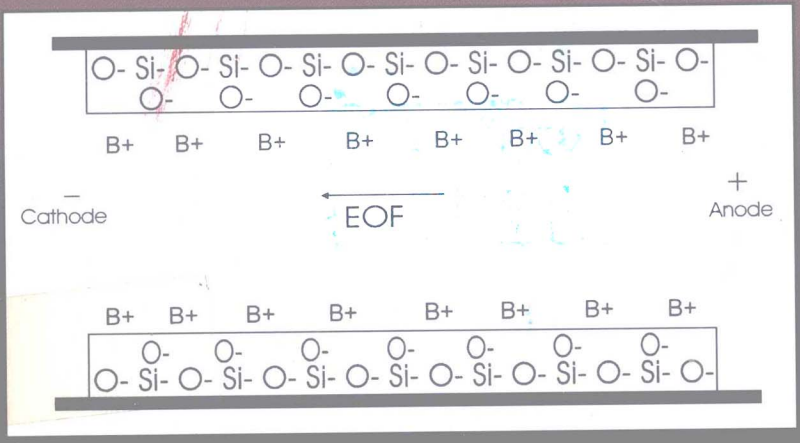
METHODS IN MOLECULAR MEDICINE™

Clinical Applications of Capillary Electrophoresis

Edited by
Stephen M. Palfrey



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Preface

The term "electrophoresis" was first used by Michaelis in 1909 to describe the migration of colloids in an electric field. The first practical electrophoresis method was described by Tiselius in 1937. He used a U-tube filled with buffer layered on top of sample; migration could be monitored using Schlieren optics. In zone electrophoresis, the U-tube was replaced by paper, a support material employed simply to prevent or minimize diffusion of ions, so that ions applied in a narrow strip to the paper will separate and remain as relatively discrete zones. Paper was superceded by a variety of other media, including cellulose acetate, hydrolyzed starch (starch gel), agarose, and polyacrylamide. The latter, in addition to being a support medium, has size-sieving properties. From the basic method of zone electrophoresis, other means of separation have been developed, including isoelectric focusing, isotachophoresis, density gradient electrophoresis, and various forms of immunoelectrophoresis.

In some ways Capillary Electrophoresis (CE) has gone full circle back to the original method of Tiselius. In its simplest form, separations occur in a buffer solution within a glass (fused silica) tube and detection occurs as sample moves past an optical window. CE has rapidly developed into a technique that rivals HPLC in its versatility. All the classical electrophoretic separations—zone, IEF, and isotachophoresis—have their counterparts in CE. Excitingly so, and authoritatively treated in *Clinical Applications of Capillary Electrophoresis*.

The addition of modifiers to electrophoresis buffers has opened up whole new separation possibilities. Adding detergents has created the technique known as Micellar Electrokinetic Capillary Chromatography (MECC); the method described in the chapter on steroids is a good example and offers an excellent description of the principles of the technique. Non-UV-absorbing species, such as anions and cations, can be detected by including chromophores in the buffer. In this instance detection occurs as a decrease in background absorbance as the ion passes the detector; detection of nitrate and oxalate use this method. Chiral separations can be achieved using additives like cyclodextrins, enabling the separation of optical isomers. One of the main driving forces of CE in fused silica capillaries is electroendosmosis; some additives like tetradecyltrimethylammonium bromide (used in the citrate/oxalate method) will reverse

the endosmotic flow. Others such as methyl cellulose will reduce it greatly, an important consideration in the separation of hemoglobins by IEF.

Further modifications are possible by applying different coatings to the internal glass surface of the capillary. Some of these coatings, such as C18, will be familiar to HPLC users. The hemoglobin and CSF methods both use coated capillaries.Taking this process one step further, capillary electrochromatography (CEC) is a technique that combines CE and HPLC. Glass capillaries are filled with HPLC packing materials, the driving force being an electrical gradient rather than high pressure.

