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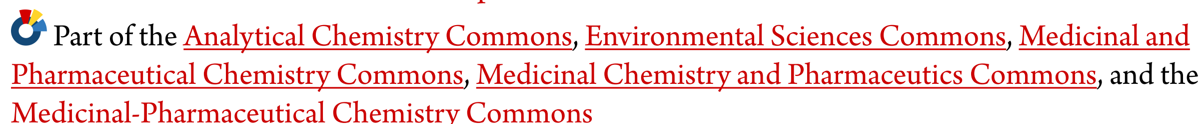

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Capillary electrophoresis separation of neutral organic compounds, pharmaceutical drugs, proteins and peptides, enantiomers, and anions

by

Wei-Liang Ding

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Analytical Chemistry

Major Professor: James S. Fritz

Iowa State University

Ames, Iowa

1998

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TABLE OF CONTENTS

ABSTRACT . . . vi

CHAPTER 1. GENERAL INTRODUCTION . . . 1
- Dissertation Organization . . . 1
- Capillary Electrophoresis . . . 1
- Micellar Electrokinetic Chromatography . . . 4
  - Type of surfactants . . . 5
    - Anionic . . . 6
    - Cationic . . . 7
    - Nonionic and Zwitterionic . . . 7
    - Mixed systems . . . 8
    - Polymers . . . 9
  - pH . . . 9
  - Organic modifiers . . . 10
- Separation of Peptides and Proteins . . . 12
  - FSCE . . . 12
  - CGE . . . 15
  - CIEF . . . 16
- Separation of Enantiomers . . . 17
  - Cyclodextrins . . . 18
  - Chiral crown ethers . . . 20
  - Chiral surfactants . . . 20
  - Carbohydrates . . . 21
  - Metal-ion complexes . . . 22
  - Proteins . . . 22
  - Antibiotics . . . 22
- High Salt Capillary Electrophoresis . . . 23
- Bibliography . . . 25

CHAPTER 2. SEPARATION OF NONIONIC COMPOUNDS BY CAPILLARY ELECTROPHORESIS USING A LAURYL POLYOXYETHYLENE SULFATE ADDITIVE . . . 34
- Abstract . . . 34
- 1. Introduction . . . 35
- 2. Experimental . . . 36
  - 2.1 Materials . . . 36
  - 2.2 Instrumentation . . . 37
  - 2.3 Synthesis of sodium Brij-30 sulfate . . . 38
- 3. Results and Discussion . . . 38
  - 3.1 Preparation and properties of Brij-30 . . . 38
  - 3.2 Effect of experimental variables . . . 39
    - 3.2.1 Brij-S concentration . . . 39

3.2.2 Applied voltage 41
3.2.3 Acetonitrile concentration 41
3.3 Separation of neutral compounds 46
4. Conclusions 57
Acknowledgements 57
References 58

CHAPTER 3. SEPARATION OF NEUTRAL COMPOUNDS AND PHARMACEUTICAL DRUGS BY CAPILLARY ELECTROPHORESIS IN ACIDIC SOLUTION USING LAURYL POLYOXYETHYLENE SULFATE AS AN ADDITIVE 60
Abstract 60
1. Introduction 61
2. Experimental 63
2.1 Capillary electrophoresis system 63
2.2 Reagents and chemicals 64
2.3 Preparation of sample solutions and buffers 64
3. Results and Discussion 65
3.1 Separation of PAH compounds 65
3.2 Separation of phenols 70
3.3 Separation of pharmaceutical drugs 74
4. Conclusions 87
Acknowledgements 88
References 88

CHAPTER 4. SEPARATION OF BASIC PROTEINS, PEPTIDES AND PHARMACEUTICAL COMPOUNDS BY CAPILLARY ELECTROPHORESIS USING A CATIONIC SURFACTANT 90
Abstract 90
1. Introduction 91
2. Experimental 92
2.1 Instrumentation 92
2.2 Samples and chemicals 93
3. Results and Discussion 93
3.1 Conditions for separation 93
3.2 Effect of pH 95
3.3 PEG additive effect 104
3.4 n-Butanol additive effect 104
3.5 Sample solutions 106
3.6 Simultaneously separation of cationic and anionic pharmaceutical compounds 106
3.7 Reproducibility 113
4. Conclusions 115
Acknowledgements 116

References 116

CHAPTER 5. STUDY OF CHIRAL SELECTOR STRUCTURE AND MIXED SELECTORS SYSTEM ON CHIRAL SEPARATION IN CAPILLARY ELECTROPHORESIS 119
Abstract 119
1. Introduction 120
2. Experimental 123
2.1 Apparatus 123
2.2 Reagents 124
2.2.1 Chemicals 124
2.2.2 Synthesis of decyl chloroformate 125
2.2.3 Synthesis of (S)-(+)-N-decoxycarbonyl-leucine 125
3. Results and Discussion 126
3.1 Effect of surfactant concentration 126
3.2 Effect of amino acids on the surfactant structure 132
3.3 Effect of surfactant chain length 132
3.4 A mixed system containing sulfated ß-CD and chiral surfactant 137
4. Conclusions 145
Acknowledgements 147
References 147

CHAPTER 6. CAPILLARY ELECTROPHORESIS OF ANIONS AT HIGH SALT CONCENTRATIONS 150
Abstract 150
1. Introduction 151
2. Materials and Methods 152
2.1 Chemicals 152
2.2 Instrumentation 152
3. Results and Discussion 153
3.1 Analysis of high-salt samples 153
3.2 Effect of BGE salt content on CE separations 157
3.3 Effect of Methanol 165
3.4 Separation of organic anions 165
3.5 Practical applications 170
4. Conclusions 173
Acknowledgements 174
References 174

CHAPTER 7. GENERAL CONCLUSIONS 177

ACKNOWLEDGEMENTS 179

ABSTRACT

Addition of a novel anionic surfactant, namely lauryl polyoxyethylene sulfate, to an aqueous-acetonitrile electrolyte makes it possible to separate nonionic organic compounds by capillary electrophoresis. Separation is based on differences in the association between analytes and the surfactant. Highly hydrophobic compounds such as polyaromatic hydrocarbons are well separated by this new surfactant. Migration times of analytes can be readily changed over an unusually large range by varying the additive concentration and the proportion of acetonitrile in the electrolyte. Several examples are given, including the separation of four methylbenz[a]anthracene isomers and the separation of normal and deuterated acetophenone.

The effect of adding this new surfactant to the acidic electrolyte was also investigated. Working at acidic condition at pH 2.4 has the advantage that electroosmotic flow is virtually eliminated. Neutral compounds are dragged by their association with the surfactant, moving as the same direction as the surfactant, resulting in fast separations. Basic compounds are also investigated under the same conditions. Excellent resolution of a mixture of 19 polyaromatic hydrocarbons was obtained in only 20 min.

Incorporation of cetyltrimethylammonium bromide in the electrolyte is shown to dynamically coat the capillary and reverse electroosmotic flow. The coating prevents basic proteins and peptides adsorption into the capillary wall, resulting in high separation efficiencies. A systematic study of experimental parameters demonstrated the importance of selecting a suitable buffer and an appropriate pH.

Chiral recognition mechanism is studied using several novel synthetic surfactants as chiral selectors, which are made from amino acids reacting with alkyl chloroformates. It was found that enantiomeric resolution can be readily manipulated by varying the alkyl groups (different chain lengths), amino acids and surfactant concentrations. Sulfonated ß-cyclodextrin is also employed as a chiral selector to compare chiral selectivity with these synthetic surfactants. A duo-chiral selectors system is further investigated for the possibility of eliminating some tedious steps in chiral compounds method development.

A satisfactory separation of both inorganic and organic anions is obtained using electrolyte solutions as high as 5 M sodium chloride using direct photometric detection. The temperature inside the capillary that resulted from Joule heating is calculated, and used to explain the unexpected fast and efficient separations. Since electroosmotic flow is suppressed, a quaternary ammonium additive (Q^+), which is normally used to reverse the electroosmotic flow in anion separations, is not utilized in our study. The effect of various salts on electrophoretic and electroosmotic mobility is further discussed. Several examples are given under high-salt conditions.

CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation begins with a general introduction containing a review of pertinent literature. This is followed by three research papers that are published or have been accepted for publication. The fourth paper is under preparation and the fifth paper has been submitted for publication. Permission from the publisher extending reproduction and distribution rights has been obtained. A general conclusion section follows these five papers. Each paper is similar to the published version, although additional figures and tables have been added. Figures and tables are contained in the text of the paper at the appropriate location. References cited within each paper are listed after the conclusions of each paper.

Capillary Electrophoresis

Capillary Electrophoresis (CE) is a technique for separating charged molecules based on their movement through a medium under the influence of an applied electric field. It was first introduced by Jorgenson and Lukacs in 1981 [1]. Over the last 20 years CE has demonstrated to be a fast and versatile analytical technique that combines simplicity with high reproducibility [2]. Narrow-diameter polyimide-coated fused silica capillaries assures flexibility and allows efficient heat dissipation, thereby permits the use of high field strength resulting in separation efficiencies over 10^5-10^6 theoretical plates. Complex mixtures of analytes can be resolved and recorded as sharp peaks due to the lower risk of zone

broadening. In its diverse modes of operation (capillary free zone electrophoresis (CZE) [3-5], micellar electrokinetic chromatography (MEKC) [6-10], capillary gel electrophoresis (CGE) [11-14], capillary isotacophoresis (CITP) [15-17], capillary isoelectric focusing (CIEF) [18-20], and capillary electrochromatography (CEC) [21-23]), CE can be applied to analyze a wide variety of analytes ranging from low molecular weight analytes such as inorganic anions [24-28], metal cations [29-32], drugs [33-35] to larger molecules such as oligosaccharides [36-40], peptides [11,41-43], proteins [5,44-46], DNA [46-50], bacteria [51,52], and single cells [53-55].

Separation by CE is based on different electrophoretic mobilities of ions (μ_{ep}, $cm^2/V{\cdot}s$), which are governed by their charge/size ratio [56],

$$\mu_{ep} = \frac{q}{6\pi\eta r} \quad (1)$$

where q is the net charge, η is the viscosity of the buffer, and r is the hydrated radius. According to Eq. 1, electrophoretic mobilities are independent of electric field (E) and capillary length (L). However, both mobilities (μ) and velocities (ν) can be measured experimentally:

$$\nu = \frac{L_d}{t_m} \quad (2)$$

$$\mu = \frac{\nu}{E} = \frac{L_d \cdot L_t}{t_m \cdot V} \quad (3)$$

where L_d is the length of the capillary to the detector, L_t is the total length of the capillary, t_m is the migration time, and V is the applied voltage.

A prominent phenomenon in CE is electroosmosis (EO). Electroosmosis occurs due to the surface charge on the wall of the capillary. An anionic charge on the capillary surface presumably owing to the ionization of silanol groups at most pH conditions results in the formation of an electrical double layer. When an electric field is applied, the layer of positive charge migrates toward the negative electrode. Since ions are solvated by water, the fluid in the buffer is mobilized as well and dragged along by the migrating cations, resulting in the bulk flow of liquid in the direction of the cathode, known as electroosmotic flow (EOF). The electroosmotic mobility (μ_{eo}) as defined by Smoluchowski in 1903 is given by

$$\mu_{eo} = \frac{\varepsilon_0 \xi}{4\pi\eta} \tag{4}$$

where ϵ_0 is the dielectric constant, η is the viscosity of the buffer, and ξ is the zeta potential on the surface. The magnitude of the EOF is largely affected by the pH of the solution. This is because the degree of dissociation of the silanol groups (which has a pK_a of 6-7) on the capillary wall is dependent upon the pH of the solution, and so is the zeta potential. Other experimental conditions, such as temperature, the buffer concentration, organic solvents concentration, and chemical additives, can also be manipulated to vary both magnitude and direction of the EOF.

The measured mobilities according to Eq. 2 are truly the sum of the electrophoretic (μ_{ep}) and electroosmotic mobilities (μ_{eo}):

$$\mu = \mu_{ep} + \mu_{eo} \tag{5}$$

Micellar Electrokinetic Chromatography

Neutral solutes cannot be separated by CZE due to their zero electrophoretic mobilities. It was in 1984 when Terabe and co-workers [6] introduced micellar electrokinetic chromatography (MEKC), and both neutral and charged solutes can be separated simultaneously using the same CZE instrumentation. An anionic surfactant, such as sodium dodecyl sulfate (SDS), is added in the buffer system. When the concentration of the surfactant exceeds its critical micelle concentration (cmc), surfactants incline to self-aggregate, such that hydrophobic tails form a nonpolar core while hydrophilic heads extend on the outer shell. This association of surfactant molecules is referred to as a micelle. For an anionic micelles, its electrophoretic mobility is toward the anode. However, under most conditions the micelle's electrophoretic velocity is not large enough to overcome the EOF, thus the micelle still migrates slowly toward the detector. This micellar medium provides a pseudostationary phase upon which analytes can interact. A cationic surfactant can be employed as well although a reversed electrode polarity has to be used.

The differential partitioning of solutes between the aqueous phase and the micelle results in different retention factors k', which is the ratio of the number of moles of solute in the micelle and aqueous phase. The migration time (t_m) for neutral compounds in MEKC can be expressed in term of retention factor k' [57]:

$$t_m = \frac{1+k'}{1+\frac{t_0}{t_{mc}}k'}t_0 \qquad (6)$$

Similarly, the resolution (Rs) is given by

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'}{1+k'}\right)\left(\frac{1-\frac{t_0}{t_{mc}}}{1+\frac{t_0}{t_{mc}}k'}\right) \tag{7}$$

where N is the number of theoretical plates, α is the selectivity, t_0 is the migration time for unretained solutes, and t_{mc} is the time for the micelles to pass the column. The difference between conventional column chromatography and MEKC is the extra term t_0/t_{mc} in the denominator. When t_{mc} equals to infinity, Equation 6 and 7 reduce to conventional chromatographic expressions.

t_{mc}/t_0 is also called elution range and has a large impact on both peak capacity (n_c) and resolution (R_s). As the elution range increases, so do the peak capacity and the resolving power. A variety of conditions have been examined to evaluate their effect on elution range, including electroosmotic flow [58], buffer concentration [59], pH [60,61], surfactant concentration [58,59,62], surfactant type [63-65], organic modifier content [62,66-68], surfactant counterion [60,61,65,69,70], and mixed micelle composition [71,72].

Type of surfactants

The development of new micelle phases continues to be a very active area of research. Since sodium dodecyl sulfate (SDS) was introduced in 1984, a large variety of surfactants have been utilized in CE, including anionic, cationic, and zwitterionic, etc. The research presented in Chapter II involved the separation of neutral compounds using a novel synthetic anionic surfactant, namely lauryl poly(oxyethylene) sulfate. This new surfactant is shown to be very effective for the separation of compounds range from highly hydrophobic polycyclic hydrocarbon compounds to small polar compounds.

Anionic

Whereas sodium dodecylsulfate (SDS) is a widely used anionic surfactant in MEKC [73-75], other anionic surfactants have been utilized including sodium pentanesulfonate, sodium octanesulfonate, sodium octyl sulfate [76], sodium decyl sulfate [64], to name a few. Generally, surfactants with alkyl tails of C8 or less do not form micelles under conditions found in capillary electrophoresis and are added as ion-pairing reagents. Alternatively, surfactants with alkyl tails greater than C14 have poor aqueous solubility.

Ahuja and Foley [70] studied the influence of dodecyl sulfate counterion on efficiency, elution range, and resolution. They found that the elution range increases in the order from lithium, sodium, to potassium. Cole and Sepaniak [77] reported that use of bile salts such as sodium cholate or sodium deoxycholate as pseudostationary phases improved separations of hydrophobic compounds, and Nishi et al. [78] utilized bile salts to separate lipophilic corticosteroids and benzothiazepine analogues.

A double-chain surfactant, disodium 5,12-bis(dodecyloxymethyl)-4,7,10,13-tetraoxa-1,16-hexadecanedisulfonate, was employed by Tanaka and co-workers [79] for several substituted naphthalene and benzene derivatives, and it was found to exhibit remarkably different selectivity and a wider migration time window compared with SDS. Likewise, Shi and Fritz [80] utilized sodium dioctyl sulfosuccinate (DOSS) in an aqueous solution containing 40% (v/v) acetonitrile. They reported that different electrophoretic mobilities for nonionic organic compounds were created as a function of the strength of the analyte/DOSS association in a nonmicellar environment.

The work of Smith and El Rassi [81,82] focused on MEKC with in situ charged

micelles. The surface charge on micelles formed by complexation between octylglucoside surfactant and alkylborate could be varied by changing the operation parameters such as borate concentration and/or pH of the buffer. This in turn created micelles with a range of hydrophobic character.

Cationic

Cetyltrimethylammonium bromide (CTAB) [63] was found to be useful for the separation of phenolic carboxylic acids [83], beta-blockers [84], flavonoids [85], and nucleic acid constituents [86]. Other cationic surfactants with similar structure, such as dodecyltrimethylammonium bromide (DTAB), dodecyltrimethylammonium chloride (DTAC), and cetyltrimethylammonium chloride (CTAC), have been evaluated in MEKC by Burton et al. [64] and Otsuka et al. [87]

Jorgenson and co-workers [88] studied electrophoretic medium consisting 0.025 M tetrahexylammonium perchlorate in 1:1 (v/v) acetonitrile/water solvent. Electrophoretic separations of polycyclic aromatic hydrocarbons (PAHs) can be accomplished by solvophobic association with tetraalkylammonium ion. Shi and Fritz [89] utilized a tetraheptylammonium salt in an aqueous acetonitrile buffer to separate nonionic organic compounds.

Nonionic and Zwitterionic

A non-ionic surfactant (Tween 20) was found to be useful for the separation of dansylamino acids by Matsubara and Terabe [90]. A zwitterionic surfactant N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (SB-12) was utilized near its cmc for a peptide separation reported by Greve and co-workers [91].

Mixed systems

Foley's group studied an anionic-zwitterionic mixed micelles system, which consists of sodium dodecyl sulfate (SDS) and N-dodecyl-N,N-dimethylammonium-3-propane-1-sulfonic acid (SB-12) [72]. The Joule heating contributed by surfactants can be minimized by partially substituting a zwitterionic surfactant for the original charged surfactant. The same group [71] reported that a nearly infinite elution range in MEKC is feasible by using a nonionic/anionic mixed micellar system, which comprised of a mixture of nonionic poly(oxyethylene)(23) dodecanol (Brij-35) and anionic (SDS) surfactants.

Separation of hydrophobic cations under the mixed micellar system composed of SDS and Tween 20 was investigated by Esaka and co-workers [92]. Likewise, a mixed micellar system of SDS and sodium cholate was evaluated for the separation of highly hydrophobic compounds such as corticosteroids [93].

Cyclodextrins (CDs) have also been used with SDS micelles to separate corticosteroids [94], aromatic hydrocarbons [94], and polyaromatic hydrocarbons [95]. This separation mode is called CD-modified MEKC. Sepaniak et al. [96] employed a duo-CD system consisting of a neutral CD and a charged CD for separation of nonionizable solutes. Neutral CDs acted as a primary phase which transported with electroosmotic flow, and charged CDs as an electrophoretically mediated secondary phase. The specificity associated with solute-CD inclusion complexation provides unique elution orders that do not follow the hydrophobicity trends of MEKC. Moreover, capacity factors can be altered in a convenient and predictable fashion simply by changing the CD phase ratio. Likewise, Szolar and co-workers [97] used a mixture of neutral and anionic ß-CDs to demonstrate the principle of

separation based on differential partitioning of analytes between the cyclodextrins, and achieved good separations for PAH isomers, including benzo[a]pyrene and benzo[e]pyrene.

