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Protocol for Resolving Protein Mixtures in Capillary Zone Electrophoresis

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The separation of protein mixtures by capillary zone electrophoresis can be plagued by wall adsorption of the protein components, causing peak broadening and distortion. A method is presented for overcoming this problem by adding ethylene glycol to the protein sample and by choosing the running buffer and protein sample to be at different pH values and molarities. This protocol appears to work for a wide class of proteins having different molecular weights and pI values. The method has been applied to the analysis of proteins in human serum. Compared to the traditional method of agarose gel electrophoresis, the present method is more rapid and offers better resolution, suggesting its potential as a clinical diagnostic of certain disease states.

INTRODUCTION

The recent technology of capillary zone electrophoresis (CZE) has been shown to separate many types of molecules quickly and efficiently (1-3). Jorgenson and Lukacs (4) noted in the early stages of (CZE) development that proteins posed a particular problem; i.e., many proteins had a tendency to stick to the walls of the capillary. The resulting slow adsorption and desorption kinetics caused extensive tailing in the protein peaks.

A number of solutions to this problem have been proposed and implemented, among which are the use of buffer pHs higher than the pI of the proteins (5), the use of low pH buffers (6), the coating of the inside walls of the capillary with materials that slow or eliminate electroosmotic flow (4, 7-9), and coatings combined with very low pH buffers (10).

We report here an alternative method that appears to be quite effective for a wide class of proteins. This procedure circumvents the difficulties of preparing coated capillaries and of using buffers with pH values so extreme that the denaturation of the protein sample may become significant. As one example of the utility of this protocol, we apply it to the separation of human serum proteins.

As early as 1937, the improved moving-boundary electrophoresis apparatus developed by Tiselius was applied to the investigation of serum proteins (11, 12). Since that time, numerous applications of electrophoresis to physiological studies of clinical significance have been made by using not only moving-boundary electrophoresis (13), but many forms

of solid matrix media including paper (14), cellulose acetate (15), and gels (starch (16), agar (17, 18), agarose (19), and polyacrylamide (20)). Agarose gel electrophoresis, as it is now used in most clinical laboratories, is a qualitative screening method useful for the detection of abnormalities of the major proteins. It quickly prompts the pathologist to seek additional confirmation of such diseases as liver cirrhosis, inflammatory response, acute nephritis, biliary obstruction, membranoproliferative glomerulonephritis, systemic lupus erythematosus, hypogammaglobulinemia, and myeloma (19). In the case of myeloma, it is necessary, at present, to perform additional immunologic procedures in order to determine the classification, e.g., to see whether the κ chain or the λ chain of the IgG is involved. If resolution of the γ region could be improved so that the various components could be separated, it might add confirmation to immunological procedures. We use a commercially available, automated capillary electrophoresis instrument. Our method provides rapid analysis time, has high sensitivity and good reproducibility, and requires very small sample volumes.

EXPERIMENTAL SECTION

Instrumentation. The CZE instrument used in this work is the automated PACE 2000 (Beckman Instruments, Inc., Palo Alto, CA) controlled by an IBM PS/2 50SX computer fitted with PACE software (Beckman Instruments, Inc.), running in a WINDOWS (Microsoft, Redmond, WA) environment, and System Gold (Beckman Instruments, Inc.). The System Gold software will integrate the area under each peak so that these area numbers can be printed along with peak heights, retention times, and height/area ratios. The electropherogram can be printed showing retention times at each peak and the names of the peaks if that option is chosen. While quantitation in terms of peak areas is easily accomplished, it would take additional software to provide grams per deciliter in the serum protein runs.

The capillary cassette used was fitted with a 75 μm i.d. fused-silica column, 37.5 cm in length (30.5 cm to the detector). Injection of sample was by pressure for 2 s avoiding any bias problems that may occur with electrokinetic injection (21). On-column detection was performed by UV absorption at 200 nm. Electrophoretic runs were made by using an applied voltage of 10 kV, and the temperature was controlled at 20 ± 0.1 °C.