For the clinical laboratory, CE is potentially a very useful tool. It enables the automation of many methods that in the past were manual or semi-automated. In addition it offers great economy of reagent usage; assays can be run with as little as 100 μL or less of buffer at each end of the capillary. This has major advantages when expensive or hazardous organic solvents are used. It requires only nanoliter quantities of sample, allowing very small scale sample preparation where expensive reagents are used, such as in PCR. Consumables are restricted to sample vials and capillaries that, if looked after, should last months. Laboratory staff generally take to the technique well and find it reliable and robust. Compared with HPLC, startup time is minimal, and if something does go wrong, the capillary can be flushed out and ready for use in seconds. In addition it is capable of some extraordinary separations; peaks separated by only 6 s can be resolved to baseline and separations of up to a million plates/meter have been reported. Figure 4 in Chapter 19 shows this power clearly; seven peaks are resolved in less than half a minute, a separation efficiency of 400,000 plates/meter. On the negative side, CE can lack sensitivity when the optical path is equal to the capillary diameter and, at the moment, there is a limited range of detectors compared to those available for HPLC.

The range of CE assays available for the clinical laboratory has grown rapidly over the last two to three years. This book tries to give a representative, rather than comprehensive, look at those assays. Most laboratories will already carry out some form of electrophoresis, such as serum and urine proteins in clinical chemistry, or hemoglobins in hematology. Serum and urine protein analyses on CE use free solution electrophoresis with UV detection. The endpoint is an electropherogram that will be familiar to anyone who has used agarose electrophoresis with scan denitometry. The separation of hemoglobins uses isoelectric focusing with detection at 415 nm. This single assay allows both the detection and quantitation of abnormal hemoglobins together with HbA_2 and HbF, replacing several manual electrophoresis assays. Other methods describe the detection of CSF proteins, lipoproteins, myoglobin, cryoglobulins, HbA_1c, and cathepsin D.

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One of the most rapidly expanding and exciting areas of CE is in DNA analysis. There are five chapters describing different aspects of this field; double-stranded DNA analysis, the prenatal diagnosis of Down's syndrome and Rh D/d genotyping, the identification of mutated p53 in cancers, the detection of microsatellite instability in cancers, and the detection of CMV. A significant advantage of CE is that it allows the injection of PCR products into the capillary without further sample cleanup.

Drug assays have long been performed in clinical laboratories, either as part of therapeutic drug monitoring (TDM) or as screening for drugs of abuse. TDM is frequently carried out by HPLC or immunoassay; CE offers a third way of performing these assays. The combination of the efficiency of CE separations coupled with diode array detection makes a powerful tool for the confirmation of the presence of drugs of abuse in urine.

Finally, *Clinical Applications of Capillary Electrophoresis* offers methods for some of the more esoteric analytes—nitrate and nitrite, oxalate and citrate, and serum polyamines—useful but not necessarily frequently requested assays.

In summary, *Clinical Applications of Capillary Electrophoresis* aims to give encouragement and guidance to laboratories new to CE and to demonstrate that the wide range of assays now available means that it is a technique tht has something to offer every clinical laboratory.

Stephen M. Palfrey

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Contents

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Clinical Applications of Capillary Electrophoresis

Margaret A. Jenkins

1. Introduction

Capillary electrophoresis (CE) is a new and innovative technique that separates charged or uncharged molecules in a thin buffer-filled capillary by the application of a very high voltage. Separations by CE are extremely fast: Some are achieved in less than 5 min, with reproducibility studies often showing coefficient of variation (CVs) of <2%. The outstanding characteristic of CE is that it is an extremely sensitive technique. Early workers reported separations greater than 1 million theoretical plates per meter by CE, which is 10× the sensitivity of high-performance liquid chromatography (HPLC). The development of automated sample injection has meant that CE can be integrated into a clinical setting in which turnaround of accurate, cost-effective results are paramount.

