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Review

Capillary Electrophoresis in Pharmaceutical Analysis

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Capillary electrophoresis (CE) is a separation technique particularly suited to the analysis of pharmaceutical compounds. This review offers a detailed discussion of the four common modes of detection coupled to CE—UV absorption, fluorescence, electrochemical, and mass spectrometry—and gives examples of the use of these methods in pharmaceutical analyses. Sample preparation and pretreatment techniques used for CE separations are described, as well as methods of preconcentration including hydrophobic retention, affinity concentration, sample stacking, and isotachophoresis. The use of affinity CE, chiral CE, and capillary gel electrophoresis for analysis of pharmaceuticals is covered in detail, and recent advances in capillary electrochromatography and CE on a chip are also discussed.

KEY WORDS: capillary electrophoresis; pharmaceutical analysis; detection; metabolites; pretreatment; preconcentration; chiral separations.

INTRODUCTION

Capillary electrophoresis (CE) is a powerful separation technique that is well suited to the analysis of small molecules, especially pharmaceutical compounds. Traditionally, pharmaceutical compounds have been separated by liquid chromatography (LC); however, CE has several advantages over conventional LC for some analyses. CE separations are generally more efficient, can be performed on a faster time scale, require only nanoliter injection volumes, and in most cases take place under aqueous conditions. These four characteristics of CE have proven to be advantageous for many pharmaceutical applications.

The purpose of this paper is to report general information concerning CE separations of pharmaceutical compounds. Previously, a review of applications of CE to pharmaceutical analysis was published in this journal (1). The current review is a continuation of the first and covers literature published from 1993 to 1996. In order to clarify the scope of this article, it is useful first to define what we mean by pharmaceutical analysis. CE separations of compounds that are marketed as drugs, are drug candidates, or are metabolites of drugs or drug candidates published during the above time frame are included in this report. In addition, CE separations employed in the study of mechanistic effects of drugs upon administration are described. Lastly, separations employed for drug screening or drug discovery work are also included.

Five classes of compounds investigated extensively by CE include amino acids, peptides, proteins, DNA, and RNA. There is a large volume of literature regarding CE separations of these five types of analytes, and this is beyond the scope of this review. In some cases, however, these compounds do qualify as pharmaceutical compounds and, in these cases, work regarding amino acids, peptides, proteins, DNA, or RNA separations will be reported.

The first half of this review covers general information regarding CE, including a description of the separation, the common modes of injection, and general information on detection systems that can be coupled to CE. The remainder covers practical aspects of pharmaceutical analyses by CE, which includes sample preparation methods and ways to decrease detection limits for samples that are concentration-limited. The use of different modes of CE for increased selectivity will also be presented.

GENERAL INFORMATION

Description of Separation Mechanism

In capillary electrophoresis, separation is driven by two factors. The first is the movement of the analyte in the capillary due to the electric field, also called electrophoretic velocity. The second is the bulk flow of solution due to the surface charge on the capillary wall, also called electroosmotic flow.

The electrophoretic velocity itself results from the equilibrium of both frictional force and electrical force. The movement of a charged analyte through a conductive solution toward or away from an electrode is dependent upon the charge of the analyte and the magnitude of the applied electric field. While the movement of the analyte through the capillary is controlled by an electrical field, the frictional forces also affect the mobility of the analyte through the capillary. Thus, a larger frictional force will impede the movement induced by the electrical field to a greater extent. This impediment of motion is dependent on the viscosity of the electrolyte and the radius of the analyte. Therefore, the final electrophoretic mobility is determined by

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a combination of electrical and frictional force contributions. This means that mobility and, hence, separation is based on the size-to-charge ratio of the analytes.

Electroosmotic flow is a second factor that affects the movement of analyte through the capillary. Surface charge on the interior of the capillary induces the formation of a double layer upon application of the electric field. In a capillary composed of fused silica, the surface possesses a negative charge over the pH range of most CE separations (approximately pH 4–12). This negatively charged surface attracts positively charged electrolytes from the bulk solution and, under an electric field, these hydrated cations migrate toward the cathode. This migration of electrolyte through the capillary leads to a bulk flow through the capillary, which occurs as a plug rather than the laminar flow that usually arises in open tubes. This bulk flow, termed electroosmotic flow, affects all analytes, regardless of charge, thus making CE advantageous for the simultaneous detection of anionic, neutral, and cationic analytes, which all migrate toward the cathode.

In an ideal CE separation, zone broadening of analyte peaks is attributed solely to molecular diffusion. This is in contrast to LC separations, where zone broadening even in an ideal case is attributed to three factors—multiple flow paths, longitudinal diffusion, and the rate of mass transfer. While CE should ideally yield efficient separations, some factors will increase zone broadening. The two main factors are sample adsorption to the capillary surface and the introduction of laminar flow through Joule heating.

A schematic diagram of a typical CE system is shown in Fig. 1. Each end of a small diameter fused silica capillary (ranging from 15–100 μm i.d.) is held in a buffer vial containing a background electrolyte. In most configurations, the system is biased so that the buffer vial into which the injection end of the capillary is inserted is maintained at positive high voltage and the buffer vial at the detection end of the capillary is maintained at ground. Alternatively for some applications, the injection end of the capillary is held at ground and the detection end of the capillary at negative high voltage. Typically, the injection end of the capillary is shielded in an insulated box equipped with an interlock switch that disables the high voltage whenever the box is opened. This ensures that the laboratory user is protected from the high voltage utilized by the system.

In conventional CE (using bare silica for the capillary and no modifiers), the injection end of the capillary is the anode and the detection end of the capillary is the cathode. This being the case, cationic analytes will migrate out of the capillary first, followed by neutral and then anionic analytes. This order of migration is, as stated previously, due to the effects of both

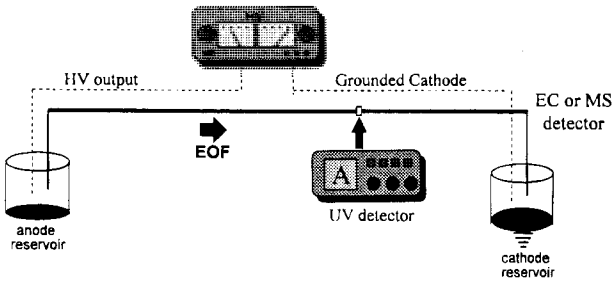

Fig. 1. Diagram of capillary electrophoresis setup.

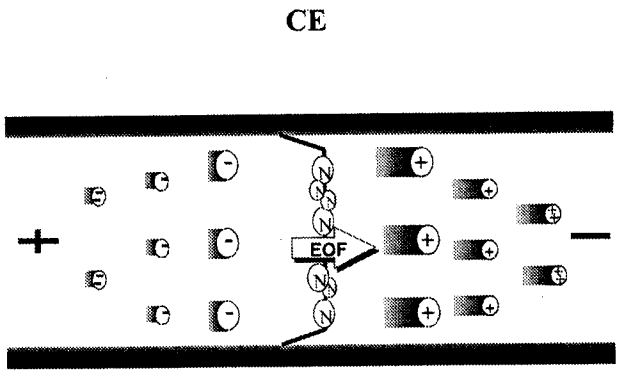

Fig. 2. Separation process in capillary electrophoresis.

electrophoretic velocity and electroosmotic flow. Cations move toward the cathode by the positive effects of both of these components, neutral compounds move toward the cathode by the effect of electroosmotic flow only; anions move according to the difference in the magnitude of electroosmotic flow directed toward the cathode and the electrophoretic flow directed toward the anode. This is illustrated in Fig. 2. It is important to note here that the polarities of the buffer vials can be switched such that the cathode is positioned at the injection end of the capillary and the anode is positioned at the detection end of the capillary. The order of elution then changes depending on whether the electroosmotic flow is suppressed or reversed.

