DETERMINATION OF CREATININE AND CREATINE

BY

CAPILLARY ELECTROPHORESIS

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ABSTRACT

The assessment of creatinine and creatine in biological fluids is important in the evaluation of renal and muscular functions. For routine creatinine determinations in the clinical laboratory, the most frequently used method is the spectrophotometric one based on the Jaffé reaction. However, this reaction is not specific for creatinine.

For this reason, several methods have been proposed, but the elimination of interferences in the determination of creatinine has still not been achieved in some of these methods; others solved this problem either with expensive equipment that does not suit routine analysis or necessitates time-waste procedures.

In this thesis capillary electrophoresis was the new tool investigated. It was applied in an attempt to achieve both the separation of creatinine from the non-creatinine 'Jaffé-reacting' chromogens and the determination of creatine in serum.

Capillary zone electrophoresis was performed with detection at wavelength 480 nm to separate creatinine from the non-creatinine 'Jaffé-reacting' chromogens in urine. The principle was based upon the different migration times due to the different molecule weights, molecular sizes and charges under the applied high voltage. The picric acid was employed as part of the running buffer to allow reaction of creatinine and picrate to take place after the sample injection. This procedure eliminated the negative influence of the reaction time that is controlled manually in the common Jaffé reaction method. Therefore, compared to the Jaffé reaction method, the new method achieved more accuracy and precision in the determination of creatinine.

Determination of creatinine in serum and urine were studied at a new wavelength 417 nm, which gave a higher sensitivity of detection than at 480 nm. This wavelength shift made the determination of creatinine in serum possible by capillary zone electrophoresis without the non-creatinine 'Jaffé-reacting' chromogens interfering. In this method, serum only needed a simple filtration before the analysis.

Creatine was discovered to have absorption at 417 nm in alkaline medium. Moreover, specific sample stacking was introduced in this method. The sample was dissolved in a mixture of two-volumes acetonitrile and one-volume 3 % ammonium chloride to give a 10-fold enhancement of detection sensitivity.

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ATP	adenosine 5'-triphosphate, disodium salt hydrate
CAPS	3-cyclohexylamino-1-propanesulfonic acid
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CI	creatine amidinohydrolase
CIEF	capillary isoelectric focusing
CITP	capillary isotachophoresis
CK	creatine kinase
CRN	creatinine
CV	coefficient variation.
CZE	capillary zone electrophoresis
EC	enzyme convention number
EOF	electroosmotic flow.
HPCE	high-performance capillary electrophoresis
HPLC	high performance liquid chromatography
HVPS	high-voltage power supply
I.D.	internal diameter
LD	lactate dehydrogenase
MECC or MEKC	micellar electrokinetic chromatography
MES	2-(N-morpholino)ethane sulfonic acid
ODS	octadecyl silica or C18 silica
PA	picric acid
PAGE	polyacrylamide gel electrophoresis
PAH	p-aminohippurate
PDA	photodiode array
PK	pyruvate kinase
Pos	positive
SDS	sodium dodecyl sulfate
SO	sarcosine oxidase
TCA	trichloroacetic acid

Tris	tris (hydroxymethyl-aminoethane)
UV	ultra violet
UV-VIS	ultraviolet-visible
WA	washing

CHAPTER ONE

INTRODUCTION

1.1 Historical background

Capillary electrophoresis (CE) is a modern analytical technique, which permits rapid and efficient separations based on the principles of charge, hydrophobicity, stereospecificity, size, or interaction with buffer additives.

The history of the development of capillary electrophoresis has been traced back to more than a century ago, as early as the late 1800s, when electrophoretic separations were attempted in free solutions as well as various gels. Many early experiments were performed using glass U rubes with electrodes connected to each of the tubes' arms as shown in **Figure 1.1**.

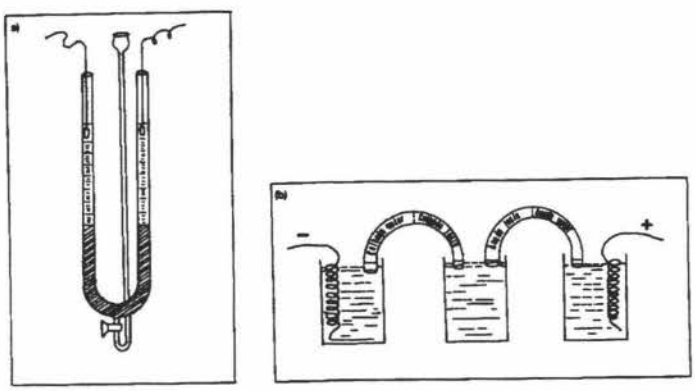


Figure 1.1 (a) a glass U tube apparatus used in early experiments with free-solution electrophoresis. Electrodes made of platinum foil were immersed in the electrolyte solution. The sample solution with indicator dye was at the bottom of the U tube. (b) an inverted U tube apparatus, which consisted of two tubes, filled with agar bridging the sample reservoir and reservoirs of distilled water (Li, 1992).

