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Review

Applications of Capillary Electrophoresis in Pharmaceutical Analysis

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The role of capillary electrophoresis (CE) in the analysis of peptides/proteins, chiral pharmaceuticals, and other small-molecule drugs has been reviewed. Potential uses of CE range from purity and structural confirmation to a micropreparative technique. Strategies for the prevention of protein wall adsorption include the use of extreme pH values, surface-modified capillaries, and high ionic strengths employing salts of alkali metals or by the addition of zwitterionic surfactants to the background electrolyte. Chiral separations of amino acids and other racemic pharmaceuticals have been achieved by micellar electrokinetic chromatography or by the introduction of cyclodextrins/modified cyclodextrins or other reagents to the running buffer. Applications of capillary electrophoresis to the analysis of small-molecule pharmaceuticals include determinations of drugs and/or excipients in various pharmaceutical preparations and the analysis of miscellaneous pharmaceuticals in standard solutions and biological fluids. The complementary nature of capillary electrophoresis and HPLC, in addition to future expectations of CE in pharmaceutical analysis, is discussed.

KEY WORDS: capillary electrophoresis; peptides; proteins; chiral separations; drugs; pharmaceutical analysis; review.

INTRODUCTION

Electrophoresis has evolved into a separation technique which is no longer used exclusively by biochemists. Open-tubular free-zone electrophoresis was demonstrated as early as 1967 by Hjerten using 3-mm-inner diameter quartz tubes (1). However, problems associated with convection within the capillary limited its acceptability. The technique did not gain widespread attention from other scientists until smaller-diameter tubes were utilized in the seventies by Virtanen (2) and Mikkers *et al.* (3). Subsequently, capillary electrophoresis was brought to the forefront in analytical chemistry with the introduction of microbore capillaries by Jorgenson and Lukacs in the early eighties (4).

The separation mechanism in capillary electrophoresis is the same as that in conventional electrophoresis. Differential migration into discrete zones is due to differences in electrophoretic mobilities, which in turn are related to the mass-to-charge ratio and conformation of the solutes. The dispersion of these bands in free-zone electrophoresis is ideally caused only by longitudinal molecular diffusion, but in actuality zone broadening can be attributed to temperature gradients within the capillary (5), sample matrix, column overloading, and longitudinal diffusion (6). Joule heat generated by the electrical current throughout the medium can escape only at the edges of the capillary resulting in parabolic thermal gradients across the bore of the capillary (7,8). As would be expected, the viscosity of the fluid inside the capillary will vary with temperature, resulting in greater electrophoretic mobilities at the center of the tube. In an effort to curtail the problem of convection in free-zone electrophoresis, Hjerten attempted to stabilize the zones through the "rotating tube method" in which the electrophoresis tube is rotated about the longitudinal axis (1). A more simplistic alternative to solving the problem of zone broadening is to evoke the "wall effect" (9), in which the cross-sectional area of the capillary is decreased relative to the surface area.

Capillary electrophoresis (CE) owes its success to the large surface-to-volume ratio, which allows a large electric potential to be applied across the capillary while maintaining effective heat dissipation. The elimination of joule heating, and thus thermal gradients, minimizes zone broadening. Very efficient separations with rapid analysis times may be obtained under these conditions (10). Another advantage of CE, dictated by the small dimensions of the capillary, is low sample consumption.

The predominant separation technique in pharmaceutical analysis in the last 15 years has been high-performance liquid chromatography (HPLC). The fact that HPLC and CE operate on different separation principles makes these two techniques potentially complementary to each other (Fig. 1) (11). The analysis of complex mixtures exhibiting a broad spectrum of physicochemical properties, as is often the case in pharmaceutical preparations, may be feasible using one or both techniques. Capillary electrophoresis has been utilized in chiral separations, formulation analysis, and purity confirmation of small molecule pharmaceuticals. Interest in the area of pharmaceutical biotechnology has increased the uti-

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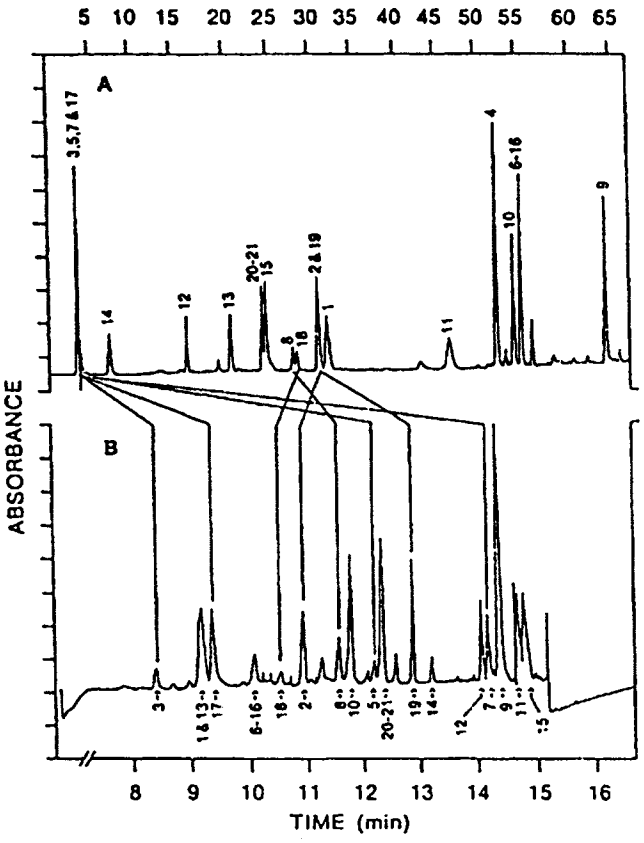

Fig. 1. Complementary separations showing the "fingerprint" of human growth hormone using (A) reversed-phase HPLC and (B) HPCE. [Reprinted with permission from *Analytical Chemistry*, Vol. 61(11), pp. 1186–1194 (1989), American Chemical Society.]

lization of CE in protein separations. The expanding role of proteins/peptides as therapeutic entities in the pharmaceutical industry demands methods to monitor the progress in the synthesis of proteins and to check the final purity of both naturally occurring and synthetic peptides/proteins. Numerous examples of protein characterization via peptide mapping and amino acid sequencing are also given in the literature and are referenced in a subsequent section.

The objective of this review is to give a general overview of the capability of CE in pharmaceutical analysis. The common modes of CE are discussed, followed by applications to the analysis of large and small molecules. The advantages and limitations of the method as well as future trends are assessed.

