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Analysis of small-scale biological compartments by capillary electrophoresis

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Abstract

Two characteristics of capillary electrophoresis make the technique attractive for the separation of the components of microscale compartments within living organisms: small sample volume requirements and direct compatibility with biofluids. Indeed, capillary electrophoresis has been used for analysis down to a sub-cellular level. There are also potentially many applications of capillary electrophoresis to biological compartments on a super-cellular scale, which are nevertheless so small that they make analysis by conventional separations techniques difficult or impractical. The analytical challenges in small-scale bioanalysis are first to develop a suitable method for collection of sample and its introduction into the separation capillary, and secondly, to achieve the required separation. Examples reviewed here will primarily focus on the analysis of tear fluid or airway surface liquid, cases in which the amount of sample that can be collected range from around 10 μl to around 100 nl.

Keywords: Small sample volume; Microscale compartments; Capillary electrophoresis; Tear fluid; Airway surface fluid; Biofluids

1. Introduction

Capillary electrophoresis (CE) has rapidly developed into a reliable micro-analytical technique for the analysis of xenobiotics and endogenous compounds in various biological fluids [1–3]. Many of the bioanalytical applications reported in these references deal with separations from biofluids which are readily available in large volumes, such as plasma or urine, and often the CE methods reported are alternatives to high-performance liquid chromatography (HPLC) methods which already exist. Some of these methods use conventional sample preparation techniques such as extractions, whilst others employ direct injection of biofluids onto the separation capillary [2]. An important factor in the use of open-tubular CE for the direct analysis of biofluids is the relatively limited impact on the separation of large sticky biomolecules such as proteins. Depending on the separation conditions used these may be solubilised such that they do not adhere to the capillary surface [4,5], the capillary surface can be modified such that the tendency to stick is reduced [6,7], or else adsorbed proteins may be washed out of the capillary between separations [8,9]. Using these approaches, sample manipulation and sample preparation steps can be minimized or completely eliminated.

One of the defining characteristics of CE is the

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small size of the separation capillary, with typically only 1 μl or less of total column volume, and nanoliter or sub-nanoliter injection volumes. This is some orders of magnitude less than the typical injection volume of a conventional-scale or even microbore HPLC system. Thus CE is particularly suited to the analysis of small volume samples. Coupled with the ability to directly inject biofluids, it is clear that CE can be a key enabling technology for furthering the understanding of biological systems on a microscale. Furthermore, determination of endogenous compounds in liquids such as sweat and tears can be important diagnostically [10], and the quantitation of drugs and metabolites in nonconventional fluids can be important in determining their availability at the site of action, or to explain unexpected toxicity at another site [11].

There is a growing body of literature which describes CE separations of microsamples of biofluids on a scale which would be highly difficult or practically impossible for HPLC. In the limit, CE has even been used successfully for analysis of the contents of individual cells of animals [12–15] or plants [16]. In this article, the application of CE to the analysis of biofluids of limited availability will be discussed, with particular reference to separations of tear fluid and airway surface fluid (ASF). The analysis of these biofluids has generally been difficult because of their their small available volume, and, in the case of ASF, inaccessibility. The scope of this article will be limited to exclude single-cell analysis or the analysis of sub-cellular components; these topics have been reviewed elsewhere [17,18].

2. Tear fluid analysis

2.1. Tear fluid

Tear fluid is produced at a rate of approximately 1 μl min^{-1} (in healthy eyes) to form a layer a few μm thick which moistens and nourishes the cornea. The tears are made up of a number of layers; mucins coat the cornea, above these is the lacrymal fluid, and an oily layer may overlay this. In general proteins are present at about one-tenth the concentration in plasma, whilst inorganic ions in tears are at concentrations similar to those found in plasma [19]. Tears play an important role in eye defense due to a variety of components such as lysozyme and immunoglobulins [20]. Some components such as mucins are produced by conjunctival goblet cells, whilst the bulk of the tears come from the lacrymal or accessory lacrymal glands. The composition of tears can be used for diagnostic purposes [15] particularly for diseases of the eye. However, since many plasma components are thought to also be present in tears, there is the potential to use tear fluid analysis for non-invasive diagnosis of systemic diseases.