The experiments were performed using a direct current of up to several hundred volts. The separation of various types of samples, such as small ions, isotopes, toxins and proteins was investigated. In order to overcome problems of convective mixing which

were encountered in electrophoretic separations performed in free solutions, various stabilizing media have been employed, such as agar, cellulose powder, glass wool, paper, silica gel and acrylamide (Li, 1992).

An alternative approach to alleviate thermal convection problems in free solution electrophoresis was the use of tubes with small internal diameters. These small tubes or capillaries dissipate heat better and provide a more uniform thermal cross-section of the sample within the tube. Provided ideal conditions can be maintained, samples migrate rapidly as a flat plug with resolution limited only to diffusion. Hence, the technique has the potential of achieving extremely high efficiency in separations.

In the early days, tube diameters of 1-3mm, were first used and then reduced in size to 200-400 μm. In 1971, Everaerts explored the separation potential of capillary zone electrophoresis (CZE) performed in 200-μm Teflon capillaries with on-column UV detection. In 1981, Jorgenson and co-workers convincingly demonstrated the analytical potential of CZE using 75-μm fused silica capillaries. The experience of this laboratory (Jorgenson, 1981) in capillary chromatography was highly useful in designing instrumentation for this new electrophoretic system. The high-resolution capability of the method was clearly shown, as illustrated in **Figure 1.2**

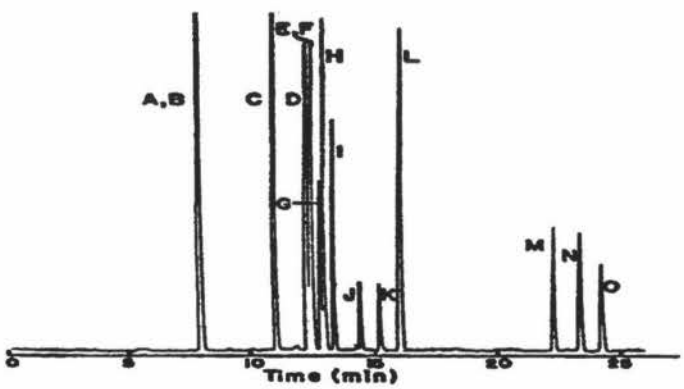


Figure 1.2. Zone electrophoretic separation of dansyl amino acids: A, unknown impurity; B, lysine; C, dilabeled lysine; D. asparagine; E, isolevcine; F, methionine; G, serine; H, alanine; I, glycine; J and K, unknown impurities; L, dilabelled cysteine; M, glutamic acid; N, aspartic acid; O, cysteic acid. The concentration of each derivative is approximately 5×10^{-4} M, dissolved in operating buffer (Guzman, 1993).

The importance of capillary operation to reduce the Joule heating effects caused by the desirable high electric fields was emphasized (Jorgenson, 1981). This work is rightly credited with the initiation of high-performance capillary electrophoresis (HPCE).

The invention of micellar electrokinetic chromatography (MECC, or MEKC), which involved adding a surfactant to the electrophoretic buffer to form micelles to enhance resolution of neutral substances (Terabe *et al*, 1984), represents another significant step forward in the development of CE. Since then, various types of modifiers for the enhancement of selectivity in CE separation have been investigated.

The developments in gel-filled capillaries and coated columns have further enhanced the scope and efficiency of capillary electrophoretic techniques (Hjerten, 1983). Theoretical plate numbers in the multimillion ranges can now be routinely achieved using gel-filled capillaries in CE separations (Cohen and Karger, 1987).

At the end of the 1980s, commercial CE instruments became available. With the rapid advances currently being made, CE is now gaining popularity as an alternative analytical tool for some routine analytical applications.

1.2 Principles of separation in capillary electrophoresis (CE)

The mechanism of separation in electrophoresis is based on the migration of charged molecules in an applied field (potential or electric field). Most electrophoretic techniques involve simple variation of the environment of the molecule to either change the charge on the molecule (i.e. isoelectric focusing) or to physically retard the movement of the molecule in the electric field (i.e. polyacrylamide gel electrophoresis, PAGE). This gives an experimenter a tremendous variety of approaches to enhance the selectivity of the separation process. The utility of the technique is so useful that it can separate anything, ranging from inorganic ions to whole cells (Camilleri, 1993).