Polymers

Palmer et al. [98] utilized oligomerized sodium 10-undecylenate to form a stable monomolecular pseudostationary phase. Tanaka and co-workers [99] employed starburst dendrimers (SBDs) (poly(amidoamines)) as a pseudostationary phase. The separation of neutral aromatic compounds was found to be influenced by the size and charge state of the SBDs.

Terabe and Isemura [100] used poly(diallyldimethylammonium chloride) (PDDAC) and (diethylamino)ethyldextran (DEAE-dextran) to separate isomeric ions having identical electrophoretic mobilities, for instance, 1- and 2- naphthalenesulfonate.

Resorcarenes, macrocyclic molecules built up by four alkylidene-bridged resorcinol units, have been employed as new pseudostationary phases to separate 12 PAHs by Bachmann and co-workers [101]. Similarly, Sun et al. [102] reported that calixarenes, a class of macrocyclic phenolic compounds with a basket-like shape, were used for separations of native and substituted polycyclic aromatic hydrocarbons.

Other types of alternate stationary phase include microemulsions [103], suspensions [104], and packed capillary [105].

pH

In chapter III of this dissertation, the use of extremely low pH (~ 2.4) for separation of neutral compounds is discussed. Again, the synthetic surfactant, Brij-S, is utilized as a pseudostationary phase.

Usually MEKC is done at basic pH, this is because stronger electroosmotic flow (EOF) is required to overcome the micellar electrophoretic mobility. Moreover, pH higher than 8 is necessary to achieve reproducible migration times. This is conformed by Shi and Fritz [80]. They found that electroosmotic flow reaches a maximum plateau above pH 8.0.

Some other pHs were occasionally utilized in MEKC depending on nature of the analytes. Fujiwara and Honda [65] employed a carrier containing 0.05 M SDS and 0.02 M phosphate at pH 11 to separate principal ingredients of antipyretic analgesics. While in another paper by Fujiwara and co-workers [64], pH 9.0 was found to be optimum to separate water-soluble vitamins.

Acidic pH system is seldom tried on MEKC. Sepaniak et al. [106] pointed out that separations at low pH (about pH 5) are impractical because the EOF is reduced, and is counterbalanced by the micelle electrophoretic mobility in the opposite direction, resulting in excessively long migration times. Terabe et al. [107] found that the poor reproducibility of migration times in bare-silica columns below pH 5.0 causes unreproducible results.

Recently, Janini and co-workers [108] demonstrated that separation of some hydrophobic compounds can be achieved at acidic pH on a polyacrylamide coated capillary (eliminates the EOF) by MEKC. Mclaughlin et al. [109] applied this mode of MEKC to separate linear alkyl benzene sulfonate (LAS) samples.

Organic modifiers

Gorse et al. [68] studied effects of organic mobile phase modifiers in MEKC. They found that the addition of 1-20% (v/v) of methanol or acetonitrile to the mobile phase is shown to extend the elution range and thus increase the peak capacity of a given system.

The efficiency of these MEKC systems is also increased with the addition of either organic modifier. Moreover, the two solvents show differing influences on selectivity depending on solute polarity. Sepaniak and co-workers [110] observed a reversal in elution order for substituted phenolic compounds using an acetonitrile step gradient. Fujiwara and Honda [111] examined the effect of addition of organic solvent on the separation of positional isomers in CZE.

Many organic modifiers are useful in MEKC. These include methanol, propanol, acetonitrile, tetrahydrofuran, dimethylformamide, and others. Adding an organic modifier to the buffer is also necessary for solutes of moderate-to-high hydrophobicity. The percent of a modifier that can be added is limited by the impact of the solvent on the micellar aggregate. Features such as the CMC, aggregation number, and micellar ionization are affected by the percent organic modifier. Since high percentage of organic solvent can disrupt micelle formation, the amount of organic solvent that can be employed in MEKC is usually limited to less than 30%. However, the elimination of micelles does not mean that separations will not occur. There are many examples [88,89,112] showing that even ionic surfactant monomers and neutral solutes can form charged solvophobic complexes that could be separated electrophoretically.

In Chapter II, the use of high percentage of acetonitrile in the separation of PAH compounds is discussed, while in Chapter III, the use of a mixture of two organic solvents is compared with one solvent alone.

Separation of Peptides and Proteins

Since the beginning of modern CE (early 1980s), the protein application was one of the first to be fully explored in the technique development. There are three basic CE techniques for protein and peptides, i.e., free solution capillary electrophoresis (FSCE), capillary gel electrophoresis (CGE), and capillary isoelectric focusing (CIEF).

FSCE

CZE is the predominant mode in FSCE for protein and peptide applications due to its simplicity, speed, and low reagent costs. Separation is based on differences in charge density. Surfactant-based MEKC is also used in this application. Although large protein molecules are too bulky to fully partition into micelles, surfactants might help to improve separation to some extent, possibly by an ion-pair or/and hydrophobic interaction between the surfactant molecule and the protein.

Proteins are polyelectrolytes, and a major concern in protein separations is that adsorption usually occurs because of coulombic attractions between the negatively charged capillary surface and the positive charges on the protein molecules. This results in either tailing peaks or even no peaks at all. There are several ways to suppress or eliminate protein adsorption, i.e., pH extremes, either dynamically or permanently coating the surface, and ion-pairing.

Luer and McManigil [113] performed protein separations at high pH. When pH is above the isoelectric point (pI) of a protein, the protein becomes negatively charged and tend to be repelled from the capillary surface.

McCormick [45] utilized low pH buffers to separate peptides and proteins. Most of

the negative charge on the capillary surface will be suppressed at low pH below 2. This means that the protein will not be electrostatically attracted to the neutral wall. A disadvantage of the extreme pH approach is that there is a wide rage of pHs which are unusable. Moreover, since proteins are either fully protonated or deprotonated at extreme pH, the separation for proteins varying only in subtle differences would be difficult.

Okafo et al. [114] utilized phytic acid as an ion-pairing reagent in order to suppress peptide adsorption. This anionic ion-pairing reagent reacts with cationic sites on the peptide molecule, making it electrophoretically negatively charged, thus eliminating its adsorption on the same charged capillary wall.

Another effective approach is to coat the capillary surface either dynamically or covalently. Covalent coating is achieved by attaching hydrophilic polymers to the silanol groups by using chemical derivatization procedures. The coatings which have appeared in the literature include methylcellulose, polyacrylamide, polyethyleneglycol, polyvinylpyrrolidone, 3-glycidoxypropyltrimethoxysilane, polyether, α-lactalbumin, etc.

Dynamic coating is performed by rinsing the capillary with an electrolyte in which various additives can be added. Green and Jorgenson [115] minimized adsorption of proteins by addition of alkali metal salts to the buffer. Since the salt competes for adsorption sites on the capillary wall with proteins, the higher the salt concentration, the higher the probability of salt adsorption. A drawback of this approach is that a high current and temperature are induced with a high concentration of the salt. Bushey and Jorgenson [116] later utilized zwitterionic salts such as tricine, CAPS, and CHES instead of ionic salts to reduce Joule heating. Since the zwitterions yield very low conductivity, up to more than

1 M of these salts may be used.

Addition of divalent amines to the buffer was found to reduce the EOF and suppress solute-capillary wall interactions [113]. This phenomenon seemed specific to diamino structure because the corresponding monoamino compounds were not effective. 1,4-diaminobutane, 1,5-diaminopentane, and 1,3-diaminopropane have been used for this purpose [117,118].

By decreasing the zeta potential on the capillary surface, protein adsorption can also be suppressed. Diverse cellulose derivatives (e.g., methylcellulose, hydroxyethylcellulose, and hydroxypropylmethylcellulose), nonionic surfactant, polyethylene oxide, polyvinyl alcohol, or polyethyleneglycol have been employed for this purpose. Moreover, these additives increase the viscosity of the buffer, therefore decrease the inclination for protein adsorption.

Cationic surfactants such as cetyltrimethylammonium bromide (CTAB) [119] may be used to reduce protein adsorption by creating a positively charged surface. Fuerstenau and co-workers [120] discovered that the zeta potential on quartz beads could be reversed in alkyltrimethylammonium salt solutions with alkyl chain lengths between C10 and C18. They postulated a concentration-dependent formation of a bilayer on the surface, or so-called hemimicelles, to explain the charge reversal.

Lucy and Underhill [121] investigated the characterization of the cationic surfactant induced reversal of EOF. They reported that nature of the anionic counterion in the solution has a strong effect on the magnitude of the reversed EOF observed.

Tsuda et al. [122] utilized the reversed electroosmotic flow by CTAB for the

separation of anionic analyte. Later, Zare and co-workers [123] investigated another additive, tetradecyltrimethylammonium bromide (TTAB) for separation of a mixture of fast and slow anions.

Zemann and Volgger [124] studied coelectroosmotic MEKC of phenols using CTAB as pseudostationary phase and acetonitrile as organic modifier. Fast separations for phenol isomers were shown.

In a study by Cifuentes et al. [125], different buffer additives, e.g., potassium chloride, morpholine, CTAB, poly(vinyl alcohol) and polyethyleneimine, were compared for the separation of basic proteins. CTAB was found to give the best separations.

Cordova and co-workers [126] examined four polycationic polymers for limiting the adsorption problem; polyethylenimine (MW=15,000), polybrene (MW=25,000), poly-(methoxyethoxyethyl) ethylenimine (MW=64,000), and poly(diallyldimethylammonium chloride) (MW=10,000). Detection of proteins with high pI was readily achieved using the first three of these polycationic polymer coating but not with the poly(diallydimethyl-ammonium chloride).

The research presented in Chapter IV dedicated to this CTAB approach. A low concentration of CTAB below its cmc was utilized to avoid protein adsorption. The experimental parameters, such as concentration of the buffer ions, types of buffer ions, organic solvent, were found to be critical in optimizing the protein resolution in our study.

CGE

In 1987 Cohen and Karger [11] first introduced crosslinked polyacrylamide gel in the capillary column to separation proteins. The gel structure creates a molecular sieving effect,

allowing the separation based on different size and charge. One drawback in the use of crosslinked polyacrylamide gels is the lack of low-UV transparency for on-column detection.

Later on, linear polymer matrices were explored, such as linear polyacrylamide, dextrans, polyethylene oxides, and polyvinyl alcohols. Not only these polymer networks allow the use in the low-UV region, they also can be easily replaced by a simple rinse. Compared to conventional SDS-PAGE slab gel electrophoresis, capillary gel electrophoresis (CGE) has roughly equivalent resolution, however it can be fully automated, the separation is fast, and electropherograms are stored permanently.

CIEF

In capillary isoelectric focusing (CIEF), proteins are separated by electrophoresis in a pH gradient [18], which is provided by carrier ampholytes consisting of polyamino-polycarboxylic acids with slightly different pI values.

The technique is developed on the basis of the difference in pI of different proteins. During a positively charged protein migrates toward the cathode, it encounters gradually higher pH, hence picking up more negative charges. Eventually, the protein stops when the net charge is zero. At this point (pI), the sample is focused in a narrow zone. Routinely, proteins differing by 0.01 pI units can be separated by CIEF.

The next step consist of transporting the focused zones past the detection point in the capillary, using either one of the three modes, e.g., chemical mobilization, hydrodynamic mobilization, and electroosmotic mobilization.

Separation of Enantiomers

Stereochemical resolution of optically active molecules remains an important and essential stage in many fields of study concerning the life sciences, as well as in the development of biological active chemical entities as potential drugs. The current analytical methods for the separation of enantiomers can be either gas chromatography (GC), high performance liquid chromatography (HPLC), thin layer chromatography (TLC), supercritical fluid chromatography (SFC), or capillary electrophoresis (CE).

Regardless of the separation technique employed, chiral recognition is obtained in one of three ways:

1. formation of diastereomers by additives to the mobile-phase or carrier electrolyte;

2. formation of diastereomers through integration with a stationary phase or heterogeneous carrier electrolyte; or

3. pre-column (capillary) derivatization with an optically pure derivatizing reagent.

In the first two cases, the techniques rely on the formation of transient metastable diastereoisomers between each enantiomer of an optically active compound and a chiral component, occurring by electrostatic and/or hydrophobic mechanisms. Chiral resolution of the two enantiomers is possible when there are sufficient differences in the free energies of formation of the two diastereoisomeric complexes. In the third case, covalently bound diastereomers are resolved on an achiral phase as a result of differences in their physicochemical properties.

Mostly, chiral resolution in CE involves the addition of chiral compounds to the buffer system. Alternatively, precapillary derivatization is also employed to create synthetic

diastereomers.

The most commonly used chiral additives in CE include: cyclodextrins (native and derivatized), metal-ion complexes, chiral crown ethers, chiral surfactants (naturally occurring and synthetic), acyclic carbohydrates (neutral oligosaccharides and charged polysaccharides), protein additives, and macrocyclic antibiotics (rifamycins and glycopeptide antibiotics).

Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides composed of between six and eight glucopyranosyl units to form a hollow cone structure. The outer surface are linked by hydrophilic primary and secondary hydroxyl groups, while the interior cavity consists of hydrophobic glycosidic bonds and carbon skeleton. Naturally occurring cyclodextrins contain either six (α-CD), seven (ß-CD), or eight (γ-CD) glucose units. There are two requirements for which the chiral discrimination can take place. One is that the analyte must have the appropriate structure geometry to fit into the CD cavity. The other is that polar groups should be in close proximity to the chiral carbon center.

Furuta and Doi [127] demonstrated that the size of the aromatic group in the analytes and the nature of the attached substituents influence the inclusion within the CD cavity. For instance, enantiomeric resolution of thiazole derivative was achieved using ß-CD and γ-CD, however, attempt to use α-CD was unsuccessful due to the poor fit of the molecule within the cavities of α-CD. As a general rule, analytes with unsubstituted aromatic rings fit perfectly in α-CD (cavity diameter, 5 to 6 Å); compounds with naphthalene groups or substituted phenyl rings suit in ß-CD (cavity diameter, 7 to 8 Å), while most complex polycyclic structures are able to include in γ-CD (cavity diameter, 9 to 10 Å).

Chemically modifying the hydroxyl groups at the 2, 3, and/or 6 positions by introducing a range of charged and uncharged functional groups can alter the physicochemical properties of CDs, such as the aqueous solubility, the overall hydrophobic character, the shape and size of their cavities, their hydrogen-bonding ability, and ultimately the enantioselectivity.

Uncharged CD derivatives can be synthesized by selective alkylation of hydroxyl groups at 2-, 3-, 6-, 2,3- or 3,6- positions by methyl, ethyl, hydroxypropyl, naphthylcaramoyl and glycosylation. Yoshinaga and Tanaka [128] compared native ß-CD with 3-methyl-ß-CD, 2,3-dimethyl-ß-CD, and 2,3,6-trimethyl-ß-CD for the separation of dansyl-DL-leucine, and found that a significant enhancement in the enantioselectivity was achieved by those derivatized CDs.

Charged CD derivatives are prepared by carboxyl-, succinyl-, sulfonyl-, aminoalkyl-, or sulfoalkylation of hydroxyl groups. One primary advantage of these charged CDs over their neutral relatives is that the separation window can be increased due to a larger difference between the electrophoretic mobilities of the analyte and that of the charged CD. Another benefit from using charged CDs is that different modes can be used depending on the pH of the buffer.

Ingelse and co-workers [129] utilized a soluble neutral ß-cyclodextrin polymer to enantiomeric resolve a range of cationic pharmaceutical racemates, e.g., selegine, clenbuterol, and beta-blockers. They found that the resolution is influenced by pH, temperature, organic solvents, and concentration of polymeric additive, as in the circumstance of their monomeric counterparts.

Chiral crown ethers

The first chiral crown ether, known as (2R, 3R, 11R, 12R)-(+)-1,4,7,10,13,16-hexaoxacyclooctadecane-2,3,11,12-tetracarboxylic acid, was reported by Kuhn et al. [130] for the separation of aromatic amino acids racemates. Enantiomeric discrimination by chiral crown ethers involves either a steric or stereoselective hydrogen-bonding interaction between the analyte and the carboxylic acids on the crown ether. A primary amino functional group in the analyte is required to allow the hydrogen bonding. This is why all enantioseparations involving chiral crown ether are carried out at low pH conditions.

Chiral surfactants

The research presented in Chapter V of this dissertation involved enantiomeric separation of pharmaceutical drugs using synthetic chiral surfactants, and studies of a mixed chiral selector system. An optically active surfactant usually possesses a chiral functional headgroup and an alkyl hydrophobic chain. The chiral discrimination mechanism is presumed to involve two steps, e.g., the first step consists of stereoselective interactions between functional groups on the chiral analyte and the optically active surface of the micelle, and the second step involves inclusion of the preferred isomer into the interior of the micelle. Bile salts [131,132], digitonin [133] and saponins [134] are examples of naturally occurring optically active detergents which have been used as chiral additives in CE.

The headgroups of synthetic chiral surfactants can either be enantiomers of amino acid derivatives, other chiral acids, or sugar derivatives. Dobashi et al. [131] reported the utilization of N-dodecanoyl-L-valinate and SDS to resolve the enantiomers of N-(3,5-

dinitrobenzoyl)-O-isopropyl esters (DNB) of four amino acids racemates. Besides L-valine, other amino acids have been used in the synthesis of chiral surfactants, such as L-threonine [136], L-alanine [137], and L-serine [137]. However, these surfactants showed lower α values for DNB-amino acids than the valine derivative.

Mazzeo and co-workers [138] introduced another class of synthetic chiral surfactants, e.g., (R)- and (S)-N-dodecoxycarbonyl-valines. These compounds have been found to have a broader enantioselectivity than N-dodecanoyl-L-valine counterpart, and shown to resolve a range of twelve pharmaceutical drugs including beta-blockers, bupivacaine, and homatropine. Reversal of the migration order of related isomers was simply accomplished by replacing an L-valine derived surfactant with the corresponding surfactant from D-valine. This is particularly critical in the determination of a small quantity of chiral impurities in a sample consisting predominantly of another isomer.

Other classes of synthetic chiral surfactant include dodecyl-ß-D-glucopyranosyl derivatives [139] and 2-undecyl-4-thiazolidine carboxylic acid [140].

Carbohydrates

These chiral selectors can be classified into two groups: noncyclic neutral oligosaccharides, e.g., maltodextrins, and charged polysaccharides, including dextran sulfate, heparin, and chondroitin sulfate C. D'Hulst and Verbeke [141] were the first to use maltodextrins to separate enantiomers of coumarinic anticoagulant drugs. Maltodextrins are formed by D-(+)-glucose units linked linearly through 1-4α glycosidic bonds. The conformation of these linear dextrins is thought to be a flexible random coil, which can change to a complete helix in the presence of an interacting analyte and buffer salts. The

flexibility of this structure allows them to interact with a wider range of molecular structures than cyclodextrins. It was found that the degree of enantiomeric resolution and selectivity is basically dependent on the degree of polymerization and molecular weight of the oligomer. Increasing the concentration of the chiral additive was also utilized to enhance the enantiomeric resolution.

