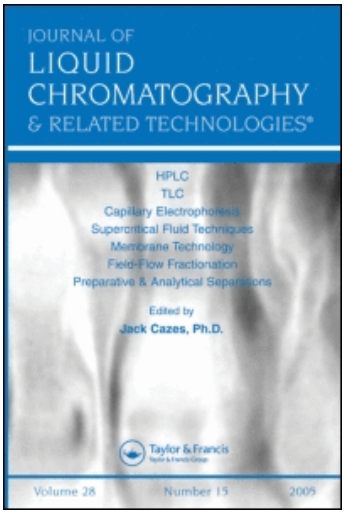

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Pharmaceutical Drug Separations by HPCE: Practical Guidelines

G. M. Mc Laughlin[a]; J. A. Nolan[a]; J. L. Lindahl[a]; R. H. Palmieri[a]; K. W. Anderson[a]; S. C. Morris[a]; J. A. Morrison[a]; T. J. Bronzert[a]

[a] Beckman Instruments, Inc., Somerset, New Jersey

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PHARMACEUTICAL DRUG SEPARATIONS BY HPCE: PRACTICAL GUIDELINES

G. M. MC LAUGHLIN, J. A. NOLAN, J. L. LINDAHL,
R. H. PALMIERI, K. W. ANDERSON, S. C. MORRIS,
J. A. MORRISON, AND T. J. BRONZERT
Beckman Instruments, Inc.
45 Belmont Drive
Somerset, New Jersey 08875

ABSTRACT

As governmental regulations become more encompassing and restrictive, it has become more important to develop high efficiency separation methods for pharmaceutical drugs. High Performance Capillary Electrophoresis (HPCE) is becoming accepted as a primary and complementary (with HPLC) separation technique. Researchers that have not previously used HPCE may not be familiar with the myriad of parameters that can be manipulated to achieve the desired efficiency, selectivity, or resolution. The purpose of this work is to develop some practical advice, guidelines, and limitations of HPCE in the separation of pharmaceutical drugs.

Several classes of pharmaceutical drugs are used to illustrate the physical and buffer parameters that can be manipulated. Some important physical parameters are discussed. Free solution concepts are illustrated using pH control, ionic strength, ion pairing reagents, and organic modifiers. Examples illustrate Micellar Electrokinetic Capillary Chromatography (MECC)-a tool for the separation of charged and uncharged species. The final major area of discussion is the separation of chiral drugs, where HPCE shows great promise. Use of cyclodextrins (natural/substituted, varying ring sizes, other buffer additives, etc.) is the primary focus.

Understanding the manipulative capabilities of these parameters offers almost unlimited potential in the separation of pharmaceutical drugs.

INTRODUCTION

High performance capillary electrophoresis (HPCE) is a new instrumental technique. It combines some of the most powerful aspects of traditional electrophoresis, chromatography, and capillary technology. HPCE appears to offer new alternatives for the separation of pharmaceutical drugs and related compounds. This study was initiated to illustrate some practical guidelines for developing methods for drug separations using HPCE.

Numerous authors have given overviews of the basics of HPCE, some equipment criteria, and the various modes [1-7]. We have attempted to give a more practical insight into several areas of HPCE. Fundamental concepts, equations, some operational modes, important physical parameters, methods development, peak identification, and quantitation concerns are discussed. The modes of HPCE discussed are free solution electrophoresis (FSE), micellar electrokinetic capillary chromatography (MECC), and the specialty separation of chiral compounds with cyclodextrins. Many different classes of drugs, pharmaceuticals, and related biomolecules are used for illustration.

Fundamental Concepts of HPCE

We have included a basic discussion of HPCE to give some insight into the subject, before delving into the application of HPCE for the separation of pharmaceuticals. An illustration of an HPCE system is shown in figure (1). The system can be as simple as a high voltage power supply connected to two buffer reservoirs that are bridged by a fused silica capillary. A section of the polyimide coating on the capillary is removed and the resultant "window" is carefully placed in a modified HPLC detector. Grounding connections, a few clamps, a plexiglass safety shield, and a strip chart recorder complete a basic unit. Sample

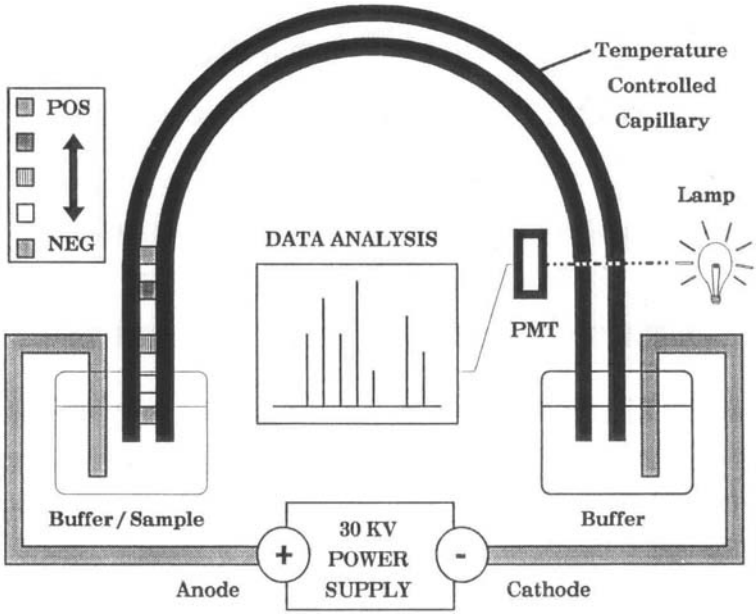


FIGURE 1 - Schematic representation of HPCE system.

introduction is accomplished by either hydrodynamic (pressure, vacuum, or gravity) or electrokinetic injection. Application of voltage starts the separation, which is monitored by the detector (e.g. UV/VIS, EC, fluorescence, radioactivity, conductivity, MS). The detector tracing, which looks like a chromatogram, is called an electropherogram.

In the FSE mode of HPCE, we can frequently take advantage of an electrophoresis phenomena known as electroosmotic flow (EOF). EOF can be introduced in a capillary at weakly acidic, neutral, and basic pH. The EOF increases as the pH increases [8]. A depiction of electroosmotic flow in fused silica capillaries is shown in figure (2). At any pH higher than weakly acidic (e.g. pH > 3) the silanols attached to the wall will have a negative charge. A

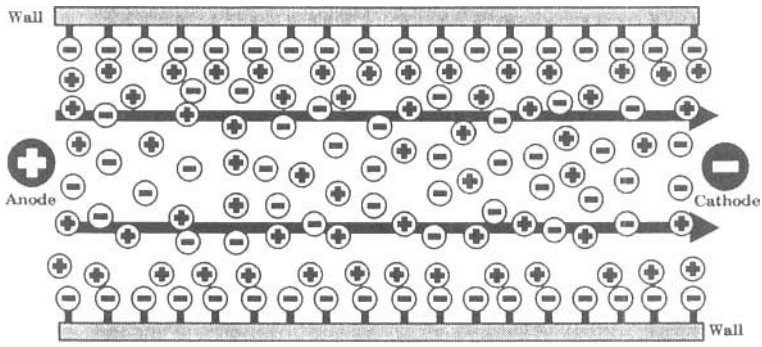


FIGURE 2 - Diagrammatic representation of electroosmotic flow in fused silica capillaries at elevated pH. Arrows indicate direction of EOF with normal polarity Negative charges attached to walls represent silanols and other positive/negative charges are from buffer ions.

double layer of positive ions is formed from the buffer ions. The inner layer will mostly be associated with the wall silanols. Some negatively charged silanols will still be available because of steric hinderance. A second layer of mobile positive charges attempts to reach those less inaccessible negative charges. When the voltage is applied, a strong flow (EOF) of the positively charged, mobile second layer migrates toward the negatively charged cathode. In most cases, the strong EOF flow is favorable and positive, neutral, and negatively charged species are swept by the detector in the same direction. The velocity of a any species will be the sum of its electrophoretic migration and EOF (Eq. 1). Positively charged solutes will move at the velocity of the EOF plus their attraction toward the cathode. Neutral solutes will move at the velocity of the EOF. Negatively charged solutes will move at the velocity of the EOF minus their attraction toward the anode. The arrows (Fig. 2) indicate direction of EOF with polarity in the normal, cathodic operating direction.

The electrophoresis term that relates velocity and voltage is mobility. Mobility relationships (for the solutes and bulk fluid movement), the effects of capillary dimensions, voltage, and time have been previously described by Lauer, et al [9] and Moring et al [10]. Equations (1-3) show the relationships for the apparent and EOF related mobilities. If a marker with a known mobility is coinjected, equation (4) can be used to determine the actual mobility (μ) of the solute. Other important factors that contribute to the electrophoretic behavior of a solute will be discussed later.

$$\mu_{app} = \mu + \mu_{eo} \qquad \text{(Eq. 1)}$$

$$\mu_{app} = \frac{V_{app}}{E} = \frac{L_d L_t}{t_{app} V} \qquad \text{(Eq. 2)}$$

$$\mu_{eo} = \frac{V_{eo}}{E} = \frac{L_d L_t}{t_{eo} V} \qquad \text{(Eq. 3)}$$

$$\mu = \left(\frac{L_d L_t}{V}\right)\left(\frac{1}{t_{app}} - \frac{1}{t_{ref}}\right) + \mu_{ref} \qquad \text{(Eq. 4)}$$

where:

μ_{eo} = electrophoretic mobility contribution from the electroosmotic flow (cm^2/V sec)

v_{eo} = electroosmotic velocity (cm/sec)

E = potential gradient (volts/cm)

L_d = distance from injector to the detector (cm)

L_t = total capillary length (cm)

t_{eo} = migration time of an electroosmotic flow marker (sec)

V = applied voltage (volts)

μ_{app} = apparent (or observed) solute electrophoretic mobility (includes EOF, if any) migrating in free solution (cm^2/V sec)

μ = solute electrophoretic mobility in free solution (cm^2/V sec)

t_{app} = apparent (or observed) migration time of solute (sec)

t_{ref} = apparent (or observed migration) time of a mobility marker (sec)

μ_{ref} = electrophoretic mobility of a mobility marker in free solution (cm^2/V sec)

v_{app} = apparent (or observed) solute velocity (cm/sec)

The reader is referred to the work of Wallingford [11], Beckers [12], Cohen [13], Jorgenson [14], Foret [5], and Hjerten [16] and coworkers for an in depth discussion of the theory of HPCE.

In some instances it is desirable to eliminate, control, or even, reverse EOF. Some EOF controlling conditions are addressed in the Results/Discussion section.

Neutral solutes will all migrate at the speed of the EOF in FSE and should not separate. Many mixtures will contain both charged and neutral solutes. Terabe and his coworkers [17,18] developed the technique of MECC to separate neutrals in the presence of charged solutes.

In MECC a surfactant such as sodium dodecyl sulfate (SDS) is added to the buffer at a concentration above its critical micelle concentration (CMC). A representation of how MECC works is shown in figure (3). It includes:

- EOF represented as a frontal zone at the right.
- SDS micelles forming and completed (center grouping).
- SDS molecules are represented by the oval (represents the hydrophilic charged sulphate group) and tentacles (dodecyl group-hydrophobic side chain).
- A representation of a neutral solute partitioning into and out of the micelle (thicker serpentine shaped objects).
- Mobility vectors:
    - μ_{Mic} = mobility of the negatively charged micelle toward anode

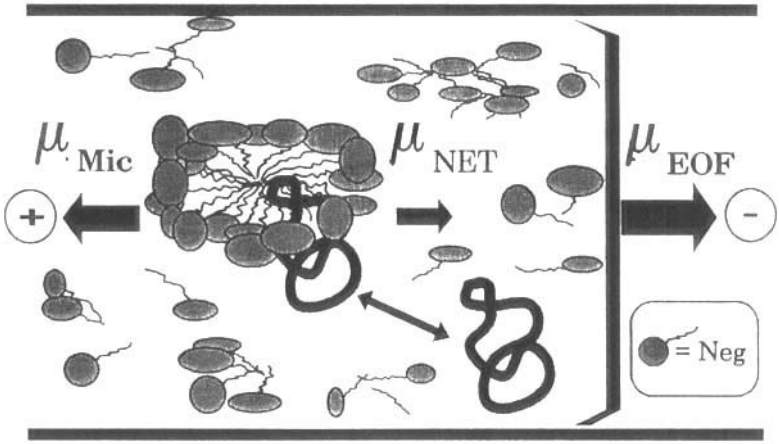


FIGURE 3 - Micellar Electrokinetic Capillary Chromatography schematic representation. Elliptical shaped objects represent sulphate moieties and attached winding lines indicate hydrophobic side chain of SDS molecule. SDS molecules are in various micellar formation stages. Thick serpentine shaped object is a molecule moving in and out of a formed micelle.