MODES OF CAPILLARY ELECTROPHORESIS

Zone Electrophoresis

Capillary electrophoresis in the zone format (CZE) tends to be the most commonly used mode. The construction and operation of the system are quite simplistic. The essential components of a CZE apparatus are illustrated in Fig. 2, where the capillary containing the running electrolyte is suspended between two buffer reservoirs. The sample is introduced at the anodic end of the capillary and a large potential is applied across the capillary. Each species will migrate at a

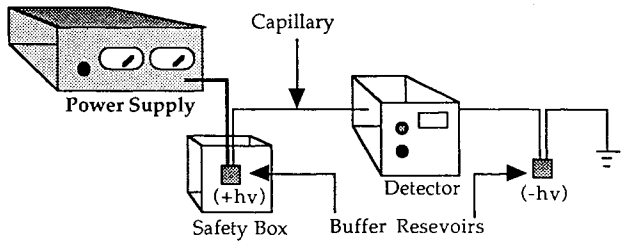

Fig. 2. Basic components of a capillary electrophoresis system.

different velocity based on its electrophoretic mobility. Over the coarse of the separation a discrete zone for each analyte will form (Fig. 3). In addition to the intrinsic mobility of the analytes, a general driving force in CZE is the phenomenon of electroosmotic flow or "bulk flow" within the capillary. Depending on the pH of the buffer, a given fraction of the surface silanols will be ionized, resulting in an overall negative charge at the wall of the capillary, which adsorbs a monolayer of cationic species from the buffer to form the Stern layer. The positive charge density decreases exponentially away from the surface to form a motionless double layer which becomes more diffuse as the distance from the wall increases (Fig. 4). The bulk movement of material within the capillary arises from the zeta potential which spans across the electrical double layer (12). The hydrated cations in solution migrate toward the cathode, effectively causing plug-like flow (13), also depicted in Fig. 3. Under conditions resulting in significant electroosmotic flow, analytes will eventually migrate past the detector, allowing the separation of all molecules in a single run regardless of the electric charge.

Separations in CZE may be characterized by a series of parameters describing electroosmotic flow rate, electrophoretic mobility, and efficiency. A detailed account of these theoretical aspects is given by Jorgenson and Lukacs (5). In HPLC it is appropriate to describe the retention time of a given solute. Similarly in CE the migration time of an analyte is given by

$$t = \frac{L^2}{\mu V} \tag{1}$$

where L is the length of the capillary and V is the applied voltage. The overall electrophoretic mobility of the solute is represented by μ, which is a composite of the electroosmotic flow (μ_o) and the electrophoretic mobility of the analyte itself (μ_e). The linear velocity (v_o) of the electroosmotic flow is

$$v_o = \mu_o E \tag{2}$$

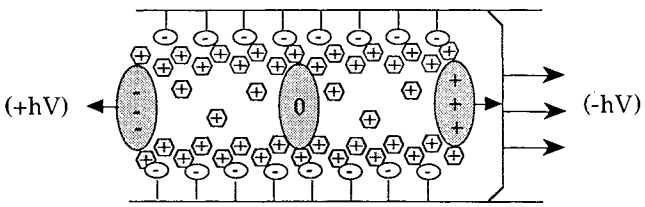

Fig. 3. Illustration of plug flow within the capillary and differential zone migration of analytes based on electronic charge.

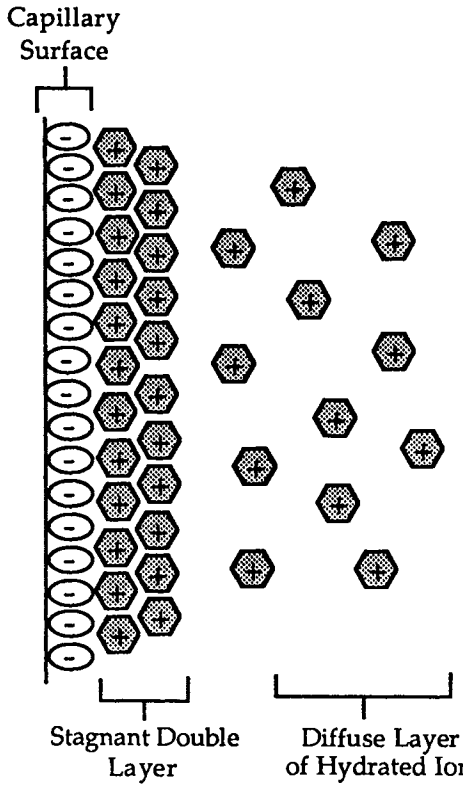

Fig. 4. Electrical double layer of cations on the charged silanol surface of the capillary and the protruding diffuse layer of hydrated cations.

in which the electroosmotic flow coefficient (μ_o) is a function of the zeta potential at the wall of the capillary. The electric field E is defined by the applied voltage divided by the length of the capillary. Likewise, the linear velocity of the analyte in the absence of electroosmotic flow is given as

$$v_e = \mu_e E \tag{3}$$

where μ_e is the electrophoretic mobility of the analyte. The overall linear velocity of the analyte is the sum of contributions from v_o and v_e:

$$v = (\mu_o + \mu_e)E \tag{4}$$

The number of theoretical plates may be calculated to give an estimation of the separation efficiency

$$N = \frac{1}{2D}(\mu_o + \mu_e)V \tag{5}$$

again, V is the applied voltage and D is the diffusion coefficient of the analyte. Equation (5) represents an ideal situation in which only one mechanism of dispersion, namely, molecular diffusion, contributes to zone spreading. Any mass transfer occurring between the bulk solution and the walls of the capillary will have severe consequences on the separation efficiency (12,14,15). The expression for resolution of two analytes in zone electrophoresis was derived by Jorgenson and Lukacs (5) and is

$$Rs = 0.177(\mu_1 - \mu_2)\left[\frac{V}{D(\bar{\mu} + \mu_o)}\right]^{0.5} \tag{6}$$

where $(\mu_1 - \mu_2)$ is indicative of the difference between the electrophoretic mobilities of the two zones and $\bar{\mu}$ represents the average of the two mobilities. The conclusion drawn from the above equations is that by increasing the applied voltage, the efficiency and, thus, the resolution will increase. This is certainly the case until a point is reached where joule heat can no longer be effectively dissipated and efficiency expectations are dispelled.

Micellar Electrokinetic Chromatography (MEC)

Capillary electrophoresis has been extended to the separation of neutral molecules through the use of micellar mobile phases. The concept of electroosmotically pumped pseudostationary phases was introduced by Terabe *et al.* in 1984 (16). A complete discussion of theory may be found in the aforementioned paper. Typically anionic surfactants, for instance, sodium dodecyl sulfate (SDS), are included in the running buffer at a concentration above that of the critical micelle concentration. The formation of micelles possessing a hydrophobic interior and an anionic exterior provide a pseudostationary phase into which analytes may differentially partition themselves. However, interactions of the analyte with the micelles are not limited to those hydrophobic in nature. Electrostatic interactions of polar molecules at the outer surface of the micelle will also influence the selectivity of a separation. Enantiomeric separations have also been successful when chiral surfactants, were employed in order to induce stereospecific interactions (17). As would be expected, anionic micelles migrate toward the injection end of the capillary; however, the stronger electroosmotic flow eventually carries the moieties past the detection window. Longer retention times are advantageous in that the time frame during which separation mechanisms can operate is increased.