Since 1937, when the original paper on electrophoresis by Tiselius was published *(1)*, many scientific papers have documented the progress of CE. Hjerten *(2)* originally suggested the usefulness of CE for zone electrophoresis and isoelectric focusing. Some excellent reviews on CE have already been published. Gordon *(3)* covered construction of instrumentation used in CE, as has Deyl *(4)*. Kuhr published a review of operational parameters and applications *(5)*. Mazzeo and Krull *(6)* reviewed coated capillaries for both capillary zone electrophoresis and capillary isoelectric focusing. In 1992, Shihabi *(7)* reviewed clinical applications of CE. Later, Jenkins et al. *(8)* and Lehmann et al. *(9)* also reviewed capillary electrophoresis applications in clinical chemistry.

2. Instrumentation

CE uses a very high voltage (1–30 kV) for the separation of analytes in the capillary, which may be either coated internally, or uncoated. Uncoated capil-

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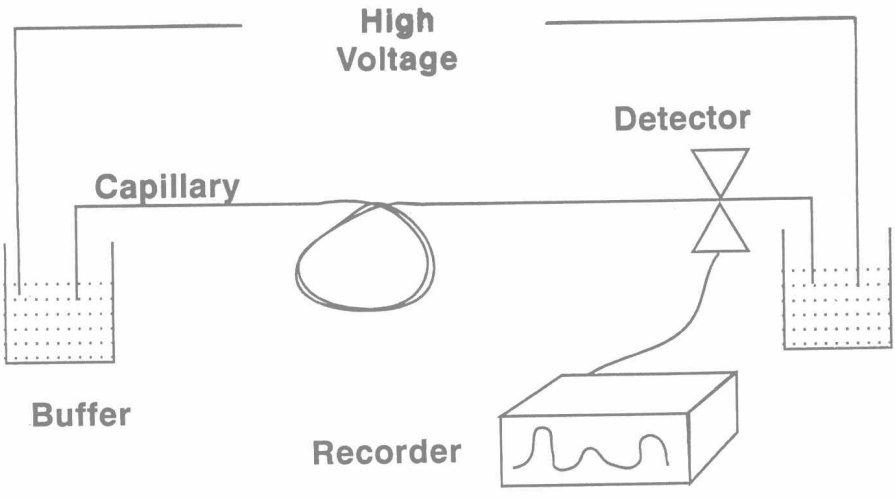

Fig. 1. Schematic diagram of CE apparatus.

laries are often referred to as fused silica capillaries. The diameter of the capillary varies between 20 and 100 μm in diameter, and is from 25 to 122 cm in length, depending on the configuration of the instrument. The ends of the capillary are placed in buffer vials, which also contain the electrodes. The narrow diameter of the capillary is important in heat dissipation from the high voltage applied, and also to decrease band diffusion. A schematic representation of a CE instrument is shown in **Fig. 1**.

Capillary columns have a polyimide outer covering, which makes the capillary mechanically stronger and protects the capillary from sudden angulation and breaking. The detector system with a CE may be variable wavelength, filter UV photometer, diode array, or a laser fluorescence detector ***(10)***. At the detector window, the polyimide coating of the capillary is burnt off to allow the light source to penetrate the capillary, and for absorbance measurements of the analytes passing the window to be made.

Two methods are usually available for introduction of the sample to the capillary: electrokinetic and hydrodynamic injection. With electrokinetic injection, the inlet end of the capillary is removed from the buffer vial, and is inserted into the sample. A voltage is applied for a time ranging from 0.5–30 s, which causes the sample to migrate into the capillary. After the injection, the sample vial is replaced with the buffer vial and electrophoresis can proceed. The amount of sample introduced can be varied by altering both the time of injection and the injection voltage. The drawback of this type of injection is that sample components with highest electrophoretic mobility will be preferentially introduced over those with lower electrophoretic mobility ***(11)***.