Injection

Routine injections on a CE system are made either hydrodynamically or electrokinetically. There are three ways to make hydrodynamic injections into a CE capillary. The first of these involves replacing the buffer vial with a vial containing the sample and applying pressure to the sample vial to force a small aliquot of sample into the capillary. After the sample is forced into the capillary, the sample vial is returned to its original position and replaced with the buffer vial and the separation is started. The second method is similar to the first except that, rather than applying pressure at the injection end of the capillary, a vacuum is applied at the detection end of the capillary. The third procedure, called gravity injection, employs a siphoning effect. That is, the buffer vial is replaced with a sample vial, which is positioned at a height greater than that of the vial at the detection end of the capillary. After the sample aliquot is forced onto the capillary, the sample vial is replaced with the buffer vial, which is returned to its original position and the separation is initiated. Electrokinetic injections are performed by replacing the buffer vial with a sample vial and briefly applying a voltage across the capillary. In conventional CE (Fig. 1), this voltage induces preferential injection of cations, then neutral compounds and, finally, anions onto the capillary. After application of this voltage, the sample vial is replaced with the buffer vial, and the separation is begun.

Detection

Four commonly used modes for quantifying analytes following CE separation are ultraviolet, fluorescence, electrochemical, and mass spectrometric detection. Given the small internal diameter of a capillary and the low flow rate of the

Table 1. Typical Ranges of Detection Limits in Cited References for Different Methods of Detection

Method of detection	Typical range of detection limit in cited references	Notes on pharmaceutical applications
UV absorption	10^{-6}–10^{-14} moles	not sensitive not selective direct and indirect
Laser-induced fluorescence	10^{-14}–10^{-18} moles	sensitive selective native detection or precolumn derivatization
Electrochemical	10^{-14}–10^{-18} moles	sensitive selective decoupler often required amperometry pulsed amperometry conductivity/ potentiometry
Mass spectrometry	10^{-12}–10^{-16} moles	not sensitive structural determination metabolic studies

solution of background electrolyte through the CE capillary, each of these detection techniques must be applied appropriately for successful quantitation. Typical ranges of detection limits for each method are listed in Table 1. A brief discussion of advantages and drawbacks of these four methods of detection follows.

Capillary Electrophoresis with UV Detection (CE-UV)

Because of its compatibility with CE, UV is still the most commonly used form of detection. The concentration limit of detection (LOD) range is usually 10^{-5}–10^{-6} M, depending on the molar absorptivity of the compound. It can be used to monitor absorbance at a single wavelength or at multiple wavelengths with a diode array detector (2). A comprehensive review of applications of CE-UV can be found elsewhere (3).

UV detection for CE is usually carried out using the capillary itself as the flow cell. This is possible because the fused silica capillaries used for the CE separation are optically transparent. A detection window is fabricated by burning off 0.5–2.0 cm of the protective polyimide coating with a flame, hot filament, or hot concentrated sulfuric acid. The use of an on-capillary detection window prevents the extra-column effects which, if a separate flow cell were used, would decrease the separation efficiency. However, a disadvantage to such an on-capillary window is that the optical pathlength is limited to the i.d. of the capillary (usually 50 or 75 μm). To increase the pathlength, some manufacturers offer bubble-shaped flow cells that are part of the fused silica capillary (4), leading to an approximately threefold increase in sensitivity. Another innovation is the Z-shaped flow cell, which also increases the pathlength and leads to an approximately tenfold increase in sensitivity over conventional CE-UV (5).

Several direct comparisons of CE-UV and LC-UV have been made. When both were evaluated for the determination of theophylline in serum and plasma, CE was found to be as reproducible as LC, but about fourfold less sensitive (6). CE and LC were also compared for the determination of felbamate in serum using UV detection (7). The linearity and precision of the two methods were similar, but the run time for the CE analysis was half that required for LC. Shihabi *et al.* used CE for the determination of xanthine in several biological fluids (8). They also found that CE-UV yielded much shorter analysis times than had been previously reported for LC-UV.

In another study, ion-pair chromatography with diode-array UV detection was compared with micellar electrokinetic chromatography (MEKC) with UV detection for the determination of several beta-adrenergic blocking agents (9). The superior efficiency of CE permitted simultaneous separation of a greater number of beta blockers. The main disadvantage of the CE-UV method was its lower sensitivity. The LODs were 100–670 ng/mL for the ion-pair chromatography method and only 1–50 μg/mL for CE-UV.

Indirect detection can be utilized in CE just as in LC with similar advantages (10,11). It provides a highly non-selective detection mode for CE, which is useful for the determination of species lacking a UV chromophore or in those cases where widely varying UV molar extinction coefficients of different compounds hinder quantitative analysis (11). Due to the high background signal with indirect detection, the LODs obtained with this approach are at least an order of magnitude worse than those achieved using conventional UV detection. In indirect UV detection, a UV-absorbing species such as phthalic acid, benzoic acid, chromate salt, quinine or anisate is added to the background electrolyte (10–15). The displacement of this chromophore by the analyte gives rise to a negative response, which is measured photometrically. The optimal properties for a UV modifier are 1) it should not absorb light at the same wavelength as the solutes, 2) it has a high molar extinction coefficient, and 3) it has an effective mobility similar to that of the analyte of interest to prevent peak asymmetry (10). Indirect detection has proven useful for CE determinations of drug compounds and spermine (13), spermidine (13), fosfomycin (14), and various ions (15) in serum.

Capillary Electrophoresis with Fluorescence Detection

Fluorescence detection in conjunction with CE is generally more sensitive than UV detection. With fluorescence detection, the intensity of the emitted fluorescent light is related to the

intensity of the incident light. This being the case, the detection limit of a particular system can be improved by increasing the intensity of the light used for excitation. In pharmaceutical analyses, most CE applications utilizing fluorescence detection employ laser sources (16–31) as these provide incident light that is monochromatic and easily focused. Visible and UV laser sources are available, although those in the UV region are quite costly. Lamp sources can be utilized for fluorescence detection in CE for the determination of pharmaceutical compounds; however, the detection limits are not as low as those of laser sources due to focusing problems.

Fluorescence detection is highly sensitive and also very selective. There are three approaches to fluorescence detection coupled with CE; these involve measurement of indirect fluorescence, native fluorescence, and fluorescence of derivatized compounds. During the time frame covered by this review, no applications of indirect fluorescence to pharmaceutical analyses have been reported. However, a publication on the determination of endogenous compounds in single erythrocytes by indirect fluorescence has appeared (32).

Fluorescence detection of compounds that possess native fluorescence, while highly useful, is limited by the number of compounds that will fluoresce at wavelengths obtainable with inexpensive lasers. Compounds that have been detected by native fluorescence include zolpidem (16), zopiclone (17), naproxen (18), heroin impurities (19), and other drug compounds (16,18,20–23).

Most reports of the use of capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) for the detection of pharmaceutical compounds involve derivatization of the compounds of interest prior to analysis (24–27). Derivatization for fluorescence detection can be performed before or after the separation. Each method has distinct advantages. With precolumn derivatization, the tagging reaction may be more complicated. The tag itself can be fluorescent since excess reagent will simply be separated from the tagged derivatives, and detection limits are often better due to lower background. In addition, because the reaction chemistry occurs prior to the separation, no band broadening results from the tagging protocol. However, because the fluorescent tag is usually large relative to the analyte of interest and is often charged as well, tagged analytes may appear more similar in size and charge than untagged analytes. This makes separation more difficult. The high efficiency of CE will often solve this problem and as a result, most of the CE separations of pharmaceutical compounds utilizing fluorescence detection have employed precolumn derivatization.

The advantages of postcolumn derivatization are that the separation is based solely on the properties of the analytes of interest and not the fluorescent tag, and that a working postcolumn system is easier to operate because the tagging chemistry is performed on-line. One disadvantage of postcolumn systems is that the tagging reagent must not be intrinsically fluorescent or there will be a large background interference due to the tag. A second disadvantage of postcolumn derivatization techniques for CE is that the analytes separated by the applied voltage are diluted during mixing with the derivatization reagents, which also results in higher limits of detection. Lastly, the derivatization reaction must be fast, and the reaction products must be stable long enough to reach the detector. Furthermore, postcolumn systems are more difficult to develop because they are more complicated to assemble.

Capillary Electrophoresis with Electrochemical Detection (CE-EC)

The coupling of electrochemical detection with CE was first reported by Wallingford and Ewing in 1987 (33). Electrochemical detection is a selective detection method that can be very sensitive. Detection limits for electroactive analytes are typically in the low nanomolar range. Through voltammetric characterization, one can also obtain information that can help to identify the analyte in a manner similar to diode array detection or wavelength ratioing. This can be accomplished by either scanning voltammetric or dual electrode amperometric detection (34).