Metal-ion complexes

Zare et al. [142] were the earliest to recognize the addition of Cu(II) and L-histidine to the buffer solution to resolve dansyl amino acids via a trimolecular complex. Later, aspartame, L-proline, and L-histidine were found to possess the structural components for chelation with the metal ion. This chiral separation method has mainly limited to the enantiomeric resolution of dansyl-DL-amino acids.

Proteins

The advantage in protein-based chiral separation is that there are many stereoselective bonding interactions involved, including hydrogen bonding, pi-pi, and steric effects. The term affinity electrokinetic chromatography (AEKC) has been applied to this mode of CE. Bovine serum albumin, α1-acid glycoprotein, human serum albumin, conalbumin, fungal cellulases, and ovomucoid have been used as protein additives in the resolution for enantiomers.

Antibiotics

Used as chiral selectors for LC, TLC, GC, SFC, and CE, macrocyclic antibiotics compounds were first introduced by Armstrong et al. [143-145]. This class of compound falls into two categories: ansamycin antibiotics (rifamycin B and SV) and the amphotiric

glycopeptide antibiotics (vancomycin, ristocetin A, and teicoplanin). Most of these antibiotics have multiple chiral carbon centers, and the presence of multiple stereogenic sites and functional groups makes them ideal candidates for stereoselective interactions.

High Salt Capillary Electrophoresis

It has been long believed that Joule heat, which is a result of the electric current passing through the electrophoresis buffer within the capillary, can have negative affect on the quality of the separation. The temperature gradients can cause natural convection, which will remix separated sample zones, distort the peak shapes, and damage the separation performance. There are several papers that devote to discussing the temperature profile inside the capillary, the thermal effects, and the zone spreading [146-148].

However, using a lower salt concentration in the sample than in the electrolyte to achieve electrostacking effect is used in many cases to improve separation efficiency and detectability. In a study by Chien and Burghi [149], an enhancement factor ~100 was achieve for the sample injection using electrostacking method.

Thornton and Fritz [28] separated inorganic anions employing an acidic buffer system, where the buffer contains 0.024 M hydrochloric acid. They reported that the greatest peak height occurred for $AuCl_4^-$ when they used a three to one ratio between the chloride concentrations of the carrier electrolyte and the sample.

Mclaughlin et al. [150] demonstrated the effect of the ionic strength of the running buffer on the migration and peak height of a standard mixture of bioactive peptides. The running buffer was sodium phosphate at pH 2.5. When its concentration went from 0.025

M to 0.125 M, a significant increase in peak efficiency and peak height was observed.

Jones [151] utilized a water jacket which surrounds a capillary to study temperature effect on seven inorganic anions. The ambient temperature around the capillary was readily changed by controlling the thermostatted water, however, the actual temperature inside the capillary was not measured. Temperature from 25°C to 60°C was studied. The fundamental effect of temperature is through the change of viscosity. It is estimated that temperature-induced changes in electrophoretic mobility are approximately 2% ~ 3% per degree. The sequence of migration remains unchanged over the entire temperature range, and apparently the resolution does not deteriorate.

Improvements in separation efficiency at a higher temperature have been observed in MEKC by Balchunas and Sepaniak [152]. NBD-amines were separated at two different temperatures (e.g., 27 and 35°C) using a mobile phase containing 0.015 M SDS and 0.005 M Na_2HPO_4. Peaks were reported to be extremely sharp at the elevated temperature.

Jorgenson and co-workers [114] utilized high concentration of metal ions to suppress protein adsorption. However, low voltage was applied in order to avoid excess Joule heat, resulting in long migration times.

Chapter VI of this dissertation discusses the impact of high concentration of buffer ions on the separation of both inorganic and organic anions. The temperature rise inside the capillary is calculated. Direct analysis of samples with high salt content such as seawater is presented.

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CHAPTER 2. SEPARATION OF NONIONIC COMPOUNDS BY CE USING A LAURYL POLYOXYETHYLENE SULFATE ADDITIVE

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Abstract

A nonionic surfactant (Brij-30) was treated with chlorosulfonic acid to form $C_{12}H_{25}(OCH_2CH_2)_4OSO_3^-Na^+$. Addition of a low concentration of this material to an aqueous-acetonitrile electrolyte makes it possible to separate nonionic organic compounds by capillary electrophoresis. Separation is based on differences in the stabilities of analyte-additive complexes in solution. Migration times of analytes can be varied over an unusually large range by varying the additive concentration and the proportion of acetonitrile in the electrolyte. This greatly expands the scope of neutral organic compounds that can be separated by CE. Several examples are given. Some very difficult separations were achieved, such as the baseline resolution of four methylbenz[a]anthracene isomers and the separation of normal and deuterated acetophenone.

1. Introduction

Capillary electrophoresis(CE) is basically a highly efficient method for the analytical separation of cations or anions [1]. Neutral compounds can be separated by adding a surfactant to form a micellar pseudo phase. The separation is based on differences in partitioning of the analytes between the solution and the charged micelle. Terabe [2,3] named this system micellar electrokinetic capillary chromatography (MECC or MEKC). Sodium dodecyl sulfate (SDS) is the most widely used surfactant [2-5], whereas bile salts [6,7], a monomolecular micelle [8], and a non-ionic surfactant, Tween-20 [9], and sodium deoxycholate [10] are examples of other systems that have been used. Although some excellent separations have been obtained, the scope of MEKC tends to be limited by two factors. One is that little or no separation occurs for the compounds which are too strongly partitioned into the micelle. A second limitation is the limited solubility of some analytes in the predominately aqueous solutions needed to maintain a micelle.

Some years ago Walbroehl and Jorgenson [11] reported the separation of neutral compounds in aqueous solutions containing 50% (v/v) or more of acetonitrile. Under these conditions the additive used (a tetrahexylammonium salt) did not form a micelle but was believed to form an association complex with analytes in solution. More comprehensive studies by Shi and Fritz showed that excellent CE separations of PAHs and other neutral compounds could be obtained in ~35 to 45% acetonitrile using a tetrahexylammonium salt [12] or sodium dioctylsulfosuccinate (DOSS) [13] as the electrolyte additive. The elution order of analytes with DOSS (negatively charged) was just the reverse of that obtained with positively charged tetraheptylammonium additive.

Li and Fritz [14,15] have recently found that low concentrations of some surfactants greatly reduce the retention times of analytes in conventional HPLC separations carried out with 60% acetonitrile-40% water (v/v) as the mobile phase. It was felt that the high proportion of acetonitrile in the mobile phase precluded any micelle formation. The surfactants tested varied widely in their effectiveness. SDS performed poorly in most instances, while Brij-30 was one of the best. The structure of Brij-30, $C_{12}H_{25}(OCH_2CH_2)_4OH$, includes repeating $-OCH_2CH_2-$ groups in addition to the C_{12} hydrocarbon chain. This combination of groups may lead to stronger interaction in solution with the polar organic compounds, while less association with highly hydrophobic solutes.

In the present work, Brij-30 was sulfonated to give it a negative charge: $C_{12}H_{25}(OCH_2CH_2)_4OSO_3^-$. This sulfonated material, referred to as Brij-S, was used in CE to separate a wide variety of non-ionic organic compounds. Migration times of the neutral analytes can be varied widely by manipulating the concentration of Brij-S added to the electrolyte and the percentage of acetonitrile that is present.

2. Experimental

2.1. Materials

Acetonitrile and sodium borate were supplied by Fisher Scientific (Pittsburgh, PA, USA). 11-ß-hydroxytestosterone, 1-dehydrotestosterone, testosterone, 17-α-methyltestosterone and testosterone acetate were purchased from Sigma (St. Louis, MO, USA). 1-methylanthracene, 2-methylanthracene, 9-methylanthracene, 5-methylbenz[a]anthracene, 6-methylbenz[a]anthracene, 7-methylbenz[a]anthracene, 12-

methylbenz[a]anthracene were kindly supplied by Dr. R. Jankowiak (Iowa State University, Ames, IA, USA). All other chemicals were obtained from Aldrich (Milwaukee, WI, USA).

2.2 Instrumentation

CE separations were performed on a Quanta 4000E CE system (Waters, Milford, MA, USA). Uncoated fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) used for CE had 50 μm i.d. or 75 μm i.d. and 60 cm long (52.5 cm effective length). Direct UV absorbance detection was performed at either 254 nm or 214 nm. Hydrostatic injection times ranged from 2 s to 30 s. Running voltage ranged from +10 kV to +30 kV. Separations were performed at 25°C. Electropherograms were collected at speed of 15 points/s and plotted by Chromperfect data acquisition system (Justice Innovations, Mountain View, CA, USA).

All standards and buffer solution were prepared with 18.3 MΩ deionized water from a Barnstead Nanopure II system (Syboron Barnstead, Boston, MA, USA). Analytes were dissolved in running buffer and sonicated for 5 minutes. Buffers were prepared by mixing sodium Brij-30 sulfate, sodium borate, acetonitrile and adjusting pH by adding 0.5 M phosphoric acid or 0.5 M sodium hydroxide.

Prior to use, capillaries were rinsed for 30 minutes with 0.1 M sodium hydroxide and deionized water for 30 minutes. Between injections, the capillaries were rinsed with 0.1 M sodium hydroxide for 3 minutes and buffer for 5 minutes.

2.3 Synthesis of sodium Brij-30 sulfate

Brij-30 9.33 g was placed in a 100 mL 3-necked round-bottom flask with a dropping funnel. The flask was placing in an ice-bath. When temperature was dropping to 15°C, 3.0 g of chlorosulfonic acid, which was placed in the dropping funnel, was added slowly into the flask over about 15 minutes with constant stirring. The ice-bath was then removed, and the reaction flask was stirred for another 5 minutes. Sodium hydroxide (1.6 g) was dissolved in 30 g of ice-slurry; and the reaction solution was added into this basic ice-slurry; adjusted pH to 8 by 3.0 M sodium hydroxide solution in order to keep the product stable. This sodium Brij-30 sulfate solution was sufficiently pure to make buffers used in CE.

3. Results and Discussion

3.1 Preparation and properties of Brij-S

Brij-S was easily prepared by reacting Brij-30 with chlorosulfonic acid at ~15°C. Details are given in the Experimental section. We were not able to recrystallize or otherwise purify the Brij-S product owing to its high viscosity. However, this type of reaction is known to proceed with high yield. Mass spectroscopic analysis showed Brij-S to be a mixture containing one to eleven ethoxy groups in the molecule. Nevertheless, several batches of sulfonated Brij-30 gave virtually identical results in the CE experiments.

Brij-S was found to be readily soluble both in pure water and in mixtures of water and acetonitrile. DOSS, which was used in a previous paper [13], required an acetonitrile concentration higher than 15-20% to dissolve enough for a good CE separation. Tetraheptyl-ammonium salts [15] require an even higher percentage of acetonitrile for dissolution.

Several non-ionic compounds (mostly PAH compounds) were separated with 50 mM Brij-S as the additive using experimental conditions similar to those previously found to be optimal for DOSS as the mobile phase additive [13]. The electropherogram is shown in Figure 1. Although the conditions are not identical, it is interesting to compare the electropherogram in Figure 1 with Figure 6 of reference 13 with DOSS as the additive. The separation with Brij-S took longer, primarily because a longer capillary was used. However, the resolution of peaks 5, 6, 7 and of peaks 19, 20 was complete with Brij-S, whereas resolution of the same compounds was incomplete when DOSS was used. The order of elution of the test compounds was identical with DOSS and Brij-S. Greater differences in migration times of adjacent peaks with DOSS and Brij-S might have been expected owing to the differences in chemical structure of the two additives.

3.2 Effect of experimental variables

Preliminary separations were performed with Brij-S as the additive in order to find reasonable conditions for CE of six neutral organic compounds: nitrobenzene, benzophenone, naphthalene, acenaphthalene, anthracene and pyrene. Then one major condition was varied while keeping the others constant. The results of this optimization follow.

3.2.1 Brij-S concentration

At 10 mM Brij-S (25% acetonitrile, pH 9.0, 25 kV), the time frame for separation was very small and the peaks were poorly resolved. Increasing the Brij-S concentration to 30 mM resulted in stronger complexation and a much better separation. The separation

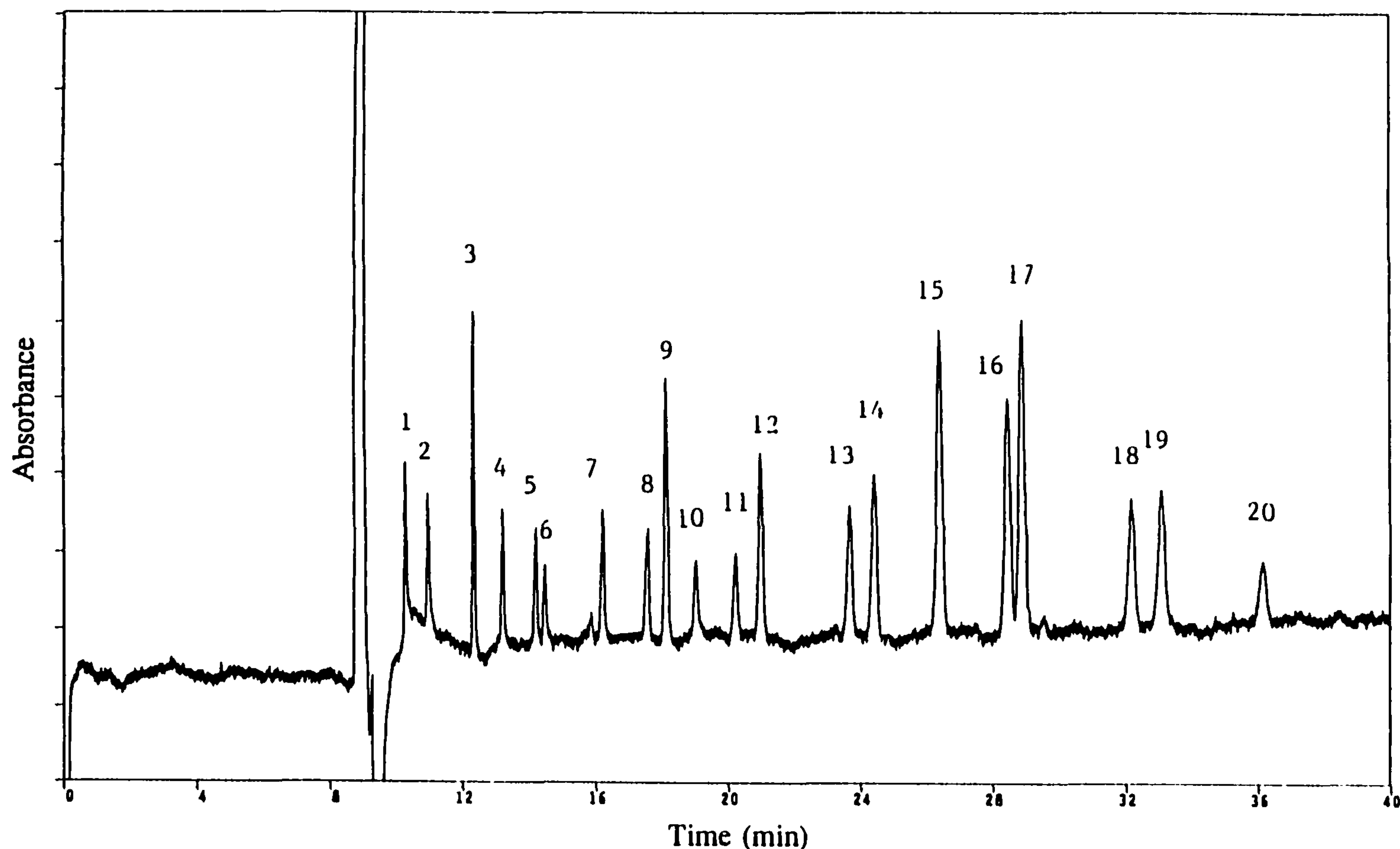

Figure 1. Separation of PAH compounds. Electrolyte, 40 mM Brij-S, 8 mM sodium borate, 40% (v/v) acetonitrile, pH 9.0; applied voltage, 20 kV; current, 68 μA; injection time 30 s; detection, 254 nm; capillary, 50 μm i.d., 75 cm length. Peaks: 1= acetophenone; 2= nitrobenzene; 3= 5,6-benzoquinoline; 4= benzophenone; 5= azulene; 6= naphthalene; 7= acenaphthylene; 8= acenaphthene; 9= fluorene; 10= 3-aminofluoranthene; 11= benz[a]anthracene; 12= anthracene; 13= fluoranthene; 14= pyrene; 15= 2,3-benzofluorene; 16= chrysene; 17= 2,3-benzphenathrene; 18= perylene; 19= benzo[a]pyrene; 20= benzo[ghi]perylene.

difference between the first and last peaks continued to increase with higher concentrations of Brij-S. Separation factors of peak pairs also increased steadily as the concentration of Brij-S increased.

3.2.2 Applied voltage

A larger applied voltage resulted in a linear increase in electroosmotic velocity from 0.10 cm/s at 15 kV to 0.25 cm/s at 30 kV. The electrophoretic velocity in the opposite direction for several test compounds also increased in approximately linear fashion with higher applied voltage, but with different slopes (see Figure 2). The time window for separation is smaller at higher applied voltages but the separation factors of analyte pairs do not vary greatly at the four voltages used. To summarize, separations are faster at the higher applied voltages but use of a lower voltage (15 or 20 kV) may be advantageous for resolution of peaks with similar migration times.