2.2. Collection of tear fluid

The thinness of the tear fluid layer and its low rate of production poses an analytical problem. Tear fluid is typically collected into a small tube or micropipet of <1 mm internal diameter. This tube is touched onto the surface of tear fluid which may collect in the outer corner of the eye or be pooled under the lower eyelid when it is pulled down. By capillary action a quantity of liquid is drawn up into the tube. The amount collected is typically in the sub-microliter to a few microliter range. Clearly, this quantity of liquid is not adequate for many analytical methods, at least unless some further sample manipulation such as dilution is performed. A couple of approaches may be used to collect more tear fluid. Stimulation of the eye may lead to greater production of tear fluid, however this may not be appropriate in many cases since the composition of stimulated tears is different from that of unstimulated ones [21]. Alternatively, repeated sampling at intervals of a few minutes can give a pooled sample of 10 μl or more of unstimulated tear fluid.

2.3. Capillary electrophoresis approaches to the analysis of tear fluid components

Protein analysis in tear fluid has typically been performed by gel electrophoresis with high resolution available from two-dimensional separations [22], or by HPLC [23]. HPLC is the separation technique of choice for small molecules [24,25] despite difficulties sometimes in obtaining adequate detection limits with small tear samples, and the fact that it is often necessary to perform extraction.

The CE papers dealing with separations of tear fluid address both large and small analytes. Varnell et al. [26] looked at a variety of different CE approaches for the characterization of rabbit tear fluid. In their work, a few μl of tear fluid was collected, and placed in microvial inserts in a commercial CE instrument suitable for holding a low-μl sample. The main difficulty with such microvials is avoiding evaporation if repeated analyses are required. A variety of approaches have been used to minimize sample evaporation in commercial instruments including good vial capping, addition of water to the main vial (in which the microvial is located) to maintain a humid atmosphere above the sample, and cooling of the sample tray; however, the effectiveness of these approaches is limited when dealing with low-μl samples.

Undiluted and diluted tear samples were analyzed [26]. A pH 2.5 capillary zone electrophoresis (CZE) experiment was able to resolve up to 35 peaks from a tear fluid sample, with 11 major peaks which were present in each of five different samples analyzed. Separation in a high-concentration borate buffer at pH 8.9 (similar conditions to those typically used for the CE analysis of major plasma proteins) revealed five peaks some of which seemed to be comprised of several co-eluting species; this was not as many resolved components as provided by silver-stained slab gels, although the CE separation was quick and required less sample [10 μl per lane was needed for the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) separations].

Phillips and Chmielinska [27] described an elegant CE method for the determination of the immunomodulatory peptide cyclosporin A in tears. Separations of tear fluid at pH 7 in a hydrophobic coated capillary revealed >20 resolved or partially resolved peaks, but cyclosporin A was impossible to quantitate at therapeutic concentrations under these conditions. To provide improved selectivity and sensitivity, an on-capillary purification and concentration approach was employed. A monoclonal antibody to cyclosporin A was immobilized onto the internal surface of a fused-silica capillary, to function as a specific adsorbent for cyclosporin A. Tear fluid was first loaded hydrodynamically onto the capillary; cyclosporin A binds strongly to the antibody and is thus recovered from the tear fluid. Then the capillary was washed out with a neutral buffer to remove unadsorbed components. Desorption and separation then took place with a pH 1.5 phosphate buffer. Fig. 1 shows electropherograms of tear fluid from patients being treated with cyclosporin A. Cross-reactivity of the antibody with cyclosporin metabolites allows their determination as well. This CE approach with immumoaffinity preconcentration provided detection limits of around 6 ng ml^{-1} using simple UV detection, and required only a small amount of sample. Correlation with a HPLC method was excellent, with the CE separation having the added advantage of being useful for analyzing metabolites as well.

