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Short communication

Automated sample introduction for an imaged capillary isoelectric focusing instrument via high-performance liquid chromatography sampling devices

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Abstract

Sample introduction of an imaged capillary isoelectric focusing (cIEF) instrument is fully automated by using commercially available high-performance liquid chromatography (HPLC) injection valves and autosamplers. Sample carryover can be controlled to under 1% when the valve and separation column are washed for 1 min between sample runs. The standard deviation of peak areas for 20 injections is 3.5%, which includes deviations created by the absorption imaging detector and the isoelectric focusing process inside the 75 μm I.D. column. Sample throughput is up to 10 samples per hour. The instrument has been applied to fast analysis of many proteins including monoclonal antibodies.

Keywords: Autosampler; Injection valve; Monoclonal antibodies; Proteins

1. Introduction

Capillary isoelectric focusing (cIEF) [1] is a powerful technique for the separation of protein mixtures based on differences in their isoelectric points (p*I*). In this electrophoretic method, a protein migrates through a pH gradient along a capillary column until it reaches the pH point at which it has zero net charge [2]. This point is the protein isoelectric point, and protein migration stops, causing the protein to be focused at the point within the capillary column. On-line, whole column imaging detectors appear to be the most suitable sensors for detection of those focused protein bands within the capillary column [3].

In the past few years, several different kinds of imaging detectors have been developed for cIEF detection [4,5]. Imaged cIEF combines separation and detection into a single step. It eliminates the mobilization process which is necessary in conventional cIEF methods to move focused protein bands past an on-column point detector. Thus, use of imaging detectors eliminates problems associated with the mobilization process, such as inaccuracies in p*I* determination [3] and uneven resolution created by uneven mobilization speed.

Sample volumes in cIEF are usually in the nl range. The sample injection and mobilization procedures of cIEF in commercial capillary electrophoresis (CE) instruments are achieved by incorporating a custom designed autosampler within the instruments. A dedicated CE autosampler must control injection pressure accurately and reproducibly, and the accurate control of the pressure is also necessary for performing mobilization in cIEF [6]. Since imaged cIEF eliminates the mobilization pro-

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cess, it may avoid the need for an expensive CE autosampler. Also, cIEF detection by whole-column imaging does not require accurately controlled sample volumes as long as the whole capillary column is filled with sample solution [4]. For this purpose, a unique capillary column cartridge was designed [7] to simplify the sample introduction procedure for imaged cIEF. With the cartridge, the capillary column can be filled with sample solution infused by a syringe pump without the need to change electrolytes [7].

In this paper, we will describe an automated sample introduction system developed in our laboratory for imaged cIEF using commercially available high-performance liquid chromatography (HPLC) injection valves and autosamplers.

2. Experimental

2.1. Apparatus

The cIEF column cartridge is shown in Fig. 1. The structure of the cartridge was reported in our previous paper [7]. The column is a 5 cm long, 75 μm I.D., 150 μm O.D. silica capillary (Polymicro Technologies, Tucon, AZ, USA). The volume of the column is 0.22 μl. The coatings on the outside of the capillary are removed for detection. The capillary is coated with noncrosslinked acrylamide to eliminate electroosmotic flow by the reported methods [1]. The ends of the column are connected to two pieces of 4 mm long dialysis hollow fiber membranes (Spectrum Medical Industries, Los Angeles, CA, USA). The two sections of the fiber are inserted into the two electrolyte reservoirs.

Fig. 1 also illustrates how the column cartridge is interfaced to an eight-port, two-position HPLC sample injection valve (Valco Instruments, Houston, TX, USA) and to a HPLC autosampler (Model ISS-100, Perkin–Elmer, Norwall, CT, USA). The injection port of the valve is linked to either a needle port for manual sample injection using a microsyringe, or the HPLC autosampler. The sample loop volume in the injection valve is 2.5 μl. The infusion pump is a syringe pump (Model A-99, Razel Scientific Instruments, Stamford, CT, USA). The syringe used with the pump can be 1–3 ml in capacity. The flow-rate