- μ_{EOF} = mobility of the electroosmotic flow
- μ_{NET} = net mobility toward cathode

The neutral solutes will partition between the micelle and the buffer depending upon their lipophilic nature. The more time a solute spends in the micelle, the later it will elute. When a completely incorporated into the micelle, all similarly affected solutes will elute as a single peak.

MECC is a good technique for the separation of neutral and charged species. It allows flexibility in altering the solute selectivity to help achieve separation. The reader is referred to the work of Wallingford [11], Terabe [17], Sepeniak

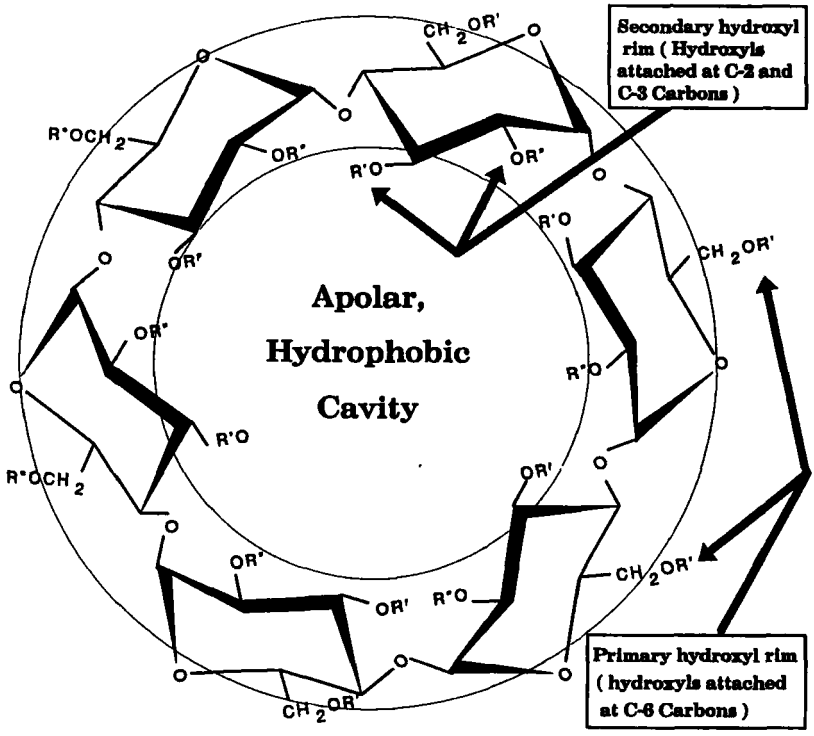


FIGURE 4 - Representation of "natural" α-cyclodextrin (R',R''=H) showing primary and secondary hydroxyl rings forming cyclodextrin complex.

[19], Fujiwara [20], Olechno [21], McLaughlin [22], and coworkers to acquire a better knowledge of MECC and its application to pharmaceuticals.

The last area discusses the separation of enantiomers with chiral additives-specifically cyclodextrin additives. Figure (4) is an illustration of a "natural" (R' and R'' are substituted with hydrogen) α-cyclodextrin showing the primary and secondary hydroxyl rings forming a cyclodextrin complex with a hydrophobic cavity. Other synthetic R group substitutions (e.g. methyl, ethyl, propyl,

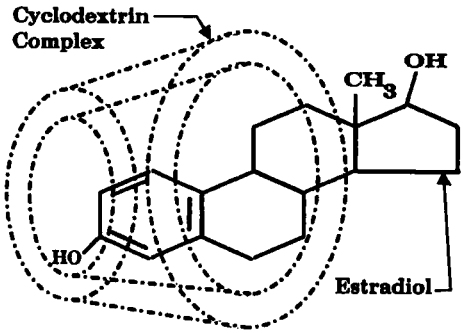


FIGURE 5 - Diagrammatic representation of estradiol inserting itself into the hydrophobic cavity of a cyclodextrin complex.

hydroxypropyl, glucosyl, and maltosyl) are used to affect the hydrophobic and steric nature of the cavity. CD's also vary in ring sizes depending upon the number of glucopyranose units combined to form a ring:

Type of Cyclodextrin	# of Glucopyranose Units in Ring
α	6
β	7
γ	8

When CD's are present some solutes will partition into the chiral hydrophobic cavity of the CD. Since CD's are electrically neutral and the outer surface is hydrophilic, no interaction with the buffer occurs. Therefore CD's migrate at the same velocity as the bulk solution. The differential migration of the solutes enantiomeric forms into the hydrophobic cavity result in a chiral separation. If the cavity is too large, or too small, the result will be no chiral recognition.

A representation of estradiol inserting itself into the hydrophobic cavity of a CD complex is seen in figure (5). The separation of chiral drugs using CD's is

an area, where HPCE shows the most promise. The reader is referred to the work of Terabe [23], Sepaniak [24], Altria [25], and Nolan [26] and coworkers to gain a better comprehension of the use of CD's for the separation of pharmaceutical enantiomers.

MATERIALS AND METHODS

The capillary electrophoresis instruments used in these studies were the PACE™ 2000 series (Beckman Instruments, Fullerton, CA) outfitted with PS2™ model 50, 50z, 55SX, or 70 computers (IBM). Some were model 2050's controlled with P/ACE software (version 2.0) operating under Windows™ version 3.0 (Microsoft Corp.). Most were model 2100's operating under the System Gold™ control, data acquisition, and analysis software (Beckman Instruments, Fullerton, CA). All data integration and mobility/area correction was performed using System Gold. All studies were performed in an automated fashion with minimum operator involvement.

We have attempted to produce figures that will visually illustrate an effect. All data was collected and translated into DIF or ASCII format using standard computer protocols. Computer programs used included: Lotus 123™ (Lotus Development), Excel™ (Microsoft Corp.), Harvard™Graphics (Software Publishing), Charisma ™ (Micrografx), and Word Perfect (Word Perfect Corporation). Overlay figures were made by adding a numerical offset to a whole data set for comparison.

Operating conditions are included with most figures. The 25μm, 50μm, and 75μm eCAP™ internal diameter fused silica capillaries of different lengths were installed in temperature control cartridges (Beckman Instruments, Fullerton, CA). Capillary nomenclature terms used are: outer diameter (od), inner diameter (id), total length (L_t), and effective length to the detector (L_d).

Most experiments used uncoated capillaries in the free solution (FSE) or MECC modes. The electrophoresis or HPLC grade buffers/chemicals employed were: common inorganics (H_3PO_4, NaH_2PO_4, Na_2HPO_4, and Na_3PO_4-all from J.T. Baker), HPLC grade solvents (CH_3CN and MeOH from J.T.Baker), ion pairing reagents (hexanesulfonic acid from Sigma Chemical), chiral additives (β-cyclodextrin and heptakis from Sigma Chemical) and HPCE grade chemicals (sodium borate, sodium dodecyl sulfate, sodium hydroxide, and urea from Beckman Instruments). A series of bioactive peptides used in several studies were acquired from Sigma Chemical (Saint Louis, MO). Peptide reference numbers, abbreviations, names, molecular weight (MW), and amino acid residues are:

1. BRAD - Bradykinin (MW=1060, 9 residues)
2. ANGII - Angiotensin II (MW=1046, 8 residues)
3. TRH - Thyrotropin Releasing Hormone (MW=362, 3 residues)
4. LHRH - Luteinizing Hormone Releasing Hormone (MW=1182, 10 residues)
5. BOMB - Bombesin (MW=1620, 14 residues)
6. LENK - Leucine Enkephalin (MW=556, 5 residues)
7. MENK - Methionine Enkephalin (MW=574, 5 residues)
8. OXYT - Oxytocin (MW=1007, 9 residues)
9. DYNO - Dynorphin (MW=2147, 17 residues)

Synthetic peptides, tryptic maps, nucleotides, vitamins, antipyretic analgesics, antihistamines, and other drug classes used in the FSE, MECC, and chiral HPCE experiments were kind gifts from researchers. They are identified on the figures and acknowledged.

RESULTS AND DISCUSSION

Many parameters are available to the analyst for HPCE method optimization. The following topics will be discussed in relationship to HPCE separations:

- Capillary dimensions
- Voltage
- Ionic strength
- Temperature
- Use of organic modifiers
- pH
- Use of ion pairing agents
- Use of cyclodextrins
- Use of other additives
- Types of injection
- Unattended methods development and peak identification
- Reproducibility

Capillary Length and Diameter

The interrelationships of capillary length, voltage, efficiency, and resolution have been given by Cohen, et al [13] and Karger, et al [27], as:

$$t = \frac{L_d L_t}{\mu_{ep} V} \quad or \quad V = \frac{L_d L_t}{\mu_{ep} t} \qquad \text{(Eq. 5)}$$

$$N = \frac{\mu_{ep} V}{2D} \qquad \text{(Eq. 6)}$$

$$R_S = \frac{\Delta\mu_{ep}\sqrt{V}}{4\sqrt{D\mu_{ep}}} \qquad \text{(Eq. 7)}$$

where t = time, μ_{ep} = solute electrophoretic ability, N = theoretical plate number, V = voltage, D = diffusion coefficient, and R_S = resolution.

The separation of enantiomers of carbinoxamine maleate on 27cm and 57cm long capillaries at nearly identical field strengths (Fig. 6) confirms the results expected (Eqs. 5,6). The theoretical plate count doubled as the capillary length was increased (2.11 fold). The resolution increase (1.41 fold) was consistent with the square root increase expected (Eq. 7). The increase in resolution, efficiency, and time (Eq. 5) can be seen in figure (7) which illustrates the effect of increasing capillary length on the separation of a tryptic digest of BSA. The field strengths are approximately equal and the lengths increased (2.11 fold). The longer capillary produced greater efficiency, increased resolution, and additional peaks.

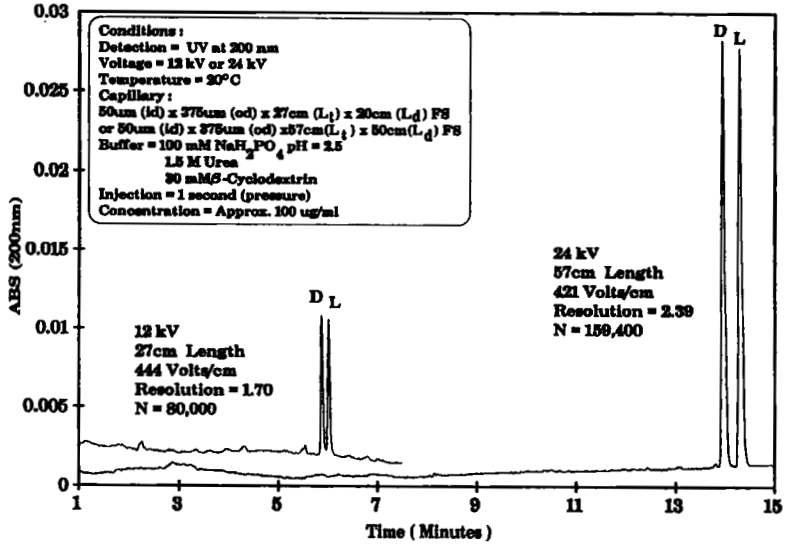


FIGURE 6 - Effect of capillary length on resolution, efficiency, and time during separation of enantiomers of carbinoxamine maleate.

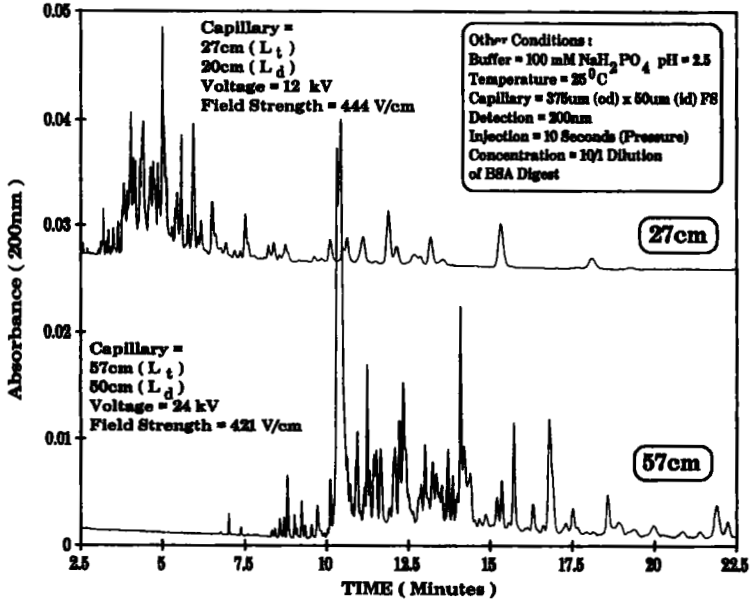


FIGURE 7 - Effect of capillary length on resolution and separation time of tryptic fragments of BSA. Field strengths are approximately equal.