3.2.3 Acetonitrile concentration

Electroosmotic velocity (30 mM Brij-S, pH 9.0, 25 kV) decreased linearly from 0.25 cm/s at 0% acetonitrile to 0.12 cm/s at 45% acetonitrile (see Figure 3). Electrophoretic velocity of the test compounds, however, was in the opposite direction and decreased in a non-linear manner (Figure 4). The net result was that the velocity difference between the first and last peaks went through a maximum between approximately 15% and 25% acetonitrile in the running electrolyte. The retention times also experienced the same trend (Figure 5). A much better resolution of the analyte peaks was obtained at 15% acetonitrile

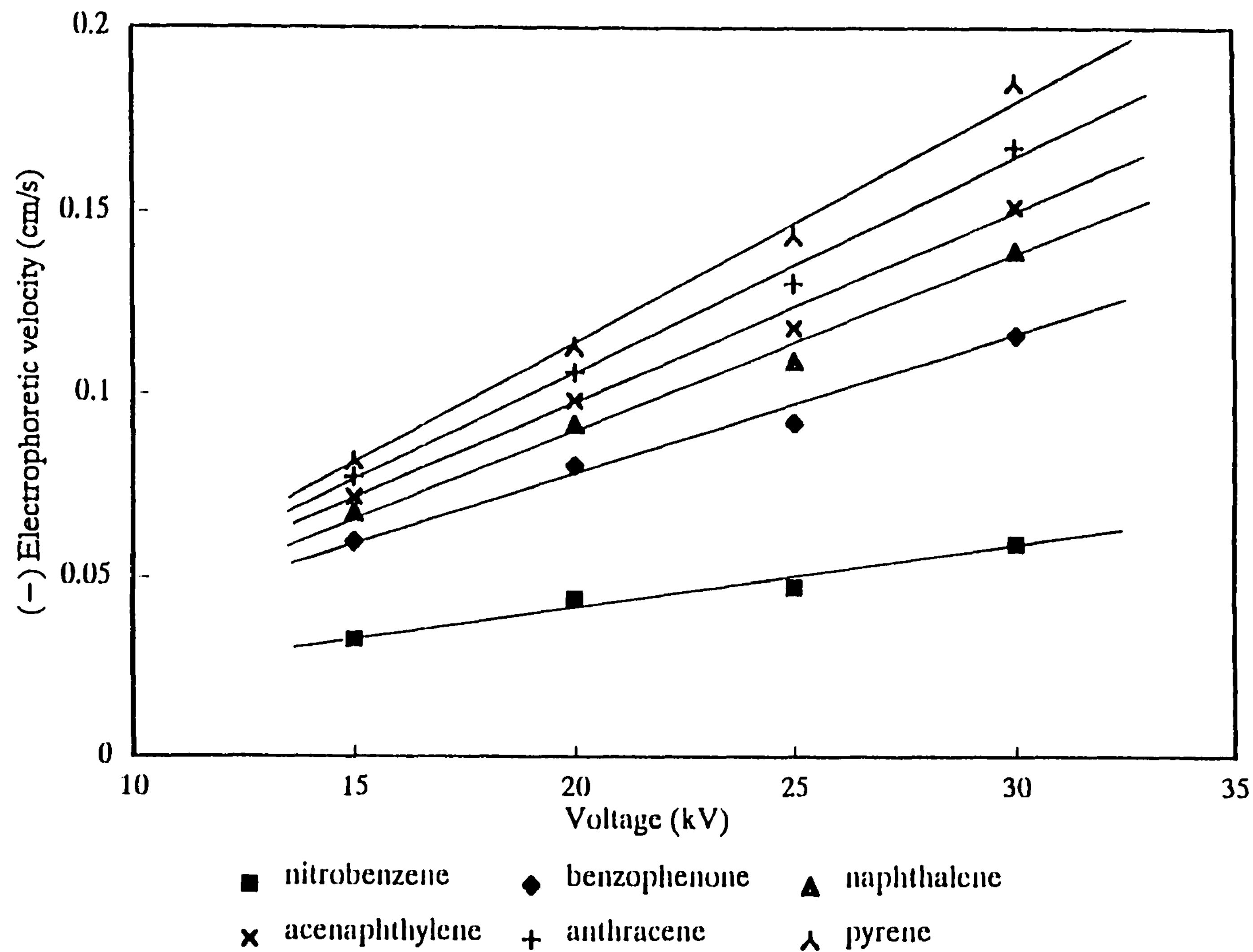

Figure 2. Plot of electrophoretic velocity of six compounds vs. voltage. Conditions: 8 mM borate, 30 mM Brij-S, 15% ACN, pH 9.0.

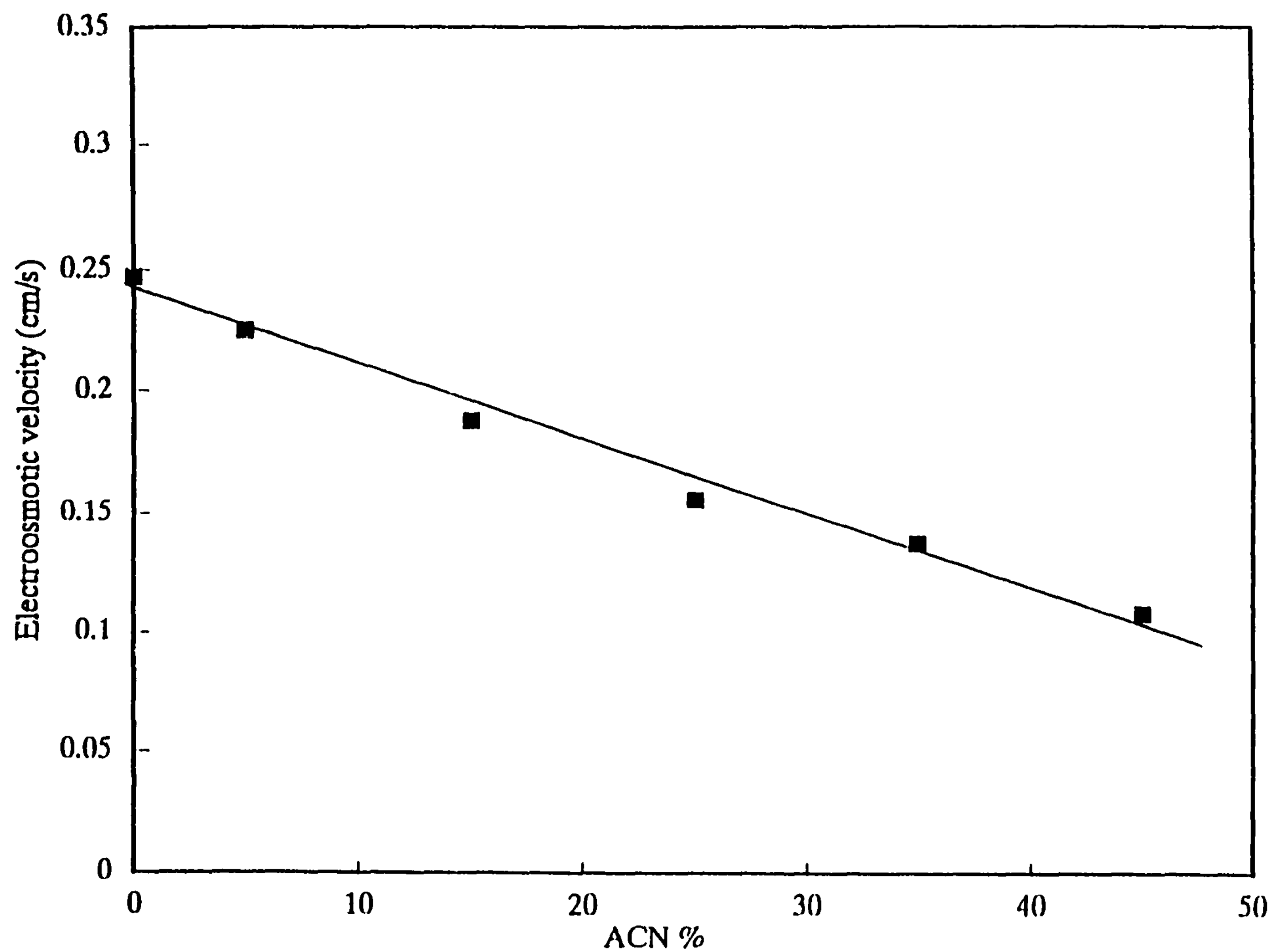

Figure 3. Electroosmotic velocity as a function of acetonitrile concentration. Conditions: 8 mM borate, 30 mM Brij-S, pH 9.0, +25 kV.

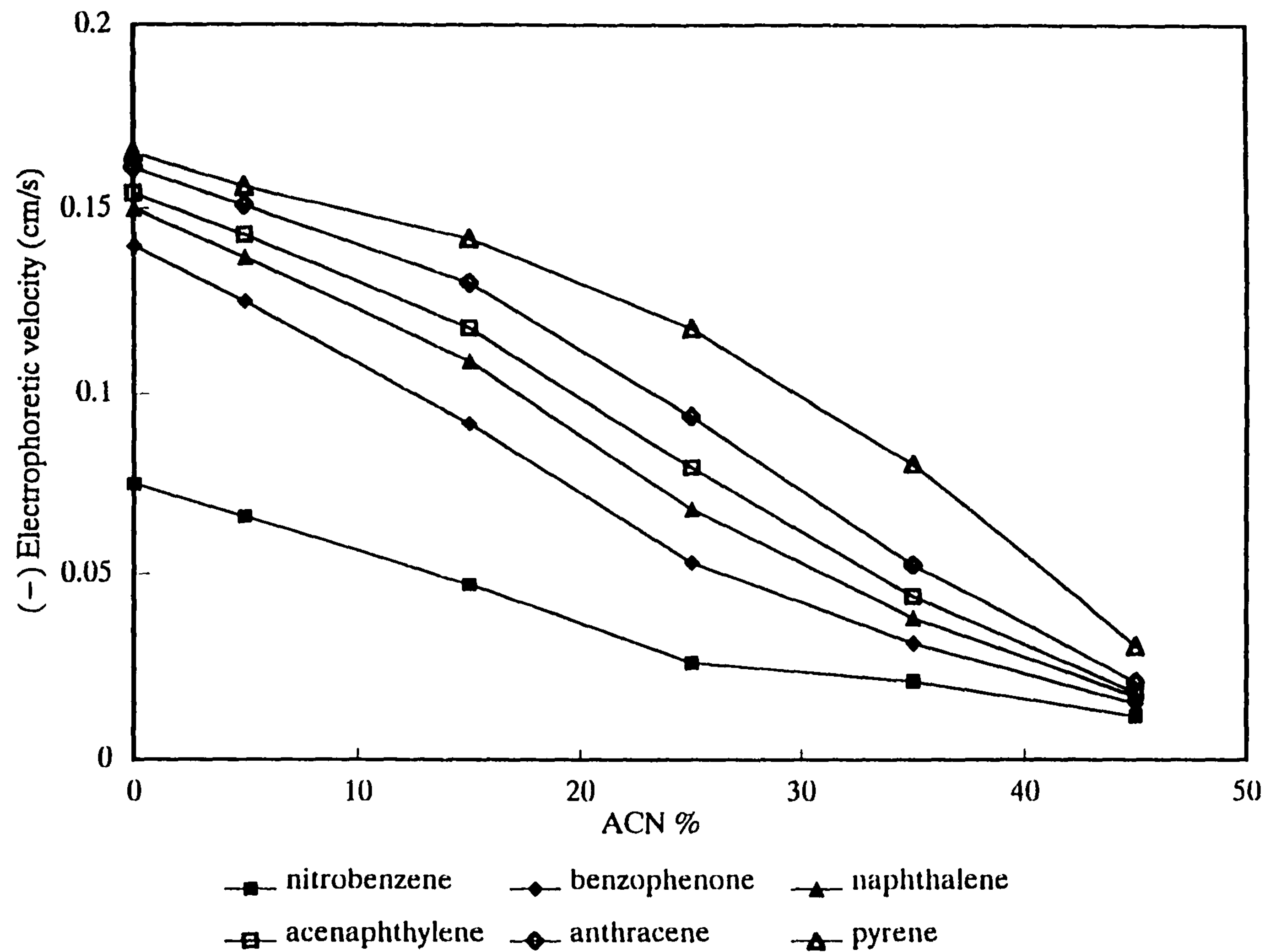

Figure 4. Electrophoretic velocity of six test compounds as a function of acetonitrile concentration. Conditions: 8 mM borate, 30 mM Brij-S, pH 9.0, +25 kV.

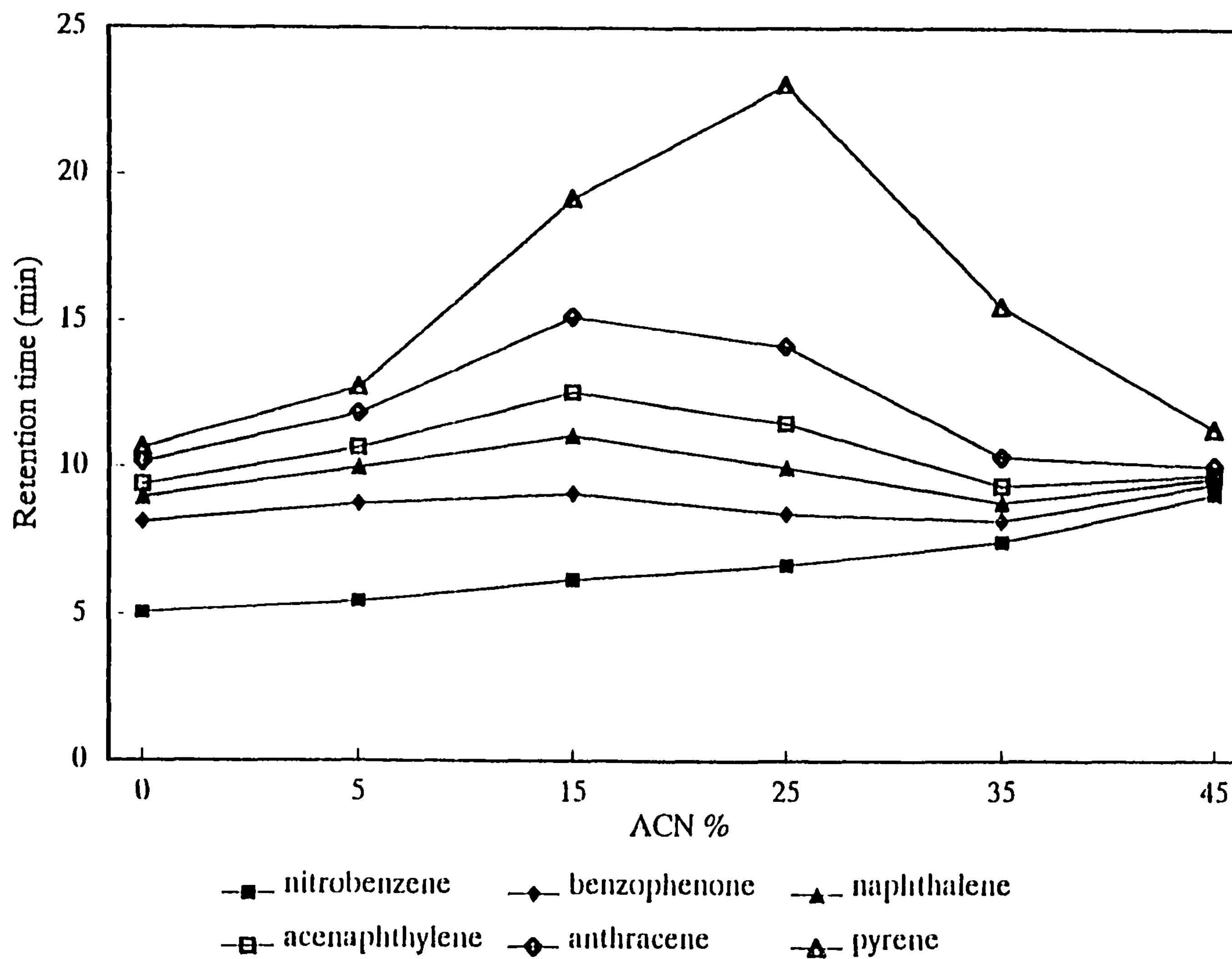

Figure 5. Retention time of six test compounds as a function of acetonitrile concentration. Conditions: see Figure 4.

than at 5% acetonitrile. The addition of 15% acetonitrile alters the partition coefficients and dramatically lowers k' for many of these components, while higher amounts of modifier may cause sufficient micellar disorder that the separation mechanism shifts over to CZE. This is indicated on the electropherogram (Figure 6) by a loss of selectivity for some solutes.

3.3 Separation of neutral compounds

The greater solubility of Brij-S made it feasible to operate at lower concentrations of acetonitrile in the capillary electrolyte than was possible with DOSS. Solvation of analytes by acetonitrile is a competitive reaction to complexation by Brij-S. Thus, by operating at a lower concentration of acetonitrile, complexation of analytes by the Brij-S anion is increased. This should make it possible to separate smaller, more polar compounds that would be too weakly complexed at higher acetonitrile concentrations. Figure 7 shows an excellent separation of 20 such compounds in 20% acetonitrile with 40 mM Brij-S as the additive.

Figure 8 shows an electropherogram for separation of five testosterones. The structures for these five testosterones are shown in Figure 9. By reducing the Brij-S concentration to only 20 mM, a complete separation was obtained in < 5 min. Compounds 2 and 3 differ only by a double bond, while compounds 3 and 4 differ only by a methyl group.

The separation of 1-methyl-, 2-methyl- and 9-methylanthracene was attempted next. This presented some challenge because the three compounds differ only in the location of the methyl group. The electropherogram in Figure 10 shows baseline resolution of the three isomers in only 11.5 min.

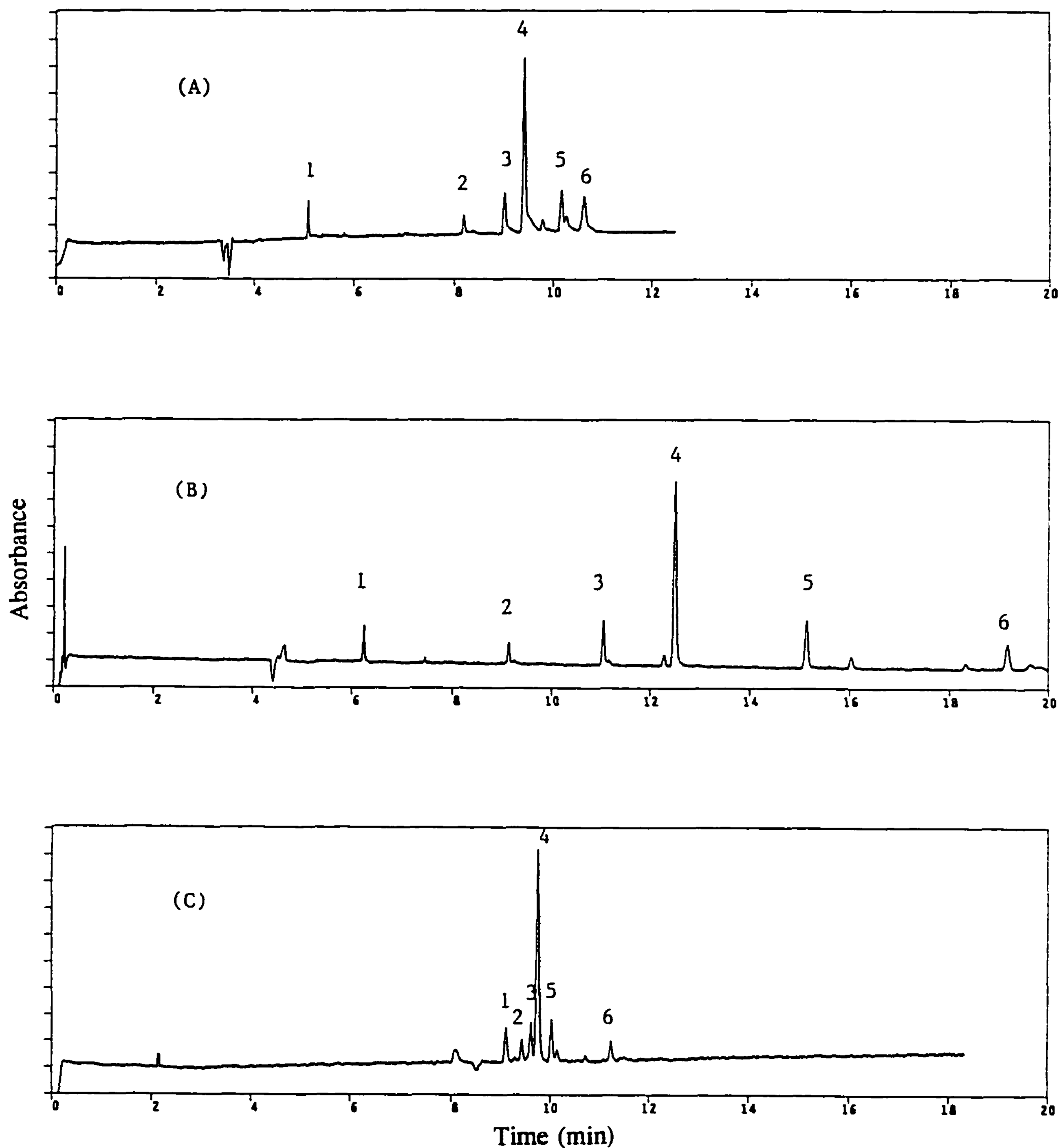

Figure 6. Electropherograms of six PAH compounds at different ACN concentration: (A) 0% ACN; (B) 15% ACN; (C) 45% ACN. Conditions: see Figure 4.