Electrochemical detection is especially suited to microcolumn separations because it is based on a reaction at an electrode surface, and the LOD often decreases as the detector size is reduced. This is in direct contrast to photometric methods of detection with CE. The selectivity of electrochemical detection arises from the limited number of compounds easily oxidized or reduced at the electrode surface; this selectivity is tunable based on the potential employed. For a more in-depth review of this technique, the reader is directed to a recent paper by Ewing *et al.* (35). In addition, pharmaceutical and biomedical applications of CE-EC have been addressed by Lunte and O'Shea (36).

Four modes of electrochemical detection that are commonly encountered include amperometric (33–38), pulsed amperometric (39,40), and potentiometric and conductimetric (41). Amperometric detection is the most common and most sensitive method. In this case, the analyte is oxidized or reduced at an electrode surface at which a constant oxidizing or reducing potential is applied. The loss or gain of electrons results in a current response at the electrode, which is proportional to the concentration of the analyte.

In potentiometric and conductimetric detection modes, the potential or conductivity between a pair of electrodes is measured as the analytical response. Lithium ion in human serum has been detected using CE with conductivity detection (38). The total sample volume requirement for this measurement was less than 20 μL with an analysis time under 4 min. The detection limit was 10^{-7} M.

Pulsed amperometric detection (PAD) is a popular mode of detection for the determination of carbohydrates, thioethers and sulfhydryl compounds. Under normal amperometric detection conditions, these analytes would foul the electrode surface, making their detection following CE separation impossible (39,40). In PAD, a three-step waveform is applied, consisting of detection, oxidative cleaning and then reactivation. The oxidative cleaning step generally takes place at a much higher applied potential than the detection, and the reactivation is usually carried out by the application of a large negative potential.

The key instrumental task in the implementation of electrochemical detection with CE is the isolation of the microamperes of separation current from the analytical signal, which, in the case of amperometric detection, is usually on the order of nanoamperes or even picoamperes. This is achieved by grounding the capillary a few centimeters in front of the working electrode, which is at or in the end of the capillary. The grounding device, or decoupler, functions by grounding the separation current through a small fracture or semipermeable membrane. Various decoupler configurations have been reported. These

include conductive polymer joints that cover a small fracture in the capillary wall or are placed at the end of the capillary (41,42), porous glass fracture joints (33), and bare fracture joints (43). CE-EC can be carried out in some cases without a decoupler, but only if capillaries with unusually small i.d. (<25 μm) are used (44). This configuration is termed end-column detection.

There are many electrode configurations that can be used with CE-EC; only a very brief summary will be given here. In general, a working electrode 25–50 μm in diameter is part of a three-electrode cell, which also incorporates a reference and an auxiliary electrode. Most of the reports in the literature use *in-capillary detection*, as defined by Zhong and Lunte (45), where the working electrode is inserted into the end of the capillary. This is usually achieved using a micromanipulator and a microscope. The electrode can also be placed at the end of the capillary (off-capillary detection) in a wall jet configuration. Lastly, the electrode can be permanently affixed to the capillary. Alignment of the microelectrode into the end of the capillary is a key issue and challenge for the general acceptance of CE-EC (46,47). An integrated *on-capillary* electrode (45) obviates this need for electrode alignment.

To expand the applicability of CE-EC, several different types of electrodes have been investigated. The use of dual electrodes in conjunction with CE allow the simultaneous detection of species at two different potentials or selective detection of chemically reversible species such as catechols or disulfides. For example, a dual gold/mercury amalgam electrode has been employed in combination with CE for the determination of cysteine and cystine in human urine (48). A dual carbon electrode has also been used for simultaneous determination of the oxidized and reduced forms of NADH (49).

It is possible to increase the selectivity of CE-EC for certain substrates using chemically modified electrodes or enzyme-based biosensors. The most common modification is incorporation of an electron transfer mediator or electrocatalyst onto the surface of the electrode (50). For example, a cobalt phthalocyanine-modified electrode has been used for selective detection of thiols in urine following CE separation. In a separate application, a glucose oxidase-based biosensor was employed with CE for the detection of glucose in blood. Enzyme-modified electrodes are generally compatible with CE-EC because most buffer systems are aqueous; thus, the enzyme does not denature as it would in reversed-phase chromatography. Chemically modified electrodes have been reviewed by Baldwin and Thomsen (51).

The selectivity and sensitivity of electrochemical detection has obvious advantages for analysis of drugs in biological fluids, especially when combined with the small sample requirements and high separation efficiency of CE. Aminopyrine and its metabolite 4-aminoantipyrine have been detected at the femtomole level in human urine by CE-EC using a carbon fiber microelectrode (52). Copper electrodes have been shown to be especially useful for the determination of amino acids and carbohydrates. A copper electrode placed in a wall jet configuration (off-column detection) has been used to detect amino acids in human urine and pentapeptides in peptide libraries (53). CE-EC with a copper microelectrode has also been employed to detect amino acids in brain microdialysates (54). In this case, a zwitterionic buffer was employed to reduce the separation current and improve the signal-to-noise ratio for electrochemical detection. For most amino acids, detection limits ranged from 10–400 nM, and the method was linear over three orders of magnitude. Lastly, CE with reductive electrochemical detection has been used for the determination of mitomycin-C in human serum (55).

Capillary Electrophoresis with Mass Spectrometric Detection (CE-MS)

Detection by mass spectrometry provides helpful structural information, which can greatly complement CE separations. However, detection limits in mass spectrometry are high relative to those obtained with fluorescence and electrochemical detection. Furthermore, mass spectrometric detection is prohibitively expensive. CE-MS has, therefore, been employed primarily for investigations of the metabolism of drugs and structural elucidation of drug products (56–65). Another important contribution of CE to MS is in the determination of chiral compounds that cannot be distinguished by MS alone (66). CE-MS instrumentation is employed extensively for peptide analyses; however, that area is beyond the scope of this review. A general review of CE-MS, which includes biological, pharmaceutical, and environmental applications, can be found elsewhere (67).

CE and mass spectrometry can be linked both off- and on-line; however, pharmaceutical applications have been limited to on-line configurations. On-line coupling is made possible through the implementation of either an electrospray or ion spray interface. Electrospray and ion spray interfaces are, in fact, quite similar. In both interfaces, the liquid delivered from the end of the CE capillary is desolvated and the separated analyte is ionized and directed toward the detector. In an electrospray interface, these processes occur by means of a potential difference across the interface. This potential difference leads to the formation of charged droplets, which are desolvated and break apart such that the separated analyte is changed to a charged gas phase state. In an ion spray interface, the formation of droplets, subsequent desolvation, and formation of gas phase ions is induced through the application of both a potential difference across the interface and the introduction of a nebulizing gas. Once the separated analyte is ionized and in the gas phase, the mass-to-charge ratio is determined. This is normally accomplished with either an electrostatic sector combined with a magnetic sector or a quadrupole instrument.

In some cases, CE-MS is not adequate for conclusive identification or structural elucidation. One means of solving difficult structural questions is through the use of tandem mass spectrometry, or CE-MS-MS. Metabolic studies utilizing tandem mass spectrometry can employ quadrupole instruments with a collision chamber between the two quadrupole analyzers. In these instruments, the parent ion is detected by the first quadrupole; this ion is bombarded with argon gas, and the mass-to-charge ratio of the resulting fragments is measured by the last quadrapole. Procedures carried out by tandem mass spectrometry coupled to CE include determination of LSD (62), anti-inflammatory drugs (63) and noncovalent complexes (68). Additional tandem CE-MS studies have employed on-line skimmer collision-induced dissociation coupled to a sector MS instrument for the investigation of mifentidine (64,65).

PRACTICAL ASPECTS OF CE SEPARATIONS

There are several characteristics of CE that make it amenable to pharmaceutical analyses. First, only nanoliter-to-picoliter

volumes are injected on a CE system, which makes it possible to analyze the same sample several times without appreciable loss in volume. This is particularly significant in cases where the analysis is sample-limited. In situations where the low injection volume leads to poor concentration limits of detection, there are several techniques unique to CE that can be used to concentrate the analyte. Second, the efficiencies exhibited by CE are generally higher than those of LC. This high separation efficiency may be required for many pharmaceutical applications, especially for the separation of drugs and their metabolites. In addition, CE is particularly well suited to the analysis of highly ionic compounds that tend to be difficult to separate by reversed-phase LC. In cases where the efficiency of the separation technique must be extremely high, the CE method can be further modified by including a second separation mechanism, such as a pseudophase in affinity and chiral CE, or molecular sieving in capillary gel electrophoresis (CGE).