Separations are accomplished through the movement of ions in an applied electric field, This movement is governed by the *mobility* of an ion (μ), where this mobility is defined as the average velocity with which an ion moves under the influence of an applied potential field. The two primary factors that affect the mobility include, first the applied

electric force (F_{EF}), which depends on the charge of the ion (q) and the electric field strength (E; in V/cm), [Eq. (1.1)];

$$\overline{F}_{EF} = q\overline{E} \tag{1.1}$$

and secondly, the frictional force (F_{FR}) that a molecule encounters as it moves through solution [Eq. (1.2)]. The simplest estimation of the frictional force (which neglects the effect of other ions in solution, and merely assumes that the total retarding force is equal to the fictional drag determined by Stoke's Law) depends on a number of parameters, including the viscosity of the buffer (η) and the velocity (v; cm/s) and size of the molecule (related to the radius of a spherical molecule, r).

$$\overline{F}_{FR} = 6\pi\eta r v \tag{1.2}$$

At equilibrium, these two forces are directly balanced and the electrophoretic velocity [v; Eq. (1.3)] depends on a number of parameters involving both the analyte and the separation column.

$$\overline{v} = \frac{q\overline{E}}{6\pi\eta r} \tag{1.3}$$

More commonly, people are concerned with the electrophoretic mobility (μ), which is just the velocity normalized to the electric field strength [Eq. (1.4)].

$$\overline{\mu} = \frac{\overline{v}}{\overline{E}} = \frac{q}{6\pi\eta r} \tag{1.4}$$

The net effect of these balanced forces results in the following properties for the mobility of an ion:

1. Mobility is directly proportional to the charge of the ion
2. Mobility is inversely proportional to the viscosity of the solvent
3. Mobility is inversely proportional to the radius of particle (represented by the diffusion coefficient)

It is important to note that all of the parameters discussed here are vector quantities, not scalar numbers. This implies that each force or velocity has a directional component (either aligned with or against the electric field). This vector representation will be seen to be even more important when we consider the mobility found in capillary electrophoresis (Camilleri, 1993).

1.2.1 Electroosmotic flow (EOF)

One of the most distinguishing features of capillary electrophoresis is electroosmotic flow (EOF). This bulk movement of solvent is caused by the small zeta potential (ζ) at the silica/water interface, which induces a minute excess of anionic charge at the surface of silica (**Figure 1.3**).

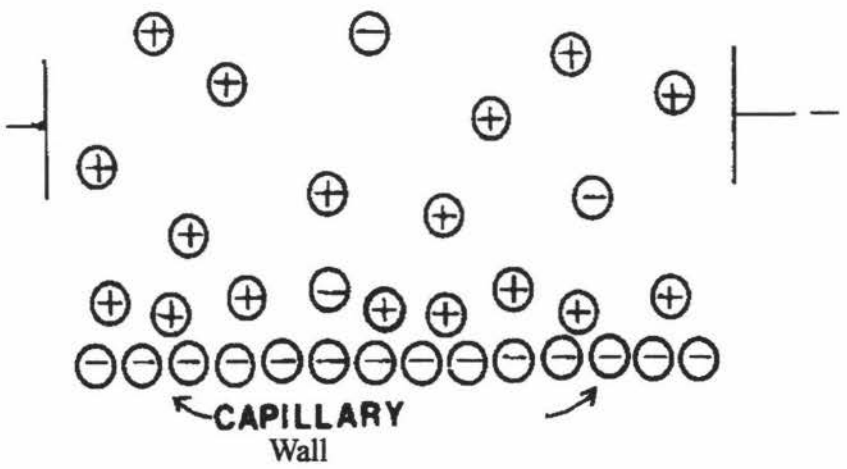
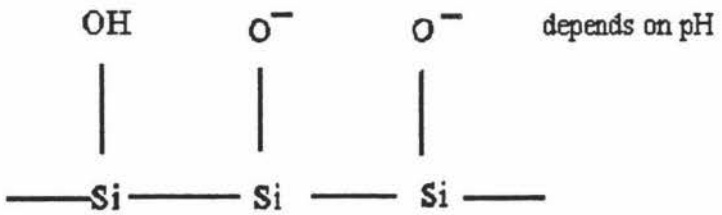


Figure 1.3 Schematic representation of ions at a silica-solution interface (Camilleri, 1993).

This charge, presumably due to the presence of dissociated -Si-OH groups on the surface of the capillary, is dependent on the pH of the buffer in the static diffuse double layer.



Titration of the silanol groups, -Si-OH + NaOH ↔ -SiO^- + Na^+ +H_2O alters the degree of dissociation of the silanol (which has a pK_a of 6 to 7), thereby altering the ζ potential. This is directly related to the velocity of electroosmotic flow as shown is Eq. (1.5)

$$\overline{\mu}_{EO} = \left(\frac{\varepsilon_o}{4\pi\eta}\right) E\zeta \tag{1.5}$$

For the silica surface, an excess of cationic species in bulk solution migrates toward the cathode, producing a net flow from anode to cathode. This means that the driving force of the flow originates at the walls of the capillary, producing a plug-like flow that has a flat velocity distribution across the capillary diameter, deviating only within a few nanometers of the capillary surface. This flat flow profile and its effect on net mobility (i.e., elution of positive and negatively charged species in electrophoresis) and its use as a pump in packed-column capillary chromatography were originally demonstrated by Lukacs and Jorgenson (1985). The flow velocity in a capillary previously exposed to acidic conditions was consistently lower than the flow from capillary previously exposed to alkaline solutions. For binary buffer mixtures containing water and a protic or an aprotic dipolar solvent, the pH of the solution was found to influence the electroosmotic flow (Schwer and Kenndler, 1991).