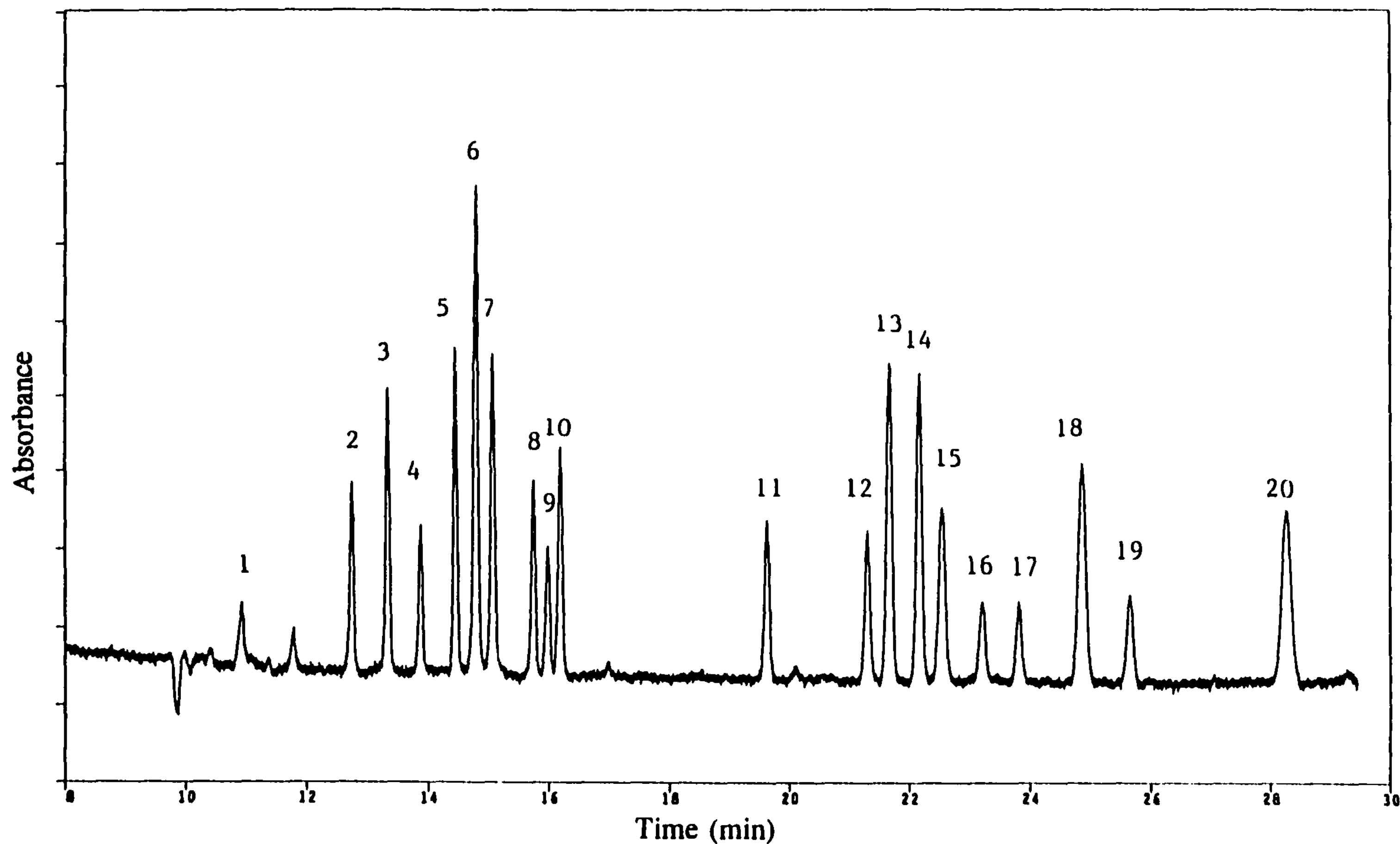

Figure 7. Separation of polar organic compounds. Electrolyte: 40 mM Brij-S, 8 mM sodium borate, 20% ACN, pH 10.0; applied voltage, 20 kV; current, 90 μA; injection time, 6 s; detection, 214 nm; capillary, 75 μm i.d., 80 cm length. Peaks: 1= benzoyl hydrazine; 2= methyl benzoate; 3= benzonitrile; 4= phenethyl alcohol; 5= phenol; 6= benzaldehyde; 7= nitrobenzene; 8= benzyl acetate; 9= benzothiazole; 10= 3'-nitroacetophenone; 11= anisole; 12= phenetole; 13= benzylchloride; 14= butylbenzene; 15= hydrocinnamaldehyde; 16= chlorobenzene; 17= benzylbromide; 18= phenylacetic acid; 19= bromobenzene; 20= 4-bromonitrobenzene.

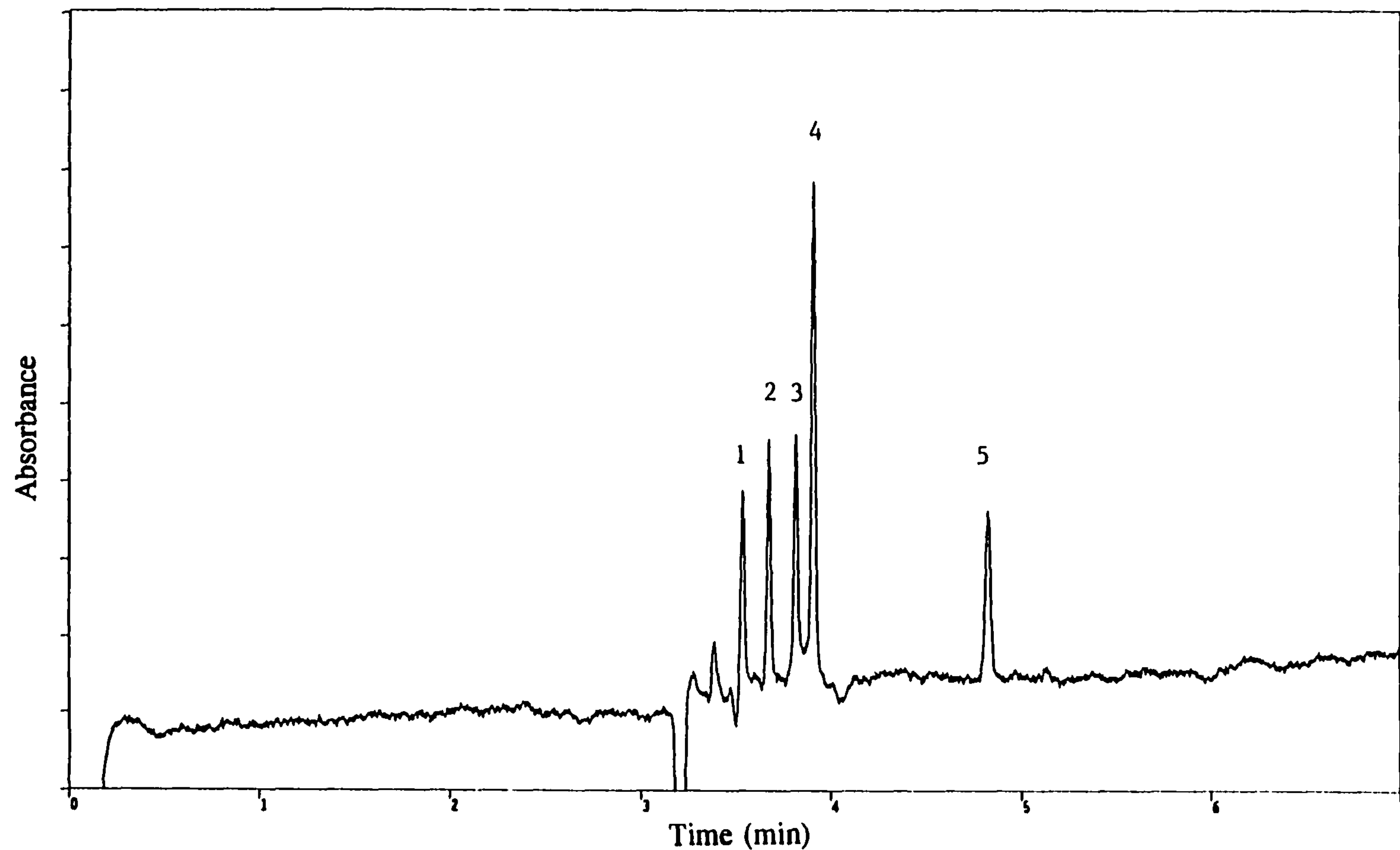

Figure 8. Separation of five testosterones. Electrolyte: 8 mM sodium borate, 30 mM Brij-S, 30% ACN, pH 10.0; applied voltage, 18 kV; current, 90 μA; injection time, 2 s; detection, 254 nm; capillary, 75 μm i.d., 45 cm length. Peaks: 1= 11-ß-hydroxytestosterone; 2= 1-dehydrotestosterone; 3= testosterone; 4= 17-α-methyl testosterone; 5= testosterone acetate.

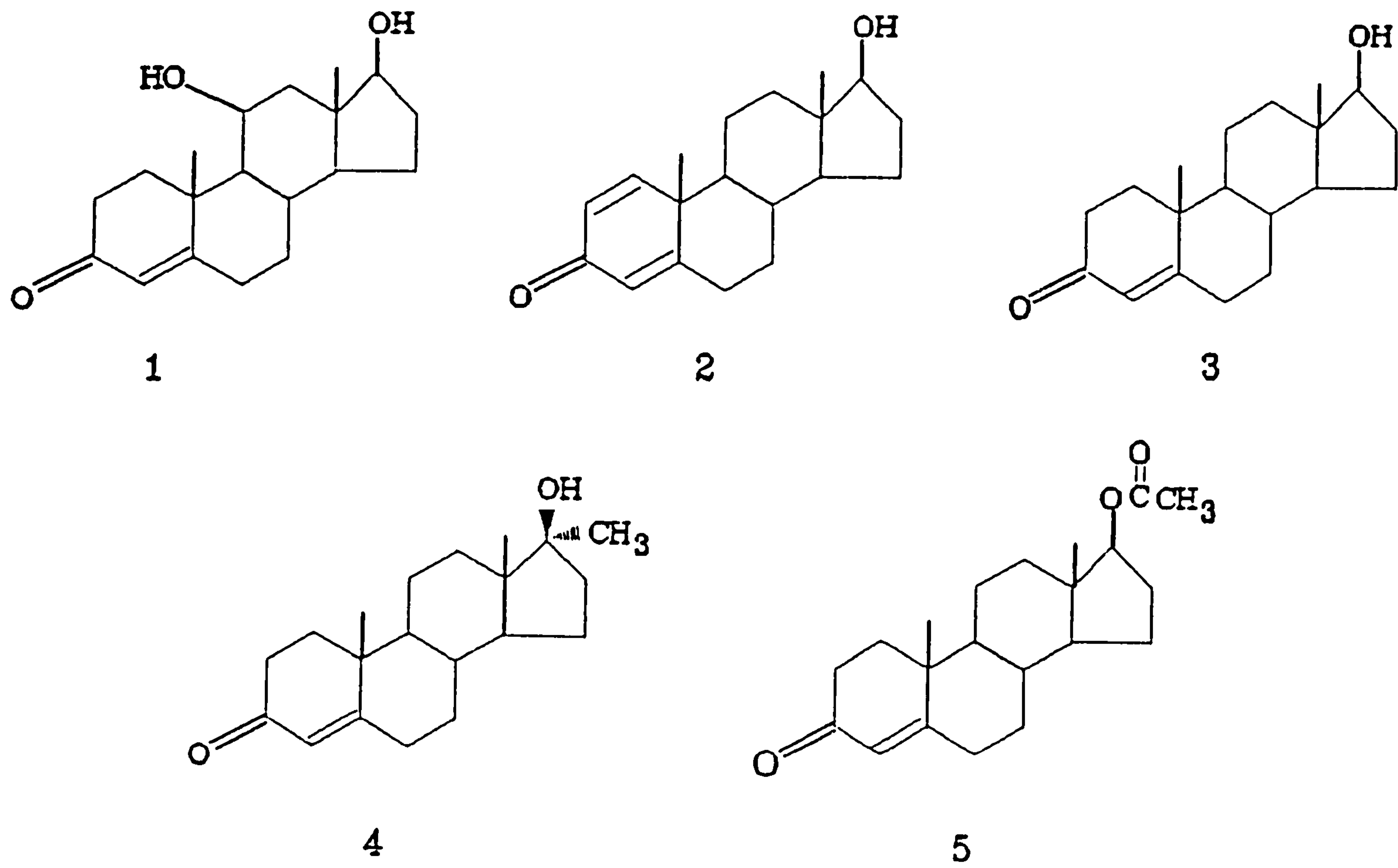

Figure 9. Structure of five testosterones.

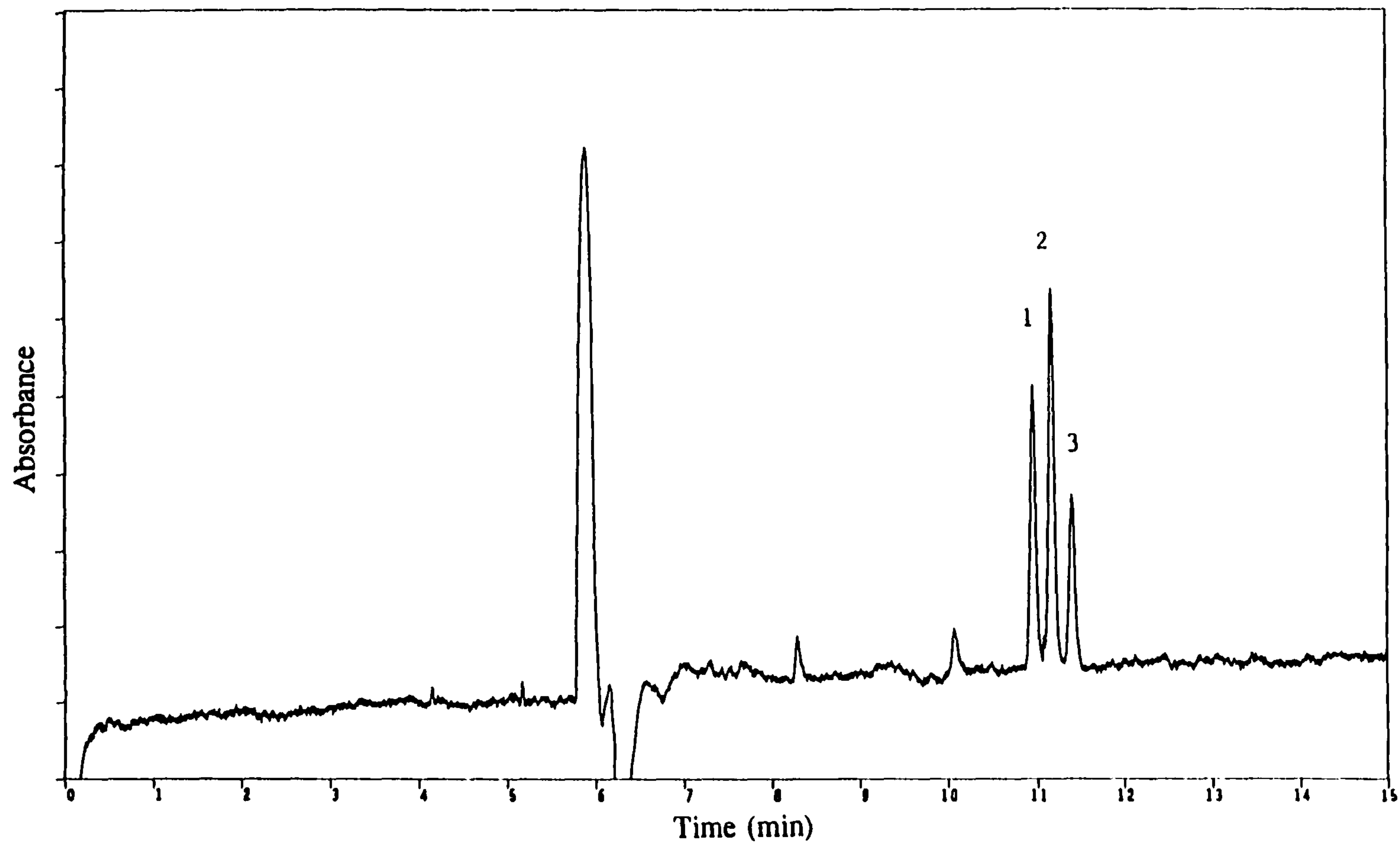

Figure 10. Separation of methylated anthracene. Electrolyte: 25 mM Brij-S, 8 mM sodium borate, 35% ACN, pH 9.0; injection time, 2 s; applied voltage, 18 kV; capillary, 75 μm i.d., 60 cm; detection, 254 nm. Peaks: 1= 7-methylanthracene; 2= 1-methylanthracene; 3= 2-methylanthracene.

Figure 11 shows the CE separation of another group of methylated PAH compounds: 5-methyl-, 6-methyl-, 7-methyl-, and 12-methylbenz[a]anthracene. Sodium dioctylsulfosuccinate (DOSS) was first tried without much success. 7-methyl- and 12-methyl-, 5-methyl- and 6-methyl- isomers coeluted (Figure 11a). Brij-S was then attempted with different concentration. A longer capillary (80 cm) was also used in order to obtain better resolution. Figure 11b shows a nearly baseline separation of these four components by Brij-S. The baseline drifting in Figure 11b was believed to be caused by low solubilities of these PAHs in acetonitrile.