Reagents. α -Lactalbumin, carbonic anhydrase, β -lactoglobulin A, chicken egg albumin, bovine serum albumin, chicken ovalbumin, jack bean urease, α -chymotrypsin (bovine pancreas), and DL-dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO). Ribonuclease A was obtained from Cooper Development (Menlo Park, CA). Ethylene glycol was purchased from J. T. Baker (Phillipsburg, NJ). Whole blood was drawn from a willing, male member of this laboratory at various intervals and used to prepare serum. Pooled serum (male) was obtained from Sigma Chemical Co. Frozen serum samples from male and female

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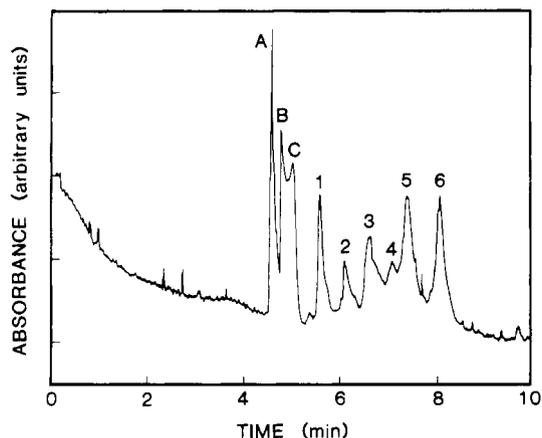


Figure 1. Electropherogram of a protein mixture with a concentration of 40 $\mu\text{g}/\text{mL}$ of each of the following: (1) carbonic anhydrase; (2) urease; (3) α -lactalbumin; (4) ovalbumin contaminant; (5) ovalbumin; (6) bovine serum albumin. Peaks marked A, B, and C are ethylene glycol. The proteins were dissolved in 20 mM boric acid, pH 4.0, containing 20% ethylene glycol. The running buffer was 50 mM sodium borate decahydrate, pH 10.0.

patients were supplied by Stanford University Hospital Clinical Laboratory. Double-distilled water was used for the preparation of buffers.

CZE Run Conditions. The buffer system used for all CZE protein separations was composed of 50 mM sodium borate decahydrate adjusted to pH 9.6, 10.0, 10.2, or 11.0 by the addition of NaOH. In some cases, the sample was prepared by mixing together the following three components in the ratio 1:3:1, namely, proteins of interest in water, 20 mM boric acid (pH 4.0), and ethylene glycol. In other cases, the proteins were dissolved directly in the 20 mM boric acid containing 20% ethylene glycol. In serum runs, the serum was diluted 40:1 with 1 mM boric acid (pH 4.5) containing 20% ethylene glycol. The capillary was filled with 50 mM sodium borate decahydrate adjusted to pH 10.0 with NaOH. After a 2-s pressure injection (about 10 nL) of sample, the same pH 10 buffer was used to follow the sample during the run. Under these conditions, all species move toward the cathode (the grounded outlet). Each run was followed by a 5-min rinse with 0.1 N NaOH, 3-min rinse with water, and 5-min rinse with starting buffer. This between-run treatment was sufficient to assure reproducibility (see below). Whenever the instrument was not to be in use for more than 2 h, the capillary was rinsed with 0.1 N NaOH for 5 min followed by a 5-min rinse with distilled water. The capillary was then left filled with distilled water. One capillary was used for this as well as two other studies for more than 15 months with no apparent degradation in performance.

A mixture of the following five proteins was made: carbonic anhydrase II (pI 6.2, 29 kD), jack bean urease (pI 5.0, 480 kD), bovine milk α -lactalbumin (pI 4.7, 14.2 kD), chicken egg ovalbumin (pI 4.6, 44 kD), and bovine serum albumin (pI 4.9, 66 kD). Note that the three albumins have a fairly narrow range of pI 's. These proteins were dissolved in the 20 mM boric acid (pH 4.0) containing 20% ethylene glycol so that the final concentration of each protein was 40 $\mu\text{g}/\text{mL}$.

Dithiothreitol (DTT) was used to reduce ribonuclease A. A volume of 50 μL of 10 mg/mL purified ribonuclease A was incubated with 50 μL of 20 mM DTT at 37 $^{\circ}\text{C}$ for 0, 1, 2, 7, and 24 h. Ten microliters of this mixture was diluted with 30 μL of boric acid and 10 μL of ethylene glycol. The final concentration of ribonuclease A was therefore 1 mg/mL. At each time point, a CZE run was made by using buffer at pH 10.2. In other experiments, ribonuclease A (10 $\mu\text{g}/\text{mL}$) was incubated with the same volume of α -chymotrypsin (200 $\mu\text{g}/\text{mL}$) at 37 $^{\circ}\text{C}$ for 0, 1, 2, 7, and 24 h and CZE runs were made at each time period.