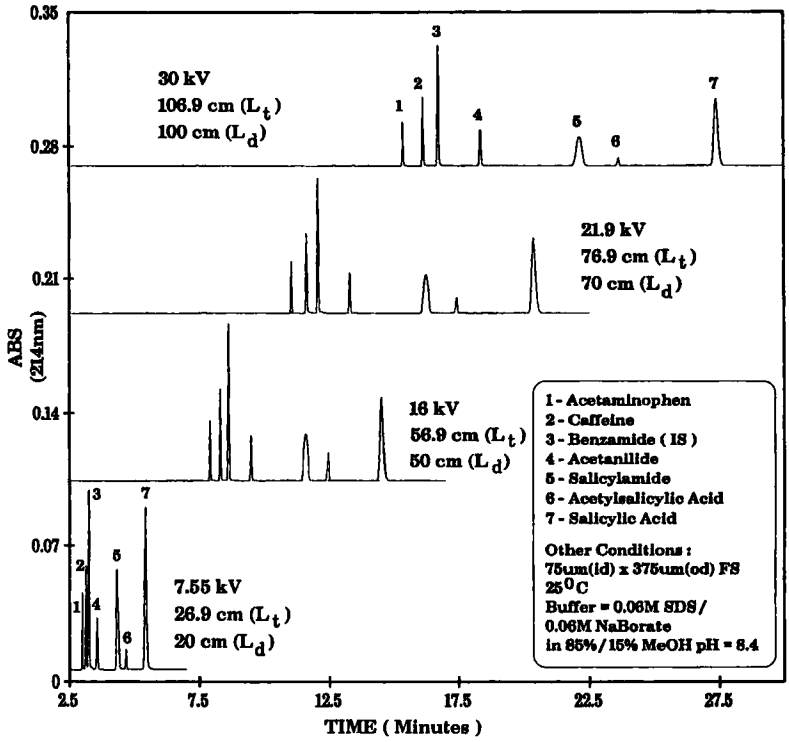


FIGURE 8 - Comparison of MECC separation of analgesic antipyretics using equal field strengths on different length capillaries. Inset equation shows relationship of time, length, and voltage between different experiments.

Figure (8) shows the MECC separation of analgesic antipyretics using equal field strengths on different length capillaries. Equation (8), below, allows convenient calculation of time, length, and voltage changes between experiments:

$$t_2 = t_1 \left(\frac{V_1}{L_{d1}\, L_{t1}}\right) \left(\frac{L_{d2}\, L_{t2}}{V_2}\right)$$

(Eq. 8)

where: t_1, V_1, L_{d1}, and L_{t1} = migration time, voltage, length to the detector, and total length for the first set of conditions, respectively. t_2, V_2, L_{d2}, and L_{t2} = migration time, voltage, length to the detector, and total length for the second set of conditions, respectively. Rearrangement of terms allows calculation of migration times, voltages, or capillary lengths for method transfer. This is only true for capillaries with identical diameters.

The main effects of increasing capillary length are:

- Longer migration times (time is directly proportional to total capillary length but distance to detector needs to be considered).
- Increased theoretical plates (directly proportional to capillary length to detector).
- Increased resolution (directly proportional to square root of length).
- Less heat is produced (current is inversely proportional to length) because resistance of the capillary is greater.

The main effects of increasing capillary diameter are:

- Increased heat (current is proportional to the diameter squared within the limits of Ohm's law) makes it more difficult to control temperature.
- Better mass loading (directly proportional to capillary length).
- Easier to align in detector window and better signal to noise (S/N) ratio.
- EOF decreases as diameter increases.

Other capillary dimension related conclusions are:

- Short capillaries are useful for: fast methods development, analysis of less complex mixtures, or when selectivity/migration times are excessive.

- Longer, or narrower, capillaries are useful for: high efficiency/resolution needs, complex mixtures, or when selectivity/migration times are inadequate.

Voltage Effects

Voltage control and maximization can result in analyses that are simultaneously faster, more efficient, and with better resolved solutes. Ohm's law, the relationship of voltage to current (power), and related factors are shown in equations (9-11) [11,13]:

$$V = IR \quad \text{(Eq. 9)}$$

$$W = VI = I^2R \quad \text{(Eq. 10)}$$

$$v_{eo} = \frac{e\zeta E}{4\pi\eta} = \mu_{eo}E \quad \text{(Eq. 11)}$$

where I = current, R = resistance, W = power, ϵ = dielectric constant, ζ = zeta potential, and η = viscosity.

As voltage is applied in a small capillary, internal heating of the buffer will occur due to resistance and viscous drag. The heating phenomenon, known as Joule heating, has been discussed by Cohen [13], Pospichal [15], and their coworkers. If the Joule heat produced is not removed, the internal capillary temperature will change. It is well known that resistance, dielectric constant, pH, and viscosity are temperature dependent. The interdependence of these terms with the velocity of EOF and the potential temperature gradient formed dictate that Joule heating must be controlled.

Proper voltage selection can be determined using an Ohm's law plot. This can be generated by placing buffer in the capillary and ramping to a series of known voltages while recording the current. Figure (9) is an overlay of current

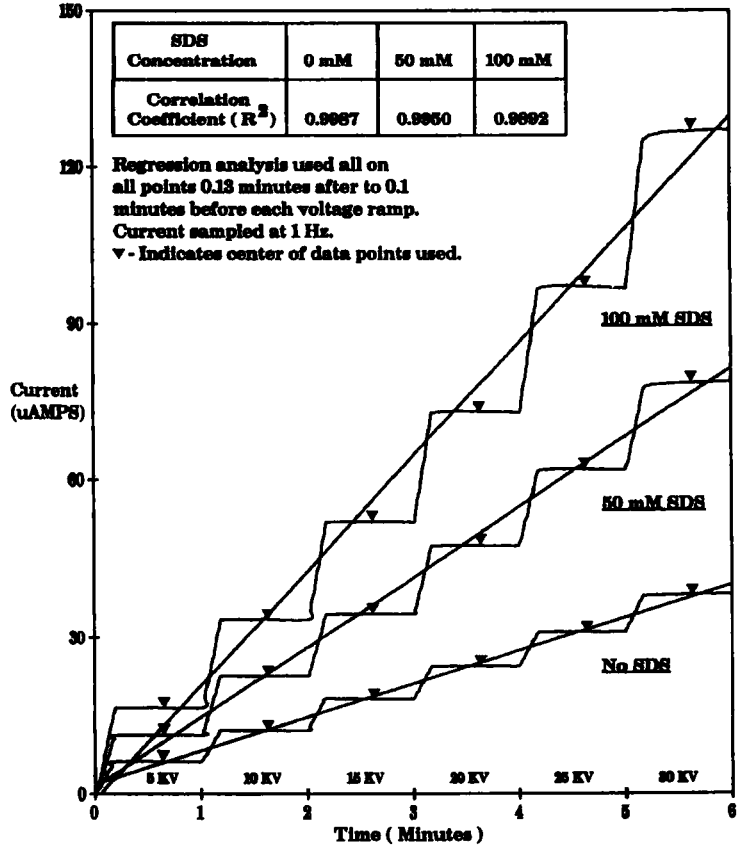


FIGURE 9 - Regression analysis of current data for Ohm's law plots at 0, 50mM, and 100mM SDS concentrations. Buffer was 50mM NaBorate at pH 8.3. Note the increase in the magnitude of the current steps above 25kV and 15kV for the 50mM and 100mM data.

data for Ohm's law plots at 0, 50mM, and 100mM SDS concentrations. The buffer was 0.1M NaBorate pH 8.3 and the experiment was run at 30°C. Regression analysis of an average of the data points was performed to achieve greater accuracy for the current values and determination of the linearity. The current steps in the 0 mM SDS from 5-30kV data are nearly equal in magnitude. The steps increased in magnitude above 25kV and 15kV for the 50mM and 100mM SDS data, respectively. A line through the center of the data points (▾) for each SDS molarity would show non-linearity above the mentioned values. Monitoring current is very useful to determine optimum voltage conditions.

The effect of exceeding the limits of Ohm's law can be seen in the separation of vitamins using MECC (Fig. 10). The time decreased as the voltage was increased. The efficiency and resolution (inset of Fig. 10) increased to a maximum, then decreased as voltage was increased. Maximum efficiency was obtained at the point where the Ohm's Law plot just deviates from linearity [12]. Peaks broadened due to diffusion (Eq. 3) at low voltage. Increased voltages yielded more plates, faster times and sharper peaks, as predicted by equations (5-7) [13,14,27].

Excessive voltage causes pronounced Joule heating, lower theoretical plates, and lower resolution. The conditions for maximum resolution are obtained when current and Joule heating are not limiting factors. Temperature control of the system is very important in attempting to use higher voltages. Temperature control is required to gain the higher field strength benefits.

It is possible to achieve a linear Ohm's law plot from with low current causing buffers such as the "Good's" series (TRIS, CAPS, etc.). A linear Ohm's law plot and overlay of the bioactive peptide series mentioned in the methods section was shown by McLaughlin, et al [28]. This data showed linear results from 5-30kV with a concomitant increase in efficiency and resolution [29].

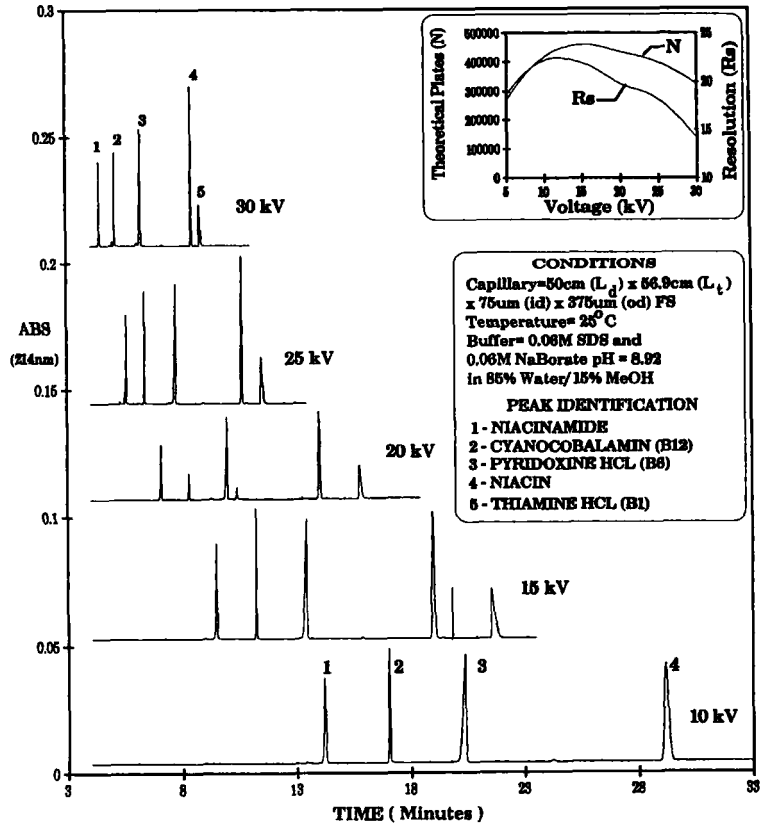


FIGURE 10 - Effect of increasing voltage on efficiency, resolution, and time during MECC separation of vitamins. Inset shows theoretical plate (N for peak 2) and resolution (R_s for peaks 2,3) versus voltage calculations from 5-30kV. The 5kV and peak 5 of 10kV data is not shown in figure for clarity.

The main effects of increasing voltage are:

- Increased efficiency and resolution (directly proportional to voltage up to the point where joule heat cannot be removed and diffusion dominates- Eq.6-7).
- Shorter analysis times (time directly proportional to voltage- Eq. 5).
- Increased heat produced (current increase directly proportional to voltage and can become a limiting factor if capillary joule heat is not removed).
- Increased need for temperature control at higher voltages.

