it did not migrate to the anode under the electrophoretic conditions. The potential gradient curves (not shown in Fig. 3) around cycles 33–37 showed much smaller slopes than the others, suggesting the presence of ions with no UV absorbance. These results showed that low-molecular-weight substances appeared after cycle 33 (elution volume *ca.* 2.3 ml).

Analysis of soluble brain proteins

Soluble proteins in bovine brain (280 μg in 30 μl) were injected into the apparatus. The conditions were the same as those for the analysis of human serum proteins. Some of the electropherograms, which corresponded to the UV peak positions in gel permeation HPLC (Fig. 4A), are shown in Fig. 4B. From the elution volumes, peaks 1–4 in Fig. 4A seem to be due to proteins and peaks 5–11

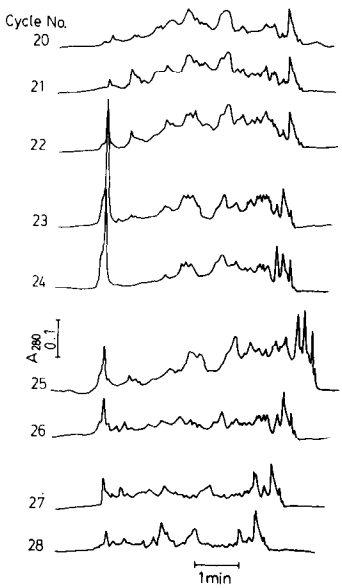

Fig. 5. Examples of sequential electropherograms (UV patterns) representing protein fractions. Electropherograms corresponding to peaks 2 and 3 in Fig. 4A were traced. In each electropherogram, 20–30 peaks or shoulders were observed.

low-molecular-weight substances. The simple UV absorption patterns of the electropherograms also suggested the absence of proteins after *ca.* cycle 37 (elution volume 2.6 ml).

Some of the sequential electropherograms (cycles 20–28), corresponding to parts of peaks 2 and 3 in Fig. 4A, are shown in Fig. 5. In each electropherogram, about 20–30 peaks or shoulders of proteins were observed.

The resolution of proteins with the combined system is determined by the resolution in each separation step. As shown in Figs. 3, 4B and 5, 20–30 protein peaks can be resolved in the capillary electrophoresis step. The HPLC packing used improved the resolution in the gel permeation chromatographic step, but still only a few protein peaks could be obtained. As improvements in the resolution of proteins in gel permeation HPLC may be restricted by the low diffusion constants of proteins and by the upper limit of the capacity factors, an HPLC system based on other separation principles (*e.g.*, reversed-phase HPLC) should also be examined for the high-resolution analysis of proteins.

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