1.2.2 Separation efficiency in capillary electrophoresis

The efficiency of any chromatographic column is a function of both the retention time and the peak width of a given analyte. This function is referred to as the number of theoretical plates, (N), a name derived from the plate model of chromatography and is defined as:

$$N = \frac{16(t_R)^2}{W^2} \tag{1.6}$$

Where t_R = retention time for analyte, W = peak width.

The maximum separation efficiency that can be obtained in the most general case is that in which an analyte is introduced at one end of a tube, separated along the length of the tube, and detected as it leaves the tube. The theoretical approach to this is quite simple. Because most of the terms associated with band broadening are absent, then the problem can be avoided simply by using smaller internal diameter (<100μm) capillaries so that Joule heating could be virtually eliminated. In fact, the only term that remains is longitudinal diffusion. The efficiency of the separation can be represented as a number of theoretical plates (N), identical in definition to that used in chromatography. Jorenson and Lukacs (1983) demonstrated that N depends only on the total electrophoretic mobility (μ_{TOTAL}, in $cm^2V^{-1}s^{-1}$), the applied voltage (V, in volts), and the diffusion coefficient of the molecule (D_o, in cm^2s^{-1}), as shown in Eq. (1.7)

$$N = \frac{\overline{\mu}_{TOTAL} V}{2D_o} \tag{1.7}$$

The flow rate gives an additional velocity component to the ions migration in the electric field, such that the total velocity (μ_{TOTAL}) of each molecule is the vector sum of the electrophoretic velocity (v) and the velocity imparted by electroosmotic flow [v_{EO}; Eq. (1.8)]:

$$\overline{v}_{TOTAL} = \overline{v} + \overline{v}_{EO} \tag{1.8}$$

Similarly, the total electrophoretic mobility is the vector sum of the electrophoretic mobility of the sample (μ) and the effective mobility due to electroosmotic flow (μ_{EO}), as shown in Eq. (1.9) and **Figure 1.4**

$$\overline{\mu}_{TOTAL} = \overline{\mu} + \overline{\mu}_{EO} \tag{1.9}$$

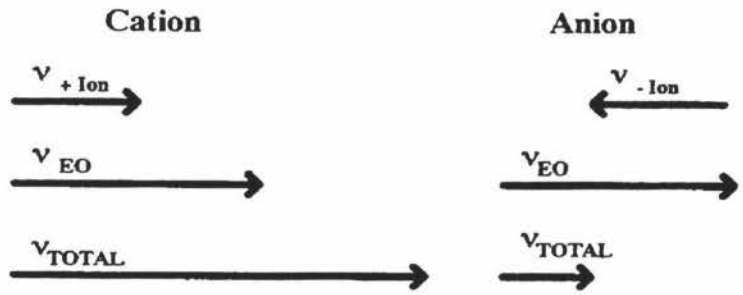


Figure 1.4 The total velocity of each molecule (Camilleri, 1993).

This can be used to great advantage in CE, because all molecules that have a net velocity toward the detector will eventually be observed. Thus, the presence of electroosmotic flow allows the separation of both negatively and positively charged species in the same run. This implies that the highest efficiency is obtained when species are migrating at the fastest velocity (i.e., have the largest value of (μ_{TOTAL}) and that we can use electroosmotic flow to help speed up the separation, and thereby increase the separation efficiency. The total number of theoretical plates is described by Eq. (1.10):

$$N = \frac{[(\overline{\mu} + \overline{\mu}_{EO})V]}{2D_o} \tag{1.10}$$

The Eq. (1.10) predicts theoretical values of the order of 10^6 for capillary electrophoresis. This compares with 5000 theoretical plates for HPLC (high

performance liquid chromatography) separations, and only 10 theoretical plats for conventional agarose gel electrophoresis (Jenkins, 1995)

.

The time that is required for the migration of a charged species in a separation capillary is simply the length of the capillary (L) divided by the linear velocity of the molecule [v_{TOTAL}; Eq. (1.11)]. If we use the electrophoretic mobility (μ) instead of linear velocity in this calculation, the elution time is minimized with a high mobility.

$$t = \frac{L}{v_{TOTAL}} = \frac{L}{\mu_{TOTAL} V} \tag{1.11}$$

The resolution, R_s, between two compounds depends not only on the distance between the peak maxima, t_{R2}-t_{R1}, where t = time, but also on the narrowness of the peaks, i.e. the average of the inverse of the peak widths. Resolution is defined as:

$$R_s = t_{R2}\text{-}t_{R1}/1/2(W_1+W_2) \tag{1.12}$$

Resolution can be increased by increasing the length of the column.