Colón and co-workers have described various

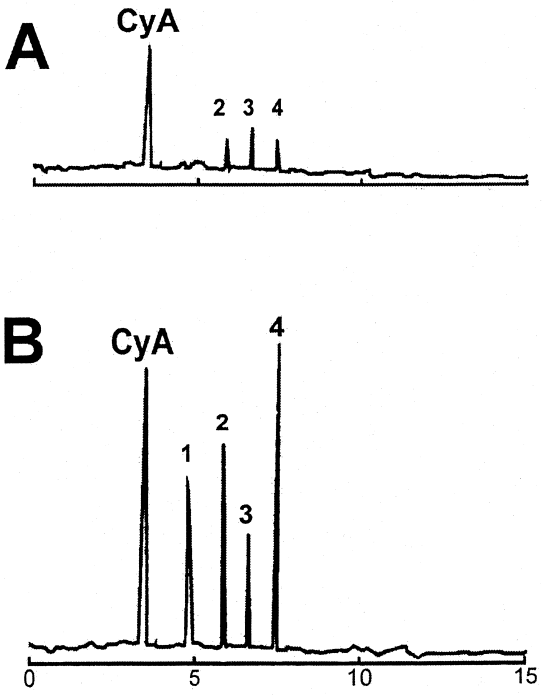

Fig. 1. Electropherograms of tear fluid from patients being treated with cyclosporin A. (A) Patient with no clinical signs of cyclosporin A toxicity, and (B) a tear sample taken during an episode of systemic toxicity. Cross-reactivity of the concentrator antibody with cyclosporin A metabolites (1–4) allows their determination as well. Conditions: capillary, first third coated with a monoclonal antibody to cyclosporin A; procedure, load tear sample, purge with pH 7.4 phosphate to remove unbound components, desorb and separate using a 100 m*M* phosphate BGE, pH 1.5 at a constant current of 100 μA; detection, UV at 214 nm. Reprinted with permission from Ref. [27], copyright 1994, Wiley.

approaches for the CE determination of sugars, and illustrated and applied these in the analysis of glucose in tear fluid [28–30]. Carbohydrates are particularly difficult analytes because they lack charge and have weak chromophores, thus they cannot be directly analyzed by CE with UV detection without complexation or derivatization. Derivatization of glucose with dansylhydrazine was reported, and an example of tear fluid analysis shown [28]. More extensive results for glucose determination in tears have been published based on an enzymatic approach [29,30]. Glucose in the presence of glucose oxidase (GOx) releases H_2O_2 giving rise to the oxidation of 4-hydroxy-3-methoxyphenylacetic acid (HVA) to the fluorescent 2,2′-dihydroxy-3,3′-dimethoxybiphenyl-5,5′-diacetic acid (HVA_{ox}) in the presence of peroxidase

$$\text{glucose} + O_2 + \text{GOx} \rightarrow \text{gluconic acid} + H_2O_2$$
$$H_2O_2 + \text{HVA} + \text{peroxidase} \rightarrow HVA_{ox}$$

HVA_{ox} can be separated and detected using laser-induced fluorescence detection. The method gave a limit of detection (LOD) for glucose of approximately 55 n*M* [30]. Although both on-column and pre-column reactions were demonstrated, the pre-column approach was preferred in a comparative study of glucose in tears and blood [29].

The determination the concentration of inorganic ions in biofluids is an important topic, and in tear fluid this can be performed by ion chromatography [31]. One CE report describes the analysis of principal cations in tear fluid, using indirect UV detection of diluted tear samples [32]. Although the tear samples were in this case diluted 1:20 before analysis, results with other biofluids suggest that direct injection should be possible. Direct analysis of inorganic species in tear fluid is likely to be an area of considerable future interest, given CE's proven abilities in inorganic ion analysis [33] and its compatibility with tear samples.