RESULTS AND DISCUSSION

The mixture of five proteins was subjected to CZE at 10 kV after a 2-s pressure injection (about 10 nL) of sample. Figure 1 shows the resulting electropherogram. The ethylene glycol emerges before any of the proteins and appears as

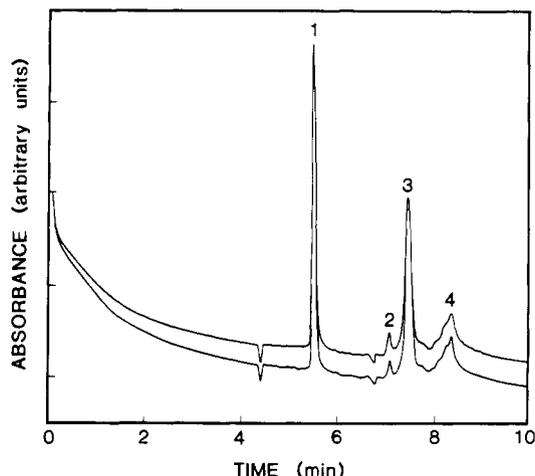


Figure 2. Two CZE runs of the same protein mixture illustrating the retention time reproducibility: (1) β -lactoglobulin A; (2) and (3) ethylene glycol; (4) carbonic anhydrase. The buffer molarities and pHs as well as conditions for sample preparation are the same as for Figure 1.

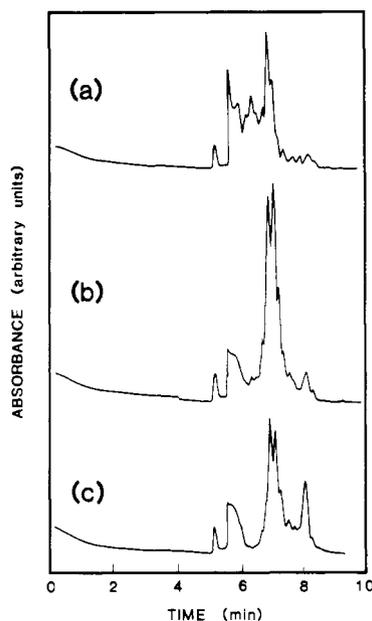


Figure 3. Electropherograms of ribonuclease A after incubation with DTT (a) 2 h, (b) 7 h, and (c) 24 h. The running conditions, sample preparation, buffer molarities, and pHs are described in the text.

multiple peaks. This behavior is observed when the sample consists only of borate buffer and 20% ethylene glycol but disappears if the borate buffer is replaced by phosphate buffer. Apparently, the ethylene glycol complexes with the borate at alkaline pH in much the same way that sugars do (22). The peaks were identified by running each protein separately. The chicken egg ovalbumin shows a minor contaminant which was seen when run by itself. Retention times are reproducible, as shown in Figure 2. The peak areas are reproducible as well.

This method also appears to be useful for following the degradation of a protein when exposed to specific cleavage chemicals or enzymes. Figure 3 shows CZE electropherograms of ribonuclease A (pI 9.4) which had been incubated with DTT for 2, 7, and 24 h at 37 $^{\circ}\text{C}$. Ribonuclease A is a single-chain molecule having four disulfide bonds in native form (23). One can see the changes with time as the reducing agent cleaves the disulfide bonds. Considering the kinetics of such cleavage, the additional peaks may be one or more disulfide bond cleavage forms. The implication is that this method can be useful for the study of structural changes of proteins. Figure

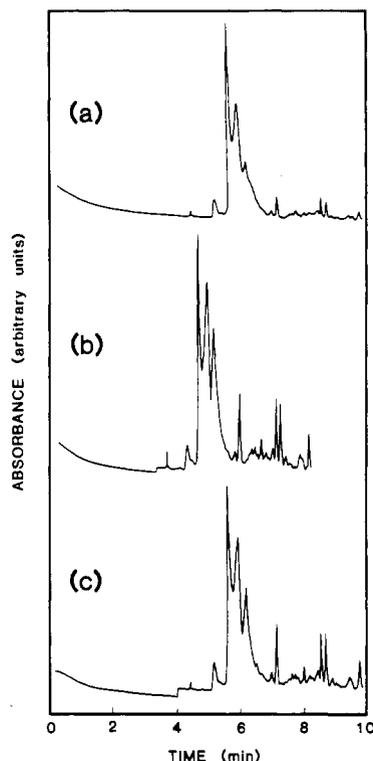


Figure 4. Electropherograms of ribonuclease A after incubation with α -chymotrypsin for (a) 2 h, (b) 7 h, and (c) 24 h. The running conditions, sample preparation, buffer molarities and pHs are described in the text.