Ionic Strength Effects

Ionic strength control is one tool that an investigator can use to improve the efficiency, sensitivity, and resolution. Cohen, et al [13], have described the interrelationships of buffer concentration, current, zeta potential, capillary radius, and viscosity, below:

$$I = \frac{\pi r^2 \epsilon E c_i \zeta_i}{\eta}$$

(Eq. 12)

where: I= current, r = capillary internal radius, ϵ = dielectric constant, c_i = molar concentration of solutes (i), ζ_i = potential (i), and η = viscosity.

Many of the effects that could be predicted to occur from equation (12) can be seen by careful inspection of figures (11) and (12). These analyses are overlays of bioactive peptide separations where the concentration of NaH_2PO_4 is varied. As ionic strength increased lower mobilities were observed (mobility is directly related to zeta potential and inversely to time). This effect was clearly seen in the 50 μm (id) capillary (Fig. 11) where mobilities decreased linearly over the buffer range of 0.025M to 0.125M before reaching a plateau. By contrast,

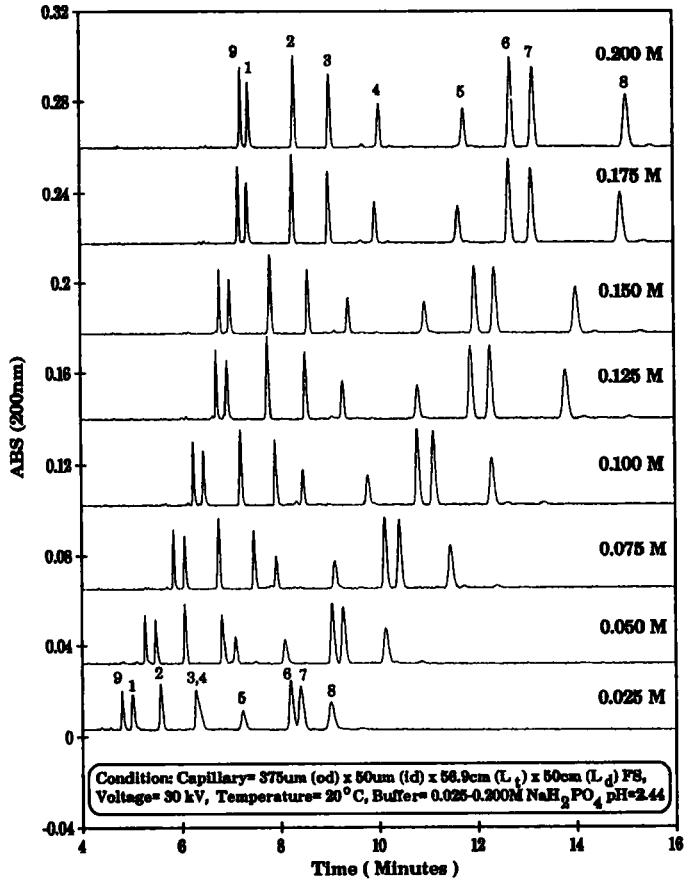


FIGURE 11 - Impact of ionic strength on the separation of bioactive peptides from 0.025M to 0.2M NaH_2PO_4 on a 50μm ID capillary. Peak height, resolution, efficiency, and time/mobility differences can be seen (Note: see materials section for identification of bioactive peptides).

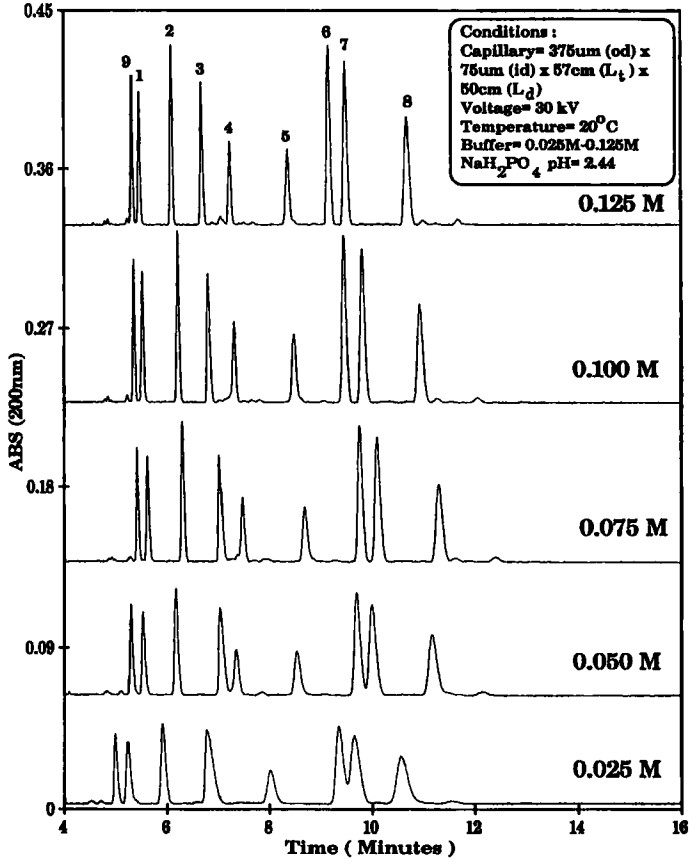
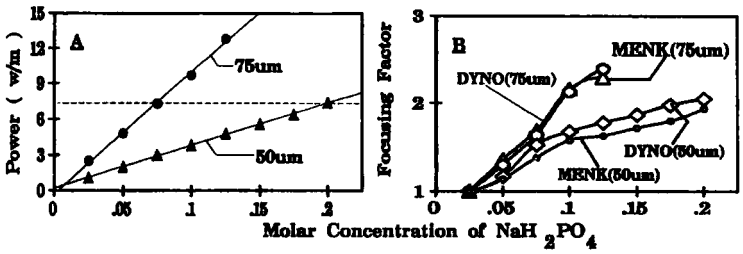


FIGURE 12 - Comparison of the impact of ionic strength on the separation of bioactive peptides from 0.025M to 0.125M NaH_2PO_4 on a 75μm ID capillary (Note: see materials section for identification of bioactive peptides).



FIGURES 13 A/B - Influence of NaH_2PO_4 concentration on power and focusing factor during separation of bioactive peptides on 50μm ID and 75μm ID capillaries. The focusing factor is defined as peak $height_{xM}$/peak $height_{0.025M}$.

migration times on the 75 μm (id) capillary increased up to 0.075M (Fig. 12). Above 0.075M migration times actually decreased. As ionic strength was increased from 0.025M, the zeta potential and electroosmotic flow decreased and lower mobilities were observed [1,15]. At higher ionic strengths (>0.075M) the results in the 75 μm (id) capillary can be explained by decreased buffer viscosity due to increased heating. In other unpublished work [29] the authors have found that shortened migration times and viscosity changes occurred also on the 50 μm (id) capillary over the range of NaH_2PO_4 concentration of 0.2M-0.45M. The plot of power versus concentration of NaH_2PO_4 (Fig. 13A) contains a dashed line that represents the practical power limit with peltier liquid temperature control.

The Ohm's law plot (not shown, Eq. 9) and power plot (Fig. 13, Eq. 10) were observed to be linear at all buffer concentrations for the 50 μm (id) and up to 0.075M for the 75 μm (id) capillaries. Thus smaller diameter capillaries permit the use of higher ionic strengths (Eq. 12) due to better heat dissipation.

Other observations from figures 11 and 12:

- As ionic strength differences between the sample (0.1% TFA) *and run buffer*

increased, the peak height increased. This is caused by injecting the sample in a low conductivity (i.e. high resistance) buffer and running it in higher (low resistance) conductivity buffers. This effect, called focusing, was the result of higher field strength in the sample injection plug than the run buffer [2,12,15]. The solutes in the sample buffer migrate very quickly because of the high electrical field, until they reach the lower field in the run buffer, where they stack. The focusing factor (peak height$_{xM}$/peak height$_{0.025M}$) vs. buffer concentration plot (Fig. 13B) shows this effect. In other unpublished work the authors [29] showed the focusing factor increased (> 10x) on the 50 μm (id) capillary at buffer strengths up to 0.45M NaH_2PO_4. Theoretical plates (N) also increased from 80,000 to 800,000. This effect can be used to increase sensitivity and sample load.

- Higher ionic strength gave better resolution (See peaks 3,4 and 6,7 in Figs. 11-12), and appeared to reduce wall interactions while increasing mass recovery. The increase in resolution over the range of 0.025-0.075M was 1.3x greater for the 50 μm (id) capillary compared to the 75 um (id) capillary.
- Low ionic strength buffers (< 0.035 M) resulted in peak broadening and solute coelution (See peaks 3,4 and 6,7 in Figs. 11-12), although shorter migration times were observed.
- Effective temperature control was essential at high ionic strengths because of the high power densities generated (12.8 W/m on the 75 μm id capillary).

The main effects of <u>increasing ionic strength</u> (Note: these effects are subject to the caveat "until the heat dissipation ability of the system is exceeded"):

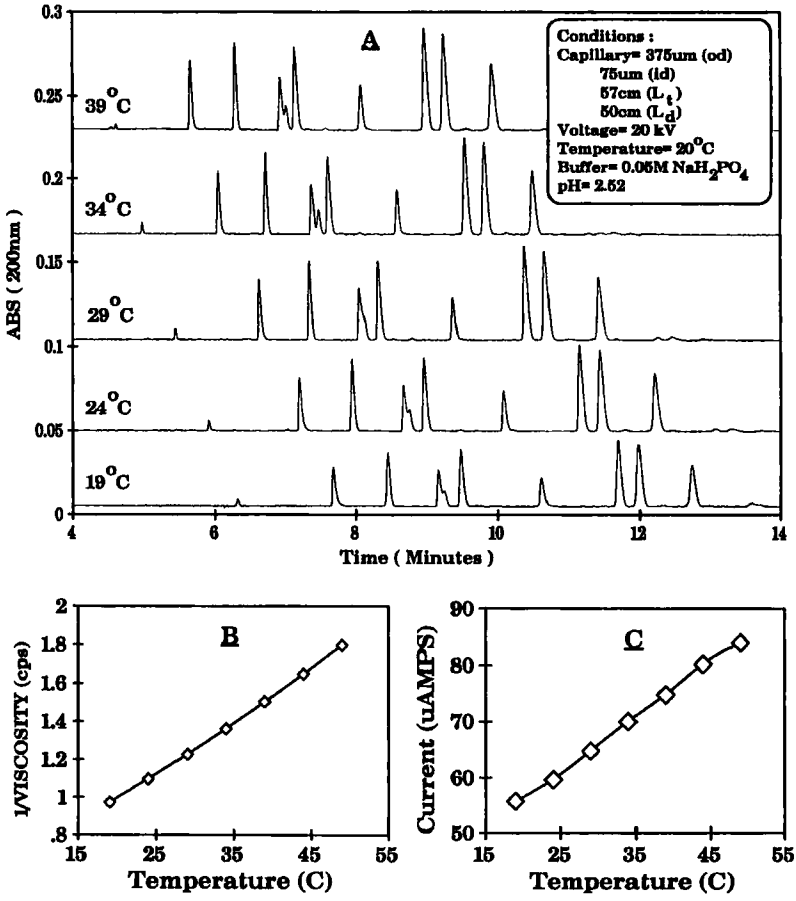
- Lowers zeta potential of capillary wall.
- Migration times increase directly proportional to ionic strength (lower mobilities).
- Increased theoretical plates, better resolution, and improved peak shape.
- More heat produced (current is directly proportional to buffer concentration).

- Effective temperature control is critical to gain the advantages of using higher ionic strengths.
- Better focusing and concentration sensitivity can be achieved.
- Smaller diameter capillaries allow use of higher ionic strengths because of their more efficient heat dissipation and ease of control (current is directly proportional to the capillary diameter squared).
- Ohm's law and power plot analysis provide a convenient way to determine ionic strength limits of the system.

Capillary Temperature Control

Temperature control of the capillary is important for better reproducibility, thermal denaturation studies, determination of thermal melting points, solubility, and shortened analysis times. There are several different approaches that have been taken, namely: none, fan blown air, high speed gas flow through a jacket, thermostated oven, direct contact peltier element, and peltier controlled recirculating liquid. The systems in this study utilized peltier controlled recirculating liquid temperature controllers. A pump circulates a non-conducting, thermal heat transfer liquid around the capillary, which is mounted in a cartridge. The importance of temperature control has been described by Nelson [30].