3. Analysis of lung airway surface fluid

3.1. Airway surface fluid

Lung ASF is a very thin (approx. 15–30 μm) low-viscosity fluid layer lining the epithelia in the conducting airways of the lung. ASF may consist of two layers, an aqueous (sol) layer with a mucus blanket (gel) layer floating on top. The mucus layer is considered to be negligible or absent in healthy individuals. Epithelial cells present on the apical side of the tracheal lumen are ciliated and these cilia project into the aqueous layer of ASF with their tips in contact with the mucus blanket. The cilia propel mucus out of the airways, a process called mucociliary clearance, to remove inhaled particulates or pathogens from the airways. Mucocilliary clearance depends on the composition and volume of ASF and is believed to be regulated by active electrolyte transport and passive water permeability of the respiratory tract fluid [34]. In airway diseases such as cystic fibrosis, an alteration of active ion transport across the epithelium can cause a change in the thickness or composition of the fluid layer due to an osmotic flow of water. This leads to impaired mucociliary clearance, bacterial colonization and infection, and finally results in lung failure. Ion transport dysfunction may also contribute to other airway diseases such as asthma and chronic bronchitis. Therefore, the study of the electrolyte composition of ASF is of considerable interest. Until recently, the composition of ASF has not been well defined due to the layer's thinness and its inaccessibility, which cause considerable difficulties in sample collection and subsequent analysis.

3.2. Collection of airway surface fluid

Bronchoalveolar lavage has been commonly used for collecting alveolar fluid and ASF from the lung and its conducting airways [35,36]. In this method, sterile physiological saline is introduced into the airways through a bronchoscope, and then the washings (lavage) are retrieved. The large volume of diluted ASF collected by lavage can be analyzed by a variety of techniques. However, there are limitations on the determination of inorganic ions due to interferences from the saline solution. Furthermore, analyses are non-quantitative due to the large, unknown dilution factor. Neither is the sampling site specific, providing information on at best a region of the airways. To overcome these limitations a variety of direct sampling techniques for ASF have been developed. Bronchoscopic brushing was used by

Jeanneret-Grosjean et al. [37] to collect mucus from human trachea. A bronchoscope is introduced orally and positioned midtrachea, whereupon a cytology brush is pushed out of the bronchoscope channel to contact the airway mucosa. The mucus-coated brush is then withdrawn through the bronchoscope. The sample obtained in this manner is generally between 1 and 2 μl in volume. The ''filter paper technique'' [38] is rather similar in that a few millimeter square strip of absorbent filter paper is placed on the epithelium for a few minutes via a bronchoscope. The filter paper absorbs ASF, and a microdroplet of liquid can be expressed from the paper and subjected to analysis.

As an alternative to the above methods, we have developed a capillary sampling procedure for the direct collection of ASF from the distal trachea which can be applied to large as well as small animals [39]. In some ways, the technique is rather similar to that described for tear collection, in that fluid is collected into a small tube by capillary action, however the process is made more complex because the site of collection is relatively inaccessible. First, an intubation tube (approx. 2 mm I.D.) is inserted into the subject's trachea. A sampling capillary, (approx. 280 μm, I.D.) is then passed through the intubation tubing (which protects it from picking up liquid in the upper airways) until it touches the epithelium. The sampling capillary is then left in contact with the epithelium for a period of 2–3 min during which time ASF enters the capillary. Typically 100–300 nl fluid samples can be collected in this way.

3.3. Analysis of inorganic ions in airway surface fluid

The most common methods of analyzing inorganic ions in ASF have been by elemental analysis using flame photometry or energy dispersive X-ray spectrometry [38] for determination of elements such as Na, K, Ca, Mg, Cl, P and S. The latter technique is most suited to analysis of ASF microdroplets obtained from filter paper. A significant limitation of elemental analysis is the inability to distinguish elemental sulfur from sulfate or from amino acids and similar problems exist for nitrogen and phosphorous analysis.