Low Sample Volume Requirements

The working volume of a CE separation can range from microliters to nanoliters, depending on the exact dimensions of the capillary. Typical injection volumes for hydrodynamic or electrokinetic injections are on the order of tens of nanoliters to picoliters. This low injection volume requirement can be quite beneficial in cases where the sample volume available for analysis is small, or where the available sample volume is moderate, but multiple analyses of the same sample would be advantageous. This is especially true for bioanalytical applications. Low volume sampling for *in vivo* monitoring of biofluids is desirable because the animal (or patient) experiences less stress, and more samples can be taken per unit time.

Another case where the low sample volume requirement for CE is beneficial is in the determination of drug compounds that are available in limited quantities, for instance, in cases where the drug is synthesized in-house or is costly. This is especially the case with combinatorial screening, which generates a large number of compounds, each of which is available in only limited quantities.

Sample Preparation or Pretreatment

Many of the problems associated with the determination of drugs in biological fluids by LC also occur with CE. Endogenous components in plasma, serum, urine and other biological matrices can lead to clogged capillaries, decreased resolution, masking of the analyte response, and poor reproducibility. The use of CE to assay drugs in a wide range of clinical and forensic samples has been reviewed extensively by Landers (69) as well as Thormann *et al.* (70).

Sample matrix effects on CE separations, including protein precipitation methods for CE, have been discussed in considerable detail by Shihabi and Garcia (71,72). Acetonitrile, alcohols, or strong acids can be used for protein precipitation (71), but acetonitrile is generally the most efficient and requires the smallest added volume. Deproteinization is usually followed by centrifugation and injection of the supernatant onto the CE system. Shihabi and coworkers (8,73,74) have described deproteinization approaches for CE analysis of serum, CSF, urine and tissue. In one example, acetonitrile was used for protein precipitation in the determination of theophylline at 4.5–20 μg/mL in plasma by CE-UV (6).

Solid phase extraction, which is discussed in a later section in more detail, is a form of "digital chromatography" in which the analytes of interest are selectively retained on the chromatographic support and then eluted. Solid phase extraction has been used for the determination of enkephalins (75) and in conjunction with CE for the determination of urinary estrogens (76). Electrically driven solid phase extraction has also been used as a sample preparation technique (77).

Liquid/liquid extraction is another form of sample pretreatment used with CE. However, it is time-consuming and can require relatively large volumes of organic solvents, leading to disposal problems. Anthracyclines in human plasma (78) and naproxen in serum (77,79) are examples of the use of solvent extraction for the analysis of drugs in biological fluids. The organic solvent is removed by evaporation, and the residue can be redissolved in the appropriate solvent for optimum CE analysis. This method is problematic for hydrophilic drugs where very low recoveries are common. This method does allow considerable preconcentration of the analyte, as a large volume of sample can be used if required.

Other methods are commonly required for analysis of drugs in tissue because tissue samples are more complex and require more rigorous sample pretreatment. For example, the use of ultrafiltration for the analysis of methionine enkephalin in samples of rat retina has been described (80).

Direct Injection

There are times when there is insufficient sample to carry out off-line sample preparation techniques, extraction recoveries are poor, or the extra time required for off-line methods is prohibitive. In these cases, direct injection of the biological sample onto the CE system without sample preparation is desirable. Direct injection should also give more accurate results and better precision because recoveries with traditional techniques add a source of variability.

Urine is the simplest biological sample matrix that can be injected directly onto a CE system. When extraction methods are used with urine, it is usually because the analyte concentration is below the limit of quantitation or a specific endogenous substance interferes with the analyte of interest. Ultrafiltration and dilution with buffer are also common approaches for the analysis of urine. They remove particulates and reduce the ionic strength of the injected solution, respectively. A method for the determination of creatinine in urine using direct injection has been reported (81). Samples were diluted 50-fold with run buffer and 2% EDTA was added to complex metals in the urine that would otherwise complex with creatinine and interfere with quantitation.

Many biological matrices are more problematic than urine because they contain greater numbers of endogenous species such as large proteins, which can block capillaries and adsorb to the capillary wall. A technique that evolved from capillary electrophoresis is MEKC, which was first described by Terabe *et al.* (82). A good overview of biomedical analyses using CE, including MEKC and its basic theory, has been given by Smith and Evans (83), while sample matrix effects and system optimization in MEKC have been covered by Shihabi and Hinsdale (84).

In MEKC, a surfactant, typically sodium dodecyl sulfate (SDS), is added to the run buffer above its critical micelle

concentration (85). This creates what has been termed a pseudophase (82) into which injected species may partition. This explains the use of the term "chromatography" in the description of this technique. The surfactant micelles can also solubilize proteins and allow direct injection of plasma and serum samples (86). MEKC is particularly useful for the separation of neutral analytes that may normally be poorly resolved. With anionic surfactants such as SDS, the micelle is negative and migrates against the electroosmotic flow in a conventional CE setup as shown in Fig. 1. Overall, however, the movement of the micelles is toward the cathode, but at a slower rate than that of the bulk solvent flow. Nonionic solutes partition into the micelles based upon their relative hydrophobicities, and thus may be separated on this basis. Ionic species can still partition into the micelles, but they will be separated by both chromatographic and electrophoretic mechanisms. SDS is the most common MEKC additive, but cationic and zwitterionic surfactants, bile salts and chiral micelles have also been employed (83).

Practical optimization of MEKC separations by manipulation of pH and SDS concentration has been studied by Wätzig and Lloyd (87). These authors discuss the manipulation of analyte mobility so that the analytes of interest migrate past the detector before the plasma proteins elute. Schmutz and Thormann (88) have also described fundamental parameters for direct injection of biological samples using MEKC. The criteria that affect the useful analytical window are the concentration of the micelle-forming substance, applied voltage, ionic strength, initial sample zone length, buffer additives, capillary length, renewal of anodic buffer, and the sample matrix. The effects of these various parameters were investigated with several model analytes, including phenobarbital, phenytoin, and ethosuximide (88).

Direct injection of rat airway surface fluid into the CE capillary using a novel sampling technique has been reported (89). Multiple analyses from nanoliter volumes were possible and several ions (Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^-) were quantitated. MEKC has been used to quantitate diastereoisomers of L-buthionine-(R,S)-sulfoxime in human plasma. The complete validated assay required about 30 min, including capillary conditioning; the limit of detection was 3.9 μg/mL (90).

MEKC and chromatographic methods have been compared for a number of pharmaceutical compounds. Shihabi and Oles (7) investigated both LC and MEKC for the determination of felbamate in serum. Deproteinization by acetonitrile was performed prior to LC analysis, but direct injection could be accomplished with the MEKC method. The two methods showed good correlation, but MEKC was less expensive, took only half the time, and required no acetonitrile. MEKC has also been used for therapeutic monitoring of the antiepileptic drugs phenobarbital, ethosuximide and primidone in human serum [91]. MEKC is much more specific and less expensive than existing immunoassays and requires no sample cleanup. A comparison of ion-pair chromatography and MEKC for the determination of beta-blockers in urine and serum indicated that the MEKC method was more efficient, as the number of theoretical plates was higher than for the ion-pair method. In addition, MEKC did not require a liquid/liquid extraction step and was less time-consuming (9). Urine samples were injected directly following filtration. Serum samples were first hydrolyzed and then subjected to solid phase extraction prior to injection onto the MEKC system.

Microdialysis

Microdialysis sampling coupled to CE has proven highly useful for *in vivo* pharmacological investigations (92–95). In microdialysis sampling, a probe constructed from a semi-permeable membrane is inserted into the physiological region of interest. Sample is administered or collected via the probe by diffusion through the membrane. Thus, sampling occurs without depletion of the fluid in the sample region, and there is minimal perturbation of the tissue of interest by the technique. As a result, sampling can be continued for extended periods of time, the animal under investigation can be used for both the experiment and the control, and measurements can be made in awake, freely moving animals.