A bioactive peptide mixture was separated using 0.05M NaH_2PO_4 between 19°-49°C. Overlays of the data from 19°-39°C are shown in figure 14A. As the temperature was increased from 19 to 39°C, the peptides migrated more quickly. While the current increased linearly with temperature (Fig. 14B), there was a parallel decrease in the viscosity (Fig. 14C) [31,32]. The observed migration times appeared directly proportional to the viscosity changes. This was not surprising since viscosity changes were significant over the temperature range studied. Small changes in resolution and efficiency were observed that were



FIGURES 14 A/B/C - Effect of external temperature control of capillary on time, selectivity, and peak height (A) during separation of bioactive peptides. Figures 14B-C show relationships of viscosity and current with capillary temperature control. (Note: Peaks elute in same order as in Figure 12 and are identified in the materials section. Data not shown for 44° and 49° experiments for clarity).

advantageous (See 3rd-5th major peaks). Although not identified in the figure, these peaks were TRH, LHRH, and an impurity. Biomolecule folding/unfolding, pH changes, differential solubilization, or other effects can be caused by temperature changes. These effects could explain the selective improvement in separation of the TRH/LHRH/impurity group at 34°C.

The main effects of increasing temperature are:

- Lowers viscosity and analysis time (viscosity and time are inversely proportional to temperature)
- Current increases (directly proportional to temperature).
- Stable temperature control becomes crucial in understanding observed phenomena.
- Selectivity differences can cause differential migration of solutes.
- Useful for denaturation studies, thermal melting studies, controlled enzyme reaction studies, efficiency optimization, and solubility.
- Can affect resolution, efficiency, and peak shape.
- Can affect buffer pH.

Effect of Addition of Organic Modifiers

Two different organic modifiers were used in these studies. They were chosen because of their differences in viscosity in aqueous mixtures over the range of 0-50%. Figure (15) shows estimated viscosity plots using varying amounts of the organic modifiers MeOH and CH_3CN [33]. They were corrected for 0.05M NaH_2PO_4 and a temperature of 30°C [34].

Addition of acetonitrile resulted in slight increases followed by steady decreases in migration times (Fig. 16). Increased migration times were seen over

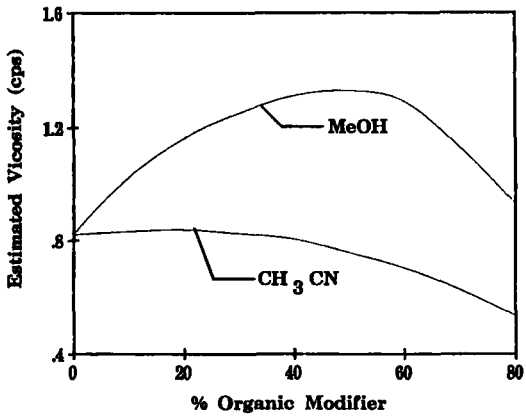


FIGURE 15 - Estimated viscosity of solutions of 0.05M NaH_2PO_4 pH 2.44 with varying percentages of MeOH and CH_3CN at 30°C [32,33].

the same concentration range with methanol (Fig. 17). Migration time behavior paralleled changes in viscosity (Fig. 15). Viscosity increased slightly up to 15% acetonitrile and decreased at higher percentages. The viscosity of methanol solutions increased up to 50%. Small changes in selectivity were observed, particularly from 0-20% organic modifier.

Equation (9) showed that mobility depends not only on viscosity but also on the dielectric constant, zeta potential, and on other factors, such as permittivity [35,36]. Plots of mobility vs. percent modifier (data not shown) indicated that mobility was directly proportional to viscosity. This observation is consistent with the results of VanOrman, et al [35]. Changes in zeta potential, dielectric constant, permittivity, and net charge also may influence results and must be considered [36]. Organic additives offer a convenient way to modify these parameters. Their effect on activity, recovery, pH, and precipitation of biomolecules or buffer salts are some potential problems that need to be considered.

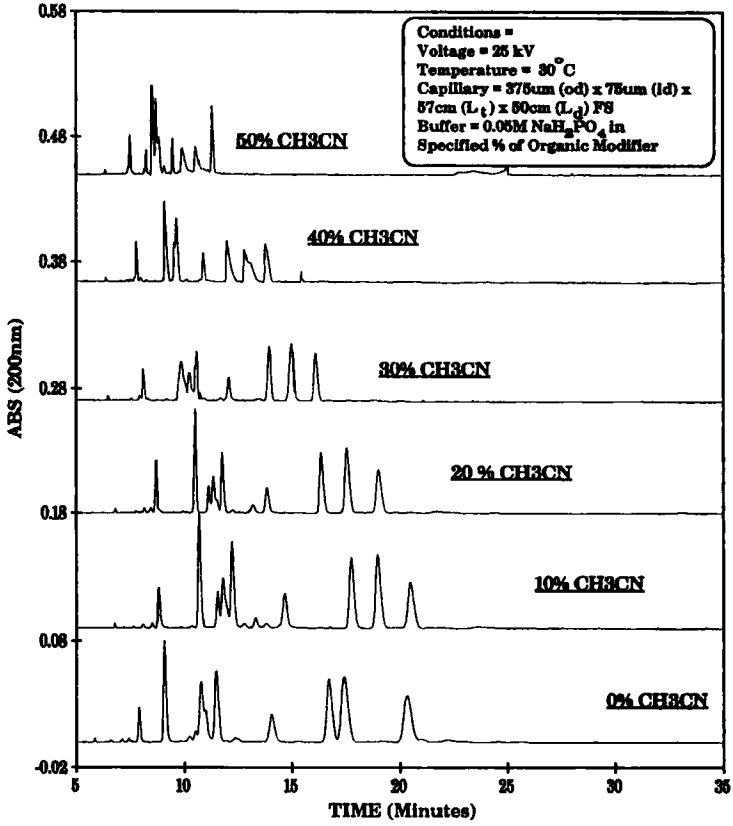


FIGURE 16 - Comparison of the effect of concentration of CH_3CN on time/ mobility, selectivity, peak height, and resolution of bioactive peptides (Note: no attempt was made to identify the elution order of the mixture of bioactive peptides, named in the materials section).

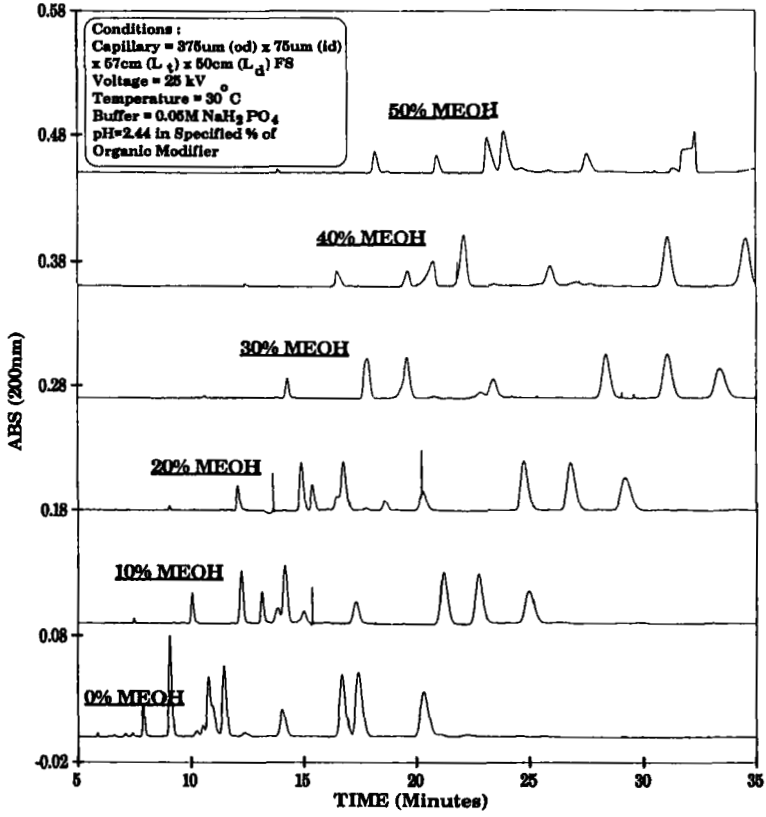


FIGURE 17 - Comparison of the effect of concentration of MeOH on time/ mobility, selectivity, peak height, and resolution of bioactive peptides (Note: no attempt was made to identify the elution order of the mixture of bioactive peptides, named in the materials section).

The main effects of using different amounts of organic modifiers are:

- Observed migration times appeared directly proportional to viscosity changes using different % organic modifiers.
- Analysis time can be increased or decreased by choosing the correct percentage of the correct modifier.
- More/less efficiency and resolution can be achieved (depends on modifier).
- Current decreases as percent organic modifier is increased (for MeOH and CH_3CN) allowing use of higher voltages and decreasing joule heating.
- Organic additives offer a convenient way to modify selectivity, solubility (frequently needed in MECC and chiral work), permittivity, zeta potential, and dielectric constant.

Effect of pH

The most important separation parameter in FSE may be pH. If the pH selection is incorrect, the likelihood of a good separation is dramatically reduced. Field, et al [37] have described the separation of a series of five peptides. They were synthesized with the same amino acid composition but different primary sequences. As the pH was increased from 2.5 to 3.5, resolution was obtained for this set of closely related analogs. Separation can be attributed to differences in the intrinsic pKa of the carboxyl terminus [37].

A series of antipyretic analgesics were separated using MECC conditions and pH range of 8.46-10.54. An overlay of the antipyretic analgesic separations is shown in figure (18). Inspection of the pH 8.46-10.04 data shows acetaminophen moved from peak 1 (pH 8.46) to coelution with peak 3 (benzamide at pH 9.42), and eventually migrated after peak 4 (acetanilide at pH 10.04). Possibly even more dramatic is the case of peak 5 (salicylamide). The peak was broad at pH

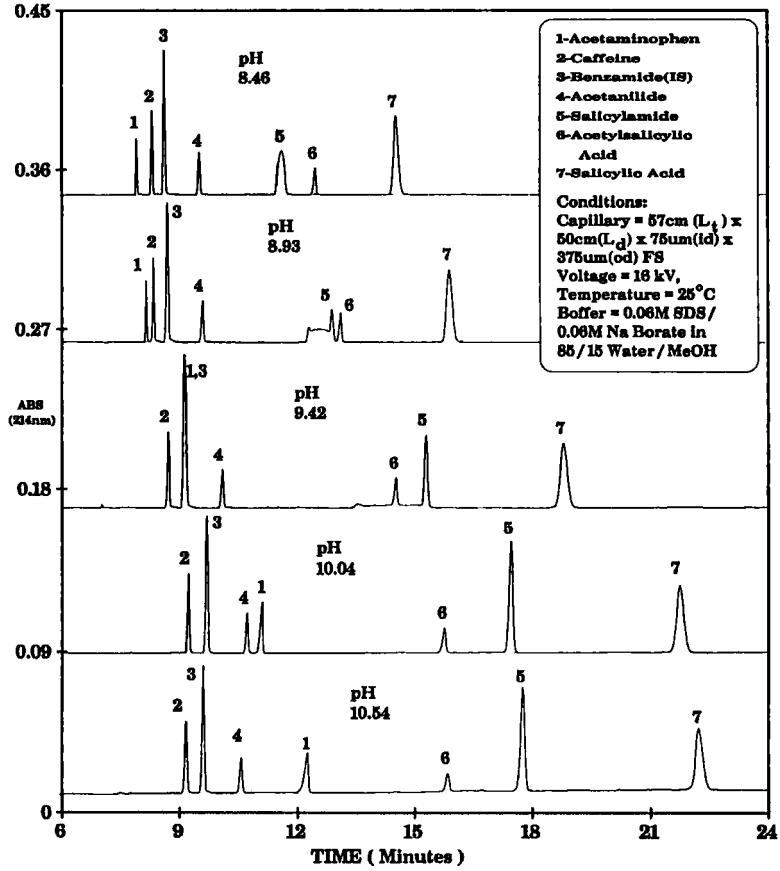
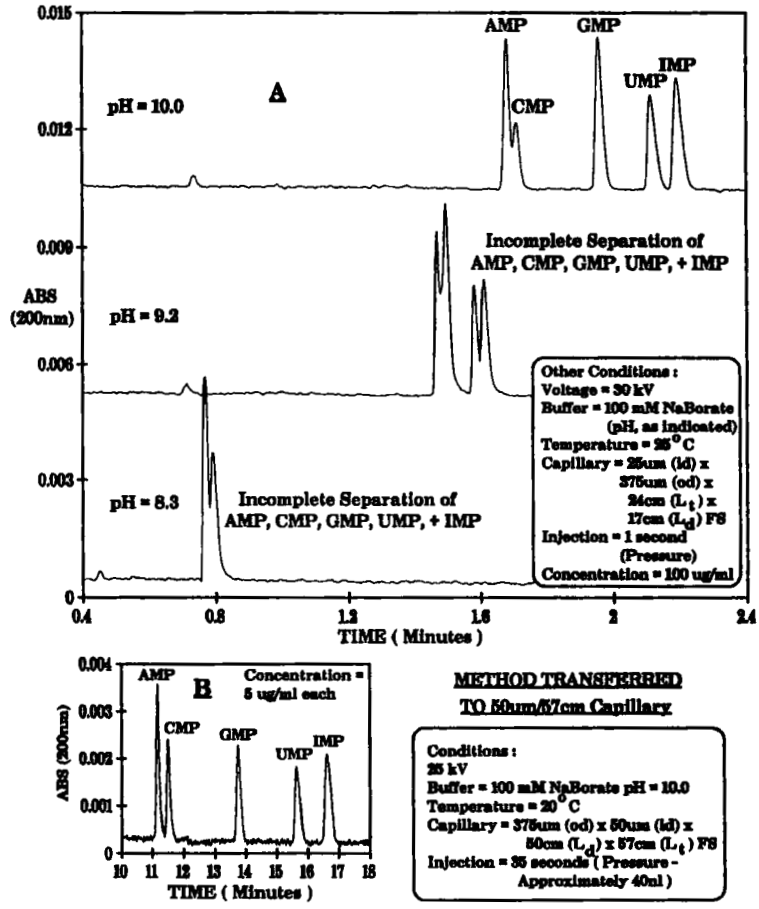


FIGURE 18 - Influence of pH (8.46 to 10.54) on resolution, selectivity, efficiency, and time during the MECC separation of antipyretic analgesics.