In addition to the previously mentioned factors affecting band dispersion in CE, several more contributions are recognized in MEC (18,19). At a low linear velocity longitudinal diffusion was determined to be the major contributor to band broadening. At higher voltages band dispersion due to slow sorption–desorption kinetics of the solute solubilization and electrophoretic dispersion of the micelles become increasingly important. As might be expected, since a chromatographic mechanism is actually occurring, albeit electrically driven, the phenomenon lends itself to the typical plate height-versus-velocity relationship illustrated in Van-Deemter plots for HPLC separations.

The addition of cyclodextrins to the mobile phase provides another means of separation selectivity manipulation (20). Inclusion complexes are formed in which the cyclodextrin serves as the host molecule and the analyte as the guest. A generalized chemical structure of cyclodextrin is shown in Fig. 5. The cyclodextrin molecules typically used range from six to eight glucose units (α-, β-, and γ-cyclodextrin), which are connected by glycosidic linkages to form a truncated cone shape structure (Fig. 5). The inner cavity is hydrophobic, while the entrance to the cavity is lined with secondary hydroxyls and is thus hydrophilic in character. Equilibrium formation of the complexes is driven by the favorable decrease in the free energy for the process. Factors such as hydrophobicity, hydrogen bonding, and size and shape of

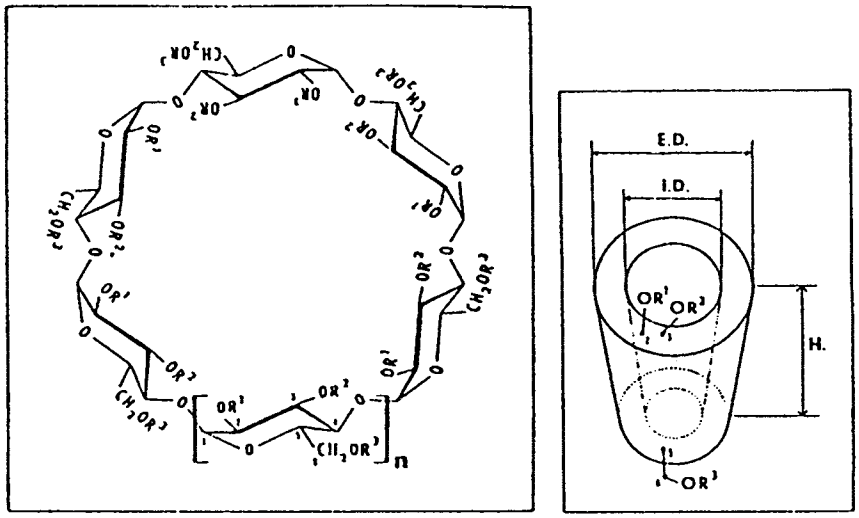

Fig. 5. (A) Chemical structure of cyclodextrin and/or analogues where n = 1, 2, or 3 (α-, β-, or γ-cyclodextrin) (B) Schematic of truncated cone structure of the cyclodextrins. Refer to the original reference for dimensions of the cavity for the cyclodextrins and derivatives. [Reprinted with permission form *Journal of Chromatography*, Vol. 452, p. 577 (1988), Elsevier Science Publishers BV.]

the guest molecule will determine the analyte's propensity to partition into the cavity (21,22). Clearly the use of micelles and cyclodextrins in the running electrolyte adds a new dimension to the electrophoretic separation and offers enhanced flexibility in the manipulation of selectivity.

Capillary Gel Electrophoresis

The use of gels in capillary electrophoresis has made a significant impact on protein/peptide and oligonucleotide separations. Gels serve as an anticonvective medium to decrease the contribution of thermal currents and solute diffusion to zone broadening. However, several other attributes of the technique make it quite attractive. Cross-linked gels may act as a molecular sieve to discriminate proteins based on size or molecular weight (23). Polyacrylamide gels are frequently used, with various amounts of bisacrylamide as a cross-linking reagent (24). The size of the channels within the gel can be customized by using various amounts of the cross-linking reagent. In the presence of SDS, denatured proteins will bind to the SDS to form a complex at a constant ratio of 1.4 g of SDS/g of protein (23). Under these conditions all proteins will exhibit the same free solution velocity and separation is based solely on the molecular weight of the protein, with the lowest molecular weight protein eluting first (i.e., seiving mechanism). The use of gels also offers the advantage of decreasing electroosmotic flow as well as the elimination of protein adsorption onto the capillary surface (25). Capillary gel electrophoresis may be utilized in a micropreparative capacity in the case of protein purification, with this aspect being addressed at a later juncture.

Isoelectric Focusing

Isoelectric focusing represents a unique separation mechanism in CE where separations are based on differences between the isoelectric points of proteins. The proteins to be separated are added to a mixture of ampholytes encompassing the desired range in pH (i.e., 3–10). A pH gradient spanning from a low pH at the anodic end to a high pH value at the cathode terminus is established during the focusing step. The process of focusing generally takes 15–20 min, during which a protein will migrate to a pH region in which the species is electrically neutral and then ceases to migrate any further. Alternatively, a permanent pH gradient may be achieved by the immobilization of buffer ions in a polymerized gel within the capillary (26). After focusing, the zones must be mobilized for detection through the use of either hydrodynamic flow or electrophoretic migration (27). In the case where gels are utilized, mobilization is not possible using hydrodynamic pumping and must be done electrophoretically. Ideally electroosmosis should be negligible during the focusing step since resolution and reproducibility may otherwise be compromised. Consequently there is great interest in hydrophilically modified capillary surfaces which prohibit electroosmotic flow (27). Polymeric additives, such as methylcellulose, in the sample/ampholyte mixture have met with some success in deterring electroosmotic flow in isoelectric focusing (28). The resolution in isoelectric focusing is a function of the linearity and the slope of the pH gradient within the capillary in addition to the magnitude of the diffusion coefficient of the proteins (24). The utilization of the entire length of the capillary and the capability to form sharply resolved zones allow large amounts of sample to be loaded onto the capillary, proving isoelectric focusing to be an excellent candidate as a micropreparative technique.

Isotachophoresis

Isotachophoresis is also a widely used technique in protein separations, however, operation is based on the development of a potential gradient rather than a pH gradient. Unlike the previously discussed modes of CE, anions and cations cannot be separated in a single run. Generally proteins are separated in the anionic form at a higher pH, al-

though operation at the high pH does not necessarily favor resolution. Isotachophoresis is characterized by the use of a leading electrolyte and a terminating electrolyte, with the former showing a higher mobility than the analytes and the latter showing a lower mobility. The mixture to be separated is positioned between the leading and the terminating electrolytes within the capillary. Once the voltage is applied the analytes will begin to migrate in order of decreasing mobility. After the potential gradient has been established a steady-state is reached in which all the zones will migrate at the same velocity. In regions where there are analytes of low mobility, the electric field is greater, thereby compensating for the lower mobility. Mikkers *et al.* (29) give an excellent theoretical account of isotachophoresis concepts dealing with resolution and separation efficiency. Theory, instrumentation, applications, and general guidelines have been laid out by Bocek *et al.* (30). As with isoelectric focusing and gel electrophoresis, isotachophoresis has also been used in a micropreparative role and in addition to other protein applications.

