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High-performance capillary electrophoresis of proteins from the fluid lining of the lungs of rats exposed to perfluoroisobutylene

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ABSTRACT

Measurements of the biochemical constituents in the fluid lining of the lung can be used for diagnosing and assessing lung disorders. To facilitate such measurements, a high-performance capillary electrophoresis (HPCE) method has been developed by which the proteins in lung fluid can be analyzed. The lung fluid was obtained by a bronchoalveolar lavage procedure using 48 ml of physiological saline to wash out the lung fluid of rats. The proteins were precipitated from the fluid with 10 volumes of acetone and concentrated by dissolution in 2 ml of water containing 0.2% of trifluoroacetic acid. Aliquots of these samples (5 μl) were then injected into a Bio-Rad HPE-100 capillary electrophoresis instrument fitted with a 50 cm × 50 μm I.D. coated capillary filled with 0.1 *M* phosphate buffer (pH 2.5). With phosphate buffer in the outlet electrode chamber (cathode) and water in the inlet electrode chamber (anode), the proteins were loaded into the capillary electrophoretically for 10 s at 10 kV constant voltage. The inlet electrode chamber was then filled with phosphate buffer and HPCE was performed at 8 kV constant voltage. Six major protein fractions were resolved in 35 min, and were detected by UV absorption at 200 nm. The procedure was used to compare the lung fluid proteins of normal untreated rats with those of rats exposed by inhalation to perfluoroisobutylene (PFIB) at a concentration of 100 mg/m^3. It was found that PFIB induced pulmonary edema involving a translocation of blood compartment proteins into the lung's alveolar compartment. Comparison of the HPCE fractions with similar fractions obtained by high-performance liquid chromatography confirmed albumin, transferrin and IgG as three major proteins translocated into the alveolar space after PFIB exposure.

INTRODUCTION

Perfluoroisobutylene (PFIB) is a toxic compound that can be generated by pyrolysis of tetrafluoroethylene polymers, such as Teflon [1]. Brief inhalation exposure to low concentrations of PFIB can result in profound lung injury, incapacitation and death. Hence there is concern about the toxicity of smoke from such polymeric materials during their thermal decomposition [1]. The hallmark feature of such acute lung injury is a breach in the permeability characteristics of the lung's air–blood barrier, which is manifested by pulmonary edema [2–5]. However, as is the case with the toxic gas phosgene [6], there can be a latency period of up to several hours following exposure to PFIB that exists before the development of clinically significant

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lung injury [2]. Such latency periods offer a period of opportunity in which therapeutic intervention could be implemented to abate the otherwise ensuing life-threatening lung damage if only the nature of the injury was understood. Hence it is important to develop new methods to study the mechanisms of action of PFIB [7,8]. With this in mind, we have recently developed a method for the analysis of lung fluid proteins by high-performance capillary electrophoresis (HPCE). This paper describes the development of the separation of lung fluid proteins by zone electrophoresis in free solution using low-pH buffers in coated silica capillaries.

EXPERIMENTAL

Animal exposure to PFIB

Adult, male Fischer 344 rats (Harlan Sprague Dawley, Indianapolis, IN, USA) were exposed to an atmosphere containing 100 mg/m^3 of PFIB for 10 min. The apparatus and operation of the exposure system are described in detail elsewhere [9]. Exposure was accomplished by withdrawing 1 ml of PFIB (Fluoro Corp., Newport, TN, USA) from its storage cylinder with a gas-tight syringe. The syringe was placed in a syringe pump and attached to a dilution air manifold. HEPA-filtered air was metered into a glove-box at 2–3 l/min and the exposure atmosphere generation began with the starting of the syringe pump, which was capable of delivering up to 0.024 ml/min of neat agent into the airstream. Downstream, a series of baffles assured complete gas mixing and an automatic sampling device withdrew atmosphere samples every 30 s for measurements of PFIB concentration using a dual-column gas chromatograph fitted with an electron-capture detector (Model 140B, Valco Instruments) sensitive to 1 ppb of PFIB. The rats were placed in Teflon animal exposure tubes, passed into the glove-box through an airlock and the atmosphere was directed to the holding tubes for exposure for 10 min. After exposure, fresh air was directed to the animals until gas chromatographic sampling indicated the absence of PFIB. The animals were then returned to their cages and provided food and water *ad libitum* for 24 h before being killed. Control animals were subjected to air only by the same protocol.

Lung fluid sampling

The extracellular lining fluid was washed from the bronchial tree and alveoli of the lungs of rats using a bronchoalveolar lavage procedure [10]. Twenty-four hours after exposure, rats were killed following intraperitoneal injections of 50 mg of pentobarbital sodium. Prior to total apnea, the rats were exsanguinated via carotid artery transection, and the trachea was subsequently cannulated with a blunt, 18-gauge needle secured with ligature. The lungs and trachea were excised from the thoracic cavity *en bloc*, and the heart and esophagus were removed.

The lavage protocol consisted of six sequential lung wash cycles with 8 ml of 0.15 *M* sodium chloride solution per wash at room temperature. This saline solution was prepared with chromatography-grade water. The lavage was performed by gently massaging the lungs during infusion and aspiration of the saline during each cycle [11]. Retrieved lavage fluid from each lung was pooled in a centrifuge tube maintained on ice. Typically, $\geq$97% of the instilled fluid volume was recovered from each lung [12]. The lavage fluid was centrifuged at 300 *g* for 10 min at 4°C to sediment the lung

free cells. The supernatant fluid was removed by aspiration, placed in another centrifuge tube and centrifuged at 2300 *g* for 10 min to sediment any remaining acellular solid material. The supernatant fluid obtained was designated the bronchoalveolar lavage fluid (BALF). This BALF was frozen in glass tubes and stored at −70°C until used for chromatography and HPCE studies.

HPLC of lung fluid samples

Reversed-phase high-performance liquid chromatography (HPLC) was performed on BALF preparations as described in detail elsewhere [13]. Briefly, a 12-ml sample of BALF from control rats or a 1-ml sample from PFIB-exposed rats was made 0.2% in trifluoroacetic acid (TFA) and 6 *M* in guanidine hydrochloride by adding these reagents to the lavage fluid. The addition of these solubilization reagents was necessary to clarify the cloudiness of the samples caused by the large amount of proteinaceous material in the fluid following acute lung injury [14]. These samples were then diluted with an equal volume of phosphate-buffered saline [13] containing 0.2% TFA and pumped at a flow-rate of 1 ml/min through the HPLC column followed by 2 ml of water containig 0.2% TFA to rinse the sample from the pumps and lines.