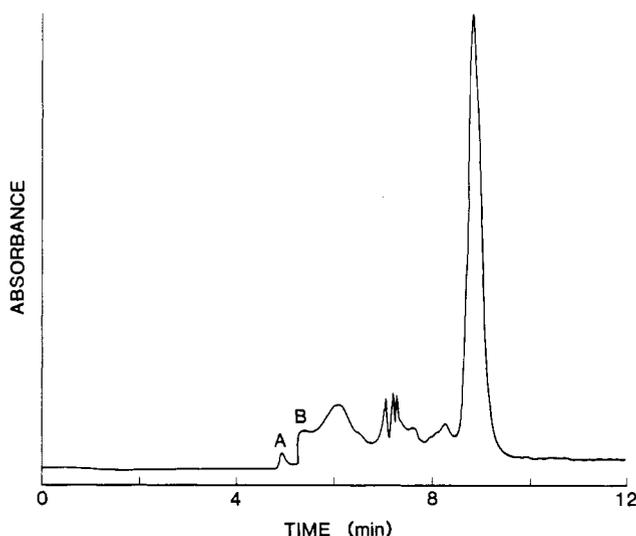


Figure 5. Electropherogram of human serum proteins from a normal male. Peaks marked A and B are ethylene glycol. The serum was diluted 40:1 with 1 mM boric acid, pH 4.5, containing 20% ethylene glycol. The running buffer was 50 mM sodium borate decahydrate. Note that the peaks come out in reverse order to that normally obtained in a clinical laboratory with agarose gel electrophoresis.

4 illustrates the ability of the method to monitor the breakdown of the ribonuclease A to peptides after incubation with α -chymotrypsin.

A CZE electropherogram of human serum from a normal individual is shown in Figure 5. Again, the ethylene glycol peaks emerge prior to any of the protein peaks. Ordinarily, in the clinical laboratory using agarose gel electrophoresis, the serum protein peaks appear in the following order: albumin, α_1 -globulin, α_2 -globulin, β -globulin, and γ -globulin. With the present method, the proteins come out in reverse order because the buffer pH is on the alkaline side of the isoelectric

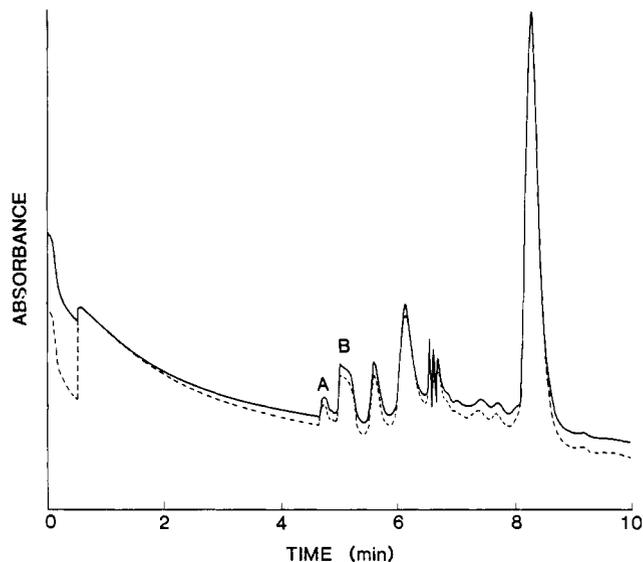


Figure 6. An overlay of two CZE runs of the same human serum protein sample showing reproducibility of retention times.

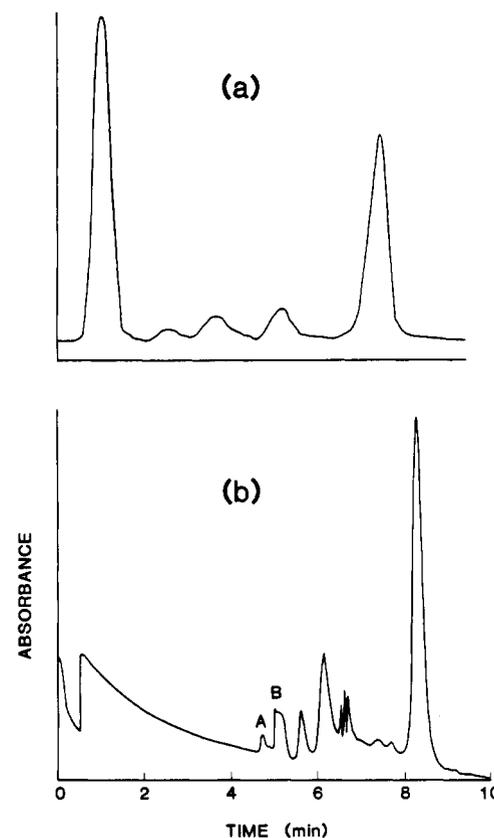


Figure 7. Electropherograms of human serum proteins (using the same conditions as for Figure 5) from a patient with multiple myeloma (IgG λ chain): (a) sample run on agarose gel by the clinical laboratory; (b) the same sample run by CZE.

points of all of the serum proteins. Figure 6 illustrates the reproducibility of retention times by presenting an overlay of two runs of the same sample. We have seen the same reproducibility from two CZE runs made 2 months apart on two different samples from the same individual. The peak areas are reproducible as well.