MACROMOLECULES

CE in Unmodified Capillaries

Capillary electrophoresis has rapidly become a valuable tool in the analysis of peptides/proteins as witnessed by the numerous citings in the literature. Unlike RPLC, the mild nondenaturing conditions used in CE are compatible with biomolecules. The small diffusion coefficients which are characteristic of macromolecules result in a poor mass transfer and thus a low efficiency in RPLC. Conversely, the same properties are advantageous in CE separations since contributions of solute diffusion to zone broadening are minimal.

Efficiency and reproducibility of protein separations in CE may be severely compromised, however, due to coulombic interactions between the protein and the walls of the capillary. Clearly the absorption of material to the walls is detrimental to quantitation, especially in cases dealing with low concentrations. The nonuniform adsorption of material on the capillary surface results in irregularities in the zeta potential across the length of the capillary. Local variations in electroosmotic flow within the capillary can lead to asymmetrical peaks (25). The adsorption of proteins onto the surface will influence the zeta potential and thus the electroosmotic flow will be variable within a given separation and in subsequent runs. The obvious result being irreproducible migration times.

Efficient protein separations in untreated fused silica capillaries have been possible in some instances by manipulation of the pH. Two strategies exist for minimizing the electrostatic interactions of the protein with the bare silica surface. The pH of the running buffer may be adjusted well above the p*I* of the protein (31). Under these conditions the protein will have a net negative charge, which will be repelled by the negatively charged surface of the capillary (11,32–34). The other alternative is to operate at a low pH at which all the surface silanols are protonated and the interaction of the positively charged protein with the surface is negligible (11,35,36). In situations where there is a wide range of p*I* values for the proteins in a mixture, difficulties may arise when determining a suitable pH to provide the required selectivity. Under these conditions elution using a dynamic change of pH in a stepwise manner may be feasible in order to resolve the analytes completely (37). Hydrophobicity has also been shown to influence selectivity through surface interactions (38), although pH is undoubtedly the most influential parameter.

Although satisfactory separations have been performed using uncoated capillaries, a number of limitations are imposed by the extreme pH conditions required. At low pH values the majority of proteins are fully protonated, thereby removing the differences in charge needed for resolution. At high pH values the large electroosmotic flow sacrifices resolution. The physical stability of the proteins at the pH extremes must also be questioned since protein aggregation and precipitation at low pH values may be problematic (39). Conformational changes at pH values far removed from the p*I* of the protein can expose inner hydrophobic residues, which may exacerbate aggregation/precipitation as well as the adsorption problem and therefore must be considered as well (40). Since the maximum stability of proteins tends to be around physiological pH, separations in the neutral region in the absence of adsorption would be preferable but not essential. The use of the entire pH range to affect protein charge without changing the electroosmotic flow would be ideal to maximize separation selectivity.

Attempts to minimize protein adsorption through the use of high-ionic strengths buffers and various additives have met with some success. Green and Jorgenson (41) employed large concentrations of alkali metal salts to compete for cation exchange sites on the silica surface. Encouraging results were observed in the presence of high concentrations of sodium and potassium, while minimal success was obtained with lithium salts. Since the conductivity of the buffer is increased at high ionic strength, operation using large voltages is not possible due to joule heating. The use of high ionic strengths aided in the prevention of adsorption, unfortunately at the expense of long analysis times. To avoid the long analysis times encountered with high ionic strengths, nonconducting zwitterionic buffers have been used to minimize both surface adsorption and protein–protein interactions (42). The combination of ionic salts and zwitterions in buffers gave efficient separations in a reasonable time frame. Separations of basic proteins at or below their p*I* values have been successful at low ionic strengths in the presence of a cationic fluorosurfactant (43). The addition of the fluorosurfactant resulted in a charge reversal on the surface of the capillary, thereby repelling the positively charged proteins. Polymeric additives (28), putrescine (44), and ethylene glycol (45) have also been included in sample matrices to impede protein adsorption.

Peak deformities of proteins may also be caused by field perturbations within the capillary. An example of poor peak shape caused by the high salt concentration within a protein sample matrix has been demonstrated (46). Experiments should be designed with buffer to sample concentration ratios in excess of 200 to avoid local changes in the conductivity and hence perturbations (3,31).

CE Using Surface-Modified Capillaries

An alternative strategy to prevent protein adsorption to

capillary walls is the incorporation of surface-modified capillaries. These efforts have also been focused on improving the reproducibility of analyte migration times. Various workers advocate significant reduction or elimination of electroosmotic flow to impart greater reproducibility since separations would then be based solely on the differential electrophoretic mobilities (36,47,48). Factors which would normally alter the electroosmotic flow by changing the zeta potential would no longer result in variable migration behavior. The elimination of electroosmotic flow is also beneficial to the improvement of resolving power over a wide range of pH values, however, such modification would preclude the option of performing anion and cation analysis in a single run. The ideal criteria when considering capillary coatings are the prevention of adsorption and other interactions of proteins/analytes with the capillary surface. The surface coating should also possess long-term stability over a wide pH range and yield reproducible separations.

The general approach has been to coat surfaces with a monolayer of a hydrophilic material to prevent sterically the interaction of the proteins with the silica surface. Table I lists some surface coatings which have been reported in the literature. In most cases the severity of the adsorption problem has been greatly reduced through the use of modified capillaries. However, the number of theoretical plates obtained were generally far fewer than the predicted values, suggesting that interactions are still present to some extent. The residual interactions may be coulombic due to insufficient coverage of the silica surface or hydrophobic in nature due to the interaction of the protein with the neutral coating. Observations regarding the nonlinear relationship of plate count versus voltage may be indicative of thermal effects. Moreover, the lower efficiency at higher voltages could possibly be correlated with the increased hydrophobic interactions as the temperature in the capillary rises (48).

The area of capillary coatings exhibits great potential in the separation of proteins and continues to expand. Investigations probing capillary surfaces which show increased stability and reproducibility as well as utility over a broad array of separation environments are ongoing.

Applications

Structure and purity confirmation is essential for the approval of proteins that are to be used as pharmaceuticals. The integrity of the protein as a bulk substance and in the final formulation must be evaluated. Numerous degradative processes, such as deamidation and oxidation/reduction, can lead to products with slight changes in conformation. For example, the introduction of a disulfide bridge upon oxidation can result in subtle conformational differences between the parent compound and the oxidized product giving indistinguishable peaks by chromatographic techniques. Grossman *et al.* have shown sequential electropherograms depicting the reduction of a peptide to give distinctly different peaks for the oxidized and reduced forms (11). Slight variations in primary and secondary structure also pose challenges in the assessment of purity. Synthetic peptides which may differ by only one amino acid residue may result in a single peak in HPLC but can actually be separated into multiple peaks by capillary electrophoresis (11). On the contrary, poorly resolved peaks in capillary electrophoresis may be completely separated by HPLC, clearly demonstrating the utility of using both techniques to confirm results. Purity control, not only subsequent to synthesis, but also throughout the synthetic and purification process, is useful in optimizing the conditions for the procedure (57). While capillary electrophoresis has proven to be an important tool in the qualitative evaluation of protein purity, the feasibility of the technique for quantitative aspects has also been demonstrated in assays deeming protein purity (11).