The semi-permeable membrane of the microdialysis probe has a molecular weight cut-off that permits diffusion of small molecules such as drugs, drug metabolites, or endogenous compounds, but prevents larger molecules such as proteins and enzymes from passing through. Because enzymes are excluded from the microdialysate, the sample acquired is not further metabolized after collection. Furthermore, the exclusion of proteins from the microdialysate facilitates direct injection of the sample onto a CE system.

Microdialysis sampling has been successfully coupled to CE both on-line and off-line. In off-line sampling, the microdialysate is collected in microliter aliquots and can be injected onto the CE capillary without further sample preparation. Off-line collection has been employed for the determination of endogenous neuroactive amino acids (92,93) in the brain under normal conditions as well as upon administration of morphine (94). It has also been utilized for the determination of the antineoplastic agent α-difluoromethylornithine (DFMO) in blood dialysates (95). The separations of a blank blood microdialysate and a sample spiked with DFMO are shown in Fig. 3. Off-line sam-

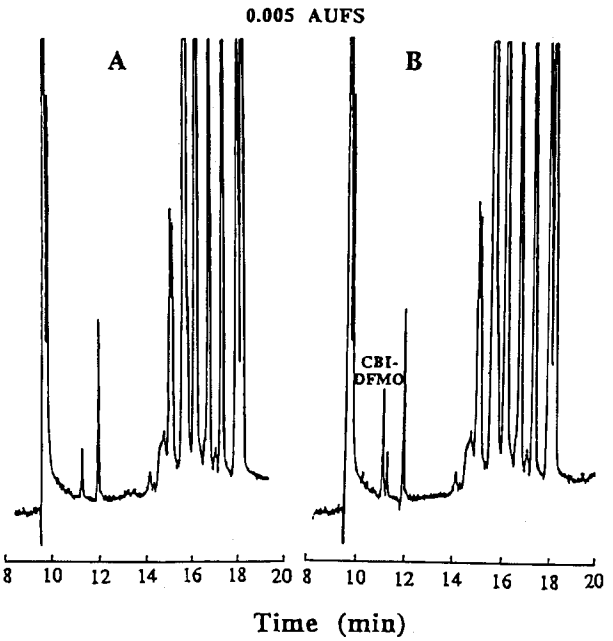

Fig. 3. Electropherogram of (A) blank blood microdialysate sample and (B) blood microdialysate spiked with 10 nM α-difluoromethylornithine (CBI-DFMO). (Reproduced from (95))

pling is practical in cases where the analyte must be derivatized before it is introduced onto the CE system (93–95).

While off-line sampling is useful, it also has a few drawbacks. First, difficulties associated with handling small sample volumes require that the volume of microdialysate collected be larger than necessary for injection onto the CE system. For the off-line systems mentioned above, samples of 4, 15 or 105 μL were collected over 15- or 16-min intervals; these large sample volumes were required for analysis even though only nanoliter volumes are required for injection onto the CE capillary. The collection of large sample volumes from the microdialysis system through implementation of high flow rates is not recommended because low microdialysis flow rates lead to better sample recovery. In addition, the collection of samples over shorter time intervals facilitates better temporal resolution of the biological event under investigation. Another disadvantage associated with off-line sampling concerns the difficulties connected with routine handling of submicroliter sample volumes. These include sample loss due to evaporation and significant loss of analyte due to adsorption to surfaces in contact with the microdialysate, as well as the physical problems associated with sample transfer.

The use of on-line microdialysis sampling overcomes the disadvantages of off-line sampling mentioned previously. On-line sampling has been utilized for the investigation of an antineoplastic agent, SR 4233, in blood (96). In this system, microdialysate samples of only 60 nanoliters were collected in a sample loop on an electronically actuated valve and injected onto the CE capillary using a specially designed injection interface (Fig. 4). In another study, on-line sampling was utilized for monitoring the levels of ascorbate and lactate in the brain (97). In this work, the transfer of microdialysate to the CE capillary was facilitated with a flow-gated interface. This interface differs from the previously described system in that a loop is not employed. Rather, a capillary used for delivery of microdialysate is positioned close to the CE capillary, the microdialysate is flushed past the CE capillary by a continuous flow of the CE buffer and, as a result, the microdialysate is directed to waste. At discrete intervals, the flow of the CE buffer is stopped and the microdialysate sample is electrokinetically injected onto the CE system. After injection, the CE buffer flow is again started, and the CE separation is performed. Both systems had similar temporal resolution—90 s for the loop system compared to 45 s for ascorbate and 125 s for lactate. In both cases, the temporal resolution for on-line microdialysis-CE analyses is significantly better than that of off-line analyses.

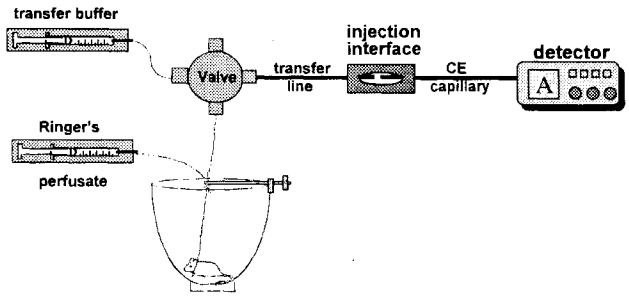

Fig. 4. Schematic diagram of on-line microdialysis-capillary electrophoresis system.

Improvement of Detection Limit Through Preconcentration

While the small sample size requirement has often been stated as an advantage for CE, there are many cases where the small sample injection size can be a disadvantage. For example, many samples from biological sources are extremely dilute and small injection volumes mean that low masses are injected onto the capillary. Injection volumes in CE usually range from 1–40 nanoliters, but several microliters may be required in order to obtain the required mass for on-capillary detection of the analyte. Often, the ideal injection volume may be greater than that of the whole capillary. In addition, limits of detection obtained with CE-UV can be higher than needed for the analyses of pharmaceuticals in biological matrices. The use of sample preconcentration provides a means of overcoming these problems. Methods of sample preconcentration include hydrophobic retention, affinity concentration, sample stacking and isotachophoresis. A summary of analyses of pharmaceutical compounds incorporating these preconcentration methods is shown in Table 2.

Sample Preconcentration Through Hydrophobic Retention

One effective means of injecting hydrophobic analytes onto a capillary is the use of a membrane for solid phase extraction (98) or an LC stationary phase for hydrophobic retention (99). Hydrophobic stationary phases are utilized in reversed-phase LC separations and, as a result, a variety of products are available. This type of concentrator can be coupled to the CE system on-line or off-line. The only limitation with this mode of preconcentration is that the analyte of interest must be hydrophobic in order to be retained by the preconcentrator.

An on-line preconcentrator employing LC packing material is made by introducing a frit at the injection end of the CE capillary to hold the packing material in place. An on-line membrane preconcentrator incorporates the membrane without a frit (Fig. 5). In the example shown in Fig. 5, the membrane is fixed between two fused silica capillaries butted together inside a piece of tubing (98). The typical protocol for use of an on-line preconcentrator involves four steps, the first of which requires pretreatment of the concentrator with an organic solvent followed by an aqueous buffer compatible with the CE. The second step involves loading of the sample, and typically permits the use of sample volumes that are large relative to the total volume of the separation capillary. The third step consists of flushing the preconcentrator to remove unretained sample and equilibration of the CE capillary with aqueous buffer. Elution of the sample from the preconcentrator with a low volume of organic solvent compatible with the CE system is the final step. In one case, the LOD for the drug verapamil was decreased 200-fold using this approach (100).

Off-line preconcentration utilizes extraction cartridges with either membrane disks or LC packing material. The protocols for off-line preconcentration are similar to those commonly employed for LC. In one example, a 50 mL sample volume of clenbuteral was reduced to 1 mL, resulting in a 50-fold enrichment, and then analyzed by CE (99).