The use of CE for the direct analysis of ASF microsamples removes the need for dilution or extraction, since nanoliter samples can be introduced directly onto the capillary. ASF sample injection is done by fitting the sampling capillary over the end of the separation capillary (relatively small 145 μm O.D. separation capillaries are used so that they will fit inside the sampling capillary). The separation capillary is inserted until it just enters the liquid within the sampling capillary (Fig. 2). Since the sampling capillary is open at both ends, the liquid is not forced into the separation capillary inadvertently when the insertion takes place. After insertion, a known amount (3–5 nl) of ASF can injected by applying a differential pressure across the system.

Indirect UV methods which provide universal detection were first used for the analysis of inorganic ions in ASF [39]. For ASF cation analysis imidazole was selected as an adsorbing background electrolyte (BGE) co-ion since it has high mobility similar to that of ASF cations of interest (Na^+, K^+, Ca^{2+} and Mg^{2+}). For anion analysis, chromate was used as an absorbing BGE as it has high UV absorption and a similar mobility to chloride which is a major component in ASF. ASF inorganic ions were identified and quantitated by using aqueous inorganic ion standards (of course, it is impossible to obtain a blank matrix for the standards). Washing with SDS solution [8,9] between runs was occasionally necessary to reduce contamination of the capillary by other components of the sample.

One of the limitations of indirect UV methods is that high concentrations of a good buffer cannot be used due to the reduced efficiency of ion-displace-

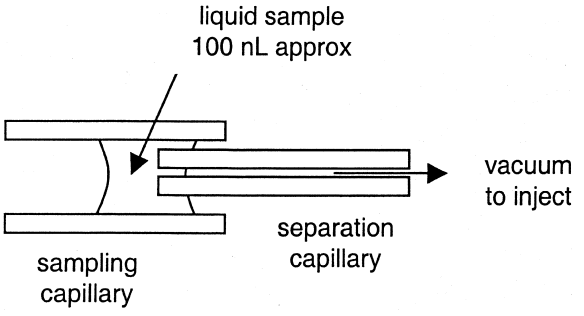

Fig. 2. Schematic diagram showing the coupling of injection and separation capillaries.

ment process necessary for detection, and further linear dynamic range of detection is also limited. The use of conductivity detection for inorganic ion analysis removes these limitations and can also improve limits of detection [40]. The ability to use a high ionic strength electrolyte with conductivity detection may also improve the resolution of the ions in matrices such as ASF where some components are present at very high concentrations. ASF analysis was performed using a commercially available conductivity detector [41]. One difficulty encountered in this application is that the cell (Concap and Contip, ATI Unicam, Boston, MA, USA) is designed to use with 365 μm O.D. capillaries, which are too large to insert into the sampling capillary used for ASF collection. Hence, a smaller O.D. (145 μm) fused-silica capillary had to be coupled to the inlet end of the Concap capillary using a butt-joint.

ASF anion and cation analyses with conductivity detection are shown in Fig. 3A and B. The relatively high concentration of electrolyte used for both anion and cation analysis is useful to achieve better peak shapes. The use of low-conductivity buffers such as 2-(*N*-cyclohexylamino)ethanesulfonic acid CHES or 2-(*N*-morpholino)ethanesulfonic acid (MES) is advantageous in minimizing background noise. The lower detection limits compared to indirect UV allowed quantitation of NO_2^- and NO_3^- for the first time.

3.4. ASF protein analysis

Excellent high-resolution two-dimensional electrophoretic separations of proteins in lavage fluid have been reported [42], and CE has also been applied to the analysis of proteins in lavage fluid [43]. The direct determination of major proteins in ASF is also possible by CE [44]. The sample collection and injection procedure used is similar to that employed for inorganic ion analysis, only the details of the separation change. Since proteins have strong UV absorption around 200 nm due to their peptide bonds, they can be detected in CE by low-wavelength UV absorption. For quantitative analysis, problems of protein adsorption to the capillary surface need to be overcome, as discussed in the Introduction [6–9]. Although the analytical resolution achievable by CE cannot compete with two-dimensional techniques, the compatibility of CE with the microsampling approach described here allows for spatial resolution of sampling within the airways, and characterization of different types of components within a single sample.