Table I. Capillary Surface Coatings

Coating	pH range	Ref. no.(s)
Polyacrylamide	2–10.5	25,39,48
Methylcellulose	8–9	25
Polyethylene glycol	3–5	40
Carbohydrates	3–7	49
Aryl pentafluoro group	7	50
C-8, C-18, hydrophilic coating	6–9.3	51
Poly(methylglutamate)	7	47
Glycol phases	7	5
Trimethylchlorosilane	7	52
Poly(vinylpyrrolidinone)	2	36
Polymethylsiloxane	7	53
Polyethyleneimine	2–12	54
Hydroxylated polyether functions	4–7.5	55
Nonionic surfactants	4–11	56

The structural identification and purity analysis of peptides/proteins are typically done by "fingerprinting." The protein is cleaved by enzymatic digests to give fragments characteristic of that particular macromolecule, which are then sequenced. Capillary electrophoresis has shown potential in the separation of these fragments, for example, shorter analysis times with increased resolution of fragments were seen from the digestion of ^{14}C-labeled carboxymethylated insulin B chain (35). Grossman *et al.* have also shown the utility of capillary electrophoresis prior to the sequencing step in order to screen the purity of fragments collected from an HPLC separation. Capillary electrophoresis has also been used in a preparative role, with fractions taken from four or five duplicate injections providing enough material for amino acid sequence analysis (35). Table II shows a comprehensive listing of references regarding applications of capillary electrophoresis to proteins and other miscellaneous references dealing with protein separations.

CHIRAL SEPARATIONS

Racemates of chiral pharmaceuticals often possess distinctly different characteristics when it comes to efficacy, toxicity, and pharmacokinetic properties. The decision of whether to develop a drug as a pure stereoisomer or as the racemic mixture is made by the pharmaceutical manufacturer after weighing factors such as the ease and cost of the large-scale synthesis of the pure stereoisomer, as well as the therapeutic/pharmacologic and toxicological characteristics of the racemic mixture. Many pharmaceuticals are currently administered as racemates, however, the tendency of pharmaceutical companies toward minimizing risk may result in the increased utilization of new drug candidates as a single isomer (89).

Table II. Applications of Capillary Electrophoresis to Proteins

Application	Analyte	Mode	Detection	Ref. no.
Amino acid sequencing	Various polypeptides, proteins	HPCE,[a] CITP[b]	Mass spectrometry	58
Amino acid sequencing, preparative	Various synthetic peptides	HPCE	UV	35
Conformation/binding studies	Calcium/zinc binding proteins	HPCE	UV	59
Microheterogeneity analysis	Monoclonal antibodies	HPCE	UV	60
Molecular weight determinations	Myoglobin fragments, lactoglobulins, pepsin, trypsinogen, various proteins	CE (SDS-gel)	UV	61
Optimization of separation conditions	Collagen (types I, II, IV, V, XI)	HPCE	UV	62
Peptide mapping	β -Casein	HPCE, HPLC	UV	63
	Biosynthetic human growth hormone	HPCE, HPLC	UV	64
	Cytochrome *c*, various proteins	HPCE	UV	65
	Human growth hormone	HPCE	UV	66
	β-Casein, serum proteins	HPCE	Indirect fluorescence	67
	Cytochrome *c*, various peptides	MEC, cyclodextrins	UV, fluorescence	22
	ACTH, enkephalins	HPCE, HPLC	Mass spectrometry	68
Prediction of electrophoretic migration	ACTH-related fragments	HPCE, MEC, HPLC	UV	69
Preparative	Nucleotides, transferrin	CITP	Immunological, radioactivity	70
Preparative, protein characterization	Oligonucleotides, Met-human growth hormone	CE (PAGE)	UV	71
Protein characterization	Recombinant insulin-like growth factor and hirudin	HPCE	Fluorescence	72
	Recombinant human growth hormone	HPCE, HPLC	UV	73
	Recombinant interleukin-3	HPCE	UV	74
	Recombinant growth hormone, tissue plasminogen activator	HPCE	UV	75
	Glycoproteins	HPCE	UV	76
	ACTH	HPCE, HPLC	UV	77
	Recombinant human growth hormone	HPCE	Mass spectrometry	78
	Synthetic peptide mixture, human hemoglobin	HPCE	Mass spectrometry	79
	Human hemoglobin	HPCE	Mass spectrometry	80
	Insulin, human growth hormone	HPCE, PAGE, HPLC	UV	81
Purity and structural confirmation	N-protected peptides	CITP, HPLC	UV	82
Purity confirmation	L-Histidyl-L-phenylalanine	HPCE, CITP	UV	83
	Glutamine-containing peptides	CITP	UV	57
	Oligo- and polypeptides	CITP	UV	84
	Various synthetic peptides	CITP	Concentration gradient	85
	Various recombinant biotechnology proteins	CE (SDS-PAGE)	UV	86
Purity confirmation, protein characterization	Glycoproteins, endorphins, biosynthetic human growth hormone, and insulin	HPCE	UV	11
Quantification in injectable dosage form	Recombinant cytokine	HPCE	UV	87
Separation of sulfonated and nonsulfonated forms	Leu-enkephalin, cholecystokinin octapeptide, hirudin	HPCE, HPLC	UV	88

[a] High-performance capillary electrophoresis (uncoated capillary).
[b] Capillary isotachophoresis.

Diastereomers possess distinct physicochemical properties leading to differing extents of interactions with stationary phases in HPLC and therefore are easily separable. Conversely, enantiomers differ only in the direction in which they rotate plane polarized light, which does not provide an adequate mechanism for separation in an achiral environment, whether it be in HPLC or CE. General approaches to chiral separations in HPLC are the derivatization of the chi-

ral analyte with a chiral reagent to form diastereomers, the addition of chiral additives to the mobile phase, and the use of chiral stationary phases. The types of variables available for manipulation in capillary electrophoresis in order to effect isomeric separations are different from those in HPLC. Interactions with stationary phases which would provide diastereomeric selectivity in HPLC are absent in capillary electrophoresis. Therefore alternative interactions which are capable of affording isomeric selectivity in capillary electrophoresis must be considered.

Many reports exist on the separation of DL-amino acids which may in turn be extended to peptide analysis. Since isomerization of amino acids within peptides may occur during or subsequent to the synthetic process, the separation of amino acid diastereomers is of practical importance in the area of controlling peptide purity. Furthermore, monitoring the conversion of L- to D-amino acids is potentially useful in following degradative processes in peptides. For example, deamidation of glutamine and/or asparagine residues results in a cyclic imide intermediate. The intermediate undergoes hydrolysis at one of two potential sites to produce either the D or the L form at the chiral center (90).