8.46. It developed a very distorted frontal zone at pH 8.93, approached a more gaussian distribution at pH 9.42, and moved past peak 6 (acetylsalicylic acid).

An example of rapid pH screening during methods development of nucleotides is shown in figure (19A). A short (17cm), narrow (25 μm id) capillary was used for the methods development. It can be seen that changing the



FIGURES 19 A/B - Use of pH variation on a short, narrow capillary for rapid nucloetide separation (A) and transfer of method to longer, wider capillary (B).

pH from 8.3 to 10.0 allowed the 5 nucleotides to resolve. The method was then transferred to a longer (57cm), wider (50 μm id) capillary for the analytical method (Fig.19B) which required more sensitivity and resolution.

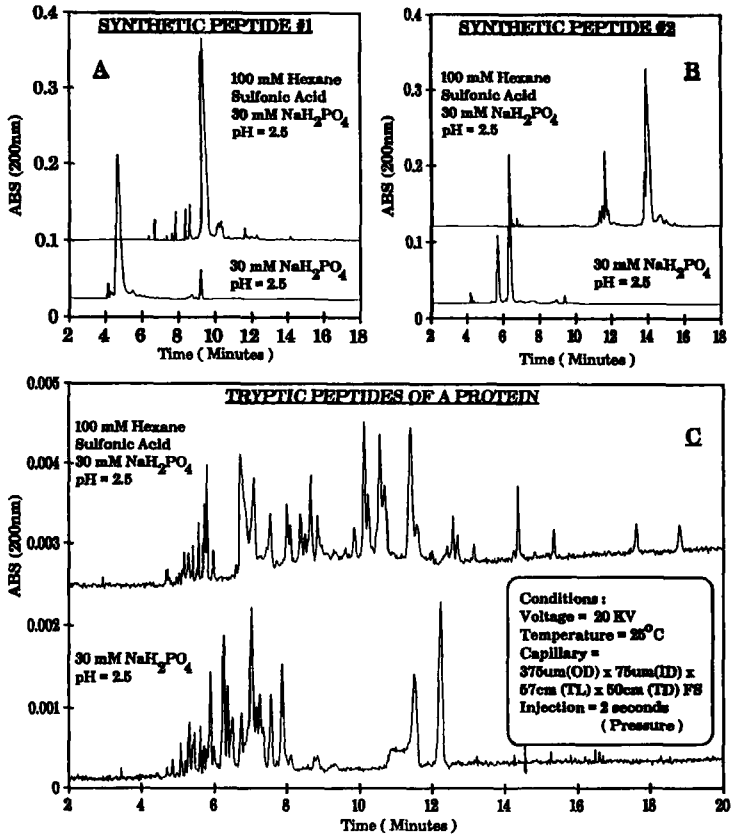
These results suggest that small differences in pKa can be the basis of separating closely related molecules by HPCE. Thus, it is equally important to investigate the high and low pH regions. An effective approach for the resolution of mixtures should involve titration using automated step changes across the pKa's of the potential side chain groups. Large changes over the range of pH 2 to 12 are recommended for unknown mixtures. Good information on buffering capacity and useable pH ranges is available in the electrophoresis literature and from vendors that sell electrophoresis chemicals, Goods buffers, and isoelectric focusing (IEF) chemicals.

The main effects of pH control are:

- Small differences in pKa can be the basis of separating closely related solutes.
- Resolution, selectivity, and peak shape can be dramatically altered.
- Temperature control is necessary to maintain pH for temperature sensitive buffers. Good's buffers are particularly temperature sensitive.
- Larger pH changes show promise for optimizing resolution and selectivity for early or late eluting components of unknowns.

Ion Pairing Agent Addition Effects

Ion pairing agents are commonly used in HPLC to alter retention characteristics of ionic solutes. Based on data of Moring and Nolan [38], we decided to apply the use of ion pairing agents in HPCE. Figures 20A-C displays the separations of two proprietary synthetic peptides and a tryptic map of a



FIGURES 20 A/B/C - Comparison of separations of two proprietary synthetic peptides (A/B) and a tryptic map of a proprietary protein (C) with and without the use of the ion pairing reagent hexanesulfonic acid.

proprietary protein. The figures show the separations of the samples with 30mM NaH_2PO_4 pH 2.5, and with the addition of 100mM hexanesulfonic acid. Observed migration times are uniformly greater with some selectivity changes realized. There are significant increases in resolution.

The hydrophobic side chain of the hexanesulfonic acid interacts with the hydrophobic portions of the peptide, thereby decreasing aggregation. Addition of negative charge to the peptides decreases their mobility and increases migration time. There may also be wall interactions that are not understood.

Some of the effects of using ion pairing agents are:

- Analysis time and zeta potential can be manipulated by the ion pairing reagent's chain length (e.g. methyl or propyl sulfonic acids) and concentration.
- Selectivity changes can be realized.
- The current increases as the amount of the ion pairing agent increases.
- Significant gains in resolution and number of components can be realized.

Use of Cyclodextrins for the Separation of Enantiomers

HPCE appears to show much promise in the area of separation of chiral compounds. Altria, et al [25] showed an exhaustive study on the use of additives for the separation of clenbuterol. They showed numerous effects due to the experimental manipulation of cyclodextrin (CD) concentration, use of substituted α, β, and γ CD's. Temperature, voltage, modifiers to increase CD solubility, pH, non-CD chiral additives (e.g. bile acid salts), and others effects were shown.

In this work we concentrated on CD's and some of their effects. Use of different ionic strengths buffers (NaH_2PO_4), CD's (β-cyclodextrin and heptakis), and CD solubilizers (MeOH and urea) in the separation of epinephrine are shown (Fig. 21). There was no separation of epinephrine without heptakis. The effects of increasing ionic strength resulted in sharper peaks and better resolution, as in

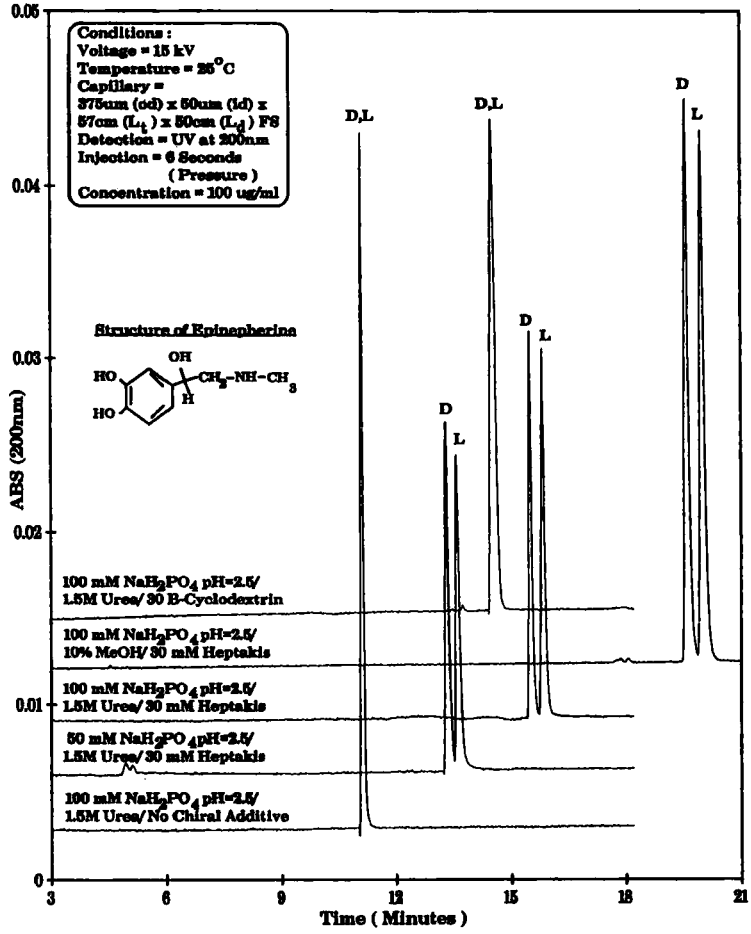


FIGURE 21 - Use of different ionic strengths, cyclodextrins, and solubilizers to effect resolution and selectivity of enantiomers of epinephrine.

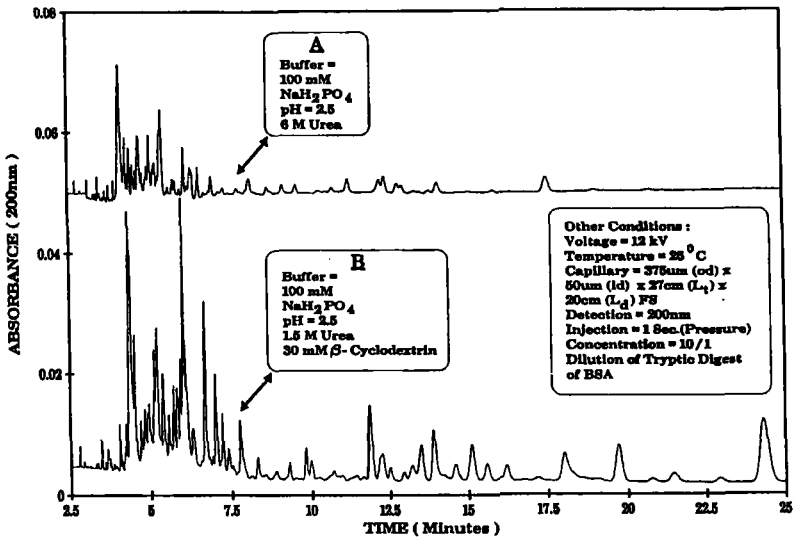


FIGURE 22 - Comparison of the separation of tryptic peptides of BSA with and without the use of β-cyclodextrin on equal length capillaries.

normal FSE. Methanol and urea both were used successfully to solubilize the chiral additive and resulted in good separations.

β-cyclodextrin was ineffective in resolving the enantiomers of epinephrine under the conditions used.

Figure (22) illustrates a superior separation of a tryptic map of BSA on a shorter capillary using β-cyclodextrin as a selectivity andresolution enhancer. Numerous additional peaks were observed using the β-cyclodextrin. Some tryptic fragments appear to have been able to enter the CD cavity, while some cannot. There may be another mechanism, but attempts at pattern recognition do not indicate a simple change in zeta potential or EOF modification. A common effect

on all peptides would lead to a recognizable pattern similar to lengthening the capillary.

Some of the effects of using CD's for the separation of enantiomers:

- Increasing the concentration of the CD by addition of solubilizers (e.g. MeOH, urea) will increase the resolution of the enantiomers. This also can be accomplished by using more hydrophilic substituted CD's in aqueous buffers.
- Lower temperatures lead to higher resolution in chiral separations (Note: This comment is based on observations of Altria, et al [25]).
- Voltage, ionic strength, and capillary length effects appear to work as in normal FSE.
- As chain length of the CD substitution increases, resolution increases to a maxima (steric effects). The size of the CD cavity and hydrophobic nature can be crucial to the success of the separation. If it is too small, neither enantiomer will be able to enter the cavity. If it is too large, both enantiomers will be able to enter the cavity undifferentiated and no separation will occur.