1.3 Instrumentation

Capillary electrophoresis involves the separation of charged molecules in a buffer-filled capillary by the application of very high voltages (1 to 30 kV). The capillary, which may be either coated or fused silica, usually varies between 20 and 100μm in diameter and between 25 and 122cm in length. The ends of the capillary are placed in buffer vials, which also contain the electrodes. The narrow diameter of the capillary is important to permit rapid heat dissipation and to decrease analyte diffusion. A schematic representation of a capillary electrophoresis instrument is shown in **Figure 1.5**

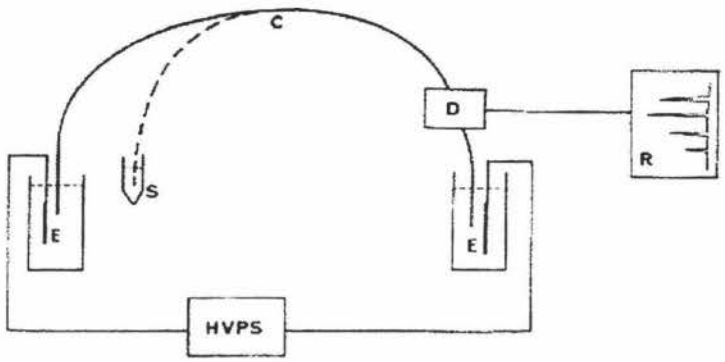


Figure 1.5 Basic scheme of the CE instrument. The separation capillary (C) is connected to the high-voltage power supply (HVPS) by electrolyte reservoirs equipped with electrode (E). The sample (S) is injected either by flow or by electromigration, and separated zones are detected on the opposite end of the capillary by a suitable detector (D). The detection signal is either recorded by a line recorder (R), or can be further evaluated by a computer (Weinberger, 1993).

Capillary columns, as indicated above, come in a wide variety of internal diameters. A polyimide outer covering makes the capillary mechanically stronger and protects the capillary from sudden angulation and breaking. Detection in CE commonly involves variable wavelength or filter UV photometry, or diode array systems. Laser induced fluorescence and mass spectrometer are available for some applications. At the detection "window" the polyimide coating of the capillary is "burnt off" to allow the light source to penetrate the capillary and for absorbency measurements to be made (Jenkins, 1995).

1.4 Detection

Capillary electrophoresis is a powerful separation technique that requires high-sensitivity detection for efficient performance. A wide range of detection techniques has been employed in CE separations. The list includes UV-absorbance, fluorescence, laser-based thermo-optical, refractive index, electrochemical, radioisotope, and mass spectrometric. Currently, the most commonly used detection systems for CE are based on UV and UV-Vis absorbance. All the standard commercial instruments are equipped with an UV detector (Li, 1992). These are similar to HPLC detectors and typically use deuterium sources with filters or a monochrometer to select the detection wavelength. Source light is partitioned via a beam splitter between a reference photodiode and the

microfocusing optics of the capillary cartridge. Scanning detectors enable spectral information to be acquired as a peak moves through the detection point. Peak spectra can be used to identify the analyte or to determine if co-migrating impurities are present in the peak. Scanning detection can be accomplished by illuminating the capillary with full-spectrum white light and dispersing the transmitted light via a polychrometer onto a photodiode array ("reverse optics" PDA detector). Alternatively, a conventional "forward optics" approach can be used, illuminating the capillary with a narrow spectral band isolated by a monochrometer placed between the light source and the capillary. Scanning is achieved by rapid computer-controlled movement of the monochrometer to sample a wide spectral range in milliseconds. This "fast-scanning" detector design produces much lower noise and, therefore, much higher sensitivity than a PDA detector (BioRad).

1.5 CE Reagents

A variety of buffers and additives can be used to achieve or enhance separations. The majority of separations use typical nontoxic biochemical buffers such as phosphate (pH 1.1 to 3.1), acetate (pH 3.8 to 5.8) borate (pH 8.1 to 10.1), and zwitterionic buffers, such as MES [2-(N-morpholino)ethane sulfonic acid] (pH 5.5 to 6.7) and Tris [(tris (hydroxymethyl-aminoethane)] (pH 7.3 to 9.3). In contrast to HPLC, capillary electrophoresis rarely requires toxic or flammable organic solvents such as acetonitrile, hexane, methanol, or tetrahydrofuran. Even when such solvents are used, only milliliter quantities are needed for many runs. The very small volumes of reagents required for CE analyses greatly minimizes the problems associated with solvent disposal and raises the possibility of using exotic solvents and additives which would be prohibitively expensive for conventional-scale separation techniques.