Various chiral additives have been utilized in capillary electrophoresis to gain enantioselectivity (Table III). The addition of cyclodextrins (CD) or modified cyclodextrins to the running buffer has been used extensively in chiral separations. The chiral selectivity via CDs originates from the chirality of the glucose units composing the cyclodextrin. Differential hydrogen bonding of the analytes with the hydroxyl groups at the outer rim of the cyclodextrin accounts for the selectivity (93). Mixed micelle systems have also been successful in the chiral recognition of substrates. Bile salts, amino acids, and nonionic chiral surfactants have been used in conjunction with SDS to obtain chiral selectivity of amino acids and pharmaceuticals based on contrasting solubilization of enantiomers within the micelle. Bile salts differ from long-chain alkyl surfactants in that they form micelles composed of approximately 10 monomers through the interaction of the "nonpolar faces" of the molecules (97). Joule heating may be problematic with the use of bile salts since large concentrations are required to reach the critical micelle concentration. Optically active amino acids or other chiral surfactants can be embedded in the SDS micelles to give differing binding affinities with racemic analytes (99). Diastereomeric interactions of enantiomers induced by exchange with auxiliary ligands of various Cu(II) complexes have also been effective in obtaining enantiomeric separations (94). The mechanism affording stereoselectivity arises from the differential stability of the ternary complex of the analyte with the Cu(II) complex. The formation of diastereomers through the use of the Cu(II) complex requires that the auxiliary ligand used to form the complex be optically pure. At neutral pH values, the ternary copper complex exhibits a positive charge, therefore the enantiomer which is more strongly associated with the copper complex will migrate at a faster velocity. In addition, copper complexes in the presence of SDS have been employed in the separation of derivatized amino acids (71). The complexation resulting in stereoselectivity in this case is believed to occur at the surface of the micelle.

The use of capillary electrophoresis in the separation of racemic mixtures appears quite attractive due to the rapid analysis times and efficiencies of the order of 1 million theoretical plates (93). Capillary electrophoresis offers advantages over current HPLC methods with respect to the lower consumption of expensive chiral reagents and the absence of costly chiral stationary phases. However, the choices available to achieve chiral separations in capillary electrophoresis are limited and more work is required to expand the technique.

SMALL-MOLECULE APPLICATIONS

The increasing complexity of pharmaceutical molecules

Table III. Chiral Separations of Pharmaceutical Molecules Utilizing Capillary Electrophoresis

Analyte	Chiral additive(s)	Mode of CE	Detection	Ref. no.
Chloramphenicol, ketotifen, thioridazine	Various CDs, heptakis(2,6-di-*O*-methyl)-β-CD	HPCE	UV	91
DL-Amino acids and peptides	DL-Marfey's reagents	HPCE, MEC	UV	90
Dansylated DL-amino acids	Bile salts/SDS	MEC	UV	92
	β-CD in PAGE	CE (PAGE)	UV	93
	Copper(II)–aspartame	HPCE	Fluorescence	94
	Copper(II)–L-histidine	HPCE	Fluorescence	95
Dansylated DL-amino acids, barbituates	Various CDs, methylated CDs	HPCE	UV	96
Diltiazem HCl, trimetoquinol HCl, and related compounds	Bile salts/SDS	MEC	UV	97
DL-Amino acid derivatives	β,γ-CD/SDS	MEC	Fluorescence	98
	N-Dodecanoyl-L-amino acids/SDS	MEC	UV	99
Phenylthiohhydantoin-amino acids	Digitonin/SDS, *N*-dodecanoyl-L-valinate	MEC	UV	100
Sympathomimetic drugs	β-CD,di-OMe-β-CD, tri-OMe-β-CD	HPCE	UV	101
Terbutaline and propranolol enantiomers	β-CD, heptakis(2,6-di-*O*-methyl)-β-CD	HPCE	UV	102

and formulation matrices poses a challenge in the area of analytical methods development. The success of HPLC in pharmaceutical applications may be accredited to the variety of formats available (reversed phase, ion exchange, etc.). However, the analysis of pharmaceutical preparations by HPLC often demands extensive sample preparation which consumes both time and resources. Capillary electrophoresis has demonstrated not only rapid analysis times but also minimal manipulation requirements for samples prior to injection (103). However, the simplicity of sample preparation in CE should not be overstated since limitations with respect to the compatibility of the injection matrix with the background electrolyte must be considered in each case. Furthermore, the lack of validated methods attests to the inexperience in this area and the hurdles left to overcome.

The same issues regarding chemical identification, purity, and structural confirmation which were alluded to earlier for protein analysis in the pharmaceutical industry may also be applied to small-molecule pharmaceuticals. Degradation of drugs in the dosage form as well as during storage is also of interest. The detection of minute amounts of closely related impurities in the presence of a large excess of parent drug requires separation techniques which are capable of resolving peaks with very high efficiencies. Although capillary electrophoresis may provide the theoretical plates required for the separation, the lack of sensitivity can be a major problem in the detection of trace amounts of impurities. The detection of minor components in matrices is complicated by the inability to inject large amounts of material without overloading the column thereby sacrificing resolution. The confidence of results in the pharmaceutical setting are often increased by using two or more techniques to verify results. Since HPLC and capillary electrophoresis are essentially orthogonal separation techniques owing to different mechanisms of selectivity, the combination of the two methods can provide data which are very informative. Table IV lists some applications of capillary electrophoresis to small molecule pharmaceuticals and excipients in various matrices.

DETECTION

The small dimensions of capillaries have pushed detection requirements to the extreme lower limits of current capabilities. The challenge in this area has attracted much attention and a brief discussion dedicated to this aspect of analysis is warranted. The small volumes associated with capillary electrophoresis translate to very little analyte on-column, which gives this technique the potential for a high mass sensitivity. Conversely, small volumes impose tight restrictions on the size of the detector flow cell since resolution acquired in the separation will quickly diminish due to extracolumn effects.

The process of "stacking" can be used for concentration enrichment preceding separation in the analysis of dilute samples without compromising resolution. Aebersold and Morrison were able to determine peptide levels in the 1–2 ng/μl range, which made possible the direct analysis of fractions collected from HPLC for purity assessment before subjecting the samples to amino acid sequencing (135). The process of zone compression, or "stacking," used in the analysis of the peptides is illustrated in Fig. 6. The first step involves the loading of a basic sample containing negatively charged peptides, by means of a pressure gradient, into the capillary containing a low-pH buffer. Upon the application of a potential gradient, the negatively charged peptides will migrate toward the anode and become electrophoretically stacked at the buffer/sample interface. The pH step gradient initially formed will disseminate and the peptides will take on a positive charge, thereby changing direction, and proceed toward the cathode. Through the use of the stacking process, the concentrations that could be analyzed were five times lower than those typically done using the conventional mode of operation. Alternatively, the same concentration enhancement has been observed by injecting a sample which has a lower conductivity than that of the background electrolyte (113).