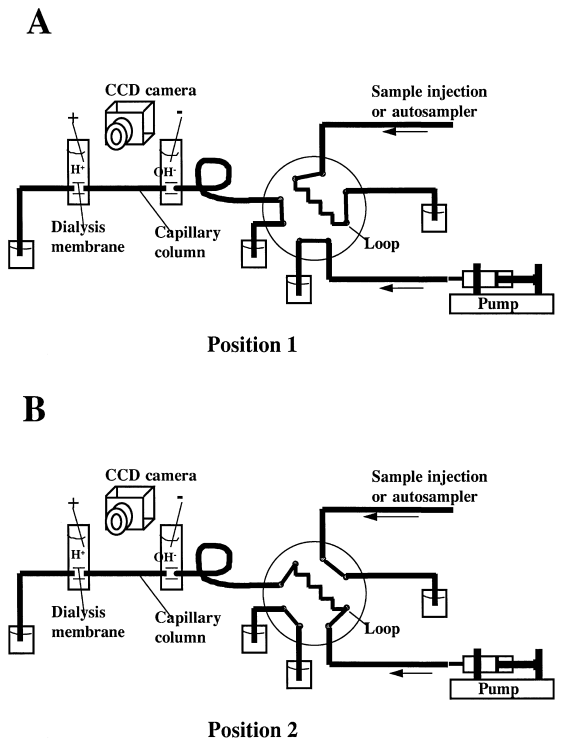

Fig. 1. Imaged capillary isoelectric focusing with automated sampling system. (A) The injection valve is at position 1. (B) The valve is at position 2.

of the pump is set at 6 μl/min. The syringe pump operates during the entire experiment. For each sample injection, 20 μl of sample is injected manually or by the autosampler to ensure that the sample loop is filled with the sample. The whole sample injection system and the imaged cIEF instrument are fully controlled by a PC computer.

The optical absorption imaging detection system is similar to our previous experiments [7]. Two detectors were used in the experiment. One was operated at 415 nm for hemoglobin detection and another at 280 nm for other proteins.

2.2. Reagents

All solutions are prepared using deionized water. All chemicals are reagent grade. Solutions of 10 m*M* H_3PO_4 and 20 m*M* NaOH are used as the anolytes and catholyte, respectively. Samples include human hemoglobin AA2 control (Helena Laboratories,

Beaumont, TX, USA) and a monoclonal antibody (anti-α_1-antitrypsin, Calbiochem–Novabiochem Co., La Jolla, CA, USA). The hemoglobin control is diluted by 200 times before being mixed with carrier ampholytes. Protein samples are mixed with the carrier ampholytes (Pharmalyte pH 3–10, Sigma) to a final ampholyte concentration of 4–8%. The final concentration of the antibody is 0.5 mg/ml.

2.3. Sample introduction and isoelectric focusing processes

As shown in Fig. 1A, at position 1, a 20 μl sample is injected into the sample loop by a syringe or the autosampler. The valve is switched to position 2 (Fig. 1B). At this position, sample solution stored in the loop will be flushed into the separation column. When the column is filled with the sample, the valve is switched back to position 1, and a 3 kV separation voltage is applied to the two reservoirs to start isoelectric focusing. The focusing finishes in 5 min. At the end of 5 min, the focused protein bands within the column are recorded by the CCD camera without mobilization. Then, the sample loop is washed by injecting 60 μl deionized water into it while the valve is at position 1. Again, the valve is switched to position 2 to wash the column for 30 s. This makes the injection system ready for the next sample.

3. Results and discussions

As shown in Fig. 1, the dialysis membrane hollow fibers used in the column cartridge isolate the sample solution inside the column from the electrolytes in the reservoirs. Small molecules, such as H^+ and OH^- ions, can still pass through the membrane freely. Thus, isoelectric focusing can take place inside the column. Samples can be directly injected into the column by a pump. After the column is filled with the sample, the injection is stopped and the separation voltage is turned on to start isoelectric focusing. At the end of the focusing process, all protein bands inside the column are detected simply by taking ''pictures'' of the column using the CCD camera.

There are two positions in the injection valve (Fig. 1). Several processes can be performed at the two positions. At position 1 (Fig. 1A), the column cartridge is isolated from the flow system. The separation voltage (3 kV) may be applied to the two electrolyte reservoirs to start the isoelectric focusing inside the capillary column. Also, at position 1, the sample loop can be washed by injecting wash solvent (deionized water, in the present experiment), or the next sample may be injected into the loop and stored there. At position 2 (Fig. 1B), the sample stored in the loop is injected into the column. Also, the capillary column can be washed if wash solvent is stored in the loop.