The HPLC system consisted of a μBondapak Radial-PAK C_{18} flexible-walled cartridge (10 cm × 8 mm I.D.) contained in a Z-Module radial compression device (Waters Assoc., Milford, MA, USA) [15] which had been equilibrated with water containing 0.2% of TFA. The BALF constituents were loaded on the top of the column by adsorption to the hydrophobic packing and the phosphate, salts and guanidine were passed through to waste. Chromatography of the BALF constituents was accomplished by a series of acetonitrile gradients and isocratic steps that progressed from water–0.2% TFA to 65% acetonitrile–0.2% TFA followed by further isocratic elution with methanol [13]. The elution solvents were pumped at 1 ml/min using a Waters Assoc. Model 6000A solvent-delivery system equipped with three pumps. Control of the gradients was achieved by computer [13], and the eluted BALF constituents were detected by UV absorbance at 206 nm using a Waters Assoc. Model 490 flow spectrophotometer attached to a strip-chart recorder.

Preparation of BALF proteins of HPCE

Our experience has been that a sample containing a complex mixture of proteins needs to have a total protein concentration of at least 0.1 μg/μl to achieve good UV detection of the individual components separated by HPCE [16]. The BALF protein concentration was too dilute for analysis by HPCE so it was necessary to concentrate the BALF proteins. Initial attempts to concentrate the sample by membrane filtration methods resulted in component loss (probably owing to membrane adsorption) and protein precipitation. We therefore attempted to use precipitation methods to remove the proteins from the BALF so they could be redissolved in a small volume of electrophoresis buffer at higher concentrations. During this early stage of our investigation we were using phosphate-buffered saline as the lavage fluid, which was customary [13,14]. This caused a serious problem when we used acetone to precipitate the proteins because the phosphate salts were also precipitated. As sodium chloride was not precipitated in acetone–water (90:10), we performed the lavage with unbuffered 0.15 *M* sodium chloride solution. With this BALF we could precipitate

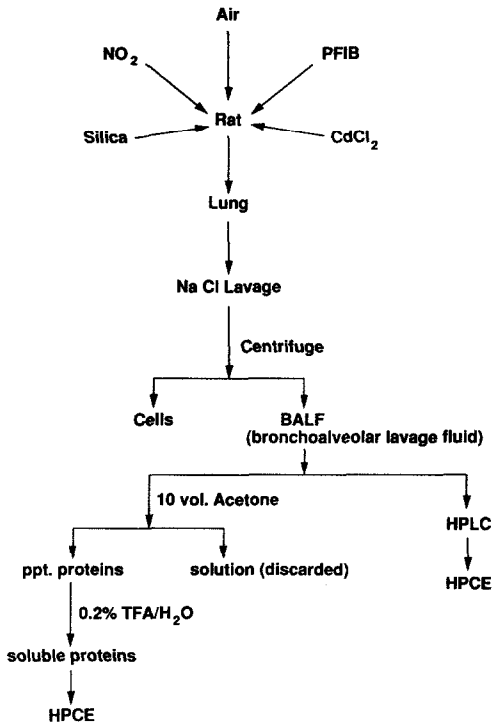

Fig. 1. Diagram of the protocol for the analysis of lung fluid proteins by HPCE and HPLC.

the proteins by adding acetone without precipitating the salt. HPLC analysis of saline lavage samples indicated that there were no differences between this lavage fluid and that obtained with phosphate-buffered saline.

The following protocol was therefore used to prepare BALF proteins for HPCE (Fig. 1). Saline BALF was obtained as described above and 10 volumes of acetone were added to 20 ml of the BALF. The proteins were precipitated overnight at 4°C. The precipitate was recovered by centrifugation at 1300 *g* for 10 min in a International Clinical Centrifuge. The supernatant acetone was discarded and the precipitate was washed once with 10 ml of acetone. The precipitate was again recovered by centrifugation. The acetone wash was discarded, the protein pellet was drained for 10 min and the proteins were dissolved in 1 ml of 0.2% TFA in water and frozen until used for HPCE. TFA is a good solubilizing agent for the proteins and it does not interfere with the electrophoresis at low pH. Because the salts in the sample have been removed, the electrophoresis sample has a low conductivity, which is advantageous for loading the sample into the capillary by electroinjection.

HPCE of BALF proteins

HPCE was performed using a Model HPE-100 high-performance electrophoresis system (Bio-Rad Labs., Richmond, CA, USA) fitted with a 50 cm × 50 μm I.D. silica capillary tube whose internal surface was chemically modified to produce a patented hydrophilic coating. Coatings of this nature have been reported by several

laboratories to eliminate electroendoosmosis and protein adsorption [17–19]. The cathode, anode and capillary buffers were 0.1 *M* sodium phosphate (pH 2.5) (Bio-Rad Labs.) unless specified otherwise. Detection of the separated proteins traversing the capillary was accomplished by in-tube monitoring of the peptide bond absorbance at 200 nm with spectrophotometer sensitivity settings ranging from 0.005 to 0.050 a.u.f.s.

To evaluate the performance of this instrument, a standard mixture of nine polypeptides was subjected to electrophoresis in a 20 cm × 25 μm I.D. coated capillary. This mixture contained bradykinin, angiotensin II, α-MSH, TRH, LHRH, [2–5] leucine enkephalin, bombesin, methionine enkephalin and oxytocin (Bio-Rad Labs.). When this standard mixture was electroinjected for 8 s at 8 kV and subjected to electrophoresis at 8 kV, nine completely resolved peaks were obtained in the order listed above.

To perform HPCE on BALF proteins, the proteins that were precipitated from 20 ml of BALF were dissolved in 1 ml water containing 0.2% of TFA and loaded into the capillaries by electroinjection [20]. To accomplish this, the cathode chamber and the 50 cm × 50 μm I.D. coated capillary were filled with 0.1 *M* phosphate buffer (pH 2.5) and the anode chamber was filled with water. Then 5 μl of sample were injected into the loading chamber at the anode end between the capillary and the water. Power was applied to the capillary for 10 s at 10 kV (constant voltage) and *ca.* 28 μA. This activity carried the cations (including the positively charged protonated proteins) into the capillary. Under these low ionic strength sample conditions the proteins were concentrated and stacked in the capillary during this loading step [21]. The excess of sample in the loading chamber and the water in the anode chamber were then replaced with 0.1 *M* phosphate buffer (pH 2.5) and electrophoresis was performed by applying power to the capillary at 8 kV (constant voltage) and ca. 23 μA. Electrophoresis normally took 35 min under these conditions. HPCE was also performed on 1 mg/ml standard solutions of rat albumin (Sigma, St. Louis, MO, USA), rat transferrin (Cappel, Westchester, PA, USA) and rat immunoglobulin G (IgG) (Sigma) dissolved in water containing 0.2% of TFA.

RESULTS

Performance of polypeptides and proteins in the HPE-100 system

The performance of the HPE-100 instrument was evaluated by subjecting a mixture of nine polypeptide standards to zone electrophoresis in a 20 cm × 25 μm I.D. coated capillary using 0.1 *M* phosphate buffer at pH 2.5. Repetitive electrophoresis of this standard produced fractions whose peak heights varied by only 3.0% (relative standard deviation). We have been unable to obtain such high quantitative precision with proteins, however. Our experience with either plasma proteins (in this paper) or with histone proteins [16] has shown that proteins in general produce much broader peaks than do small polypeptides and that electroinjection produces more variation in the quantity of loaded proteins than it does in the quantity of loaded quantity polypeptides.