The following discussion is limited to the more commonly used modes of detection. A more descriptive and comprehensive overview of detection methods used in capillary electrophoresis and the limits of detection associated with each method are given in several reviews (136,137). Table V lists some references which focus on detector development and application of the various detection systems to the analysis of pharmaceutically relevant molecules.

Spectrophotometric Methods

A simple way to prevent the loss of resolution due to extracolumn effects in capillary electrophoresis is to utilize the capillary itself as a flow cell. A detection "window" can easily be incorporated by removing a 1- to 2-cm region of the polyimide coating and then wiping the area clean. The coating may be burned off by means of a flame or chemical methods (hot concentrated sulfuric acid), the latter of which affords a window without loss of capillary flexibility. Detection windows as small as 1 mm have been produced using a heated filament while maintaining the flexibility of the capillary (153). Spectrophotometric methods such as fluorescence and UV are amenable to on-line detection in capillary electrophoresis and therefore are frequently used. Although UV detection is probably the most commonly used, detection sensitivity suffers with the use of capillary columns due to the small path length of the flow cell. In general, the lower concentration limit using UV is approximately 10^{-5} to 10^{-6} *M*. Fluorescence affords greater detection sensitivity and selectivity since most molecules are not inherently fluorescent. The sensitivity of fluorescence detection is anywhere from 100- to 1000-fold greater than UV due to the dark background. In situations where the analyte does not fluoresce, selective tagging of the analyte with a chromophore can provide the detection handle needed (98,143,154). Derivatization has been performed in a pre-, on-, or postcolumn fashion (10,155,156), but derivatization is highly analyte dependent and therefore is not a general solution. Fluorescence detection has also been utilized in the analysis of chiral molecules via fluorescence-detected circular dichroism (157). Indirect fluorescence detection provides an alternative to derivatization for nonfluorescent analytes (141). A fluorescent electrolyte is included in the running buffer and detection of zones is accomplished through the displacement of or interaction with the background chromophore. The nonspec-

Table IV. Small-Molecule Applications of Capillary Electrophoresis

Analyte	Matrix or application	Mode	Detection	Ref. no.
Analgesics	Standard solutions	HPCE, MEC	UV	104
Antibiotics	Tablet and standard solutions	HPCE	UV (photodiode array)	105
Antibiotics (β-lactam)	Chemical and pharmaceutical preparations	CITP	UV	106
Antibiotics (penicillin, cephalosporins)	Standard solutions	MEC with tetraalkylammonium salts	UV	107
β-Phenylalkylamines	Pharmaceutical preparations	CITP	Conductivity	108
Barbiturates	Human serum and urine	MEC	Multiwavelength detection	109
Benzylpenicillin	Tablet and injectable forms	HPCE	UV	110
Cefpiramide	Human plasma	MEC	UV	111
Cefuroxime axetil and cefuroxime	Pharmaceutical preparations	MEC	UV	103
Cimetidine	Tablet, liquid, and injectable preparations	HPCE	UV	112
Cytosine-β-D-arabinoside	Plasma	HPCE	UV	113
DNA sequencing	DNA fragments from DNA sequencing reactions	CE(GEL)	Fluorescence	114
Flavonoids	Plant extracts	CITP	Conductivity, differential conductivity	115
Flavonol-3-*O*-glycosides	Plant extracts	MEC	UV	116
Ingredients of antipyretic analgesic preparations	Commercial tablet	MEC	UV	117
Methotrexate and major metabolite	Serum	HPCE	Fluorescence	118
Minoxidil	Compressed tablets and topical solutions	CITP	UV	119
Monoclonal antibodies	Micropreparative	HPCE	UV, fluorescence	120
Nucleic acid constituents (modified)	Standard solutions	MEC	UV	121
Nucleosides, bases, and oligonucleotides	Standard solutions	MEC and metal additives	UV	122
Nucleotides	Blood, liver, and kidney	HPCE with ethylene glycol	UV	123
Oligonucleotides	Standard solutions	MEC, HPCE with buffer additives	UV	124
Penicillin antibiotics	Standard solutions	MEC	UV	104
Quinine, quinacrine, and proflavine	Standard solutions	HPCE	Fluorescence	125
Purines	Standard solutions	MEC	UV	126
Ranitidine (zantac)	Pharmaceutical preparations	HPCE	UV	103
Riboflavin-5′-phosphate	Purity control of chemicals	HPCE	Fluorescence	127
Thiamine, rubidium, sodium, and calcium	Eyedrop formulation	CITP	Conductivity	128
Tricyclic antidepressants	Standard solutions	HPCE	UV	129
Various drugs	Solutions for intravenous injection	CITP	UV	130
Vitamers (B6)	Urine	MEC	Fluorescence	131
Vitamin metabolites, derivatized amino acids	Whole blood	MEC	UV, fluorescence	132
Vitamins (water- and fat-soluble)	Standard solutions	MEC	UV	133
Vitamins (water-soluble)	Standard solutions	MEC (tetraalkylammonium salts)	UV	107
	Standard solutions	MEC	UV	104
	Standard slutions	MEC	UV	134

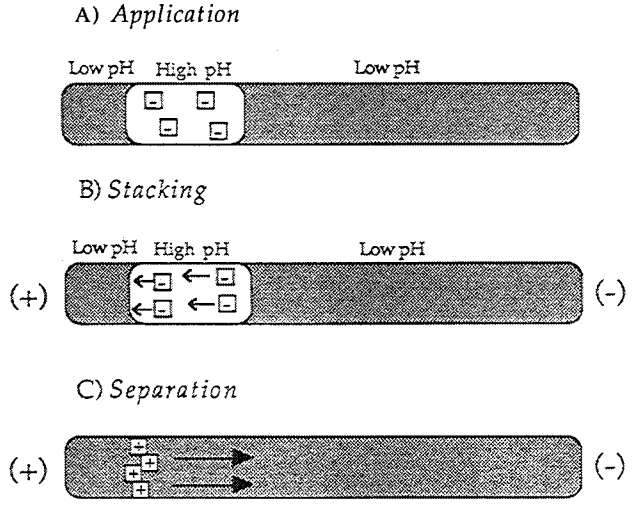

Fig. 6. Stacking process in capillary electrophoresis. (A) High-pH sample is loaded onto the capillary using a pressure gradient. (B) Zone compression step, in which analytes are electrophoretically stacked at the sample/buffer interface. (C) Species in concentrated zone acquire a positive charge and reverse the direction of migration for the separation process.

ificity of the method precludes any detection selectivity. Laser-based excitation has been used in both direct and indirect (140) fluorescence. Laser sources are ideal for use with capillaries since the monochromatic beam can be focused onto a very small area on the capillary. The beam is collimated and introduces an intense source of radiation. Sensitivity can be substantially improved since the fluorescence signal is directly proportional to the incidence radiation. Unfortunately, lasers are available only for selected wavelengths, which restricts the number of analytes which can be excited by the source. Helium–cadmium lasers are routinely used for LIF detection but supply lines only at 325 and 442 nm, whereas the lines frequently used on an argon ion laser are 458, 488, and 514 nm. A helium–neon source, which emits incidence radiation in the red at 633 nm, has been utilized in capillary electrophoresis for laser-based refractive index detection in the determination of underivatized carbohydrates (158).