The volume of the sample loop is more than 10 times that of the separation column, which ensures that the column will be filled with the sample solution at a time after the valve is switched to position 2. The time needed to flush sample from the loop to the column can be calculated from the flow-rate and the volumes of the cartridge inlet tubing if the flow-rate of the syringe is controlled accurately. However, considering the use of the low pressure syringe pump and the different back pressure created by different capillary column cartridges, to make reproducible and bubble-free sample injections into the column, a monitoring method is still needed. In our system, current passing through the column is used to monitor the injection when a 500 V voltage is applied to the column. The stability of the current can also show whether there are air bubbles within the column. Since a capillary column is usually kept dry after use, air bubbles may occasionally hide at capillary connectors inside the switch valve even after the whole column and valve are filled with liquid. The bubbles may cause current to fluctuate.

Fig. 2 shows the current changes when a hemoglobin sample is injected into the column after the valve is turned to position 2. The peak of the curve indicates the moment when the separation column is filled with the sample. At that time, the sample injection should be stopped by switching the valve to position 1. The current stability in the plateau at the curve peak shows whether there are air bubbles inside the column. Thus, there are two criteria to determine the moment when the injection should be stopped: (1) the current reaches its maximum value and (2) the current is stable at this maximum value

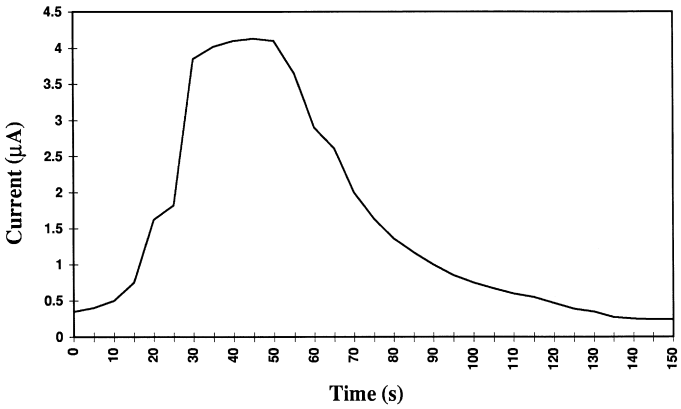

Fig. 2. Current changes inside the column when the hemoglobin sample is injected into the column from the sample loop. The flow-rate of the injection is 6 μl/min.

for a certain period of time. For the 2.5 μl loop used in the system, as shown in Fig. 2, the period of time is about 15 s. We can use ''stable for 5 s'' as the second criterion. This value depends on the volume of the loop. The first criterion is set to ensure that the concentration of the sample inside the column is the same as that in the sample vial. The second one is used to ensure bubble-free injections.

The choice of injection time can be done by a computer. A computer program was developed in our laboratory to do this job. Fig. 3 shows the steps of the program for sample injection. Three parameters in the program need the user's input. The first is the peak current. This value depends on the carrier ampholytes' concentrations and the sample concentrations. It should be obtained from experiments. For the 8% carrier ampholytes concentration used in the experiment, at 500 V, the maximum current value should be higher than 3 μA. The second parameter is stability. Usually, the fluctuation of the current is less than 0.1 μA after it reaches the maximum value if there is no air bubble inside the capillary column. We entered 0.1 μA as the tolerance for the fluctuation. The third parameter is waiting time. Here it is set at 1 min. If in 1 min the current cannot reach a value higher than 3 μA and the 5-s stability, which means there are air bubbles inside the column, the computer will consider it a ''bad'' injection and will reinject the same sample. The waiting time may also be longer if a longer sample loop is used in the switch valve. This procedure ensures that the concentration of the sample solution along the whole column is the same as that in the sample vial. It also makes bubble-free injections.

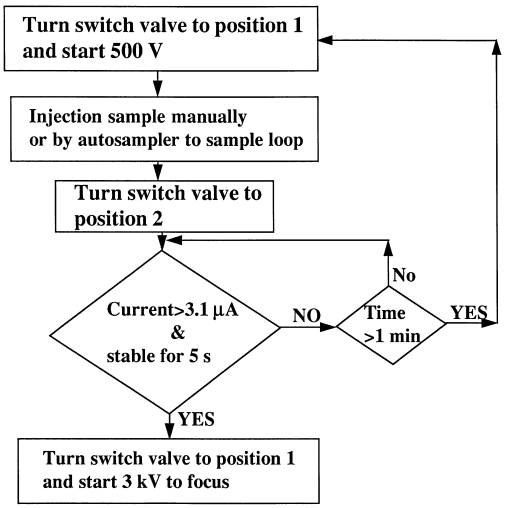

Fig. 3. Steps needed to ensure the concentration reproducible, bubble-free sample injections to the column.