Separation of deuterated organic compounds from hydrogen isomer represents a real challenge in chromatography. In counter-current CE, separations of closely-related substances become better as the migration times are increased. This can be accomplished in two ways. One is to increase the Brij-S concentration. This will decrease the electroosmotic flow (Figure 12) due to a decrease in surface charge and an increase in viscosity of the buffer. Since acetophenones are quite small compounds, partition of the acetophenones into the micellar pseudophase must be relative low. As we demonstrated early, increasing surfactant concentration will improve the interaction between small molecules and micelles. Figure 13 shows that by increasing Brij-S concentration from 50 mM to 100 mM, the resolution of acetophenone and acetophenone-d_3 was dramatically improved. Another way to slow down electrophoretic velocity is to increase buffer ionic strength owing to the same reason as we explained above. Figure 14 shows an increase in the phosphate buffer concentration giving rise to a great improvement in the resolution, and a separation of acetophenone-d_3 from ordinary acetophenone was achieved with a resolution

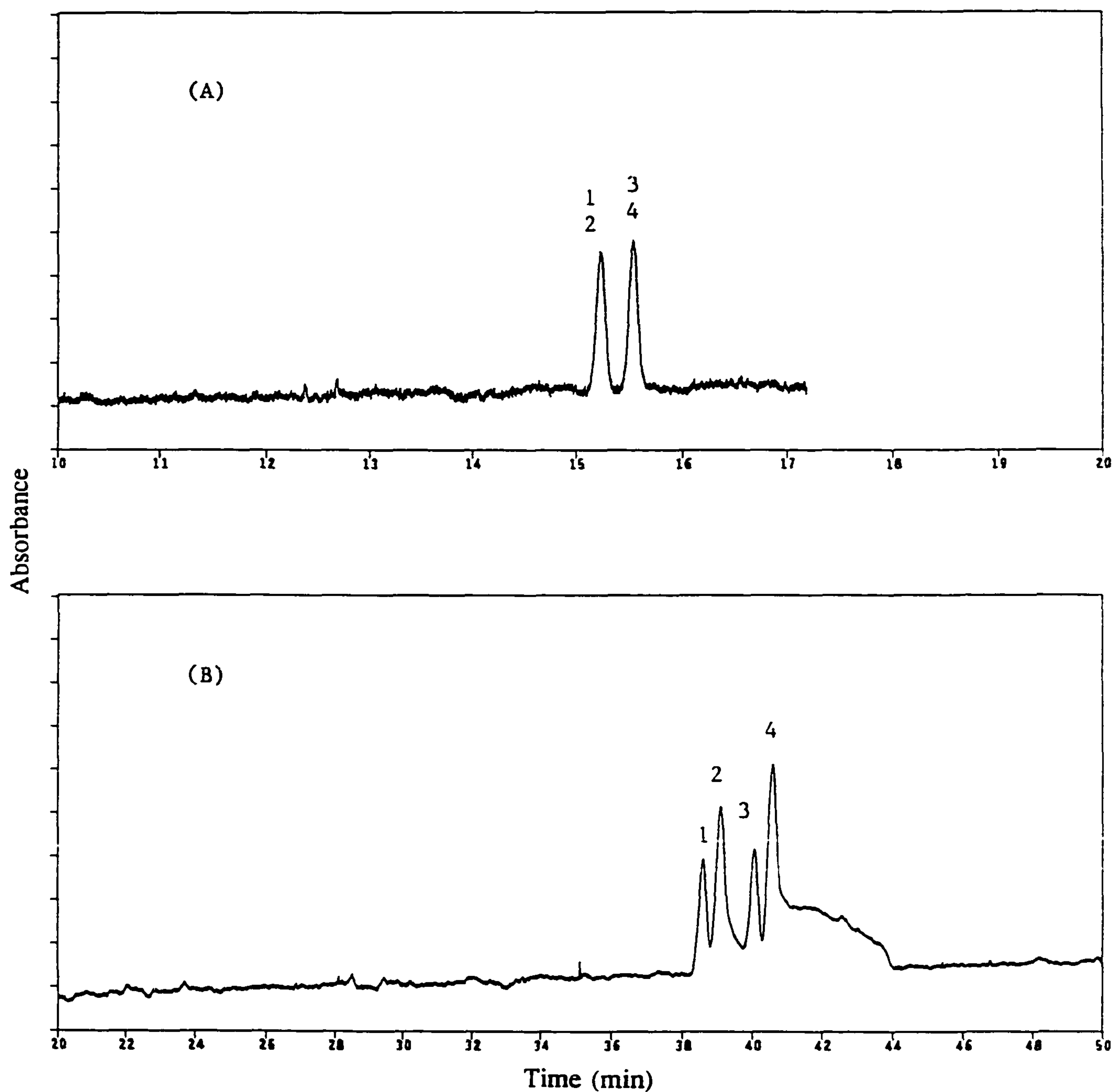

Figure 11. Separation of methylated benz[a]anthracene. Electrolyte: (A) 8 mM borate, 40 mM DOSS, 30% ACN, pH 9.0; (B) 25 mM Brij-S, 8 mM sodium sulfate, 34% ACN, pH 10.0; applied voltage, +20 kV; injection time, 6 s; capillary, 75 μm i.d., (A) 60 cm and (B) 80 cm length; detection, 254 nm. Peaks: 1= 12-methylbenz[a]anthracene; 2= 7-methylbenz[a]anthracene; 3= 6-methylbenz[a]anthracene; 4= 5-methylbenz[a]anthracene.

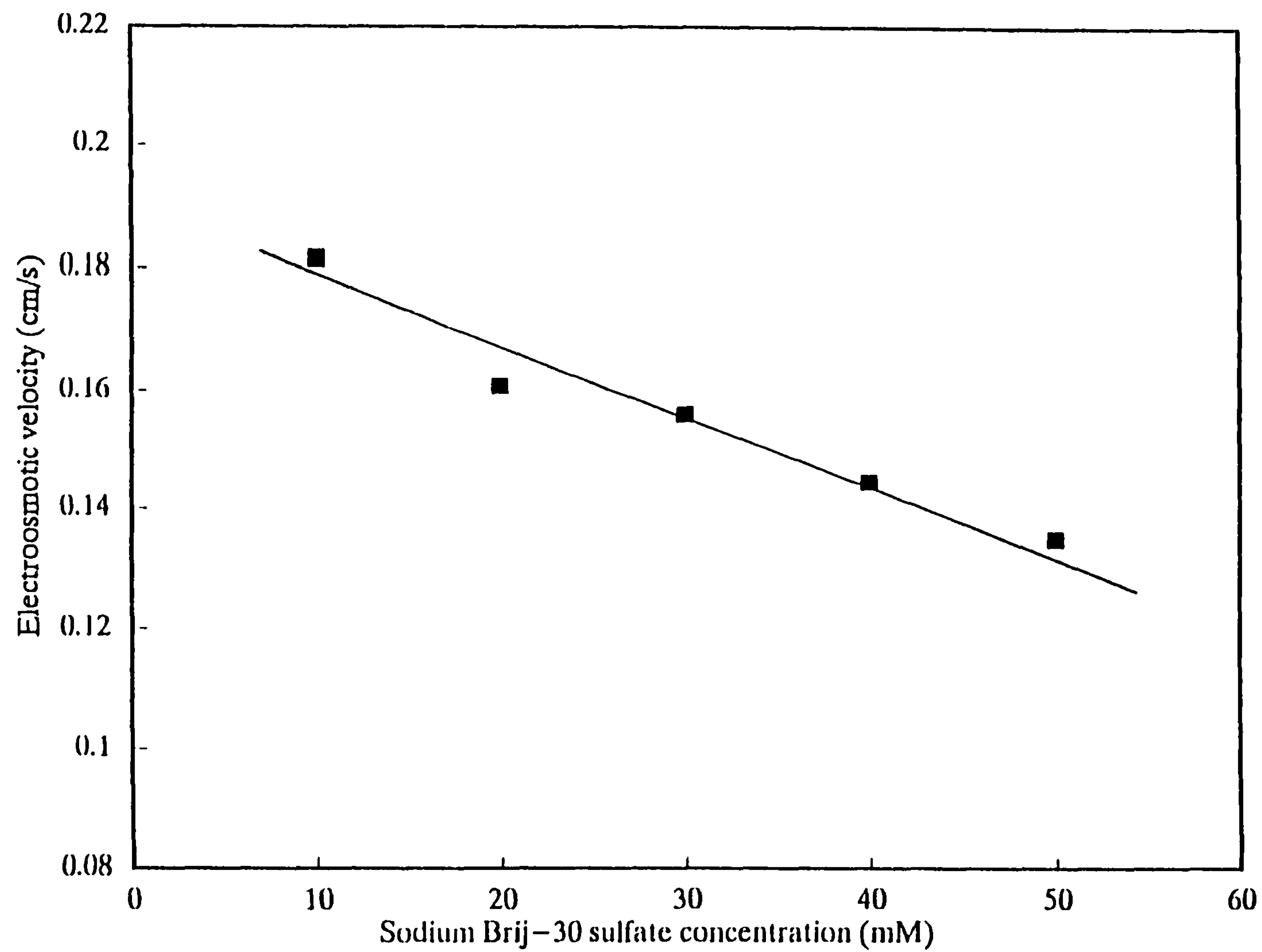

Figure 12. Electroosmotic velocity vs. Brij-S concentration.

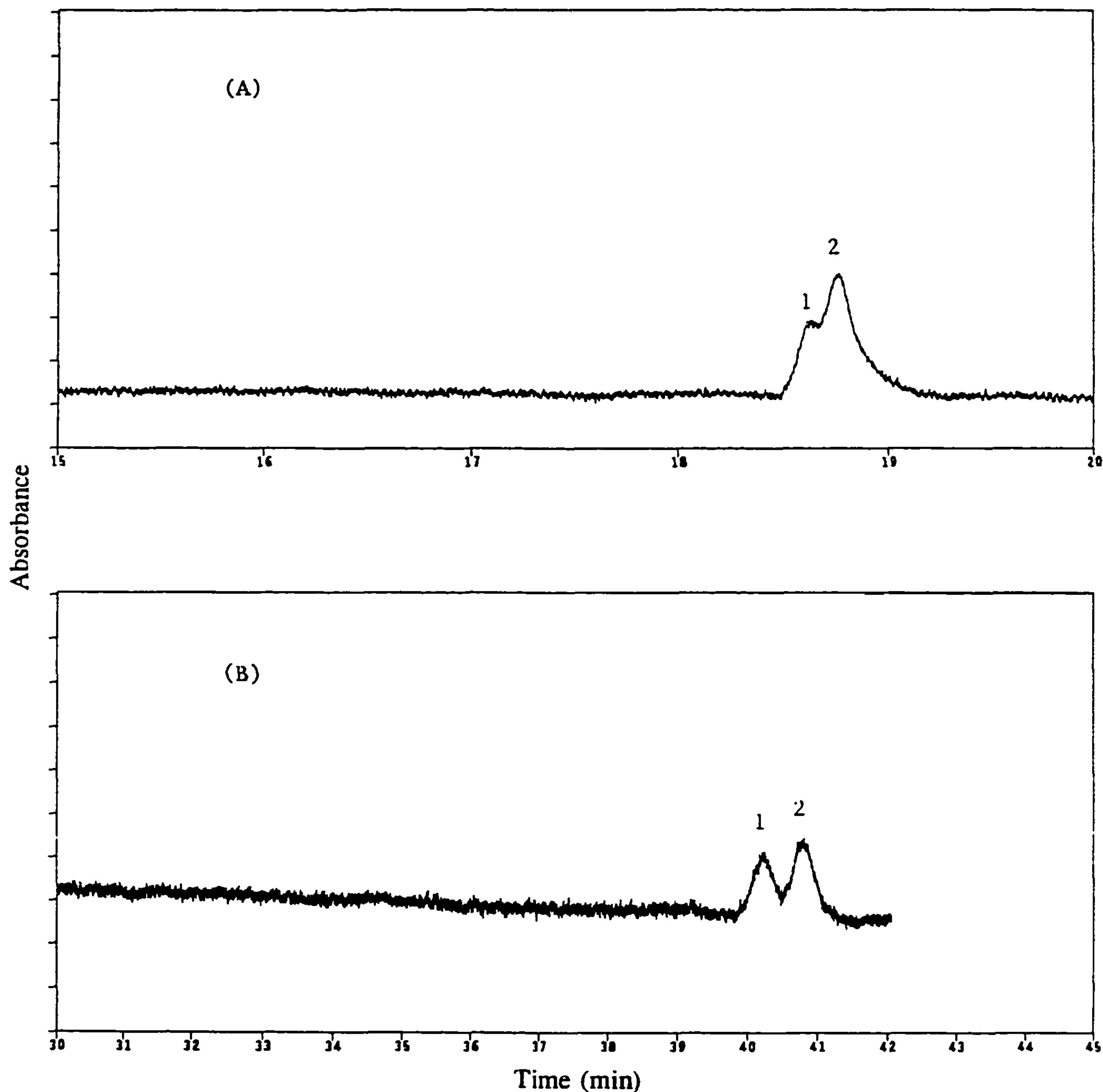

Figure 13. Separation of deuterated compounds. (A) 50 mM Brij-S; (B) 100 mM Brij-S. Electrolyte: 25 mM sodium borate, 25 sodium phosphate dibasic, pH 9.1; injection time, 10 s; applied voltage, 10 kV; capillary, 50 μm i.d., 60 cm length; detection, 214 nm. Peaks: 1 = acetophenone-d_3; 2 = acetophenone.

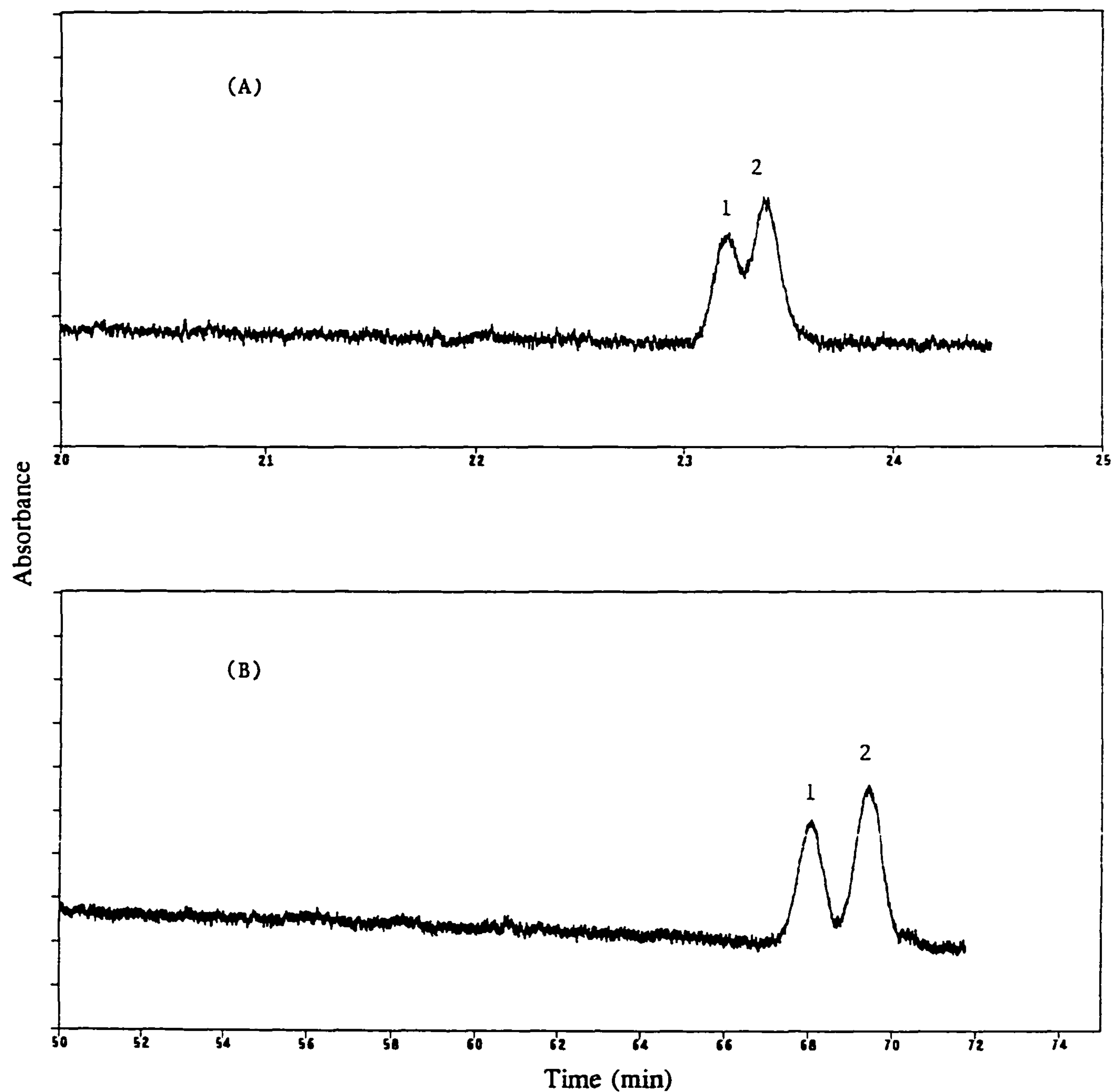

Figure 14. Separation of deuterated compounds. (A) 5 mM phosphate; (B) 80 mM phosphate. Electrolyte: 5 mM sodium borate, 100 mM Brij-S, pH 9.1. Other conditions and peak identities: see Figure 13.

of 1.20.

4. Conclusions

Separation of neutral organic compounds by CE is based on differences in their interaction with a negatively-charged additive called Brij-S. A counter-migration mode is used in which electroosmotic flow is toward the detector and electrophoretic migration of the analytes is in the opposite direction. Migration times of analytes can be varied over a broad range by manipulating two experimental parameters. Increasing the concentration of Brij-S in the electrolyte results in stronger complexation and increases the migration times. Increasing the percentage of acetonitrile decreases complexation and causes shorter migration times.

The flexibility inherent in this method promises to enhance significantly the scope of separating neutral compounds by CE. It now becomes feasible to separate analytes ranging from fairly small, polar compounds to large, hydrophobic substances.

The extraordinary separation possibilities of counter-migration in CE have been demonstrated again. By adjusting conditions so that the opposing migration vectors are almost the same, separation of compounds of very similar chemical structure becomes possible. The separation of acetophenone and acetophenone-d_3 is a case in point.

Acknowledgments

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CHAPTER 3. SEPARATION OF NEUTRAL COMPOUNDS AND PHARMACEUTICAL DRUGS BY CAPILLARY ELECTROPHORESIS IN ACIDIC SOLUTION USING LAURYL POLYOXYETHYLENE SULFATE AS AN ADDITIVE

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Abstract

Working at pH 2.4 with uncoated silica capillaries has the advantage that EOF is virtually eliminated. Excellent separations of protonated organic bases were obtained when ethanesulfonic acid was added to the running electrolyte to coat the capillary surface by a dynamic equilibrium. The effect of adding a new surfactant, sulfonated Brij-30, to the acidic electrolyte was also investigated. Use of this surfactant in acidic organic-aqueous solutions changes the elution order of many organic cations and also permits the separation of neutral organic compounds. Excellent resolution of a mixture of 19 PAHs and similar compounds was obtained in 40% organic solvent in only 20 min. The largest organic compounds form the most stable association complexes with the sulfonated Brij-30 and thus have the shortest migration times. It is shown that the type and concentration of surfactant, as well as the

composition of the aqueous-organic solution, are conditions that can be varied over a broad range to obtain superior separations of both neutral and cationic organic compounds. The type of organic solvent is yet another condition that can be manipulated advantageously. For example, the use of equal volumes of acetonitrile and 2-propanol in water-organic solutions can give better resolution of neutral organic analytes than either solvent used alone.

1. Introduction

Capillary electrophoresis (CE) has been successfully used for a wide range of samples, and is a complementary technique to liquid chromatography [1-3]. Neutral compounds do not separate by capillary electrophoresis because they migrate together at the velocity of the electroosmotic flow (EOF). Terabe et al. [4-5] introduced micelle electrokinetic chromatography (MEKC) to address this problem. In MEKC, a surfactant such as sodium dodecyl sulfate (SDS) is added to the electrolyte at a concentration above its critical micelle concentration (CMC).