Sample Preconcentration Through Affinity Chromatography

One sample preconcentration method for CE is based on affinity chromatography. In this approach, the attraction of the

Table 2. Drugs Analyzed with the Preconcentration Techniques Discussed in the Text

Drug compound	Preconcentration technique	Matrix	Concentration or enhancement	Ref.
Haloperidol	on-line hydrophobic retention and stacking	aqueous + MeOH	380 nM	98
Verapamil	on-line hydrophobic retention	plasma	5 nM detection limit 200-fold	100
Clenbuterol	off-line hydrophobic retention	tablet	1.1 μg/mL 50-fold	99
Levothyroxine	off-line hydrophobic retention	tablet	4.0 μg/mL 20-fold	99
IgE	affinity	serum	N.R.[a]	101
Insulin	affinity	serum	200–1000-fold	103
Narcotine	stacking	aqueous + MeOH	240 ng/mL	106
Clenbuterol	stacking	aqueous + MeOH	92 ng/mL	106
Flurazepam	stacking	aqueous + MeOH	180 ng/mL	106
Codeine	stacking	aqueous + MeOH	108 ng/mL	106
Pethidine	stacking	aqueous + MeOH	100 ng/mL	106
Flurazepam	stacking	urine	1 μg/mL	106
Neostigmine	isotachophoresis	urine	10 μg/mL	108
Homatropine	isotachophoresis	urine	10 μg/mL	108
Scopolamine	isotachophoresis	urine	10 μg/mL	108
Clenbuterol	electroextraction-isotachophoresis	aqueous + MeOH	2 nM	113
Salbutamol	electroextraction-isotachophoresis	aqueous + MeOH	2 nM	113
Terbutaline	electroextraction-isotachophoresis	aqueous + MeOH	2 nM	113
Fenoterol	electroextraction-isotachophoresis	aqueous + MeOH	5 nM	113
Verapamil	isotachophoresis	aqueous	80 ng/mL detection limit	116
Verapamil	isotachophoresis	serum	420 ng/mL detection limit	116

[a] N.R. = not reported.

analyte for the affinity phase is the basis for collection and concentration of the analyte. The sample is pumped through the affinity preconcentrator. Typically, the preconcentrator is then washed, the analyte of interest is eluted, and the analysis is completed by CE.

Affinity sample preconcentration methods apply to a more specific interaction than that used in most other solid phase preconcentration steps. The use of monoclonal antibodies is a logical choice for creating an affinity preconcentrator. Guzman described the use of monoclonal antibodies against IgE to concentrate injected IgE from serum (101). The antibodies were used to "affinity capture" the analyte in a highly selective way in a specially fabricated analyte concentrator-reaction chamber. The reactor consisted of an immunoaffinity solid support inserted at the inlet of the capillary. The reactors were 1–5 mm in length and 25–400 μm in diameter. Two designs were discussed by Guzman—one used multiple capillaries in a bundle and the other a solid glass rod with several small diameter (25 μm) holes laser drilled into it. Twenty microliters of serum were injected; this was followed by a wash step to remove salts and other serum consituents. An elution buffer was then used to elute the IgE. At this point the separation voltage was applied and analytes were monitored with UV detection at 214 nm.

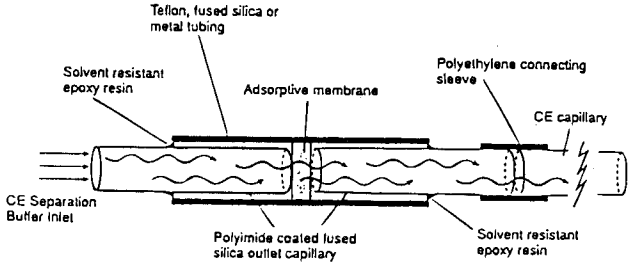

Fig. 5. Schematic diagram of a membrane preconcentration-capillary electrophoresis cartridge. (Reproduced from (98))

Guzman et al. (102) also reported the use of immunoaffinity concentration for the purification and collection of pharmaceutical compounds by CE. In this case, antibodies that were directed against methamphetamine were employed for the determination of the drug in human urine.

A different approach to immunoaffinity preconcentration was reported by Cole and Kennedy (103), who employed capillaries (150 μm i.d.) packed with a protein G chromatographic support. After an antibody to the analyte of interest was loaded onto the column, it could be used as an immunoaffinity precon-

centrator. Bovine insulin spiked in serum was selectively retained and preconcentrated. Both the antibody and antigen were eluted from the preconcentrator with a desorption buffer at a lower pH and then separated by CE. The authors illustrate its use in both on-line and off-line modes. A 1000-fold preconcentration of insulin samples was obtained with this system.

Sample Preconcentration Through Sample Stacking

Sample stacking is an on-column sample concentration technique. In this approach, the analyte is compressed into a narrow band due to differences in conductivity between the matrix of the sample plug and the background electrolyte. As mentioned previously, CE can exhibit a low mass LOD due to the small sample volumes employed, but it does not possess a low concentration LOD because of the short optical path defined by the column diameter when using UV detection. The LOD can be decreased by sample stacking, but is limited by the amount of sample that can be injected. The concentration of analyte in the sample zone can be increased 10–1000-fold by stacking, depending on how much sample is injected and the mode of injection (104). This concentration technique is applicable only to a sample of very low ionic strength because the sample must have a lower conductivity than the background electrolyte.

In sample stacking, the sample is introduced by either hydrodynamic or electrokinetic injection. When voltage is applied, the sample zone is of higher field and lower conductivity than the remainder of the capillary. The ions concentrate at the front or back of the sample plug due to the higher mobility of the ions in the sample plug compared to the bulk electrolyte. The stacking occurs at the point where the ions meet the background electrolyte. For example, if the anode is at the injection end, the cations have increased mobility and concentrate in front of the sample plug due to the lower field strength. The electroosmotic flow then carries the analytes to the detector.

Ionic-strength mediated stacking employs dilute run buffer or water as the sample matrix. For optimum sample stacking, the sample should be prepared in buffer that is tenfold more dilute than the background electrolyte (105). Although water is often used, it is not usually recommended as a sample matrix. It creates a high field over the sample plug and generates osmotic pressure on the fluid in the capillary because the electroosmotic flow is suppressed in the sample plug (105). Both of these effects can cause band broadening, and sample degradation is possible in the high field.

An alternative form of stacking is pH-mediated stacking, which is often used for the separation of zwitterionic peptides (105). The peptides are prepared in ammonium hydroxide and the run buffer is acidic. When a sample is introduced and a high voltage is applied, the anions migrate toward the anode until they reach the run buffer. If the peptide contacts the acidic run buffer, its charge changes and the ion reverses its electrophoretic migration toward the cathode. The result of these processes is that the plug collapses upon itself, creating a narrow, positively charged band.

Sample stacking can be helpful in bioanalysis to concentrate analytes. Large-volume sample stacking CE was recently compared to conventional CE for the determination of drugs of forensic significance in buffer and urine samples (106). In this application, the LODs for narcotine, clenbuterol, flurazepam, codeine, pethidine, hydroxyethylflurazepam, and desalkylflurazepam were improved approximately tenfold. Sample stacking was also shown to decrease the concentration detection limit by as much as three orders of magnitude for DNA adducts of polycyclic aromatic hydrocarbon (PAH) metabolites (107).

Sample Preconcentration Through Isotachophoresis

Isotachophoresis (ITP) is a technique that can be used to separate and focus compounds into small, highly concentrated zones prior to CE (108,109). It is most useful in concentrating dilute samples prior to CE separation. Both coupled and single capillaries can be used. ITP allows the injection concentration to be increased by a factor of 50–100, and it can be performed on commercially available equipment (110). The technique of ITP has been described at length by Mikkers and Everaerts (111). It involves a discrete amount of sample containing the analytes of interest (separands) at the interface of two different electrolytes: the leading electrolyte and the terminating electrolyte. The simplest form of ITP involves the leading and terminating electrolyte each containing only one ionic constituent of the same charge as the separands and a counter constituent to preserve electroneutrality. The effective mobility of the leading electrolyte should be greatest, followed by that of the separands and then the terminating electrolyte. In one form of ITP, the capillary is first filled with the leading electrolyte. The sample is then injected and the terminating electrolyte is placed in the run buffer reservoir. At this point, a voltage is applied and, once a steady-state is reached, the separands are in a contiguous zone in order of mobility. At this stage, the leading electrolyte is introduced into the inlet of the capillary. The contiguous zone is then relaxed as the leading electrolyte migrates through the zone and the sample ions are separated. The degree to which the sample is concentrated is a function of the leading electrolyte; at equilibrium the zones are often of different size, but their concentrations are approximately equal (112). Thus, in ITP, the major analytes of the sample are diluted while trace analytes are concentrated. Anions and cations cannot be separated in a single ITP run.