1.6 Different modes of CE

Different modes of capillary electrophoretic separations can be performed using a standard CE instrument. The origins of the different modes of separation may be attributed to the fact that capillary electrophoresis has developed from a combination of many electrophoretic and chromatographic techniques. In general terms, it can be

considered as the electrophoretic separation of a number of substances inside a narrow tube.

The distinct capillary electroseparation methods include: **(Figure 1.6)**

Capillary zone electrophoresis (CZE)
Capillary gel electrophoresis (CGE)
Micellar electrokinetic capillary chromatography (MEKC or MECC)
Capillary electrochromatography (CEC)
Capillary isoelectric focusing (CIEF)
Capillary isotachophoresis (CITP)

1.6.1. Capillary zone electrophoresis (CZE)

The separation in CZE is based on the differences in the electrophoretic mobilities resulting in different velocities of migration of ionic species in the electrophoretic buffer contained in the capillary. The separation mechanism is mainly based on differences in solute size and charge at a given pH. Most capillaries used for CE today are made of fused silica, which contains surface silanol groups. These silanol groups may become ionized in the presence of the electrophoretic medium. The interface between the fused silica tube wall and the electrophoretic buffer consists of three layers: the negatively charged silica surface (at pH > 2), the immobile layer, and the diffuse layer of cations (and their sphere of hydration) adjacent to the surface of the silica that tend to migrate towards the cathode. This migration of cations results in a concomitant migration of fluids through the capillary. This flow of liquid through the capillary is called electroosmotic (or electroendosmotic) flow.

The electroosmotic flow in uncoated fused silica capillaries is usually significant with most commonly used buffers. In many cases, it is also significantly greater than the electrophoretic mobility of the individual ions in the injected sample. Consequently, both anions and cations can be separated in the same run. Cations are attracted towards the cathode and their speed is augmented by the electroosmotic flow.

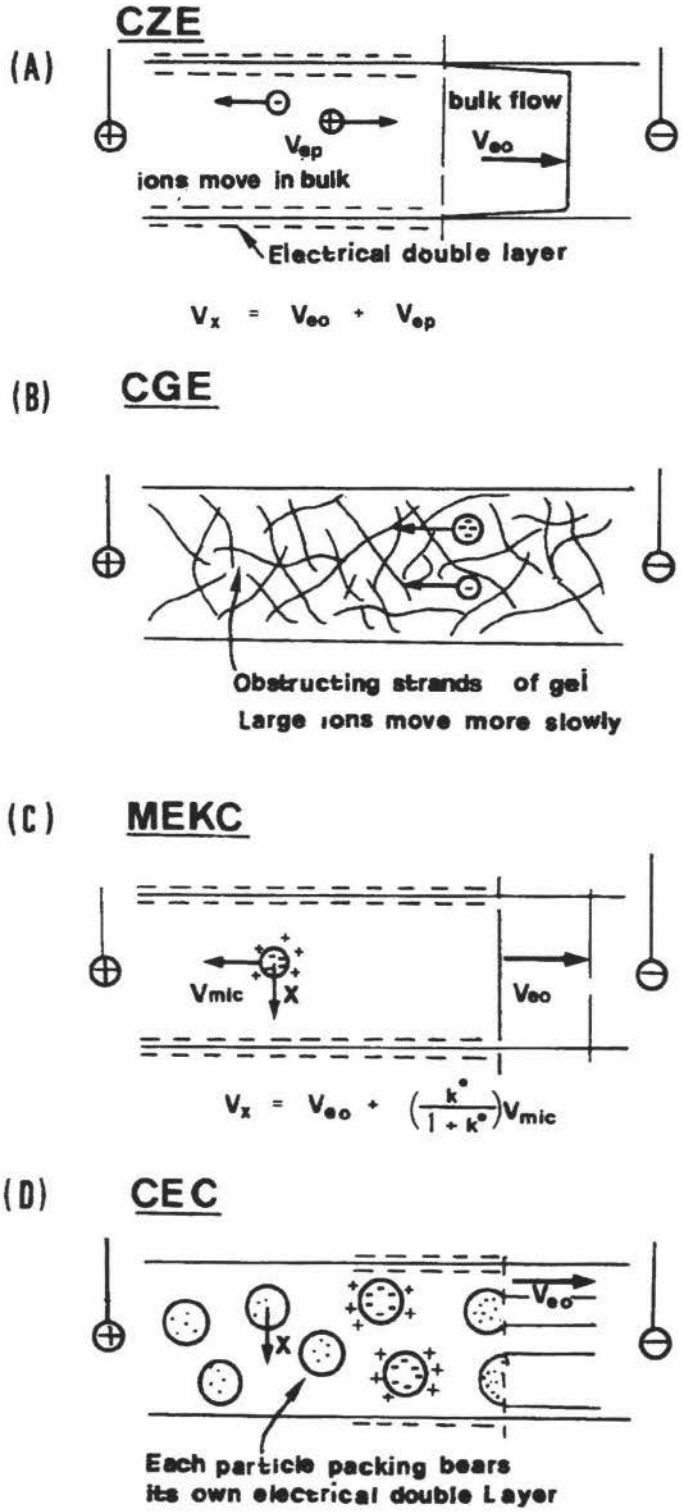


Figure 1.6 Diagrammatic representation of (A) capillary zone electrophoresis (CZE), (B) capillary gel electrophoresis (CGE), (C) micellar electrokinetic capillary chromatography (MEKC or MECC), and (D) capillary electrochromatography (CEC). V_x is the linear migration velocity of the analyte X. V_{eo} is the electroosmotic velocity, Vep is the electrophoretic velocity and k' is the phase capacity ratio (Li, 1992).