Electrochemical Detection

The measurement of solution conductivity is a general means of detection which is nonspecific. The signal is generated from the difference between the conductivity of the background electrolyte and that of the analyte/electrolyte zone. The nonspecificity makes it a universal detection method, however, the sensitivity is not exceptionally high (10^{-6} *M*) (159). Huang *et al.* have adapted an on-line conductivity detector for capillary electrophoresis by positioning the electrodes in laser-drilled holes on either side of the capillary (160).

Oxidative electrochemical detection is much more specific than conductivity because not all molecules are electroactive. The interfacing of electrochemical detection with capillary electrophoresis is complicated by the presence of the high electrical field associated with the method. Wallingford and Ewing (139) designed an off-column configuration for the analysis of catechol and catecholamines in which the detector is isolated from the current in the capillary. The same off-column electrochemical sensor was used with a 9-μm capillary to give a greater mass sensitivity, with a detection limit of 4.0×10^{-8} *M* (138). More recently, another electrochemical detector design has been described which employs an oncolumn nafion joint to isolate the detector from the applied voltage (161).

Mass Spectrometry

Qualitative information regarding the structural characterization and identity of analytes is invaluable. Mass spectrometry is an information rich method of detection with high sensitivity capabilities. Capillary electrophoresis has successfully been coupled to mass spectrometry through an electrospray ionization (ESI) interface and applied to the analysis of enkephalins and polypeptides (162). Typical limits of detection were found to be in the femtomole range. Steps have been taken to improve the interface by using a

Table V. Detection in Capillary Electrophoresis

Detection	Analyte	Ref. no.(s)
Electrochemical	Serotonin and dopamine	138
	Catechol and catecholamines	139
Indirect fluorescence	Native amino acids	140
	Nucleotides, nucleosides, and lysozyme	141
Laser-induced fluorescence		
Precolumn derivatization	Primary amines	142, 143
On-column derivatization	Conalbumin, bovine serum albumin, and β-lactoglobins	144
MS, coaxial CF-FAB	Chemotactic peptides	145
	Neuropeptides	146
MS, coaxial CF-FAB with tandem MS	Various peptides and angiotensins	147
	Morphiceptin, proctolin, and various peptides	145
MS, electrospray ionization	Nucleotides, nucleotide coenzymes, various bioactive oligopeptides	148
	Cytochrome *c*, myoglobin, and other oligopeptides	149
	Various small peptides/proteins (insulin, bradykinin, myoglobin, etc.)	150
MS, ion spray tandom MS	Dynorphins and leucine enkephalin	151
UV, diode array	Water-soluble vitamins	152

sheath flow of liquid at the capillary terminus to minimize the dead volume (163).

Mass spectrometry and tandem MS (MS-MS) have been utilized for labile biomolecules using a coaxial continuous-flow fast atom bombardment interface with capillary electrophoresis (147). Separation efficiencies for some smaller peptides were over 500,000 plates. The utility of the system was also demonstrated for larger peptides such as angiotensin I and *N*-acetylangiotensin I.

CONCLUSIONS

The versatility of capillary electrophoresis in the analysis of a wide array of pharmaceutically relevant analytes varying in polarity, size, and stereochemistry has been represented through the numerous accounts available in the literature. The future role of CE in the area of drug analysis was evaluated by Steuer *et al.* and the following conclusions were made (164). The high efficiencies obtained in CE are well suited for complex mixtures in which resolution of a large number of peaks in a short analysis time is desirable. The dimensions of the capillary and the small loading capacity potentially create an arena for high mass sensitivity. However, in drug analysis the emphasis is often placed on concentration rather than mass sensitivity. Advancements in detection systems tailored for capillary electrophoresis, such as laser-induced fluorescence and electrochemical detection, make low concentration sensitivity possible but limit the general applicability. Over the course of the development of capillary electrophoresis, a number of parameters available for manipulation of selectivity have been exploited (pH, buffer species and concentration, surfactants, cyclodextrins, etc.), however, the variable showing the greatest overall effect on selectivity is the pH, which influences the ionization of the surface silanols and/or the analyte.

The general consensus among separation scientists seems to be that capillary electrophoresis will not replace existing separation techniques but, instead, take on a complementary role. Capillary electrophoresis may be used independently of HPLC (165) or in tandem with HPLC as in the case of two-dimensional systems for the analysis of complex protein mixtures (166,167).

The widespread acceptance of any analytical technique depends not only on the attributes of the method but also on the availability of automated instrumentation. Although great strides have been made in the automation of capillary electrophoresis, commercial systems are still first-generation instruments, with improvements in hardware, such as detectors, injectors, and fraction collectors, likely to appear in the foreseeable future. Advancements are being made in column technology in the area of surface coatings in order to produce stable surfaces that provide reproducible results. Different capillary geometries are being investigated in order to improve the surface/volume ratio to effect heat dissipation and to enhance sensitivity by increasing the path length of the flow cell. Lauer and Ooms have provided a useful discussion on the future trends in capillary electrophoresis regarding aspects of separation and instrumentation (168).

Capillary electrophoresis has yet to reach the full stage of development as witnessed by the exponential rise in the number of journal publications over the last decade (Fig. 7). Currently there are very few validated methods which utilize CE, however, continued efforts aimed at improvements in separation capabilities, detection sensitivity, reliable quantitation, and commercial instrumentation will surely encourage the widespread acceptance of this technique in pharmaceutical analysis.

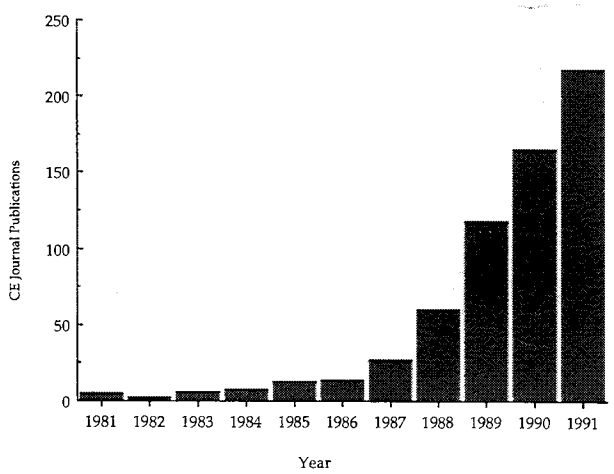

Fig. 7. Exponential growth in the number of journal publications featuring capillary electrophoresis over the last 10 years. Capillary isotachophoresis articles were excluded from the search.

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