Repetitive electrophoresis revealed a variability of 7.2% in the mobility of the nine polypeptide standards. This variability was also experienced with proteins, which made it impossible to identify unequivocally protein fractions by comparing

their mobilities with those of external standards. Therefore, to identify the peaks in a complex protein mixture, it was necessary to "spike" samples with an internal standard protein to facilitate assignments of known proteins to specific peaks in an electropherogram.

From previous experiments we expected BALF from PFIB-treated rats to have >10-fold increases in plasma proteins [8]. We therefore examined the effect that protein sample concentration might have on a protein's mobility. From our experience with histone proteins [16] we had determined that proteins could not be resolved in the small 20 cm × 25 μm I.D. capillaries used for polypeptides. Therefore, this experiment was performed in a 50 cm × 50 μm I.D. coated capillary in which transferrin was loaded for 10 s at 10 kV and then subjected to electrophoresis at 12 kV. It was found that the mobility of transferrin was reduced by only 1% by a fivefold increase in load, whereas it was reduced by 15% by a tenfold increase in load. Hence there appears to be a threshold in the 0.5–1.0 μg/μl range above which there is a significant decrease in protein mobility.

These experiments served to define the limits of performance for the electrophoresis of proteins in the HPE-100 system. Most important, they illustrate the great differences in performance experienced between the electrophoresis of polypeptides and that of proteins in this HPCE system.

HPCE of BALF proteins under acid conditions

Initial experiments on the HPCE of BALF proteins indicated that electrophoresis in 25-μm capillaries resulted in frequent capillary plugging. As a result, we chose to conduct our studies using the 50 cm × 50 μm I.D. coated capillary in which we could conduct 20–50 runs before noticing any degradation of the electropherograms. These studies also indicated that electrophoresis at 12 kV, the operational upper limit for the HPE-100, produced frequent sparking which resulted in degradation of the electropherogram. Consequently, we chose to conduct our studies by loading the BALF proteins at 10 kV for 10 s and then performing electrophoresis at 8 kV.

To determine the optimum pH to resolve the BALF proteins under acid conditions, samples were subjected to electrophoresis in various 0.1 *M* phosphate buffers having a pH range of 2.5–5.0 (Fig. 2). We found that the proteins ran the fastest at pH 2.5 and also achieved the best resolution at this pH (Fig. 2A). Higher pH runs produced peak tailing (Fig. 2B) and at pH ⩾3.75 the fractions moved so slowly that resolution was totally lost due to diffusion (Fig. 2C–F). Therefore, pH 2.5 was found to be the optimum condition for the electrophoresis of this set of proteins under acid conditions in phosphate buffer.

HPCE of the major blood compartment proteins

Albumin, transferrin and immunoglobulin G (IgG) are the major plasma proteins expected to be found in BALF proteins [22]. These proteins were subjected to HPCE (Fig. 3a–c) and the electropherogram of their mixture (Fig. 3d) was compared with that of BALF proteins (Fig. 3e). The order of mobility of the plasma proteins (from fastest to slowest) was albumin, transferrin and IgG. Albumin ran as a single peak while transferrin contained two components. IgG ran as a slow-moving broad fraction. Owing to the 7% variability in mobilities in this HPCE system (discussed above), it was impossible to determine unequivocally which fractions in the BALF

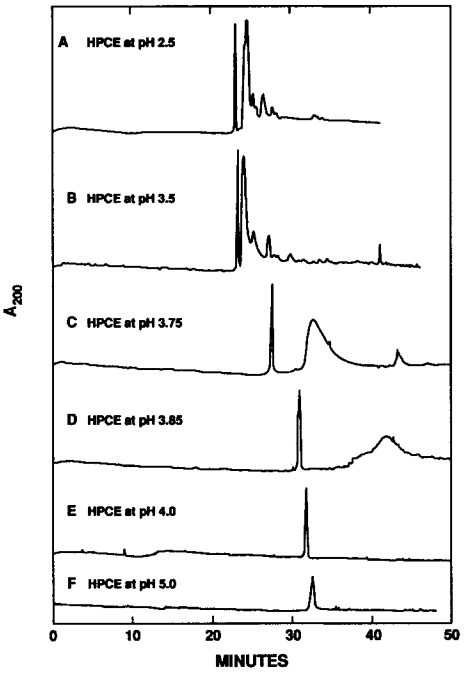

Fig. 2. HPCE of BALF proteins under acid conditions. Rat BALF proteins dissolved in 0.2% TFA were loaded by electroinjection for 10 s at 10 kV into a 50 cm × 50 μm I.D. coated capillary fitted in a Bio-Rad Labs. HPE-100 instrument. Electrophoresis was then performed at 8 kV (constant voltage) in 0.1 *M* phosphate buffer of pH 2.5–5.0.

electropherogram were these plasma proteins by comparison with the external standard runs.

Identification of blood compartment proteins in BALF protein samples

To identify the blood compartment proteins in the BALF protein electropherogram, albumin, transferrin or IgG was individually added to different samples of BALF proteins. These "spiked" samples were then subjected to HPCE. Each plasma protein in the electropherogram of the whole BALF protein sample was located by the increase in the fraction's peak height (Fig. 4b–d). The location of these three plasma proteins was identified in the BALF protein electropherogram (Fig. 4a). The second and largest BALF peak was found to contain albumin. The first transferrin peak was found to be a trailing shoulder on the third BALF peak and the second transferrin peak was found to be in the fourth BALF peak. IgG was not detectable in the BALF proteins (Fig. 4a). However, the area of the electropherogram trailing the fifth BALF protein peak (>26 min) is usually above the baseline. Because of the broad nature of the IgG peak (Fig. 4d) a small amount of IgG might be difficult to detect.