Most applications of MEKC are done at basic solutions because of the requirement of a stronger EOF. Sepaniak et al. [6] pointed out that separations at low pH (about pH 5) are impractical because the EOF is reduced, and is counterbalanced by the micelle electrophoretic mobility in the opposite direction, resulting in excessively long migration times. MEKC was attempted at pH below 5.0 with reversed power supply, but without success [5]. Terabe et al. [5] claimed that the poor reproducibility of migration times in bare-silica columns below pH 5.0 was one of the reasons for the failure. Coated capillaries were used in some applications in acidic conditions to improve reproducibility. Kiessig et

al. [7] performed chiral separations using acidic buffers and different cyclodextrins as chiral selectors in a polyimide-clad coated fused-silica capillary. More recently Janini and co-workers [8] demonstrated fast separation of some hydrophobic compounds using MEKC on a polyacrylamide coated capillary (eliminates the EOF) after reversal of the polarity. This mode of MEKC was also used to separate linear alkyl benzene sulfonate (LAS) samples by McLaughlin and co-workers [9,10].

Using a very acidic pH to suppress the EOF has been used in the separation of proteins and peptides [11-14]. Interaction between proteins and the capillary wall is prevented and good reproducibility may be obtained. A recent study by Thornton and Fritz [15] showed that metal ions could be separated at pH 2.0 using only hydrochloric acid as an aqueous buffer. However, when Thormann et al. [16] tried to separate hydrophobic, positively charged substances at pH 2.2 by using SDS as an additive in MEKC, they claimed that this mode was unsuitable for highly hydrophobic compounds.

In the present work excellent separations of both organic cations and a variety of neutral organic compounds were obtained with bare fused-silica capillaries at pH 2.4. A new negatively-charged surfactant was used as a solution additive. When SDS is used as an additive, many hydrophobic compounds have been shown to be totally incorporated into the micelles and therefore cannot be separated [17-22]. Only by using a high concentration of SDS and other additives such as organic solvents, urea and cyclodextrins [23-26], have separations of these hydrophobic solutes become possible. However, the use of large amounts of SDS and other additives results in longer migration times and reduced sensitivity [27]. In a previous study [28] we used a novel surfactant named lauryl polyoxyethylene(4)

sulfate $C_{12}H_{25}(OCH_2CH_2)_4OSO_3^-Na^+$ (Brij-S), and found that this new surfactant is much more effective than SDS in separating both highly hydrophobic compounds and small polar compounds. We have shown that 20 PAHs were nicely separated by using only 40 mM Brij-S with 40% (v/v) acetonitrile, moreover, structural isomers (methylated benz[a]anthracenes) were baseline separated without adding any cyclodextrins [28]. Separation of these PAHs are based on different strengths in solvophobic association between Brij-S and PAHs [21-22,28-29], due to the absence of micelles at high ACN content in aqueous solutions. This new surfactant (Brij-S) was further studied here in acidic conditions for several group compounds including PAHs, phenols, and basic drugs. Low pH 2.4 was used to eliminate the EOF. After reversal of the polarity, the more hydrophobic and positively charged solutes which more strongly associate with Brij-S are eluted first, and the least hydrophobic solutes are eluted last. This new surfactant showed excellent selectivity not only for the highly hydrophobic PAHs but also the hydrophilic drugs. Sharp peaks were observed because of the fast separation period.

2. Experimental

2.1 Capillary electrophoresis system

CE separations were performed on a Quanta 4000E system (Waters, Milford, MA, USA). Uncoated fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) used for CE had 50 μm i.d. and 45 cm length (37.5 cm effective length). Direct UV absorbance detection was performed at either 254 nm or 214 nm. A voltage range of -20 to -30 kV was applied. The time of hydrostatic injection was from 6 s to 10 s. The temperature of the

capillary was kept at 25 °C. Electropherograms were collected at a speed of 15 points/s and plotted by Chromperfect data acquisition system (Justice Innovations, Mountain View, CA, USA).

Before first use, a new capillary was preconditioned by rinsing with 1 M NaOH for 0.5 h, followed by a 0.5 h rinse with deionized water. At the start of each day, the capillary was conditioned with 1 M HCl for 10 min. Between introduction of samples, the capillary was rinsed with 0.1 M HCl for 2 min, and the operating buffer for 2 min.

2.2 Reagents and chemicals

All chemicals were of analytical reagent grade and the organic solvents methanol, acetonitrile, 1-propanol, and tetrahydrofuran (THF) were HPLC grade. Brij-S was made by sulfonation of Brij-30 ($CH_3(CH_2)_{11}(OCH_2CH_2)_4OH$) with chlorosulfonic acid according to the procedure in ref. 28. The analyte chemicals and buffer additives were all used as obtained from Aldrich (Milwaukee, WI, USA), J. T. Baker (Phillipsburg, NJ) USA) and Sigma (St. Louis, MO, USA).

2.3 Preparation of sample solutions and buffers

Stock solutions of PAHs and phenols were prepared in acetonitrile in a concentration of 5.0 mg/mL, while solutions of drugs were in methanol by 10.0 mg/mL. The sample solutions were prepared by adding appropriate aliquots of the stock solutions to buffer, resulting in solute concentrations of 20-100 μg/mL. The running buffer was prepared by adding appropriate aliquots of 1.0 M HCl, 250 mM Brij-S stock solutions into water or into

mixtures of water and organic solvents. The pH was adjusted as required, by adding aliquots of 0.1 M HCl or 0.1 M NaOH stock solution, and measured by a pH meter. A Barnstead Nanopure II system (Syboron Barnstead, Boston, MA, USA) was used to further deionize distilled water for all buffer and sample mixtures.

3. Results and Discussion

3.1 Separation of PAH compounds

Neutral organic compounds that have no charge can be separated by micellar electrokinetic chromatography (MEKC). However, PAH compounds and other large, hydrophobic molecules are all strongly attracted to the micelle and are therefore difficult to separate by normal MEKC. Incorporation of ~40% acetonitrile in the capillary electrolyte weakens the attraction of PAH compounds for the charged surfactant, probably by breaking up the micelle into smaller, less organized fragments. Excellent separations of PAH compounds have been obtained in acetonitrile-water using DOSS [22] or sulfonated Brij-30 (Brij-S) [28] as an additive and a basic pH. However, these separations required a rather long analysis time because the electroosmotic and electrophoretic vectors were in opposite directions (counter migration).

By working at a very acidic pH, such as 2.4, the EOF is much smaller than the electrophoretic migration of the analyte-Brij-S complexes. A reversed power supply was used so that the net migration of analytes was toward the positive electrode. Separations were performed in 40% organic-60% aqueous solution containing 30 mM Brij-S as the additive. An apparent pH of 2.4 could be maintained with 10 mM hydrochloric acid as the

only buffer. Under these conditions, nineteen PAH and related compounds were separated in 20 min (Figure 1). The peaks were sharp because of the relatively short migration times. The largest PAH compound, rubrene which has eight benzene rings, was the most strongly associated with Brij-S and was therefore the first peak to appear in the electropherogram. The other analytes migrated in roughly the order of decreasing size and hydrophobic character, which is just the opposite to the elution behavior usually obtained in chromatography.

The use of Brij-S at an acidic pH has a big advantage over CE separations in basic solution, especially for analysis of compounds of high molecular weight. The same nineteen compounds were separated in basic solution with a positive power supply. The migration order was exactly the reverse of that in acidic solution and the separation took more than 60 min. Rubrene and benzo[ghi] perylene could not be detected in the run in basic solution.

A brief study on the effect of Brij-S on separation was conducted on electrolytes containing Brij-S up to 70 mM with 20% (v/v) acetonitrile + 20% (v/v) 2-propanol. No peaks were detected for 4 test PAH compounds (9,10-diphenylanthracene, benz[a]pyrene, anthracene, benzophenone) at 10 mM Brij-S in 40 min. At this low concentration of Brij-S, the association with solutes is very weak. At 20 mM Brij-S migration times for anthracene and benzophenone were still quite long. Between 50 mM and 70 mM Brij-S migration times were short as a result of stronger association and some peaks were only partially resolved. For these reasons, an intermediate concentration of 30 mM Brij-S was chose for further studies.

The concentration of organic solvent in the BGE also affects the migration of neutral

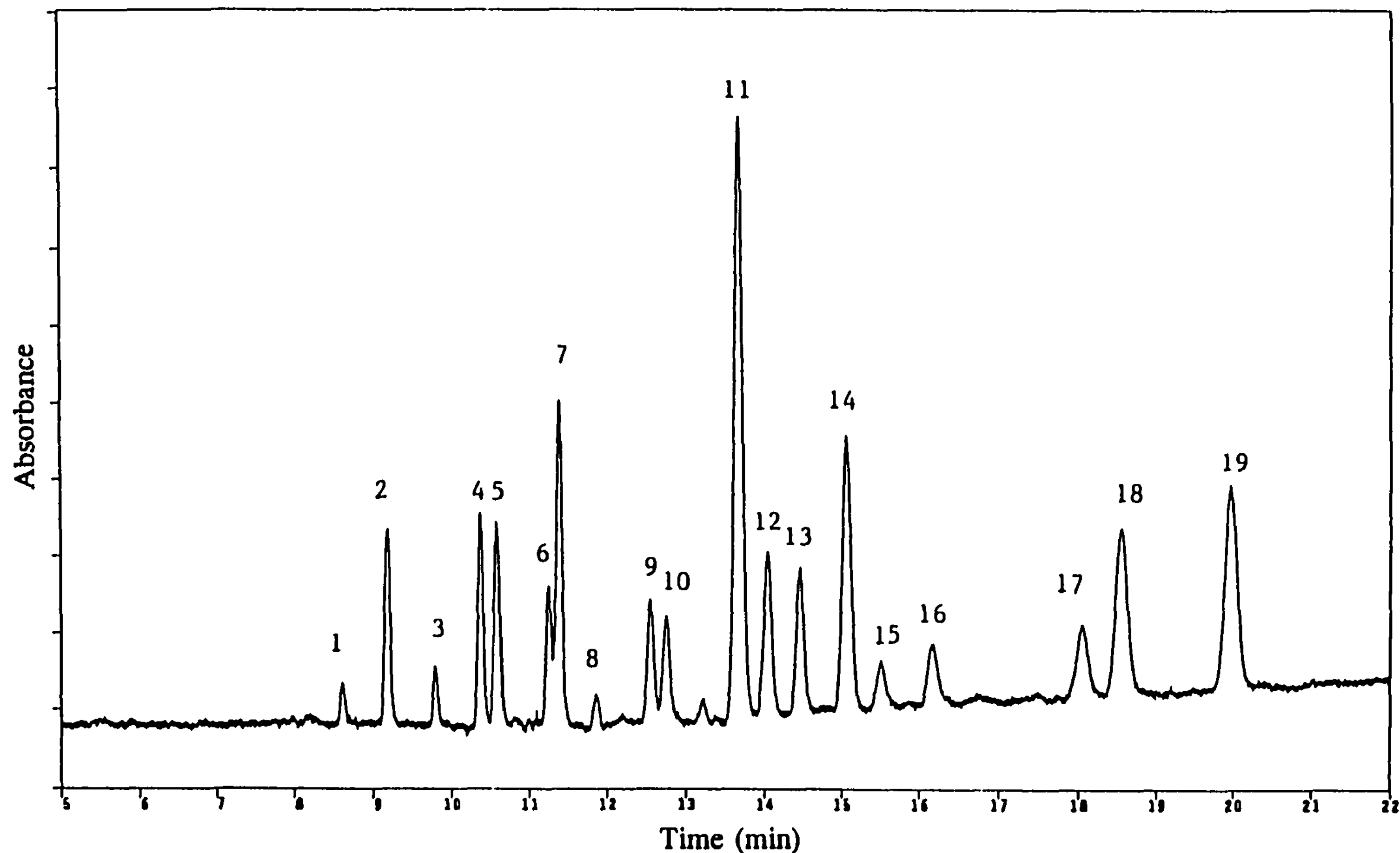

Figure 1. Separation of PAH compounds in acidic condition. Electrolyte, 30 mM Brij-S, 10 mM HCl, 20% (v/v) acetonitrile, 20% (v/v) 2-propanol, pH 2.40; applied voltage, -22 kV; current 50 μA; injection time, 10 s; detection, 254 nm. Peaks: 1= rubrene; 2= 9, 10-diphenylanthracene; 3= benzo[ghi]perylene; 4= benzo[a]pyrene; 5= benzo[e]pyrene; 6= benz[a]anthracene; 7= chrysene; 8= 2,3-benzofluorene; 9= pyrene; 10= fluoranthene; 11= anthracene; 12= phenanthrene; 13= 1-hydroxypyrene; 14= fluorene; 15= acenaphthene; 16= 1-methylnaphthalene; 17= naphthalene; 18= azulene; 19= benzophenone.

analytes. Figure 2 shows that there is no separation for anthracene, benz[a]pyrene and 9,10-diphenylanthracene at 10% acetonitrile due to strong participation into micelles. More organic solvent will weaken this interaction resulting in a resolution of these three components. Notice here that a higher percentage of organic solvent is required to get a good resolution of analytes than in basic solution. This can be explained by the existence of a countermigrated electroosmotic flow in basic solution, and this will weaken the interaction between micelles and analytes.

In aqueous-organic solution an analyte will be solvated primarily by the organic solvent. Addition of Brij-S results in an equilibrium in which part of the solvated analyte (AO) is converted into a charged Brij-S complex (AB^-)

$$AO + B^- \rightleftharpoons AB^- + O \qquad (1)$$

Electrophoretic migration of the analyte depends on the fraction present as AB^-. Equation 1 shows again that a higher concentration of organic solvent (O) in the BGE will shift the equilibrium to the left and decrease the migration rate.

The type of organic solvent used, as well as the total concentration, can also have an impact on a separation. The solvating power of organic solvents for a general class of analytes is apt to differ. In addition, differences in solvation for individual analytes within a general class may lead to differences in the fraction present as AB^- in Equation 1. These solvation differences constitute an additional parameter that can be manipulated to obtain better resolution of individual analytes.

These concepts were tested on the separation of benzo[ghi]perylene and perylene. Attempts to do this separation with 30 mM Brij-S were not successful in 40% (v/v)

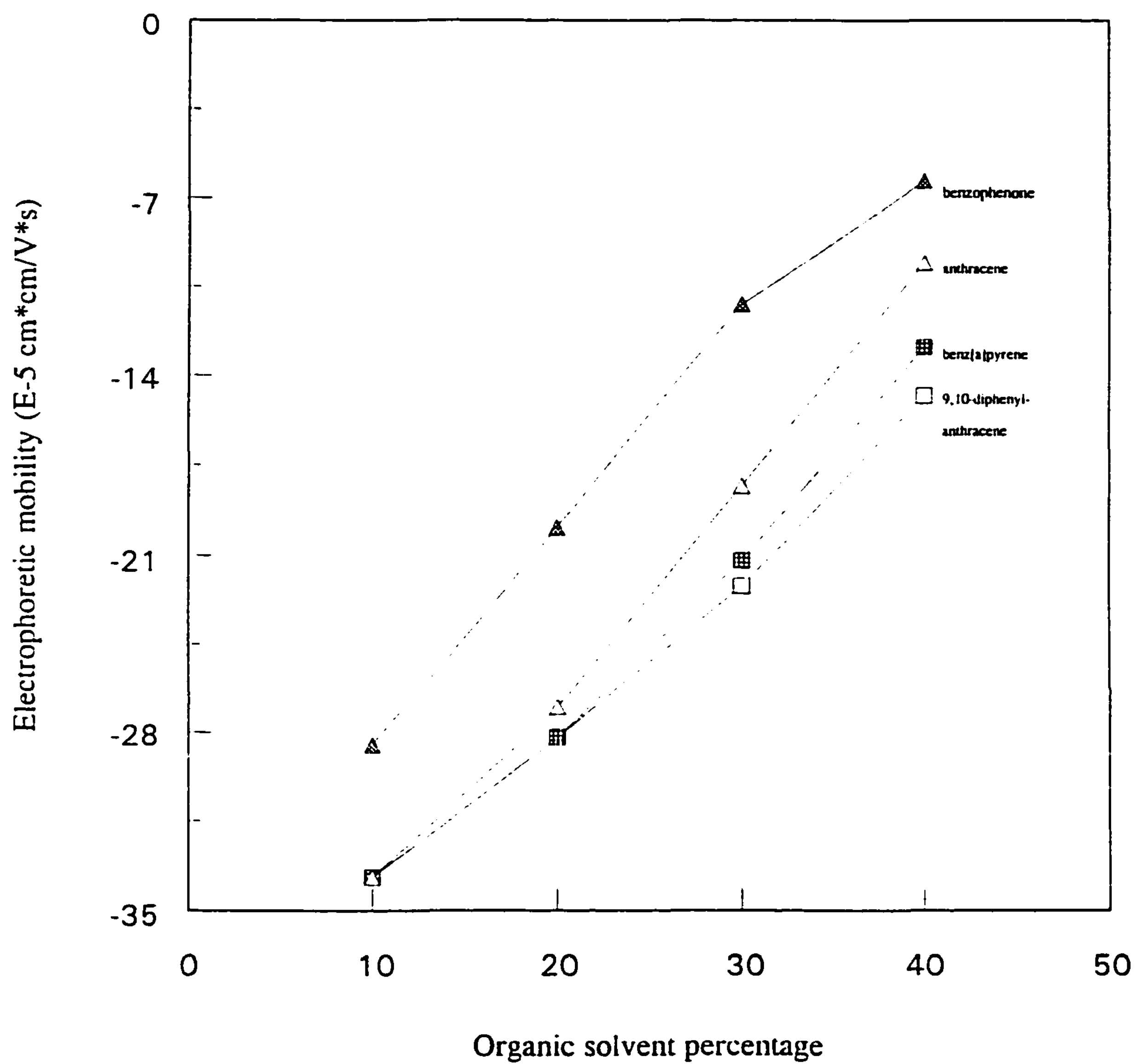

Figure 2. Organic solvent effect on electrophoretic mobility.

acetonitrile, methanol or 2-propanol alone. Then we studied combination of two different organic solvents (20% v/v each): acetonitrile with methanol; acetonitrile with 2-propanol; acetonitrile with THF; 2-propanol with THF; 2-propanol with methanol and methanol with THF. Only 2-propanol with THF gave a bad baseline; the other combinations permitted separation of benzo[ghi] perylene and perylene. A mixture of 20% acetonitrile and 20% 2-propanol gave the best separation of these two compounds (Figure 3) and was used for separation of other PAH compounds.

3.2 Separation of phenols

Small polar neutral compounds tend to have weaker complexation with surfactants than highly hydrophobic compounds. This weak interaction can be improved by using less organic solvent composition or by increasing surfactant concentration, as we discussed above. By reducing the acetonitrile concentration in the electrolyte to 10% and again using 10 mM hydrochloric acid (pH 2.40) and 30 mM Brij-S, an excellent separation was obtained for ten phenols (Figure 4). The entire separation took less than 10 min. By using an internal standard, the migration times were very reproducible, with a relative standard deviation of less than 1% in 8 consecutive runs.