Anions, although electrophoretically attracted toward the anode, are swept towards the cathode with the bulk flow of the electrophoretic medium. Under these conditions, cations with the highest charge/mass ratio migrate first, followed by cations with reduced ratios. Then the unresolved neutral components migrate as their charge/mass ratio is zero. Finally, the anions migrate. Anions with lower charge/mass ration migrate earlier then those with greater charge/mass ratio. The anions with the greatest electrophoretic mobilities migrate last. One important point to note is that it is possible to change the charge/mass ratio of many ions by adjusting the pH of the buffer medium to affect their ionization and hence electrophoretic mobility.

CZE was used exclusively in this study; the other methods are briefly described below for completeness.

1.6.2 Capillary gel electrophoresis (CGE)

The main separation mechanism in capillary gel electrophoresis (CGE) is based on differences in solute size as analytes migrate through the pores of the gel-filled column. Gels are potentially useful for electrophoretic separations mainly because they permit separation based on "molecular sieving". Furthermore, they serve as anti-convective media, they minimize solute diffusion, which contributes to zone broadening, they prevent solute adsorption to the capillary walls and they help to eliminate electroosmosis. However, the technique is subject to the limitation that neutral molecules would not migrate through the gel, since electroosmotic flow is suppressed in this mode of operation.

1.6.3 Micellar electrokinetic capillary chromatography (MEKC)

The main separation mechanism of micellar electrokinetic capillary chromatography (MEKC) is based on solute partitioning between the micellar phase and the solution phase. The technique provides a way to resolve neutral molecules as well as charged molecules by CE.

Micelles form in solution when surfactant is added to water in concentration above its critical micelle concentration (cmc). Micelles consist of aggregation of surfactant

molecules with typical lifetimes of less than 10μs. MECK is most commonly performed with anionic surfactants, especially sodium dodecyl sulfate (SDS). In the case of anionic surfactants, the hydrophobic tail groups tend to be orientated toward the center and the charged head groups along the surface of the micelle. The MEKC system contains two phases: an aqueous phase and a micellar phase. The surfaces of SDS micelles have a large net negative charge. The micelles therefore exhibit a large electrophoretic mobility (μ_{ep}) toward the anode, which is in the opposite direction to the electroosmotic mobility (μ_{eo}) towards the cathode present in most commonly used buffer systems in CE. The magnitude of μ_{eo} is slightly greater than that of μ_{ep}, resulting in a fast-moving aqueous phase and a slow-moving micellar phase. Solutes can partition between the two phases, resulting in retention based on differential solubilizaton by the micelles. Consequently, the MEKC technique provides a means of obtaining selective separations of neutral and ionic compounds while retaining the advantages of the capillary electrophoresis.

1.6.4 Capillary electrochromatography (CEC)

In capillary electrochromatography (CEC), the capillary is packed with a chromatographic packing which can retain solutes by the normal distribution equilibria upon which chromatography depends and is therefore an exceptional case of electrophoresis. In CEC the liquid is in contact with the silica wall, as well as the particle surfaces. Consequently, electroosmosis occurs in a similar way as in an open tube due to the presence of the fixed charges on the various surfaces. Whereas in an unpacked tube the flow is strictly plug flow, and there is no variation of flow velocity across the section of the column, the flow in a packed bed is less perfect because of the tortuous nature of the channels. Nevertheless, it approximates closely to plug flow and is substantially more uniform than a pressure-driven system. Therefore, the same column ends to provide higher efficiency when used in electrochromatography than when used in pressure-driven separations.

1.6.5 Capillary isoelectric focusing (CIEF)

Another separation method, which can be conveniently performed using a capillary electrophoresis instrument, is isoelectric focusing. Here polyionic substances (e.g. proteins) are separated on the basis of their isoelectric points or pI values. In this technique, the protein samples and a solution that forms a pH gradient are placed inside a capillary. The anodic end of the capillary is placed into an acidic solution (anolyte), and the cathodic end in a basic solution (catholyte). Under the influence of an applied electric field, charged proteins migrate through the medium until they reside in a region of pH where they become electrically neutral and therefore stop migrating. Consequently, zones are focused until a steady state condition is reached. After focusing, the zones can be migrated from the capillary by a pressurized flow, e.g. simply by lifting the height of one end of the capillary and permitting the sample to flow through the detection cell.