HPLC of BALF proteins

Our laboratory has previously developed an HPLC method for the analysis of

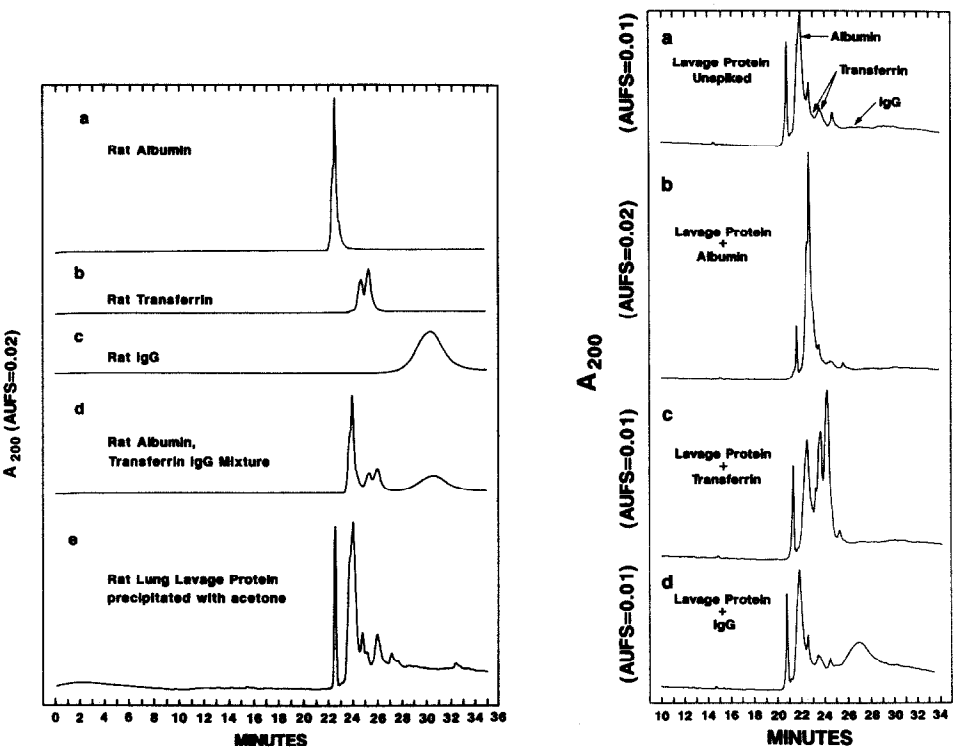

Fig. 3. HPCE of major blood compartment proteins. Rat (a) albumin, (b) transferrin and (c) IgG dissolved in 0.2% TFA at 1 mg/ml were subjected to HPCE at pH 2.5 as described in Fig. 2. (d) A mixture of these blood proteins was compared with (e) BALF proteins.

Fig. 4. Identification of blood compartment proteins in the HPCE electropherogram of BALF proteins. (a) BALF proteins were subjected to HPCE at pH 2.5 as described in Fig. 2. (b) Albumin, (c) transferrin and (d) IgG were individually added to different samples of BALF proteins. The fractions in the BALF electropherogram that contained these proteins were identified by the increase in the peak height of the fraction.

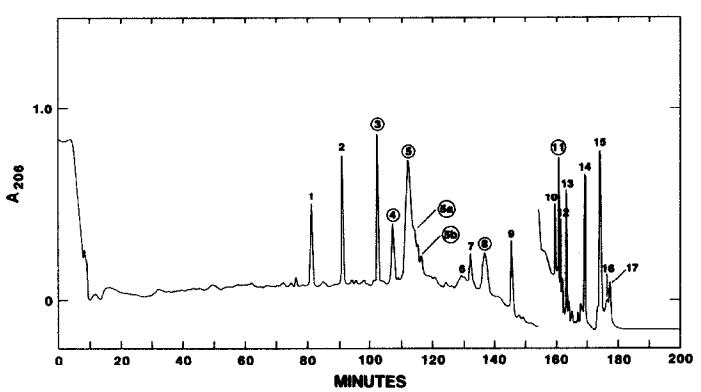

Fig. 5. HPLC of BALF proteins. A 12-ml sample of BALF was loaded directly onto a μBondapak Radial-PAK C_{18} reversed-phase column. Fractions were eluted at 1 ml/min with a series of acetonitrile gradients and isocratic steps that progressed from water–0.2% TFA to 65% acetonitrile–0.2% TFA in 155 min. Following this, additional fractions were eluted with methanol (the break in the baseline at 155 min is the result in a baseline adjustment due to the change in solvents). Fractions of 1 ml of effluent were collected and dried for HPCE analysis. Fractions circled were detected by HPCE.

BALF proteins [13]. Nine of the HPLC fractions were found to contain protein [13]. To determine the identity of the HPLC fractions in the HPCE electropherogram, the HPLC fractions were prepared directly from BALF. This was accomplished by subjecting 12 ml of BALF to reversed-phase HPLC (Fig. 5). Fractions of 1 ml of the column effluent were collected for 200 min and evaporated to dryness. Each of these samples was then dissolved in 30 μl of water containing 0.2% of TFA and used for HPCE analysis. In this chromatogram, transferrin has been shown to elute in peak 4, albumin in peak 5 and IgG among the cluster of peaks 6–9.

HPCE of the HPLC fractions of BALF proteins

Each of the seventeen HPLC fractions marked in Fig. 5 were subjected to HPCE (Fig. 6). HPLC fractions 3, 4, 5, 5a, 5b, 8 and 11 produced significant HPCE electropherograms. HPLC peak 3 was found to produce a single peak by HPCE, indicating that it is probably homogeneous as it elutes from the HPLC column. HPLC peak 4 (the transferrin peak) was found to be a single peak in the BALF. Commercial preparations of transferrin prepared from plasma contained two HPCE peaks (Fig. 3b). HPLC peak 4 was also found to be contaminated with some albumin migrating ahead of the transferrin.

HPLC peak 5 (the albumin peak) was found to contain two HPCE fractions. Commercial preparations of albumin prepared from serum contain one HPCE peak

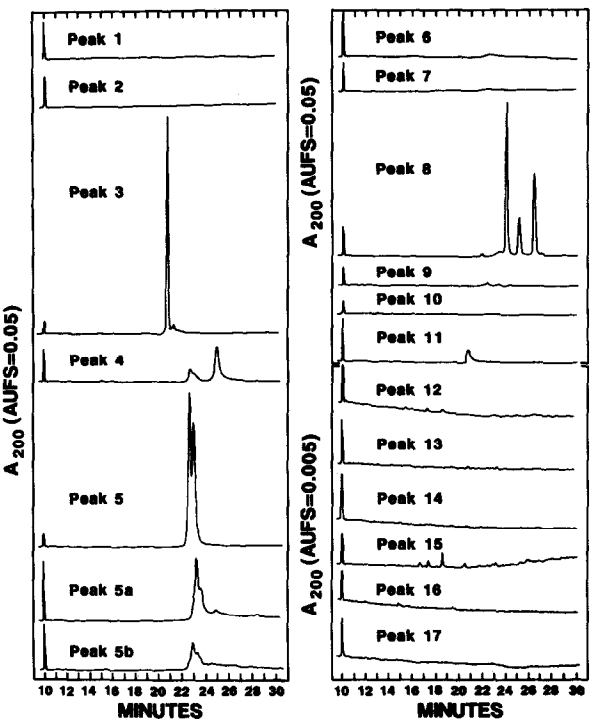

Fig. 6. HPCE of HPLC fractions of BALF proteins. The HPLC fractions (numbered 1–17 in Fig. 5) were subjected to HPCE at pH 2.5 as described in Fig. 2. The peak at 10 min in these chromatograms is produced by guanidine eluted from the HPLC column.