Use of Other Additives and Wall Coatings

Many different types of additives and coatings can be used in HPCE. The following is a partial list of reasons additive or wall coating are used:

- EOF modification or elimination.
- Elimination of interactions with capillary wall surface.
- Alteration of separation selectivity.
- Change hydrophobic/hydrophilic nature of buffer.
- Introduction of sieving or size based mechanisms.
- Sensitivity enhancement by the addition of chromophores or fluorophores to use indirect absorbance or fluorescence techniques.
- Addition of a charge carrier for non-aqueous HPCE separations.

Some wall coatings have been described in the literature and introduced commercially. They control or eliminate EOF, or eliminate binding and interactions with the capillary wall surface. Some of the fused silica wall coatings or covalently bound moieties are: polyacrylamide [39,40], polyethyleneimine [41], polyethylene glycol [42]. Others are: epoxy diol [43], and bonded phases similar to traditional HPLC packings with covalently bonded C1-C18 alkyl side chains [44]. Some of these coatings or bonded phases have been successfully used for isoelectric focusing of proteins [39,40,45], polyacrylamide gel electrophoretic (PAGE) sizing separations (DNA fragments [46], DNA sequencing [47-50], oligonucleotide purity [51], peptides with SDS [52], proteins with SDS [53], anionic surfactants/sulfoureas [54]). Better recovery of proteins [44] was another area of application.

McCormick, el al [55] showed promising separation of proteins on a PVP phase at low pH (<2.0). An interesting example of EOF control was shown recently by Cooke, et al [53]. They used a covalently bound quaternary amine capillary to reverse and accelerate EOF for fast (< 1 minute) drug separations.

Atamna, et al [56] provided a comprehensive comparison of the use of different buffer cations. Buffer additives with pharmaceutical implications have being used in several recent publications or presentations. Use of sodium alkyl surfactants and Brij 35 was shown by Rasmusson, et al [57]. Palmieri, et al [58] showed the separation of a few closely related analogs of Growth Hormone Releasing Peptide. Addition of small amounts of POE 10 (a non-ionic "Brij like" surfactant), to take advantage of small differences in hydrophobicity, promoted resolution of the previously unseparated analogs. Bullock, et al [59] dramatically improved efficiency of basic proteins by adding 1,3-diaminopropane (30-60mM) plus moderate levels of alkali metal salts to suppress analyte-wall interactions over the pH range of 3.5-9. Swedberg [60] applied non-ionic and zwitterionic surfactants for the enhancement of selectivity of tricyclic antidepressants and

heptapeptides. The use of cetyltrimethylammonium bromide (CTAB) to modify EOF was shown by Tsuda [61]. Addition of CTAB slows and, in high enough concentrations, reverses EOF. Merion [62], Rohde [63], and coworkers used proprietary buffer additives for better recovery and peak shape of proteins.

Modification of capillaries by masking or deactivating surface silanol groups by physical coating of the capillary wall with hydroxy methyl cellulose (HMC) was described by Hjerten [64]. Zhu [40], Schwartz [65], Ulfelder [66], and coworkers have applied the use of HMC to the FSE separation of DNA restriction fragments. The fragments were up to several thousand base pairs and separated by apparent size. The further use of ethidium bromide (as an intercalator) with the HMC buffer allowed the separation of very closely related (< 10 base pairs) fragments [66]. Other additives used to achieve separation of analytes by apparent size were shown. VanOrman, et al [67] separated polystyrene particles using ACES, while Righetti, et al [68] was able to resolve immobiline buffer polymers using Ficoll-400.

Kuhr et al [69,70] have described the addition of chromophores and fluorophores to buffers to achieve better sensitivity in the UV and fluorescent regions for numerous biomolecules by indirect techniques. One other use of additives and indirect detection is in the development of ion analysis by HPCE. Jones, et al [71] have incorporated buffers including hydroxyisobutyric acid, chromate, and proprietary EOF modifiers to achieve separation and detection of common ions.

Some of the effects of using other additives and wall coatings are:

- EOF modification/elimination to affect migration time and resolution.
- Elimination of interactions with capillary wall surface for better recovery, peak shape improvement, and recovery.

- Alteration of separation selectivity or hydrophobic/hydrophilic nature of buffer to achieve better resolution, efficiency, and improved separations.
- Introduction of sieving/size based mechanisms for separations by apparent size.
- Sensitivity enhancement by the addition of chromophores or fluorophores to use indirect absorbance or fluorescence techniques.

Types of Injection

One of the main benefits of HPCE is the ability to inject extremely small volumes of sample. Typical injection volumes are from picoliters to hundreds of nanoliters. There are two different classifications of injection for HPCE: electrokinetic and hydrostatic. It is important to recognize the benefits and shortcomings of each injection type, and how each technique effects quantitation.

Electrokinetic injections are performed by simply turning on the voltage for a certain period of time. The amount of sample injected and its composition will be determined by a combination of EOF, the individual analyte's electrophoretic attraction toward an electrode, and the electrical resistance of the medium dissolving the sample. The amount injected using an electrokinetic injection is given (Eq. 13):

$$Q_i = (v_i + V_{eo})\, c_i \pi r^2 t \qquad \text{(Eq. 13)}$$

where: Q_i = amount of species (i) introduced into the capillary, c_i = concentration of species (i), and V_i and V_{eo} are the velocity of species (i) and (eo), respectively [72]. As discussed by Huang et al [73], this injection technique can cause a sample bias. During sample injection, based on differences in charge, sample components move into the capillary at different velocities. With no EOF

this injection technique will allow selective loading of either anions or cations. Neutrals, anions, and cations will be loaded at different rates, if EOF is present. One additional problem of electokinetic injection is that the presence of high amounts of salts in the sample buffer can lead to extremely irreproducible quantitation. Schwartz et al [65] and Ulfelder et al [66] discussed this problem relevant to DNA fragment quantitation. Electokinetic is the preferred means of injecting onto pressure sensitive gel filled capillaries. It is easier to apply extremely small samples (pl) by using a low injection voltage for short periods.

Hydrostatic injections can be performed in three different manners. Pressure injections involve application of a known pressure to a capped vial for a predefined time to move sample into the capillary. Vacuum injections apply a known vacuum to the outlet of the capillary, the inlet of which, is inserted into a sample vial for a predefined time. Gravity injections are performed by physically raising the sample container with the capillary immersed in it to a predefined height for a predifined time. The amount injected (hydrodynamic injections) is given by the Poiseuille equation (Eq. 14):

$$V_c = \frac{\Delta P \pi r^4 t}{8 \eta L_t} \qquad \text{(Eq. 14)}$$

where: V_c = calculated injection volume amount of species, ΔP = pressure drop across the capillary, t = injection time, η = viscosity of the electrolyte, and L_t = total capillary length [72]. Hydrodynamic injections allow injections of uniform composition of any species. Other benefits of hydrodynamic injections are: lack of sample decomposition/evaporation from applied voltage and the ability to more easily apply large injection volumes during micropreparative work. Some of the shortcomings are: inability to control extremely small injection volumes and cannot be used on pressure sensitive gel capillaries. Sample evaporation is a common injection related problem. It is relatively easy

to inject from less than 5ul of sample on modern instruments. It is not easy to prevent evaporation of the sample, however. The problem of evaporative losses through the use of capped vials was addressed by Ohms [74]. Extremely small samples are usually placed in a microvial insert inside a larger vial. The sample buffer can vaporize and redistribute from the microvial insert into the autosampler vial. Nelson [75] devised a means of avoiding this problem in capped vials by incorporating buffer into the outer vial chamber. Open tubes are frequently used, but not recommended, as sample containers.

Some of the effects of using different types of injection are:

- The amount injected using an electrokinetic injection can cause a sample bias.
- Electokinetic is the preferred means of injecting onto pressure sensitive gel filled capillaries and is easier to use in applying extremely small samples.
- Hydrodynamic injections offer uniform solute composition, lack of sample decomposition/evaporation, and the ability to apply large injection volumes.
- The shortcomings of hydrodynamic injections are inability to control small injection volumes and cannot be used on pressure sensitive gel capillaries.

Unattended Methods Development and Rapid Peak Identification

Unattended methods development and rapid peak identification are desired criteria for development of pharmaceutical methods. Several instrument features were utilized for rapid peak identification. The ability to load samples from either end of the capillary, to reverse the polarity, and to program multiple injections/coinjections were used. In a typical experiment the capillary was equilibrated with a set of buffer conditions and a sample mixture was injected. Peaks were identified by automated spiking by coinjection of individual components and the mixture under the same conditions. The same sequence of

injections of the mixture and coinjection of mixture/individual components were then performed under a different set of experimental parameters.

Spiking of individual components for peak identification is shown in the separation of vitamins overlay (Fig. 23). The results clearly show a gain in peak height for the component coinjected. Figure (24) depicts runs using reversed polarity and injection onto the short outlet end of the capillary (effective length of 6.9cm). Rapid analysis times resulted. Using multiple injections and varying injection times allowed discrimination of closely eluting components. Although short (effective length to the detector) capillaries offered less theoretical efficiency and resolution, they were adequate for peak identification and resulted in faster run times. The total time for these 8 runs was less than 60 minutes, including setup, programming, rinses, and separations. Use of short length capillaries can be a very effective means of performing rapid methods development, when mixtures are analyzed which do not require maximum efficiency or resolution.

Another example of rapid methods development was previously shown in figure (19). The method used pH changes on a short, narrow capillary to resolve the individual components of the mixture in less than 3 minutes. The buffer system used was based on the work of Tsuda, et al [76].

Some Unattended Methods Development and Rapid Peak Identification conclusions are:

- Spiking by coinjection is a convenient means of tracking peak identity during methods development.
- Fast methods development can be accomplished in 2-3 minutes on short effective length capillaries. Use of short capillaries is an effective method of rapid buffer screening and identifying peak elution order.

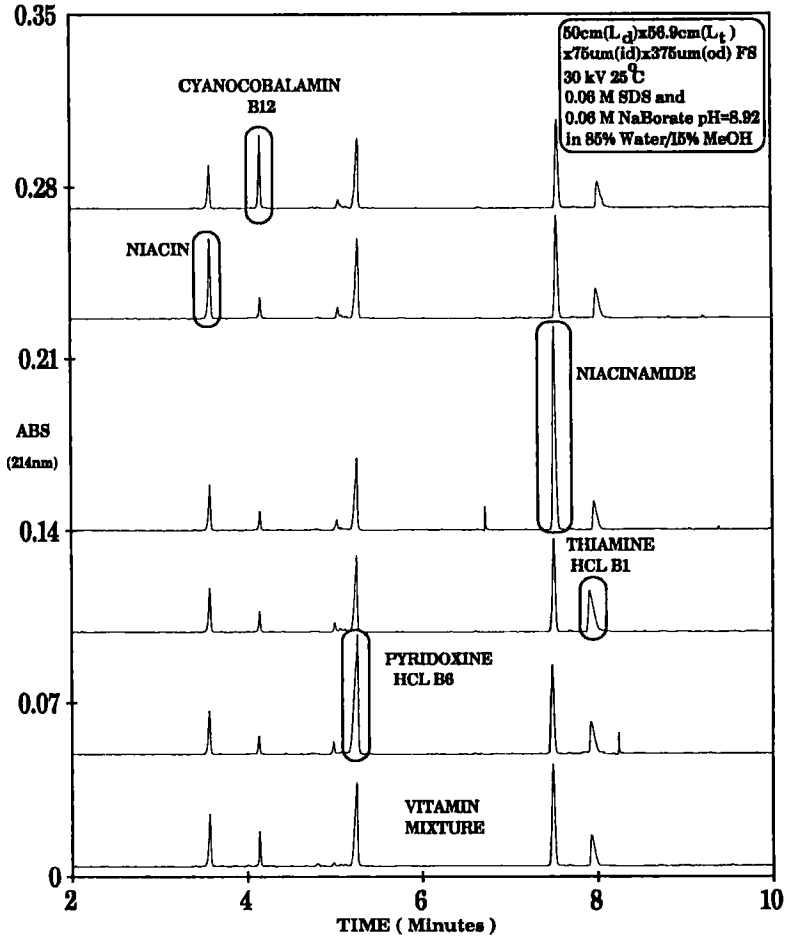


FIGURE 23 - Utilization of automated spiking by coinjection of mixture/ individual components for identification of vitamins in a mixture.