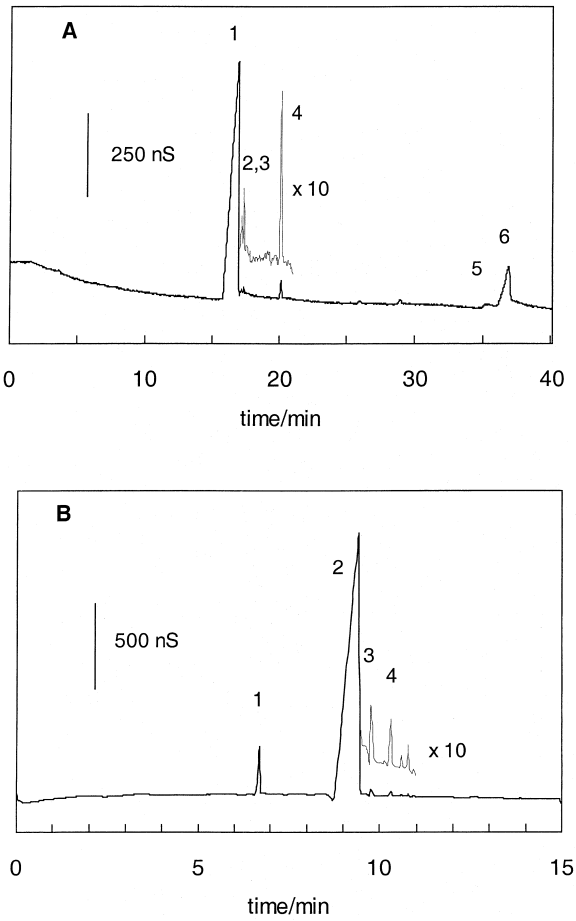

Fig. 3. Analysis of anions (A) and cations (B) in rat ASF. Separation conditions: 90 cm long fused-silica capillary (50 μm I.D.) with end-capillary conductivity detection cell with (A) BGE, 100 m*M* CHES, 40 m*M* LiOH (pH 9.3)–2-propanol (92:8, v/v) (propanol improves resolution of nitrate/nitrite from chloride) with 80 μ*M* spermine additive to eliminate EOF; field strength, −278 V cm^{-1}; (1) Cl^-, (2) NO_2^-, (3) NO_3^-, (4) SO_4^{2-}, (5) PO_4^{2-}, (6) HCO_3^-. (B) BGE, 100 m*M* MES, 100 m*M* histidine, pH 5.6, with 20 m*M* α-hydroxyisobutyric acid as a complexing agent to modify selectivity; field strength, 222 V cm^{-1}; (1) K^+, (2) Na^+, (3) Ca^{2+}, (4) Mg^{2+}. Reprinted with permission from Ref. [41], copyright 1997, American Chemical Society.

For the analysis of proteins in ASF without sample preparation, two different approaches have been

adopted [44]. A moderately high ionic strength borate buffer with higher pH (9.1) and a low pH (2.5) phosphate buffer with an additive, such as spermine, for dynamic coating of the capillary surface have been used. Under these conditions protein adsorption is reduced and there is good resolution and reproducibility. Albumin, transferrin and globulins were seen by direct CE analysis to be the major protein components in rat ASF (Fig. 4), with peak identification performed by the comparison with protein standards under the two different separation conditions used. Lysozyme, lactoferrin and mucins are also thought to be present in ASF. In these studies lysozyme was identified as a minor