1.6.6 Capillary isotachophoresis (CITP)

The main feature of CITP is that it is performed in a discontinuous buffer system. Sample components condense between leading and terminating constituents, producing a steady-state migrating configuration composed of consecutive sample zones.

1.7 Capillary electrophoresis as a clinical tool

Basic knowledge in immunology, biochemistry, physiology, and pharmacology related to normal physiological activities and human diseases has expanded greatly in the past two decades. Consequently, advancements in basic scientific developments are a necessity for the analysis of biochemical substances present in biological fluids, blood cells, and tissue specimens.

Much information about a disease state has been gained by measuring a certain number of biochemical substances that are used as indicators of the disease activity, as well as providing information about the intensity of the whole pathological process. Therefore, the clinical chemistry laboratory is an important contributor to the medical team

involved in the diagnosis and treatment of disease, and physicians rely heavily on laboratory test results before making decisions (Guzman, 1993)

Clinical chemistry has been defined as "the study of the chemical aspects of human life in health and illness and the application of chemical laboratory methods to diagnosis, control of treatment, and prevention of disease" (Guzman, 1993). Thus, clinical chemistry is a fundamental science when it seeks to understand the physiological and biochemical processes operating both in the normal state and in disease. It is an applied science when analyses are performed on body fluids, or tissue specimens to provide useful information for the diagnosis or treatment of disorders.

Capillary electrophoresis (CE) is a relatively young branch of separation science. Many observations of the potential importance of capillary electrophoresis were made by analytical chemists during the last decade. Comparative studies have been carried out to learn more about the potential of high-performance capillary electrophoresis (HPCE) in the clinical laboratory.

In the modern medical environment, patient diagnosis and treatment relies heavily on investigation procedures. With the introduction of productivity-based funding of laboratories, they are now required to provide rapid, cost-effective tests. This is often best achieved by the use of automated methods.

Capillary electrophoresis (CE) involves a separation of charged molecules in a buffer-filled capillary by the application of a very high voltage. The outstanding characteristic of CE is the high separation efficiency, which can be achieved: 10^6 theoretical plates are able to be attained, at an applied voltage of 30kV. This allows an analysis time in the order of minutes (Jenkins and Guerrilla, 1996).

With commercially available CE instrumentation, automation of the assays is now available. Some instruments provide up to 50 position sample carousels, thus allowing sufficient space for a full overnight run. (Jenkins and Guerrilla, 1996).

It is now possible to determine nanoliter to picoliter quantities of material and to reach detection sensitivity levels of less than 3 zeptomoles (1 zeptomole = 10^{-21} mol = 600

molecules), and uses only nanolitres of buffer. The main consumable costs relate to the capillaries and sample cups. The automation of the system allows for considerably lower labour costs compared with other electrophoretic methods, including agarose gels.

The analytical capabilities of CE allow clinical analysts to consider automation of tests previously performed only on a manual basis. These include serum and urine protein electrophoresis, hemoglobin electrophoresis and vitamin studies. Assays for several newer drugs by CE could have significant advantages over the sometimes-difficult HPLC techniques currently used.

1.8 Summary

Capillary electrophoresis employs the separation mechanisms of conventional electrophoresis in a capillary format. Basic CE offers the ease and speed of HPLC while eliminating the problems of HPLC associated with column packing, toxic solvents and their disposal, and provides quantitative analysis and automation. Separated components are identified by on-line detection during the analysis, so, in contrast to slab gel electrophoresis, time-consuming staining and destaining steps after the separation are not required. Results are obtained in a matter of minutes, while conventional electrophoresis typically requires hours to days before results are available. (BioRad)

Rapid micro-volume analysis is one major advantage of CE over HPLC. Since only nanoliter of sample are consumed in each analysis, literally hundreds of CE runs can be performed from the microliter sample volumes typically consumed for a single HPLC injection.

The simplicity and separative power of capillary electrophoresis is another additional key advantage of the technique in contrast to HPLC. As CE separation technology evolved it become apparent that tremendous flexibility in separation selectivity can be achieved by simple changes in the composition of the electrophoresis buffer. This enables a single capillary to be used to obtain separations based on a wide range of molecular properties (e.g. size, charge, chirality, hydrophobicity) which would require thousands of dollars invested in HPLC columns. Separation methods can be quickly

developed and optimized by rapid scouting experiments with simple buffer changes, shortening method development time from a matter of weeks or months to a few hours or days.

The attributes which have made capillary electrophoresis such a successful tool in basic research are identical to those attracting clinical laboratories: speed (more efficient, less labor-intensive), low cost (minimal buffer consumption), small sample volume (reduced blood collection volume from patient), increased selectivity (determination of multiple solutes in one run), and versatility (detection of analytes over the wide range of molecular masses and chemical composition).