ITP can be used to concentrate trace analytes from large sample volumes and to clean up the biological matrix prior to electrophoretic separation. ITP can be performed on-line in combination with CE to increase both the separation capability and loading. When liquid-liquid electroextraction (EE) is performed on-line with ITP and CE, the focusing time can be reduced. In EE, the analyte of interest, dissolved in organic solvent, is electrokinetically injected into the aqueous CE system. The low conductivity of the organic phase relative to the aqueous phase leads to a higher electric field strength, which in turn leads to a high extraction rate of the analyte into the CE system. EE-ITP-CE has been shown to be an effective focuing and separation technique prior to mass spectrometry to improve sample concentration detection limits. With this technique, β-agonists have been detected at the $2–5 \times 10^{-9}$ mol/L level (113). Also, recent publications indicate a wide range of applications of ITP for biological samples, including the separation of urinary thiols (114); the determination of ascorbic acid in a cell-free system, neuroblastoma cell extracts and urine (115); and an assay for verapamil in serum (116).

Variations of CE

The many advantages of CE make the separation technique amenable to pharmaceutical research. However, conventional CE (as defined previously) is inadequate for some important types of pharmaceutical work; in these cases, modified CE can be utilized. Affinity CE, for example, involves ligand receptor binding through the use of specific buffer additives. Chiral separations often cannot be achieved with conventional CE. However, the use of certain buffer additives has led to the successful implementation of CE for these separations. A third type of modified CE, capillary gel electrophoresis, which incorporates the process of molecular sieving, is effective for the separation of oligonucleotides or oligonucleotide analogs. In fact, capillary gel electrophoresis has been invaluable in phosphorothioate research.

Affinity Capillary Electrophoresis

The determination of binding constants is helpful for understanding pharmacological events and for predicting the actions of drugs and drug candidates against various compounds. Affinity CE is particularly useful for the measurement of binding constants. In affinity CE, the migration of analytes of interest is determined in the presence of a charged substrate. If the analyte interacts with the substrate, the migration time of the analyte will be shifted relative to the migration times of analytes separated in the absence of substrate. If electrophoretic separations are performed at different substrate concentrations, and the migration time of bound and free substrates is determined for each of these different substrate concentrations, a Scatchard plot can be made. The binding constant of the added substrate to the analyte is derived from the Scatchard plot.

There are advantages to performing binding studies using affinity CE. Conventional methods involve the separation of bound and free molecules through the use of filtration or equilibrium dialysis and subsequent detection by fluorescence or scintillation counting. The compounds of interest are radioactively or fluorescently labeled. A review of techniques for drug binding studies can be found elsewhere (117). The analysis time for affinity CE is shorter than for conventional methods. The main advantage of CE is that the amount of receptor and ligand required for the binding study is much smaller than for conventional methods. This permits the use of receptors and ligands that are available in limited quantities due to prohibitive cost or difficulty of preparation. In addition, analytes do not require pretreatment or purification before injection onto the affinity CE system, as CE will typically separate the analyte of interest from other species. Furthermore, simultaneous measurements of the binding constants of several ligands to the same receptor can be made.

Studies with affinity CE have been used for rapid screening as well as for determination of binding constants of particular ligands and receptors. During the time frame of this review, affinity CE has been used for the screening of peptide libraries for affinity to vancomycin (118,119). It has also been employed for determination of the binding constants of peptidoglycan precursors in bacterial species to vancomycin (120) as well as those of heat shock protein Hsc70 and its peptide fragment with the immunosuppressant deoxyspergualin (121).

Chiral Separations

The determination of chiral purity of pharmaceutical compounds is important as a compound can exert physiological effects different from those of its enantiomer. While CE is a highly efficient separation technique, in general, the efficiency of an unmodified CE capillary is inadequate to separate chiral compounds. However, it is possible to modify the electrophoretic mobility of a compound relative to its enantiomer through the addition of buffer additives or derivatization, making the separation more selective. With indirect separation, the enantiomeric compounds are derivatized with a tag that produces diastereomers that can then be separated. In a successful indirect technique, the mobilities of the two diastereomers will be different enough to permit separation of the two chiral compounds from each other. However, this method has not been widely used in pharmaceutical analysis as the use of the derivatization reaction can make the analysis more complicated.

The second approach to chiral separations in CE is to employ additives in the background electrolyte that will significantly change the mobility of the compound of interest relative to its enantiomer such that the compounds can be separated and quantified. Chiral separations in CE facilitated through the use of additives have been reported extensively in the literature. In the time period of this review, several different additives have been used for chiral separations of a variety of pharmaceutical compounds (Table 3). These include cyclodextrins, antibiotics, polysaccharides, anionic surfactants and crown ethers, as well as a few other unusual additives. General reviews of chiral separations by CE can be found elsewhere (122,123).

Cyclodextrins are by far the most widely used additives for chiral drug separations. Cyclodextrins are cyclic oligosaccharides consisting of 6, 7, or 8 glucose units. The cavity size can vary, depending on the number of glucose units, to yield α–,β–, or γ-cyclodextrin. The use of different cavity sizes is advantageous for different enantiomer separations. While most of the reported research reported in this review employed β-cyclodextrin (either unmodified or modified) (17,26,99,124–135), some separations that involve the use of γ-cyclodextrin have been reported (136,137). The separation of zopiclone and its metabolites in human urine, shown in Fig. 6, utilizes β-cyclodextrin as an additive. In addition to differences in cavity size, cyclodextrins can be positively or negatively charged or neutral. An interesting variation of the use of cyclodextrins is the application of polymeric cyclodextrins for the separation of several enantiomeric drugs (132,138).

Two other classes of macrocyclic additives used for separations of chiral drug compounds are antibiotics and crown ethers. Macrocyclic antibiotics possess different cavity dimensions, as well as different charges. Various pharmaceutical compounds have been separated using the following antibiotic additives with CE: vancomycin (139–143), rifamycin (144), teicoplanan (145), and ristocetin (146). A crown ether, 18-crown-6 tetracarboxylic acid, has been utilized as an additive for the separation of aminotetralin enantiomers (147,148).

In addition to macrocyclic compounds, several other classes of compounds have been utilized as additives for chiral CE. The polysaccharides heparin (133, 149, 150) and dextran sulfate (150) and the maltooligosaccharide maltodextrin (151) have yielded good separations of various drug enantiomers. Surfactants, specifically glucopyranoside-based (152) and cys-

Table 3. Separation of Representative Chiral Drugs

Drug class	Buffer additive	Ref.
Anthelmintics	β-CD	133
	heparin	133
Anticoagulants	vancomycin/SDS	142
	maltodextran	151
Anticonvulsants	glucopyranoside-based surfactant	152
Antidepressants	β-CD	124
Antihistamines	β-CD	131
	γ-CD	137
	heparin	149,150
	dextran sulfate	150
Antimalarials	β-CD	132
	γ-CD	132
	heparin	149,150
	dextran sulfate	150
Antimuscarinics	γ-CD	137
Antineoplastics	vancomycin	140
Cardiac drugs	β-CD	99
	γ-CD + MECC-polymerized micelle	136
	β-CD polymer	138
	rifamycin B/30% 2-propanol	144
Hypotensive agents	rifamycin B/30% 2-propanol	144
Nonsteroidal anti-inflammatory drugs	β-CD/low viscosity polymer	125
	β-CD	128
	vancomycin	139,140
	vancomycin/SDS	141
	teicoplanin/acetonitrile	145
	teicoplanin	145
	ristocetin A	146
	bovine serum albumin	155
Opiate agonists	β-CD/1.2% methanol	135
Quinolines (anti-infective)	bovine serum albumin	154
Respiratory/cerebral stimulants	β-CD	124,129
	β-CD/1.2% methanol	135
	rifamycin B/30% 2-propanol	144
Skeletal muscle relaxant	γ-CD	137
Sympathomimetic agents	β-CD	124,126,130,131,134
	β-CD/1.2% methanol	135
	β-CD polymer	138
	rifamycin B/30% 2-propanol	144
	glycopyranoside surfactants/10% methanol	152

Note: β-CD represents either unmodified or modified β-cyclodextrin.

teine-related (153), have also proven useful for chiral separations. In addition, bovine serum albumin has been utilized for the separation of ofloxacin and DR-3862 (154) and ibuprofen and leucovorin (155).