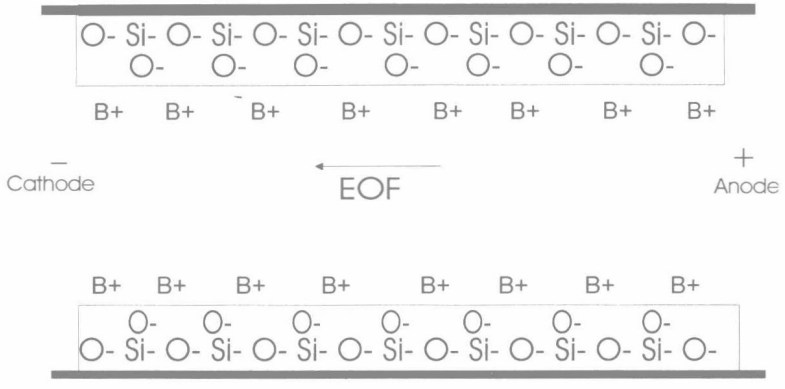

Fig. 2. Diagram showing cause of electroosmotic flow. Positively charged buffer ions, adjacent to the exposed negatively charged silanol ions of the fused silica wall, are attracted to the cathodes.

With hydrodynamic injection, the sample vial is raised above the capillary to a predetermined height, and the sample pours into the capillary for a defined period of time. Alternatively, the sample may remain at the same height as the outlet end of the capillary, and either positive pressure is applied to the sample vial or a vacuum is applied to the outlet electrode container. Hydrodynamic methods of sample introduction are not affected by the sample composition.

The type, pH, and ionic strength of the buffer are critical for the separations obtained ***(12)***. Buffers may be made from a single component, such as phosphate, or may be quite complex, using two or more anions (borate–phosphate is a frequent combination). The pH of any buffer used in CE needs to be carefully optimized and maintained, to ensure reproducibility. The length of the capillary used, and the voltage applied, also influence the time of separation.

Electroosmotic flow is an important phenomenon in CE that can assist in the separation process ***(13)***. The internal surface of fused-silica capillaries is negatively charged because of exposed silanol ions when the buffer is above pH 2.0. When an electric field is imposed, it causes hydrated ions in the diffuse double-layer adjacent to the silica wall to migrate toward the oppositely charged electrode, dragging solvent with the ions. This is termed electroosmotic flow, and can be used to advantage (*see* **Fig. 2**). The net flow of ions past the detector will reflect the balance between the electrophoretic and electroosmotic forces within the capillary. By adjusting the pH of the buffer in the capillary, electroosmotic flow can either enhance or oppose electrophoretic migration. Electroosmotic flow may also be decreased either by increasing the ionic strength of the buffer or by increasing the viscosity of the buffer by the

addition of polymers, small amounts of organic solvents, or molecules such as glucose. Electroosmotic flow decreases with decreasing surface charge on the capillary, either by decreasing the pH of the buffer, or, alternatively, by decreasing the applied voltage *(14)*.

3. Modes of Separation

There are four major modes of separation by CE.

3.1. Capillary Zone Electrophoresis (CZE)

In free-solution capillary zone electrophoresis (CZE) a thin plug of sample is introduced into a buffer or gel-filled capillary. Under the influence of an external field, this yields discrete zones, which may be measured as they pass an in-line detector. Solutes are separated in this technique on the basis of differences in charge-to-mass ratio. In gel- or polymer-network-filled capillaries, solutes are separated, by the process of sieving, on the basis of their size.

Coatings for capillaries used in free-solution separations must be chemically stable and reproducible. For optimal separation, the surface modifications, which may be neutral or charged, should only partially inhibit electroosmotic flow. Examples of neutral coatings are polyacrylamide, methylcellulose, or polyethylene glycol. Charged coatings include quarternary ammonium functional groups bound to the capillary surface, or a small-mol wt polyethyleneimine coating that is suitable for basic proteins.

3.2. Isoelectric Focusing

Gel isolectricfocusing (IEF) can separate proteins that differ by as little as 0.001 of a pH unit *(15)*. As with gel IEF, capillary isoelectric focusing (CIEF) utilizes ampholytes that span the pH range of interest. These ampholytes facilitate high resolution separation of protein and peptide mixtures. CIEF usually uses a coated capillary; however, if the electroosmotic flow is sufficiently reduced by the use of methylcellulose or hydroxypropylmethylcellulose, then CIEF can be carried out in a fused-silica capillary. In CIEF, the capillary is filled with a mixture of protein sample and ampholytes. At the cathode, a basic solution (usually sodium hydroxide) is used, and an acidic solution (often phosphoric acid) is used at the anode. When an electric field is applied, the proteins migrate to the position at which the pH equals their respective pIs. When focusing has been completed, the current drops within the capillary to a minimal level.