Since the column volume is only in the high nanoliter range, carryover may be a big problem when using HPLC switch valves and connectors which may have microliter dead volumes. In our sampling system, between sample runs, the loop and the separation column are washed to eliminate the carryover by injecting deionized water into the loop and the column. The carryover is evaluated by running hemoglobin AA2 control sample followed

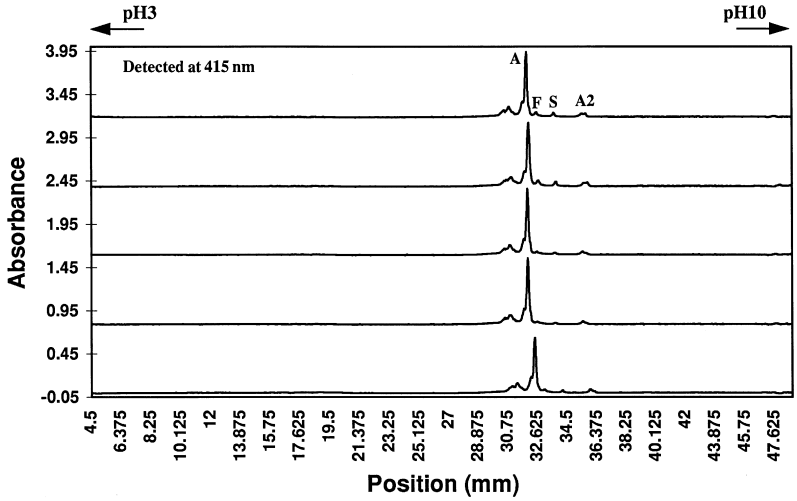

Fig. 4. Electropherograms of five consecutive injections of a hemoglobin AA2 sample separated by the imaged cIEF instrument.

by carrier ampholytes blank. As illustrated in Fig. 1, between runs, 60 μl water is injected into the loop to wash it at position 1. Then, the valve is switched to position 2 to wash the column for 30 s at a flow-rate of 6 μl/min. By doing this the carryover is less than 2.5% for the hemoglobin sample. The carryover can be controlled under 1% if necessary when the wash time increases from 30 s to 1 min. However, this will decrease the sample throughput.

The design of the capillary column cartridge ensures reproducible sample volume for each run because the isoelectric focusing always occurs between the two dialysis hollow fiber sections, as shown in Fig. 1. The automated sampling system provides concentration reproducible, bubble-free sample injections. The combination of both ensures reproducible results for quantitative analysis of protein samples. Fig. 4 shows electropherograms of five consecutive injections of a hemoglobin sample. For 20 injections, the relative standard deviation in the

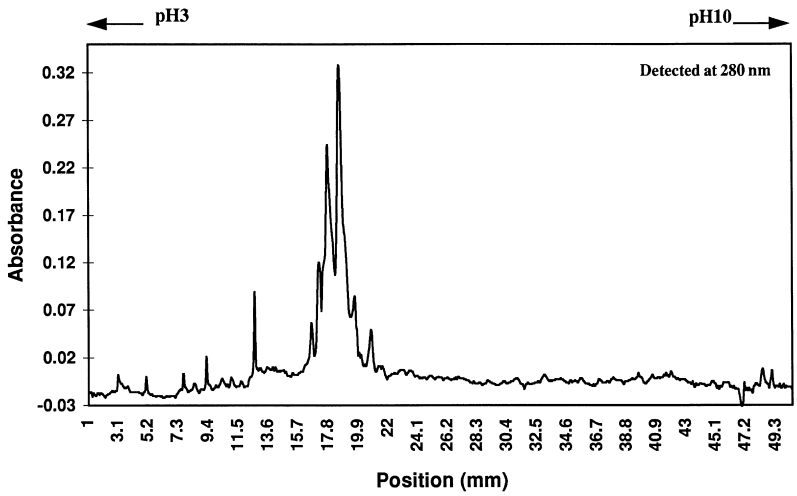

Fig. 5. Electropherogram of a monoclonal antibody sample (anti-α_1-antitrypsin) analyzed by the imaged cIEF instrument.

hemoglobin A peak area (Fig. 4) is 3.5%. This is just a rough estimation of the injection reproducibility, since the result should also include the deviation of the imaging detector and isoelectric focusing process.

The imaged cIEF instrument has been applied to fast analysis of many protein samples. With the sample introduction system, sample injection for the imaged cIEF can be fully automated using commercial HPLC injection valves and autosamplers. The sample throughput of the imaged cIEF is up to 10 samples per hour including separation and detection. Fig. 5 shows an example of applying the instrument to monoclonal antibody analysis. The electropherogram shows microheterogeneity of the sample.

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