Capillary Gel Electrophoresis

Gel electrophoresis is electrophoresis in a nonconductive polyacrylamide gel. The purpose of performing electrophoresis in a gel is to decrease zone spreading caused by Joule heating. The gel containing the solute(s) of interest is usually immersed in a conductive aqueous buffer solution and an electric field is applied to perform the separation. Gel electrophoresis performed within a capillary is known as capillary gel electrophoresis (CGE). CGE has been utilized for a variety of separations. Although a complete review of the various uses is beyond the scope of this article, one novel area of separation research—CGE for the determination of oligonucleotide phosphorothioates—will be covered.

CGE has been used for the separation of phosphorothioates (156). Oligonucleotides of various classes exhibit potential therapeutic applications, including the inhibition of the papilloma virus, cytomegalovirus, the inhibition of the expression of human intracellular adhesion molecule, the inhibition of HIV replication and the development of anti-tumor compounds (157). A number of phosphorothioates are now in clinical trials and preclinical development. As the development of this line of therapies progresses, analytical methods must be developed that can quantitate intact oligonucleotides as well as metabolites of the oligonucleotides differing by only one or two nucleotides. Such methods using CGE with UV detection for various oligonucleotides in serum, plasma, urine or buffer have recently been published (156–158). Since the capillary is gel-filled, it

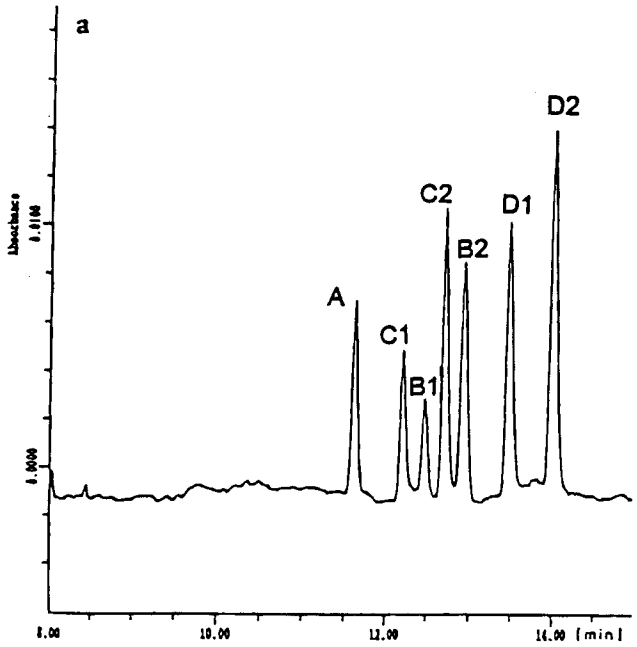

Fig. 6. Electropherogram of human urine 6 h after oral administration of zopiclone. Peaks: (A) zolpidem, internal standard; (B1) R-(−)-zopiclone; (B2) S-(+)-zopiclone; and metabolites (C1) R-(−)-N-desmethylzopiclone, (C2) S-(+)-N-desmethylzopiclone, (D1) R-(−)-zopiclone-N-oxide, and (D2) S-(+)-zopiclone-N-oxide. (Reproduced from (17))

is necessary to introduce a sample by electrokinetic injection instead of pressure injection, in order to avoid disruption of the gel. The quantity of sample injected is therefore a function of the electrophoretic mobilities of the solutes present in the sample. A sample pretreatment step to remove proteins and salts is necessary in each case. Also, an external reference standard in a sample matrix identical to that of the sample containing the drug of interest is crucial for accurate quantitation of the drug. An internal reference standard is also necessary to correct for differences in migration time and peak area (156). Limits of detection as low as 50 ppb were found for antisense phosphorothioate DNA (158).

RECENT ADVANCES

New uses of CE in the pharmaceutical industry and other areas of bioanalysis are continuously being developed as improvements are made in established techniques. Capillary electrochromatography and capillary electrophoresis on a chip are two such advances.

Capillary Electrochromatography (CEC)

CEC is a separation technique that combines electrophoretic and chromatographic separation for charged and neutral species. Fused silica capillaries are packed with reversed-phase particles like those used in LC columns. CEC efficiency can be substantially greater than that seen with LC columns for two reasons. First, it is possible to use sub-micron particles as packing material since electroosmotic flow produces no hydrostatic pressure. Second, there is a plug-like flow profile from the electric field driving the mobile phase instead of high pressure. The resulting theoretical plate values are often >300,000 plates per meter with reduced plate heights <1 (159). The choice of packing methods and mobile phase buffer should be given special attention before performing CEC; these have been discussed by Boughtflower *et al.* (160).

Capillary Electrophoresis on a Chip

The miniaturization of a CE system on a chip has been accomplished through micromachining. Photolithography followed by etching has been used to create channel systems on small silica or glass chips (Fig. 7). Capillary electrophoresis is well-suited for miniaturization for two reasons. First, large pumping systems are not necessary since the run buffer is driven by an applied electric field. Second, separation efficiency and peak capacity are primarily dependent on the absolute value of the voltage applied across the separation capillary and not on its length. If band broadening from injection, detection and Joule heating can be minimized, long channels are not necessary.

CE on a chip has the potential to be very useful for bioanalysis. It can provide increased sample throughput by employing chips with multiple channels, decreased analysis time (to seconds) and reduced sample waste. Several applications of microchip CE have been published. These include the determination of cortisol in serum by CE-LIF in less than 30 s (161), and the separation of phosphorothioate oligonucleotides ranging from 10 to 25 bases in less than 1 min (162). Advanced designs reported by Jacobson *et al.* make it possible to perform pre- (163) and postcolumn chemical reactions and CE on a chip (164).

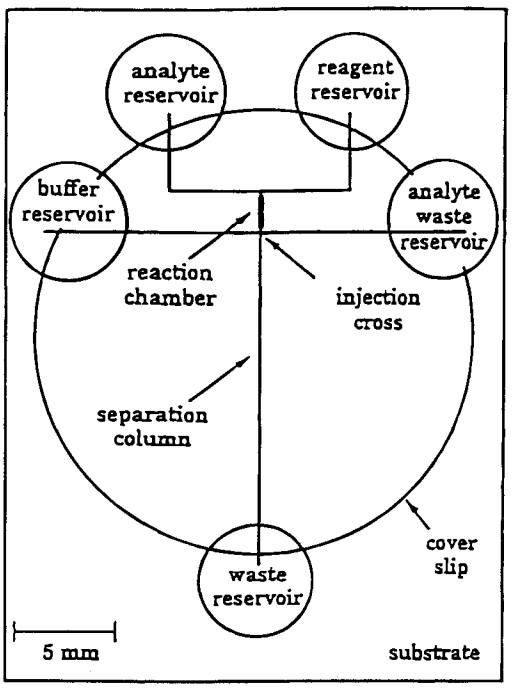

Fig. 7. Schematic diagram of a microchip with integrated precolumn reactor. (Reproduced from (163))

CONCLUSIONS

The utility as well as the versatility of capillary electrophoresis for bioanalyses is clear. CE can be used for the separation and quantitation of pharmaceuticals or pharmaceutically related compounds in a variety of biological matrices. CE coupled to mass spectrometry is valuable for mechanistic studies and structural elucidation. Samples can be injected into the separation capillary with conventional pretreatment techniques, such as extraction or precipitation. Sample can also be injected directly on the capillary with MEKC, or with the combination of microdialysis and CE. Separations by CE employ low volume samples, but with the use of a preconcentration technique such as affinity or hydrophobic interaction, larger sample volumes can be analyzed. In addition, the use of preconcentration techniques leads to signal enhancement and extends the usefulness of CE for pharmaceutical analyses. Variations of CE such as affinity CE, chiral CE and capillary gel electrophoresis have further extended the applications of CE in pharmaceutical analyses. In the future, capillary electrochromatography and CE on a chip are certain to create increased interest in the use of CE for bioanalyses. These areas will inevitably provide exciting new avenues of research.

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