Mobilization of peaks past the detector may be achieved by several methods. The first is electrophoretic mobilization, which involves adding salt to one of the electrolytes; for example, the addition of 80 m*M* NaCl to 20 m*M* NaOH *(16)*. Alternatively, mobilization of focused peaks may be achieved by the appli-

cation of a vacuum to the capillary, as well as maintenance of the high voltage *(17,18)*. The third alternative is the recording of the pH gradient without mobilization. In practice, this is achieved by imaging the whole length of a short glass capillary *(19)*. Recently, cathodic mobilization has been achieved by replacing the catholyte with a proprietary zwitterionic solution (Bio-Rad, *20,21*), and by using gravity mobilization *(22)*.

3.3. Capillary Isotachophoresis

This mode of separation which employs stacking of dilute components, is not widely used, but in certain instances has useful applications. The stacking is achieved by using a small (e.g., 2 s) plug of water on either side of the injected sample. The result is that the sample becomes insulated from the buffer, resulting in sharper separations of dilute solutions.

An alternative isotachophoretic approach may involve using different leading and terminating electrolytes for focusing and preconcentration. After this step, the terminating electrolyte is replaced with the leading electrolyte for the remainder of the separation *(23)*.

3.4. Micellar Electrokinetic Capillary Chromatography

The essential characteristic of this type of separation, first described by Terabe in 1984 *(24)*, is the use of buffer containing surfactants at concentrations above their critical micelle concentration in fused silica capillaries. Thus micellar electrokinetic capillary chromatography (MECC) is a modification of CZE. Inside the capillary tube, there are two phases: a pseudostationary phase, which is an electrophoretically migrating micellar or slow moving phase, and an aqueous phase, with the electroosmotic force at a velocity higher than that of the micellar phase.

To be suitable for MECC, the micellar phase should be a surfactant that is highly soluble, and the solution must be UV-transparent and homogeneous. Examples of micellar systems include sodium dodecylsulphate (SDS), sodium deoxycholate, or SDS-tetra-alkylammonium micelles.

4. Clinical Separations

The number of scientific papers describing specific disorders diagnosed using CE has increased dramatically since 1990. These include DNA diagnosis of Down's syndrome *(25)*, adenylosuccinate lyase deficiency *(26)*, and P53 oncogene analysis *(27)*. Well-documented scientific papers are available on topics such as lipoprotein analysis by CE *(28)*, oxalate/citrate analysis *(29)*, plasma nitrate/nitrite *(30)*, organic acids in urine *(31,32)*, drugs of abuse in urine, anticonvulsants *(33)*, and urinary steroids *(34)*. CE techniques for serum proteins *(35–37)*, urine proteins *(38,39)*, hemoglobin variants *(40–41)*,

cryoglobulins *(42)*, enzymes, cerebrospinal fluid (CSF) protein electrophoresis *(43)*, and HbA_1c *(44)* are also available.

Verification of a CE method, so that it can be introduced as a routine clinical method, involves testing at least 300 samples by both CE and conventional methods. The samples tested should be all the samples which come into the laboratory for that analyte, and must include at least 20% normal samples. If gross differences between the CE method and the conventional method are found during the testing of these 300 samples, then further samples should be assayed (up to 1000 samples) to show the proportion of these differently-behaving samples in everyday, routine testing. Statistical analysis of the two methods should be employed to show the correlation between the two methods, as well as the line of best fit.

The CE methods presented include the modes of free solution, IEF, micellar chromatography, and isotachophoresis.

One of the most informative aspects of this MIMM series is the Notes section, in which authors have indicated any problems or faults that can occur with their technique, and how these problems have been identified and overcome. This publication is aimed at scientists with no previous CE experience. The information contained within each chapter will allow validated methods to be successfully used by other laboratories keen to be involved with the rapid, sensitive, and extremely useful technique of capillary electrophoresis.

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