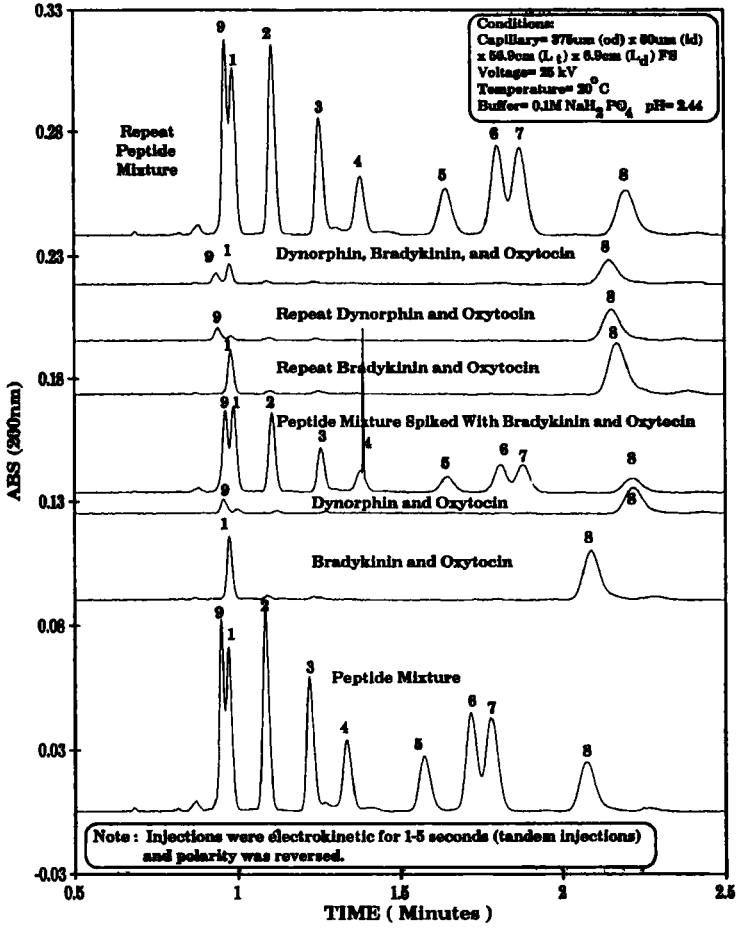


FIGURE 24 - Fast peak identity/verification of bioactive peptides by automated spiking and injection time variation on a very short length capillary using polarity reversal (Note: see materials section for identification of bioactive peptides).

- Short capillaries offer less theoretical efficiency/resolution but provided a very quick means for identification.

Reproducibility, Mobility Correction, and Quantitation

Reproducibility and quantitation in HPCE pose some unique problems. Inability to maintain a constant temperature within the capillary, changes in viscosity, pH variation, ion depletion, and any other factor that changes mobility must be considered. When operating with EOF all species will be migrating past the detector at different velocities. This is much different from HPLC where the normal situation is constant velocity. Changes in mobility can lead to misidentification of peaks and improper quantitation.

Mobility corrections for changes in migration time and therefore area can be essential to provide the analyst with an accurate quantitative answer. Moring et al [10] and Grossman et al [77] described the mathematical basis of mobility corrections on migration time and area. Huang et al [78] provided additional mobility definition. Palmieri et al [79] and McLaughlin et al [28,80] applied mobility to correction to a wide variety of different problems. Some of the data sets shown in this work were used to test the limits of mobility correction.

The System Gold software that was used in our work does the following to help achieve accurate quantitation:

- Allows entry of capillary dimensions.
- Reads voltage from instrument.
- Finds actual migration time/mobility marker (based on peak height, actual or corrected area) and calculates corrected migration time.
- Identifies peaks correctly from their calculated corrected migration times.

- Reports the mobility and apparent mobility of solutes.
- Calculates corrected peak area, multiplying the actual area (mAU/minute) by the analyte velocity (cm/min).
- Calculates concentration based on corrected area, actual area, or height.

The effect of mobility corrections for changes in migration time and area are shown for integrated data from figures (10,14, and 25). Figure (14) shows the separation of bioactive peptides over the temperature range of 19-39°C. Average % RSD'S for all peptides at 19°-39°C, excluding TRH, were: peak height= 16.96%, migration time= 11.24%, corrected migration time= 0.77%, peak area= 12.06%, concentration= 1.22%, and concentration error= 1.20%. The calculations used area correction and internal standard method to compensate for the changes in time and volume injected. These results indicate that good precision and accuracy can be obtained even under extreme conditions.

Eight consecutive runs of a bioactive peptide mixture (Fig. 25) were run to demonstrate reproducibility at constant temperature. Previously published results on this same data set from Anderson et al [81] indicated good migration time/area reproducibility. Average % RSD'S for all peptides, excluding TRH, were: peak height= 2.85%, migration time= 0.56%, corrected migration time= 0.28%, area= 2.25%, and concentration= 1.40%. This example illustrates the principle of buffer depletion. The inlet/outlet buffers were changed every fourth run. The effect of changing the buffer on migration time can be seen by observing some of the smaller peaks (between peaks 5/6). The repeat runs are marked (▲) to indicate the runs on which the buffers were changed.

A plot of percent relative standard deviation (RSD) vs. migration time (data not shown) indicated that RSD's of less than 1% could be obtained. The percent RSD increased with migration time, indicating that mobility was not constant. Mobility corrected results indicated that using a single mobility marker

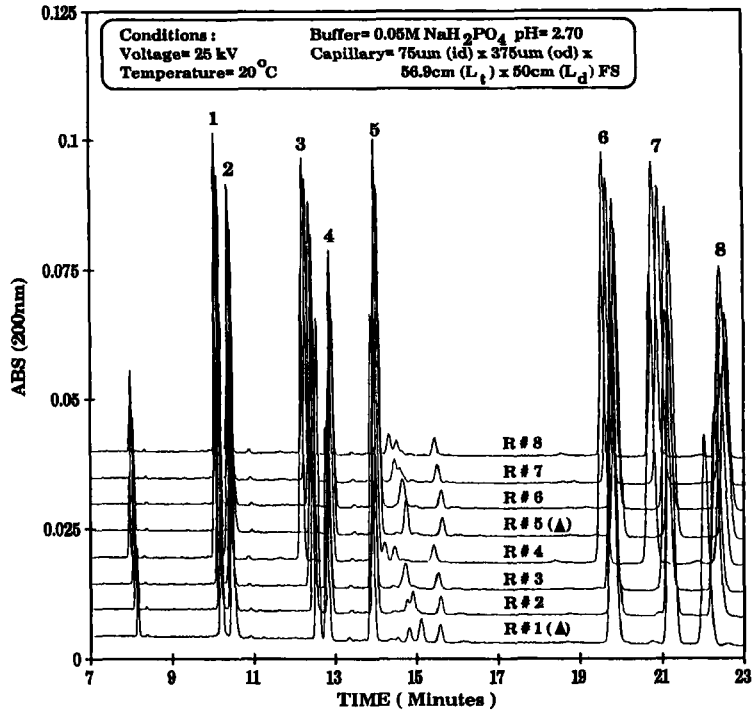


FIGURE 25 - Reproducibility comparison of 8 consecutive runs of a bioactive peptide mixture (Notes: inlet/outlet run buffers were changed at run indicated by (▲). (Note: see materials section for abbreviations of bioactive peptides).

(Dynorphin) improved precision but did not eliminate the variation with time. Multiple mobility markers may be required, if mobility changes within a run.

An extreme example of mobility correction was shown by Palmieri et al [79] using the separation of vitamins (Fig. 10) data. Quantitation of the vitamins run at 5-30 KV indicate large (>80%) actual time and area variance. The corrected

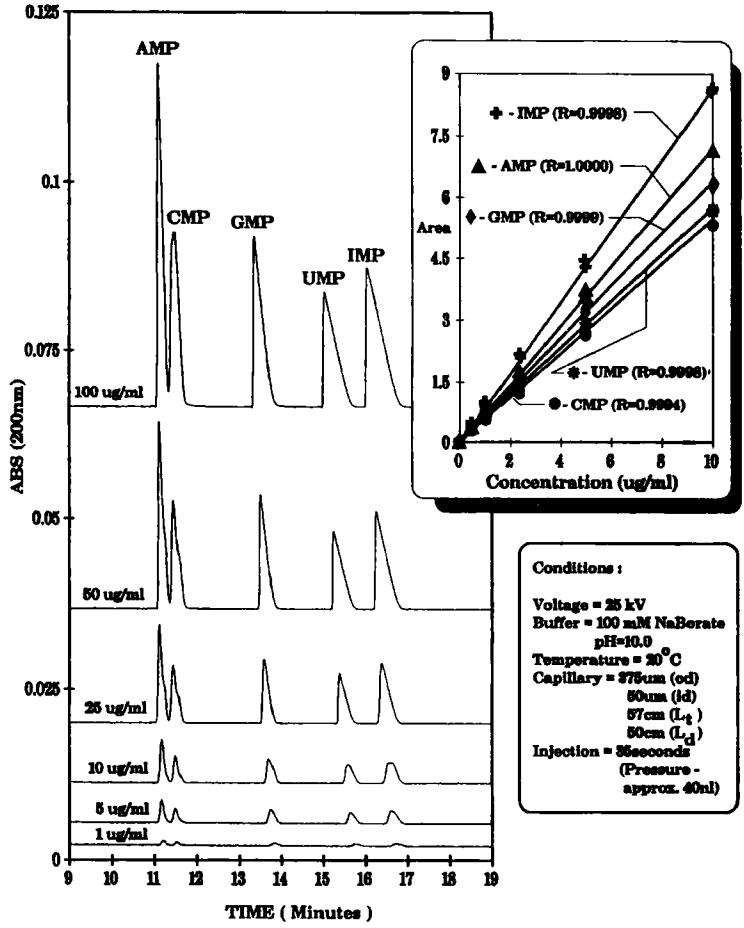


FIGURE 26 - Overlay of separations (Concentration range= 1-100 ug/ml) and a calibration plot (Concentration range= 1-10 ug/ml) of nucleotides at various concentrations.

values were significantly improved (average values for both corrected migration time, area, and concentration were from 1.5 to 2.3%).

Figure (26) shows achievement of linear-quantitative results at very high sensitivity for a series of nucleotides. The overlays show the electropherograms for the 1-100 ug/ml standards. The inset plot shows linearity (R= 0.9994-1.0000) for the five nucleotides in the concentration range of 0.5-10 ug/ml.

These examples used more severe conditions than would be required for many assays. The following are some of the tactics that can be used to improve reproducubility and quantitation:

- Minimization of evaporative losses by sealing or cooling samples.
- Use of low heat producing buffers. Reproducibility of migration times on the order of 1% or lower can be obtained with no correction at low power densities (<1 W/m) with good temperature control.
- Temperature dependent variables (e.g. viscosity, pH, etc.) can be eliminated or minimized through effective temperature control. This may be the most important factor in reproducibility.
- Reproducibility of migration times (<1-2% RSD) requires frequent, automated buffer regeneration protocols.
- Mobility and migration time variability caused by certain factors (e.g. temperature, capillary lengths, ionic strength, viscosity, EO modifiers, etc.) can be compensated for by using the corrected migration time parameter.
- Proper identification of the mobility marker and internal standards relies on location of reference peaks. Proper peak identification relies on the automatic calculation of corrected migration time during post run analysis.
- Changes in area due to differences in analyte mobility can be corrected for during post run analysis. This correction plus the addition of an internal standard will lead to proper integration and accurate concentrations.

CONCLUSIONS

Many of the conclusions have been presented in the results and discussion of individual experiments or sections. Some of the major benefits that HPCE offers to the pharmaceutical method development chemist are:

- Methods can be developed and parameters optimized rapidly.
- HPCE can handle ionic/neutral solutes and appears to offer significant advantages in methods development of highly charged solutes.
- Automation of methods development can be obtained using commercially available hardware. Some systems offer instrument control and HPCE specific-data calculation ability.
- The extremely high efficiency and resolving power of the technique offer significant potential to resolve impurities never achieved.
- The technique offers advantages in solvent savings, speed of analysis, limited mass requirements, and sensitivity.
- Time savings and increased sample throughput.
- Reproducibility can be achieved that will meet governmental regulations.
- Separation of chiral compounds is a strong point of HPCE.
- The unique complementary selectivity differences of HPCE and HPLC make them an ideal combination for cross confirmation of purity.

The final and most important conclusion that can be drawn from this work is that high performance capillary electrophoresis presents a new array of tools for the development of quantitative methods for pharmaceutical drugs and related compounds!

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