(Fig. 3a). Thus, either BALF modifies the albumin in some way to produce an albumin component with an altered electrophoretic mobility, or the BALF contains another major component (not yet identified) that elutes with albumin from the HPLC column. The trailing shoulders of HPLC peak 5 (peaks 5a and 5b in Fig. 5) appeared also to have slower moving HPCE components. For example, HPLC peak 5a appears to contain the second HPCE albumin peak plus a slower moving shoulder and HPLC peak 5b appears to contain the second HPCE albumin peak plus two slower moving shoulders. Hence the albumin-containing HPLC peak 5 probably contains at least four different components.

Among the cluster of peaks eluting from the HPLC column at high acetonitrile concentrations (peaks 6–9 in Fig. 5), only HPLC peak 8 produced an HPCE electropherogram. This HPLC fraction produced three well resolved HPCE fractions that eluted between 24 and 27 min. We know from previous experience that IgG and IgM will elute from the HPLC column in this part of the chromatogram [13–15]. However, the HPCE components of HPLC peak 8 (Fig. 6) do not resemble the HPCE components of IgG (Fig. 3c) in any way. The HPCE components are well defined and move significantly faster than the poorly defined, slower moving IgG.

Among the HPLC fractions eluted with methanol (peaks 10–17 in Fig. 5) only peak 11 produced a fraction detectable by HPCE (Fig. 6). This was expected as protein was found previously in this cluster of HPLC fractions (peaks 10–13 in Fig. 5) [13]. However, it was surprising that no HPCE fractions were found in the last cluster of HPLC fractions (peaks 15–17 in Fig. 5). These HPLC fractions have been shown to contain significant amounts of both protein and phospholipid [13]. Consideration should therefore be given to the possibility that phospholipids might interfere with the HPCE of those proteins, perhaps by hydrophobic interactions with the capillary wall.

Identification of HPLC fractions in the HPCE electropherogram of BALF proteins

Each HPLC fraction that was detected by HPCE is circled in Fig. 5. To determine where those HPLC fractions occur in the HPCE electropherogram of whole BALF proteins, samples of BALF proteins were "spiked" with HPLC fractions 3, 4, 5, 8 and 11 and then subjected to HPCE. The fractions in the BALF electropherogram that contained the HPLC components were identified by the increase in the HPCE peaks (Fig. 7). The first HPCE peak was identified as HPLC peak 3 (Fig. 7b). The fourth HPCE peak was identified as HPLC peak 4 (Fig. 7c). As the mobility of HPCE peak 4 and the elution of HPLC peak 4 correspond to those of transferrin, it is reasonable to identify those fractions as containing transferrin.

The second, large HPCE peak was identified as HPLC peak 5 (Fig. 7d). The trailing shoulder of HPLC peaks 5a and 5b were also identified in the second HPCE peak (Fig. 7e and f). As the mobility of HPCE peak 2 and the elution of HPLC peak 5 correspond to those of albumin, it is reasonable to identify these fractions as containing albumin.

The three HPCE fractions found in HPLC peak 8 were identified in the BALF protein electropherogram as the third, fourth and fifth HPCE peaks (Fig. 7g). This means that the fourth HPCE peak contains not only transferrin, but also some other component. The HPLC peak 11 was found to have an electrophoretic mobility faster than the first HPCE peak (Fig. 7h). As this fraction is not normally seen in the HPCE

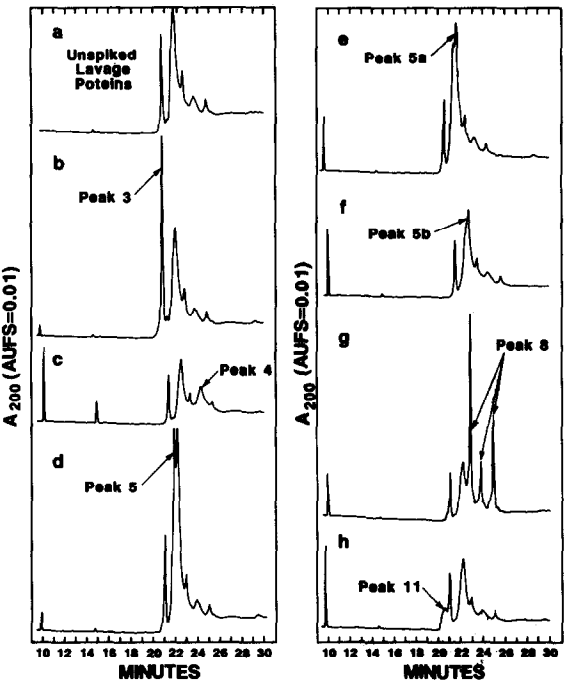

Fig. 7. Identification of HPLC fractions in the HPCE electropherograms of BALF proteins. Each HPLC fraction circled in Fig. 5 was detected by HPCE in Fig. 6. These seven HPLC fractions (peaks 3, 4, 5, 5a, 5b, 8 and 11) were individually added to separate samples of BALF proteins and subjected to HPCE. The fractions in the BALF electropherogram that contained the HPLC components were identified by the increase in peak height in the HPCE fraction. The peak at 10 min in these chromatograms is produced by guanidine eluted from the HPLC column.

of BALF proteins (Fig. 7a), this component is either present at concentrations too low for detection in the HPLC system or it is not recovered by acetone precipitation during the preparation of BALF proteins for HPCE.

Guanidine hydrochloride in HPLC fractions

During the course of these experiments is was observed that all HPCE electropherograms of samples prepared by HPLC contained a sharp peak at 10 min (Figs. 6 and 7). As guanidine hydrochloride was added to BALF as a solubilizing agent before performing HPLC on these samples, we subjected a guanidine hydrochloride standard to HPCE to determine its electrophoretic mobility in our system. It was found to migrate at the position of the 10-min peaks seen in Figs. 6 and 7 and had a high sensitivity for detection at 200 nm. Its presence as a contaminant from the HPLC system was surprising as it is highly charged under the acid conditions of HPLC (pH 2) and was expected to pass through the hydrophobic C_{18} column in the void volume.

Another surprising feature was that the guanidine was present in all fractions regardless of their position in the HPLC chromatogram (Fig. 6). Hence it was necessary to determine whether the guanidine was adsorbed to the proteins being eluted from the column or whether it was adsorbed directly to the column itself and bled from the column throughout the entire elution gradient. To do this, a new C_{18} col-

umn that had never been exposed to proteins was injected with sample solvent containing guanidine and eluted with the acetonitrile gradient containing TFA. Column effluent taken at 115 min (the time albumin normally elutes) was found to contain the 10-min guanidine peak, thus demonstrating that the guanidine was adsorbed directly to the HPLC column. Purging the C_{18} column with 100% acetonitrile removed all guanidine from the column (data not shown) and also removed any TFA from the column [13].

This suggests that the adsorption of guanidine to the C_{18} packing may be facilitated by ion pairing between TFA and guanidine, which formed an uncharged more hydrophobic species capable of interacting with the C_{18} column. Equilibrium of guanidine between the TFA on the column and that in the solvent might account for the constant bleeding of guanidine into the eluting solvents and the inability to flush out the guanidine before the TFA is removed from the system at the end of the HPLC trace.