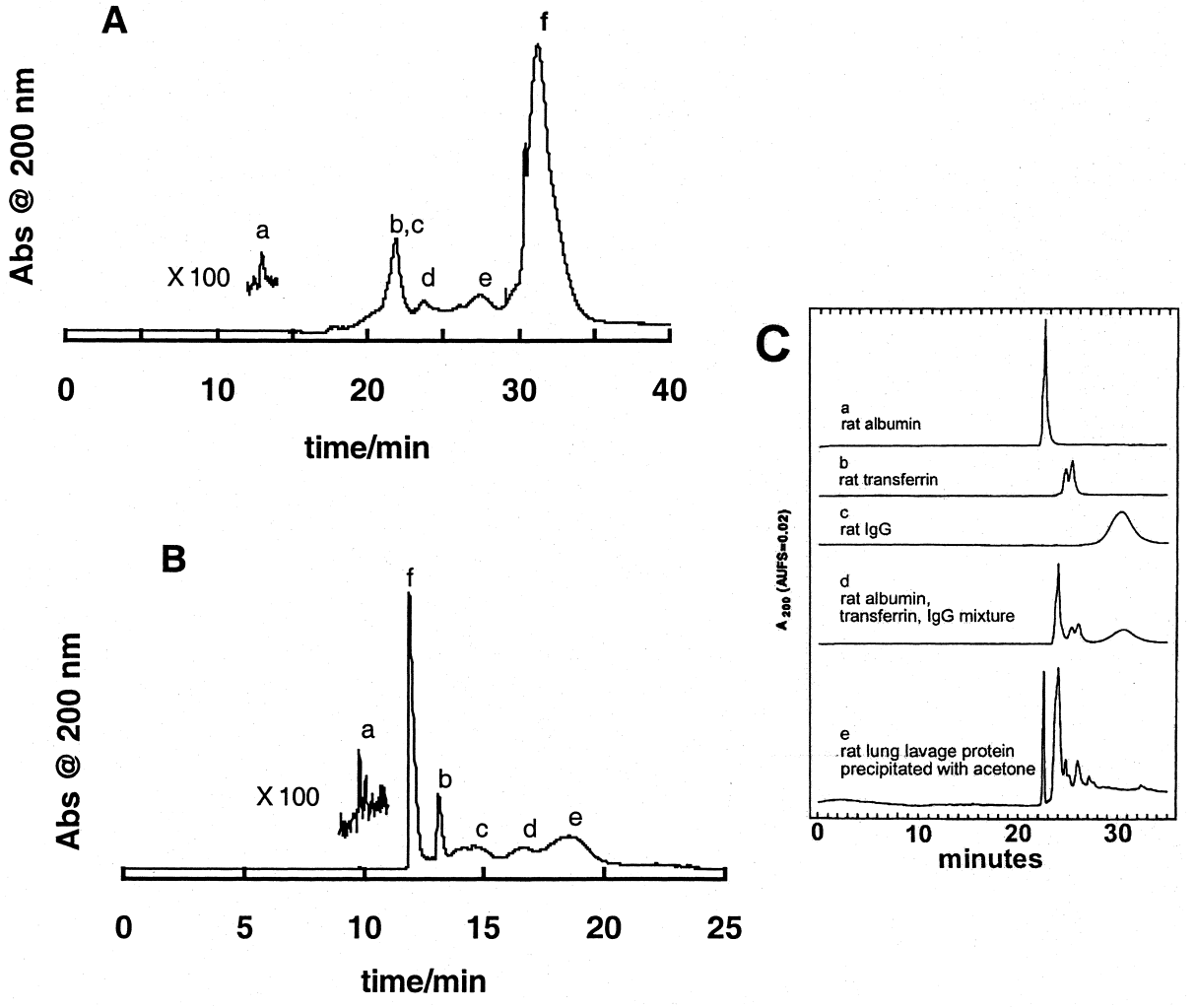

Fig. 4. Separations of major proteins in rat ASF. (A). Direct-injection of ASF with high-pH separation in an uncoated capillary. Conditions: fused-silica capillary 72 cm long (50 cm effective length)×50 μm I.D.; BGE, borate, pH 9.1; field strength, 167 V cm^{-1}. (B). Direct injection of ASF with low-pH separation in a dynamically-coated capillary. Conditions: capillary as above; BGE, phosphate, pH 2.5 with 0.5 m*M* spermine additive; field strength, 250 V cm^{-1}. Peak assignments (a) lysozyme, (b) transferrin, (c) γ-globulin, (d) β-globulin, (e) α-globulin, (f) albumin. (C). Separation of rat plasma protein standards (a–d) and rat ASF proteins sampled by lavage (e). Conditions: capillary, 50 cm×50 μm I.D. fused-silica with covalently-bound neutral hydrophilic coating; BGE, phosphate, pH 2.5; field strength, 160 V cm^{-1}. (A) and (B) reprinted with permission from Ref. [44], copyright 1998 Elsevier; (C) reprinted with permission from Ref. [43], copyright 1991, Elsevier.