Eight serum samples from different patients with multiple myeloma were run with this method, and the resulting electropherograms of two of them are shown in Figures 7 and 8 along with the clinical laboratory's results from agarose gel electrophoresis. It is obvious that the resolving power of

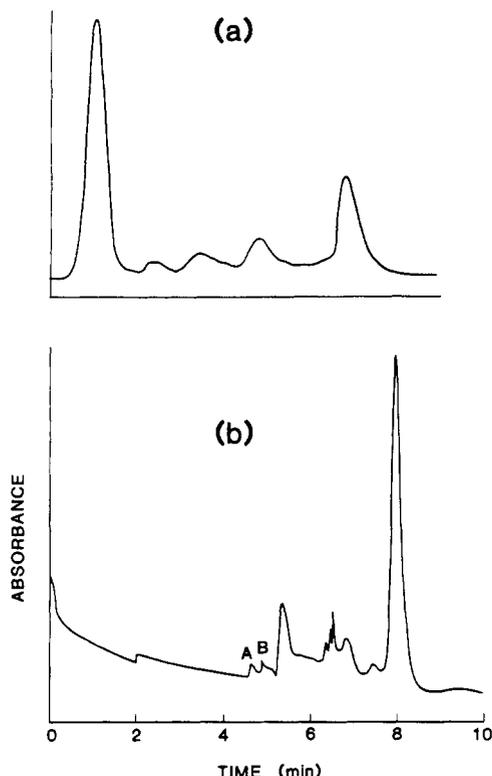


Figure 8. Electropherograms of human serum proteins (using the same conditions as for Figure 5) from a different multiple myeloma patient (IgG κ chain): (a) sample run on agarose gel by the clinical laboratory; (b) the same sample run by CZE.

our method with CZE is superior to that with the standard agarose gel.

In order to understand better the contributions of ethylene glycol and alkaline pH (pH 10.0), a serum sample was run five times with the ethylene glycol. The resulting electropherograms show excellent reproducibility for retention time and peak area. The same sample was run five times under the same conditions but without the ethylene glycol. The first run has most of the peaks separated. The second through fifth runs show the retention times increasing with run number and the peak areas changing from run to run. In other words, without ethylene glycol in the protein sample the electropherograms are not reproducible. When a serum sample with ethylene glycol is run in a barbital buffer, pH 8.6, the separation of peaks, as detected by UV absorption at 280 nm, is unsatisfactory, but that pattern is very reproducible in subsequent runs. However, if the sample without ethylene glycol is run in the same buffer, the electropherogram is similar to the runs with ethylene glycol, but the second run shows marked distortion of the pattern, which worsens with subsequent runs.

In conclusion, a protocol is described for analyzing by CZE the protein components in human serum. This procedure allows the major protein groupings, albumin and α_1 -, α_2 -, β -, and γ -globulins, and some of their subgroups to be resolved and quantitated. Moreover, compared to agarose gel electrophoresis, the present method has shorter analysis times and superior resolution.

Coating the inside wall of capillaries is, in some cases, more time consuming and in other cases is a labor-intensive process.

Subjecting proteins to pHs of 11 or higher will induce some alkaline hydrolysis of some proteins, and pHs of 2 also cause some proteins to degrade. This protocol avoids both the chore of coating capillary walls and the possible drawbacks of using extreme pHs. The ethylene glycol is very effective in preventing the proteins from adsorbing to the walls. Even in a barbital buffer at pH 8.6, a pH which would not be expected to prevent protein sticking, we can obtain reproducible patterns when ethylene glycol is present in the sample at the 20% level, even though the components may not be well resolved. We explored adding ethylene glycol to the running buffer as well as the protein sample. However, the sensitivity is greatly reduced because ethylene glycol absorbs broadly in the UV region. Ethylene glycol may also reduce protein-protein interactions.

The addition of ethylene glycol to the sample does not in itself account completely for the gain in resolution reported here. In addition, resolution is being enhanced by the use of higher pH and borate molarity in the running buffer than in the protein sample so that pH gradients, viscosity gradients, and conductivity gradients are being established during a run. The gradients may sharpen or broaden the peaks of various components, but the separation is improved in any case. The mechanism is presently not understood and likely to involve several interactions. Whatever the mechanism responsible for this gain in resolution, the present protocol is simple to apply and appears to work satisfactorily for proteins with differing pI and molecular weight values.

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