HPCE of BALF proteins from PFIB-treated rats

Having characterized the HPCE profile of BALF proteins from control (unexposed) rats, HPCE was performed on the BALF proteins of rats exposed to PFIB (Fig. 8). Because of the acute edema in the lungs 24 h after PFIB treatment, we expected to find a great increase in plasma proteins in the PFIB samples [8]. Thus the

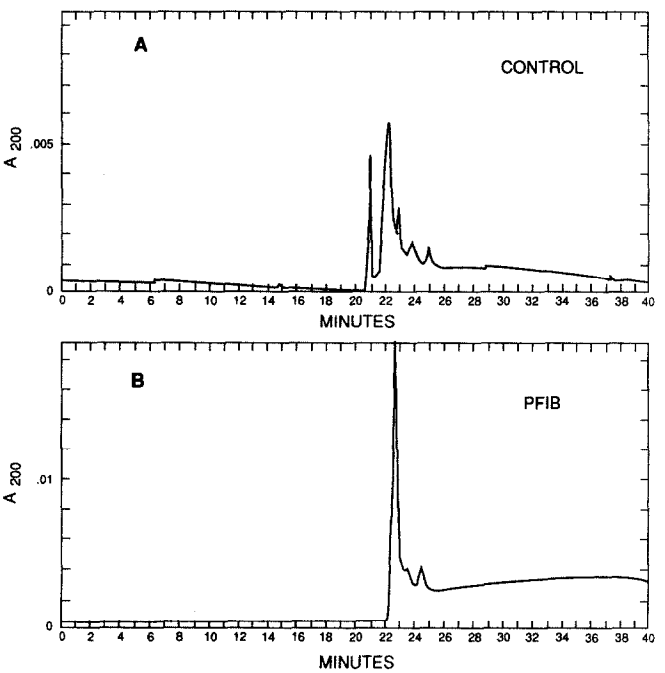

Fig. 8. HPCE of BALF proteins from control and PFIB-treated rats. Proteins were precipitated with acetone from the BALF of control (air-exposed) and PFIB-exposed rats. The amount of BALF used to prepare the protein sample from the PFIB-exposed rat was one tenth of that used for the preparation of the control sample. (A) HPCE of BALF proteins from control rats; (B) HPCE of BALF proteins from PFIB-treated rats.

PFIB proteins subjected to HPCE (Fig. 8B) amounted to the protein taken from one tenth the amount of BALF which was used for the HPCE of control proteins (Fig. 8A). This was necessary to keep the protein concentration in the PFIB sample within the concentration range of the analytical instrument. Examination of the electropherogram of the PFIB sample revealed that the first HPCE peak was missing while the second HPCE peak (albumin) was greatly increased. The increase in peak height was four times that of the control. Since one tenth the amount of BALF was used for the PFIB sample, this increase amounts to a *ca.* 40-fold increase in albumin. There was also an increase in the peak height of the PFIB transferrin peak (fourth peak at 23.5 min) to three times that of control. This indicated that transferrin was increased roughly 30-fold. A similar increase was observed for the fifth peak at 24.5–25 min. Following these peaks there was a high background in the electropherogram of the PFIB sample in the region from 26 to 30 min where one would expect to find IgG.

From this HPCE profile it is concluded that the first HPCE peak is not increased by PFIB treatment. It may either be lost from detection by the reduction in the PFIB sample size or it may actually decrease owing to PFIB treatment. The great increase in plasma proteins in the PFIB sample detected by HPCE confirmed similar increases observed in these proteins in PFIB samples analyzed by HPLC in our preliminary studies [8].

HPCE analysis of HPLC fractions of BALF proteins from PFIB-treated rats

Because of the profound changes in the HPCE profile of the BALF proteins from PFIB-treated rats observed in Fig. 8, a more detailed analysis was performed by comparing the HPCE analysis of individual HPLC fractions obtained from the BALF of treated and untreated animals. To do this, 12 ml of BALF from a control rat and 1 ml of BALF from a PFIB-treated rat were subjected to HPLC fractionation (Fig. 9A and E and Fig. 10A and G). The numbered fractions in these chromatograms were collected and subjected to HPCE (Fig. 9B–D and F–J and Fig. 10B–F and H–L).

The HPLC profile (Fig. 9E) confirmed the conclusion drawn from the HPCE profile (Fig. 8B) that the HPLC peak 3 did not increase after PFIB treatment. In contrast, there was a very large increase in the amounts of HPLC peaks 4 (transferrin) and 5 (albumin) and the peak cluster 6–10 that followed. In addition, two new fractions, A and B, were observed to appear in the HPLC profile after PFIB treatment. We have observed the appearance of these two new peaks during other cases of pulmonary edema caused by agents such as cadmium and nitrogen dioxide [14].

We were unable to detect the new A and B fractions by HPCE (Fig. 9F and G). However, small amounts of contaminating transferrin (HPLC peak 4) and albumin (HPLC peak 5) were observed in the A and B samples. HPLC peak 3, which migrates at 20 min by HPCE (Fig. 9B), was undetectable after PFIB treatment (Fig. 9H). This result was the same as that observed when performing HPCE on acetone-precipitated proteins (Fig. 8B). We conclude that this fraction is decreased by PFIB treatment because, if it remained the same as the control, we would expect to see a small HPCE peak at 20 min in Fig. 9H. This fraction (HPLC peak 3) was contaminated with transferrin and albumin (Fig. 9H).

Transferrin (HPCE peak 4) was greatly increased by PFIB treatment (Fig. 9E). HPCE analysis of this HPLC fraction revealed that, in addition to transferrin, this