component. A lactoferrin standard was found to migrate between albumin and transferrin at pH 2.5, but was not observed in rat ASF. Mucins are heterogeneous glycoproteins which are principally thought to be present in diseased airways, and have not been observed in the studies described here. By way of comparison, Fig. 4C shows a CE analysis under similar low-pH separation conditions of rat protein standards and rat ASF obtained by lavage [43]. In this case, the ASF proteins were prepared for analysis by precipitation from the lavage fluid with acetone, followed by reconstitution in 0.2% trifluoroacetic acid in water. It can be seen that the microsample and the lavage sample electropherograms are of fairly similar quality in terms of peak shapes and signal-to-noise ratio. The absence of immunoglobulins in the lavage sample compared to the microsample may reflect a locally higher concentration at the site of collection of the microsample (compared to the average concentration in the large region of the lung washed by the lavage), losses during the sample preparation performed on the lavage fluid, or may simply be due to inter-subject variation.

3.5. Quantitative aspects of ASF analysis and composition of ASF

The parameters related to quantitation such as minimum detectable concentrations (MDCs) and reproducibility have been examined. The MDCs for inorganic ions using indirect UV detection were in the range 20–100 μM for cations, and around 200 μM for Cl^- [39]. With conductivity detection, the MDC was <100 μM for all species measured [41]. For protein analysis, the MDC is around ~1 mg ml^{-1} [44]. Intra-day variability of the method was determined for each ion and protein. It was not possible to perform a large scale validation using actual ASF samples, because of limited volume of the ASF available in each sampling. Replicate analyses (~6–10) were made by injecting from sampling tubing which contained a standard solutions of inorganic ions within the calibration range (0.04–1.0 mM) or proteins (2–40 mg ml^{-1}). In all cases, the relative standard deviation (RSDs) in peak areas were $<10\%$ with the higher values being observed using the coupled-capillary conductivity detection system. For protein analysis, the RSDs for peak areas were 9–11% for albumin, 4–12% for transferrin and 4–14% for globulin. For migration times, the RSDs were typically $<1\%$ for the ions using both indirect UV and conductivity measurements and $<2\%$ for protein analysis using direct UV method.

4. Conclusion

The examples given above have focused on CE analysis of tear fluid and airway surface fluid, and serve to illustrate the ability of CE to characterize microliter and sub-microliter biofluid samples. CE has also been applied to the analysis of other volume-limited biological samples, for example synovial fluid [45] and liquid collected from renal tubules [46]; the technique has also found application in analysis of small-volume microdialysis samples, a topic reviewed recently [47]. Given that CE uses only nanoliters of material per injection, each microliter sample may be repeatedly analyzed by CE to determine a variety of different components, e.g., inorganic anions, cations and proteins. This ability to perform essentially non-destructive analyses of diverse components on microliter or sub-microliter samples sets CE apart from other separation techniques. Furthermore, spatial information may be obtained by microsampling from different sites, or temporal information may be obtained by taking multiple samples over time. Such information is often not obtainable if the whole biological compartment of interest is disturbed by a macroscale sampling technique.

It is often the case that our understanding of biological systems is limited by our ability to make measurements in them, and conversely, some systems are well studied simply because we have the tools to do so (for example, it can be argued that electroactive neurotransmitters have been the subject of intense scrutiny not solely because they have an important physiological role but also because high-sensitivity electrochemical detectors with the ability to detect them at low concentrations have been available for some time). Because of the unique characteristics of CE, the analysis of many small biological systems and compartments which previ-

ously posed intractable problems has now become possible, if not facile.

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