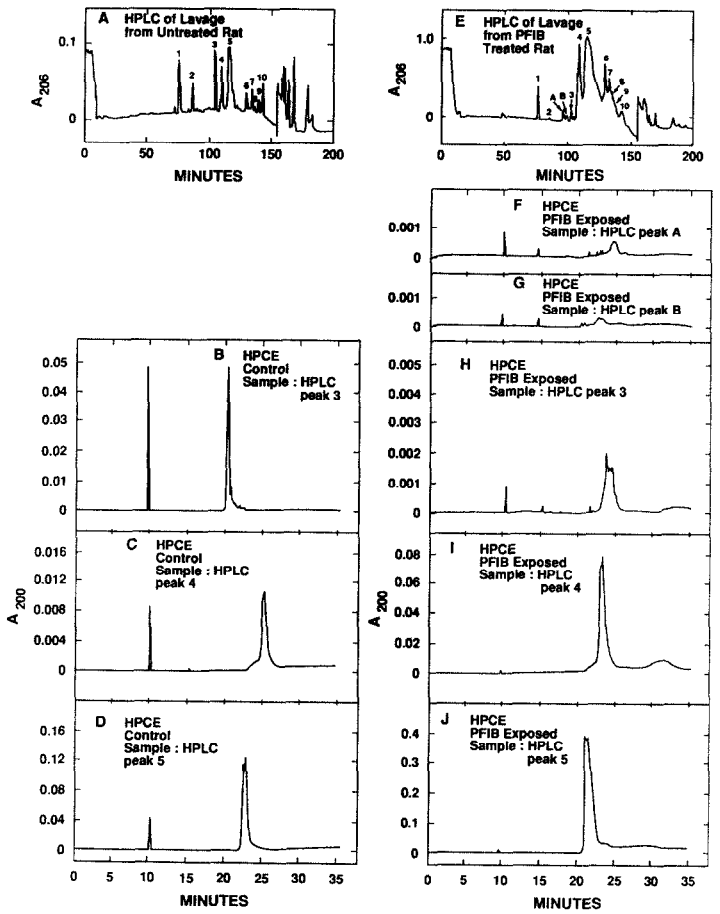

Fig. 9. HPCE of HPLC fractions of BALF taken from unexposed rats and from rats exposed to PFIB. BALF from unexposed and PFIB-exposed rats were subjected to HPLC (A and E, respectively). HPLC peaks 3, 4 and 5 from untreated rats were subjected to HPCE (B, C and D, respectively). HPLC peaks A, B, 3, 4 and 5 from PFIB-treated rats were subjected to HPCE (F, G, H, I and J, respectively).

HPLC fraction contained an HPCE fraction at 30 min that resembled IgG (Fig. 9I). This was surprising as IgG is normally eluted in the HPLC peak cluster 6–10 [13–15]. Albumin (HPLC peak 5) was also greatly increased by PFIB treatment (Fig. 9E). HPCE analysis of this HPLC fraction showed no significant amount of IgG-like material in this fraction (Fig. 9J).

PFIB treatment increased the amount of material in the HPLC peak cluster 6–10 (Fig. 10G). HPCE analysis of these peaks indicated that all these HPLC peaks from PFIB-treated animals were contaminated with albumin and perhaps transferrin (Fig. 10H–L). Hence the HPLC peaks 6–10 are riding on top of the tailing shoulder of the massive albumin peak (Fig. 10G). Even though most of these HPLC peaks are undetectable by HPCE (Fig. 6 and Fig. 10B–F), the large increase in the peak heights of these fractions in Fig. 10G suggests that these fractions are probably increased by PFIB treatment (Fig. 10G). No IgG-like peaks were observed in the HPCE analysis of this peak cluster (Fig. 10H–L).

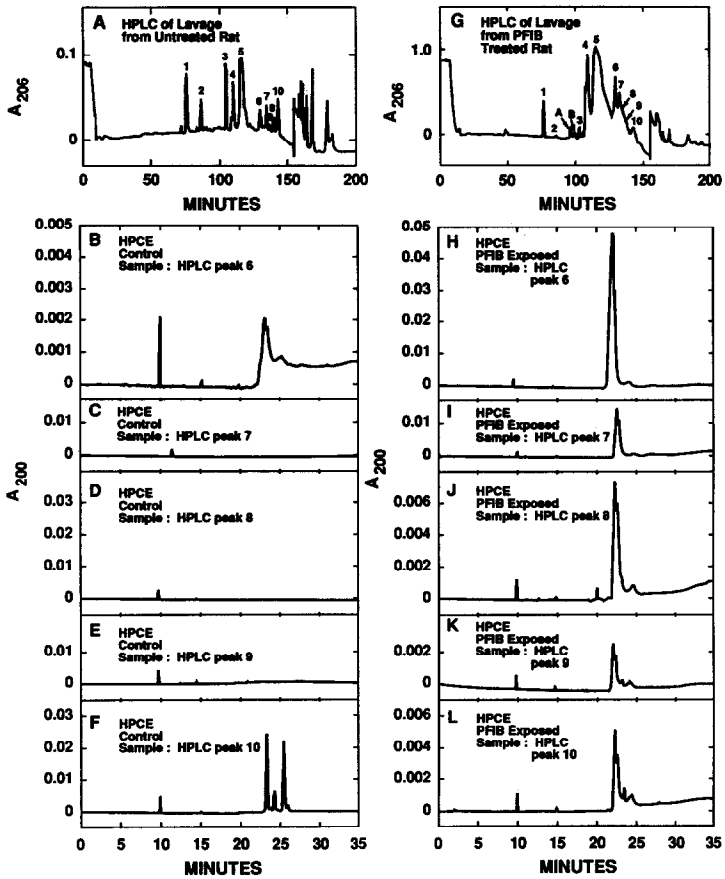

Fig. 10. HPCE of HPLC fractions of BALF taken from unexposed rats and from rats exposed to PFIB (this is a continuation of Fig. 9). BALF from unexposed and PFIB-exposed rats were subjected to HPLC (A and G, respectively). HPLC peaks 6, 7, 8, 9 and 10 from untreated rats were subjected to HPCE (B, C, D, E and F, respectively). HPLC peaks 6, 7, 8, 9 and 10 from PFIB-treated rats were subjected to HPCE (H, I, J, K and L, respectively).

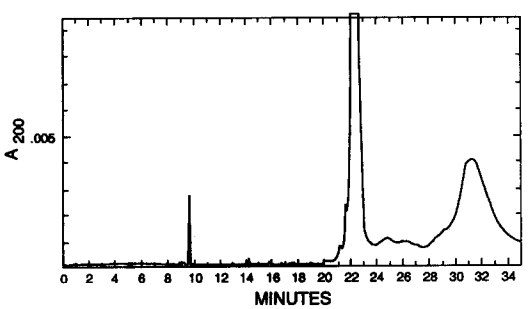

Fig. 11. HPCE of the HPLC transferrin peak from a PFIB-exposed rat. The HPLC transferrin fraction from a PFIB-exposed rat (such as peak 4 in Fig. 9E) was collected and subjected to HPCE. Guanidine eluted at 9.6 min, transferrin at 22.3 min and the IgG-like protein at 31.2 min.

To confirm that an IgG-like substance was induced in the transferrin HPLC peak by PFIB, the BALF of another PFIB-exposed rat was subjected to HPLC and its transferrin peak was subjected to HPCE (Fig. 11). Again the IgG-like peak was observed late in the electropherogram after the transferrin peak. Thus, HPCE provides the only method for detecting the appearance of this IgG-like substance during lung injury.

DISCUSSION

In this paper we have described the development of an HPCE system for the analysis of BALF proteins, identified the major blood compartment proteins in the electropherogram, characterized the HPCE fractions with respect to the BALF proteins fractionated by HPLC and demonstrated the usefulness of HPCE in analyzing BALF protein changes induced by a hazardous toxic gas such as PFIB. The most effective application of HPCE was found to be its use as a second-dimensional analysis system following a first-dimensional fractionation of BALF proteins by HPLC. An excellent example of this application was the discovery of an IgG-like protein hidden in the HPLC transferrin fraction of PFIB-treated rats. The absence of this component in controls (untreated rats) indicates that PFIB has mediated a major breach in the air–blood barrier of the lung allowing the escape of large proteins into the alveolar space.

Another example of the usefulness of HPCE in the second dimension was the discovery that a single peak which was eluted at high acetonitrile concentrations from the HPLC column actually contained three components that were well resolved by HPCE. In a similar manner, the single albumin peak obtained by HPLC was found to be subfractionated into two major and two minor components by HPCE. An interesting reversal of this observation was demonstrated with transferrin. Using HPCE, serum-derived transferrin standards were found to contain two components, but only one component was found in BALF proteins.

Another effective application of HPCE was found to be its use as a quality control system for analysis of the purity of BALF components prepared by HPLC. Because of the large amounts of albumin and transferrin in the BALF protein mixture, the preparations of other BALF components were sometimes found to be contaminated by these proteins. Such contamination will be detrimental to the accurate amino acid analysis, amino acid sequencing and immunological analysis which will be necessary for the identification of the unknown components in BALF. HPCE will provide a rapid and simple method to document the purity of the preparations used for such endeavors.

The use of HPCE in this manner has also revealed an unexpected contaminant in the HPLC fractions of BALF proteins. This contaminant is guanidine, which was added to the BALF as a solubilizing agent. This reagent has been used for some time to dissociate complex mixtures of proteins for HPLC fractionation. Because of its highly charged nature, it has been presumed that all the guanidine was eluted immediately from the hydrophobic reversed-phase HPLC column leaving the proteins guanidine free when they were eluted. With HPCE we have shown that this is not so. Under many circumstances the presence of residual guanidine in the HPLC fractions may be irrelevant. However, if guanidine is detrimental to the further use of these HPLC preparations, HPCE can be used to determine quickly whether is is present.

Another important application of this HPCE system will be its use as an assay method to guide the development of new HPLC systems for the isolation and preparation of the subfractions of BALF proteins which are unresolved by present HPLC systems. HPCE will be particularly attractive in this role as it is fast and can be automated for use with the large number of fractions generated by HPLC.

The HPCE system has its share of limitations as an analytical method at this early stage in its development. Quantitative reproducibility is the most serious. Quantitative analysis appears to be fairly good for polypeptides. However, HPCE does not meet the standards of reproducibility for proteins that is expected by most researchers. The reasons for this difference remain unclear. This is not simply a problem encountered only with BALF proteins. We find the problem equally a nuisance when attempting to perform HPCE on histone proteins which have a very different nature from BALF proteins [16]. We suspect that the problem involves variations in electroinjection, but we have not yet been able to identify the varying parameter.

Another limitation of HPCE is sample concentration. While sample volumes required by the system are very small (5 μl for the HPE-100), the concentration of the protein in the sample must be high if UV detection is used to monitor the electrophoresis. For complex mixtures of proteins, this concentration should be at least 0.1 mg/ml in order to detect most of the components. With BALF proteins, we found it necessary to concentrate the samples by either precipitation, evaporation or reversed-phase HPLC before we could use HPCE for analysis. In addition to these concentration steps, electroinjection itself produces another helpful concentration by stacking the proteins in the capillary [21]. If some other method of injection is employed for HPCE, one should expect to have to use protein sample concentrations considerably greater than 0.1 mg/ml.

The reproducibility of mobilities cannot be relied upon for identification of closely migrating components. This is a general problem with HPCE, which we found to be as true for polypeptides as for proteins. In chromatography we have commonly used radiolabeled internal standards to circumvent this problem, but the present lack of sensitive radioactivity detection for HPCE systems makes this impractical. However, we found that identification of the various components could usually be made by "spiking" the samples with a known internal standard and detecting the fraction in the electropherogram by the increase in its peak height.

A number of BALF fractions which were isolated by HPLC were not detectable by HPCE. This may be due to the presence of hydrophobic interactions between some lung constituents and the coating of the capillary. The resulting adsorption might remove the more hydrophobic components, such as lipids and surfactants, from the sample during electrophoresis. Another possible explanation for the loss is that some components were not charged under the acidic conditions of this HPCE system. The absence of electroosmotic flow caused by the capillary coating would prevent uncharged components from reaching the detector. Hence it may be necessary to analyze some HPLC samples in uncoated capillaries which produce electroendoosmosis.

While the shortcomings of the HPCE of proteins need to be clearly recognized, we have nevertheless, found that this new technology offers effective new ways to analyze a complex mixture of proteins. With the BALF proteins, HPCE has significantly pushed back the frontier of analysis of lung fluid samples.

The use of HPCE together with HPLC has enabled us to characterize the effects

of PFIB on the fluid lining of the lung. We found that, 24 h after PFIB exposure, the resulting pulmonary edema is accompanied by a massive outpouring of plasma components into the alveolar compartment. BALF albumin was increased >40-fold and BALF transferrin was increased >30-fold. There was also a significant amount of an IgG-like protein in the BALF after PFIB treatment that was undetectable in controls. The great increase in these proteins in the BALF indicate that PFIB has caused a breach in the air–blood barrier in the lung that is large enough to permit the translocation of large macromolecules such as IgG from the bood to the alveolar compartment.

Two new unidentified components (labeled A and B in the HPLC traces) were found in the BALF after PFIB treatment. These two components have been found in BALF after other treatments that cause pulmonary edema [14]. Hence it is suspected that their origin may also be the blood compartment.

There was one major component of normal BALF that was found to be lost from the BALF of PFIB-treated lungs. This component has not yet been identified. It is easily detectable by both HPLC and HPCE. It elutes early from the reversed-phase C_{18} HPLC column, indicating that it is not very hydrophobic. It is the fastest migrating component in BALF proteins subjected to HPCE, indicating that it is highly positively charged. Previous work has shown that this component contains protein [13,14].

When we exposed rats to other edema inducing agents this component has not increased greatly like albumin and transferrin [14]. There was a small loss of this component in cadmium-injured lungs and a small increase in nitrogen dioxide-injured lungs [14]. Hence this component does not appear to be derived from the blood compartment, but rather is likely a product of the lung cells and/or the lung's extracellular matrix. Its loss after PFIB treatment may reflect damage to the cells producing it. HPCE may play an important role in uncovering the nature of IgG in BALF. When serum-derived IgG standards are subjected to HPLC they elute late in the chromatogram [13,14]. However, the only IgG-like substance we observed by HPCE was found in the early-eluting HPLC transferrin peak from PFIB-treated rats (Fig. 11). The late-eluting components in the HPLC chromatogram contained three HPCE peaks that did not have the electrophoretic properties of IgG. We therefore suspect that IgG may be altered or degraded in normal lung fluid. HPCE should provide a useful new method to examine this possibility and to determine if toxic agents such as PFIB interfere in the process.

This work demonstrates that HPCE can be effectively used for the analysis of lung proteins. It is expected that in the future this method will be refined to a point where it will be a major analytical tool by which the pulmonary biologist, biochemist and toxicologist can elucidate the mechanisms of lung injury.

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