Changing the type of organic solvent also affects the migration of phenolic analytes. By using 5% acetonitrile plus 5% 2-propanol instead of 10% acetonitrile, longer migration times were observed (Table 1). Replacement of part of the acetonitrile by 2-propanol increased the solvation of the phenols with the organic solvent and decreased the association between Brij-S and phenols owing to a stronger solvation effect by 2-propanol. The net

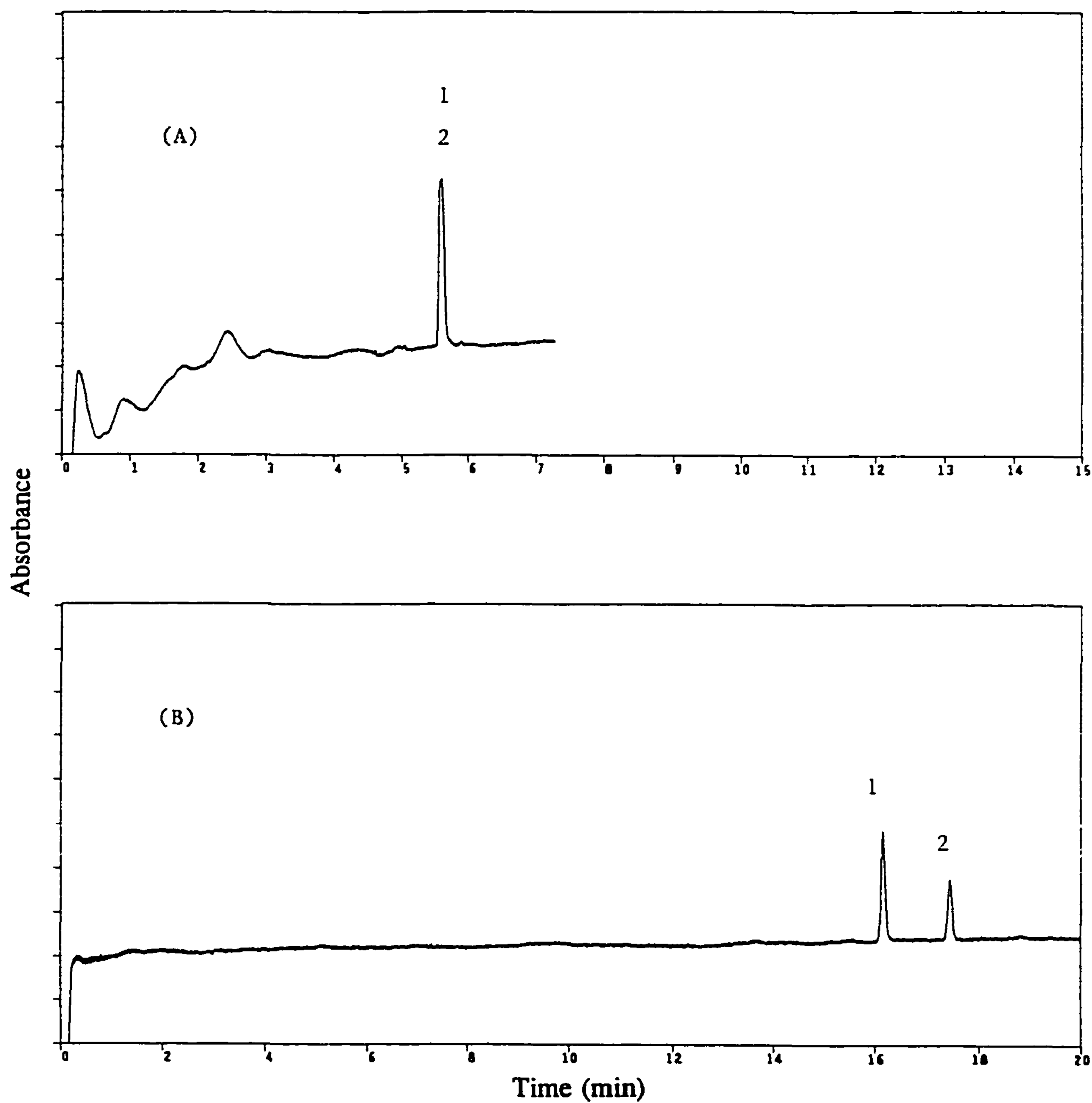

Figure 3. Comparison of different solvent system: (A) 40% acetonitrile; (B) 20% acetonitrile and 20% isopropanol. Other conditions as in Figure 1, except -20 kV is used. Peaks: 1= benzo[ghi]perylene; 2= perylene.

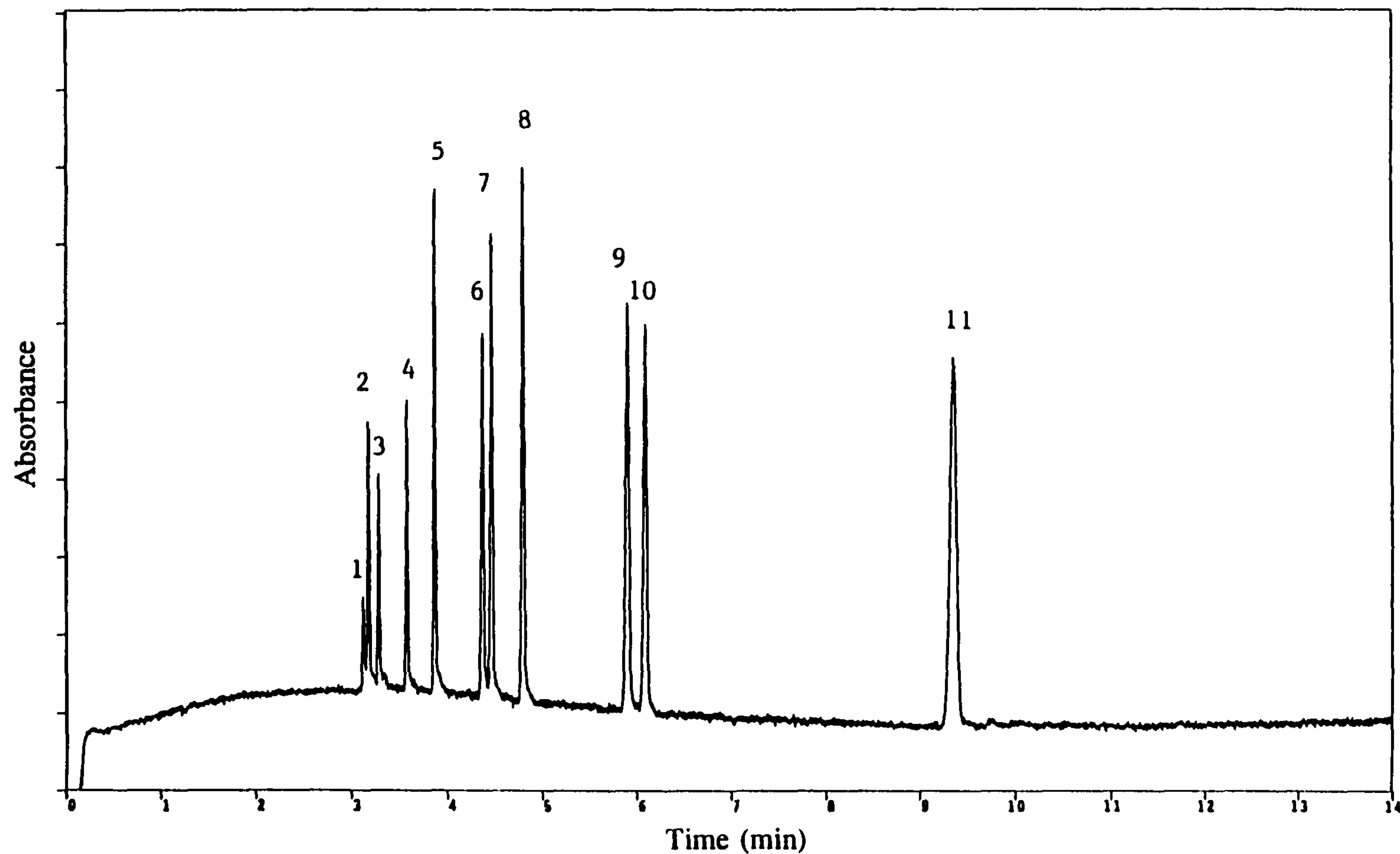

Figure 4. Separation of phenols in acidic condition. Electrolyte, 30 mM Brij-S, 10% (v/v) acetonitrile, 10 mM HCl, pH 2.4; sample injection time, 6 s; applied voltage, -20 kV; detection, 214 nm. Peaks: 1= impurity; 2= 4-amyl phenol; 3= 4-butyl phenol; 4= 4-propyl phenol; 5= 2,4,6-trimethyl phenol; 6= 4-ethyl phenol; 7= 2,5-dimethyl phenol; 8= 2,6-dimethyl phenol; 9= o-cresol; 10= m-cresol; 11= phenol.

Table 1. Migration time change in different solvent. Buffer: 30 mM Brij-S, 10% organic solvent, pH 2.4.

	Migration time (min)		
Compounds	10% ACN	5% ACN + 5% 2-PrOH	percent change
4-Amyl phenol	3.17	3.59	+ 13.2%
4-Butyl phenol	3.28	3.73	+ 13.7%
Propyl phenol	3.57	3.73	+ 4.5%
2,4,6-Trimethyl phenol	3.87	4.59	+ 18.6%
4-Ethyl phenol	4.37	5.26	+ 20.4%
2,5-Dimethyl phenol	4.47	5.42	+ 21.4%
2,6-Dimethyl phenol	4.80	5.91	+ 23.1%
o-Cresol	5.91	7.44	+ 25.9%
m-Cresol	6.10	7.72	+ 26.5%
Phenol	9.36	12.35	+ 31.9%

result was a decrease in the effective electrophoretic mobilities, thus longer migration times.

3.3 Separation of pharmaceutical drugs

Although the pharmaceutical industry depends heavily on HPLC for their analyses, the use of capillary electrophoresis is gaining in popularity. At pH 2.4 many basic drugs exist as protonated cations which should be separable by free solution CE. Preliminary investigations showed that quinidine and procainamide could be separated in aqueous solution containing 20% acetonitrile at pH 2.4 with a phosphate buffer, but the peaks were relatively broad (Figure 5a). Recent work has been shown that CE separation of protonated amino acids is greatly improved by adding some ethanesulfonic acid (ESA) to the capillary solution [30]. Addition of ESA to the phosphate buffer also resulted in considerable sharpening of the quinidine and procainamide peaks (Figure 5b and 5c). Under the same conditions an excellent separation was also obtained for the seven drugs investigated (Figure 6).

Pertinent data for the separation of protonated base cations at pH 2.4 in 20% acetonitrile are given in Table 2. A small electroosmotic mobility was measured with formamide using the accelerated EOF measurement method [31] but the electrophoretic mobilities were 10 to 16 times higher for the compounds investigated. Thus the migration is controlled mostly by electrophoretic behavior at the very acidic pH employed. However, 30 mM ethanesulfonic acid (ESA) must be present to give a good separation with sharp peaks. Previous work indicated that ESA improves CE results by coating the capillary surface [30]. However, it is likely that ESA also modifies the migration of analyte cations by weak ion-pair formation. The effect of ESA on the observed mobilities of several cations

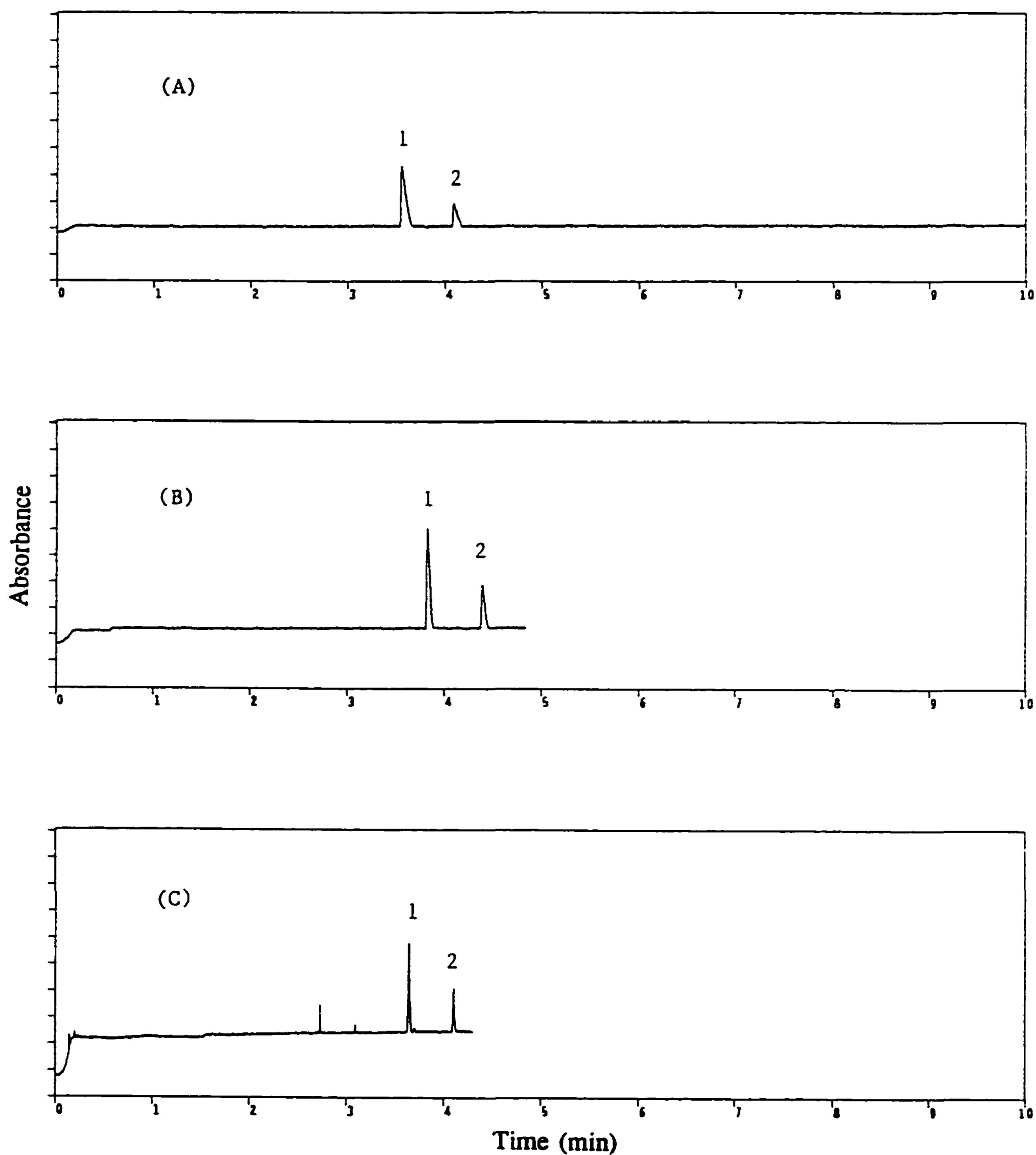

Figure 5. Comparison of different buffers at pH 2.40: (A) 10 mM phosphate; (B) 10 mM ethanesulfonate and phosphate; (C) 30 mM ethanesulfonate and phosphate. Peaks: 1= quinidine; 2= procainamide.

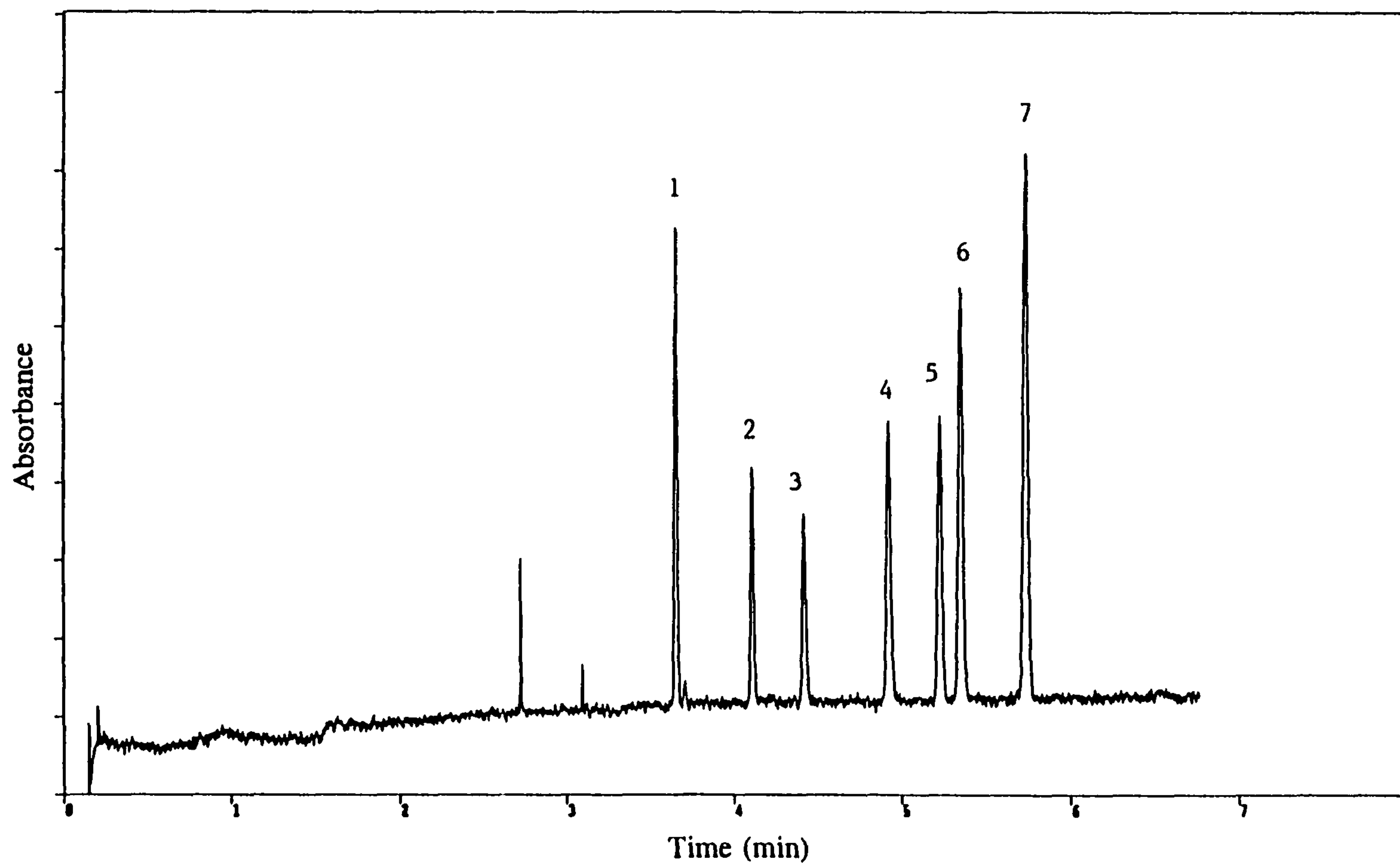

Figure 6. Separation of basic drugs in free solution CE. Electrolyte, 30 mM ethanesulfonic acid, 20% acetonitrile, pH 2.40; injection, 3 s at 4 kV; detection, 214 nm; applied voltage, 20 kV. Peaks: 1= quinidine; 2= procainamide; 3= phenylpropanolamine; 4= chloramphenicol; 5= lidocaine; 6= imipramine; 7= trimethoprim.

Table 2. Comparison of migration times of drugs in different solvents (without Brij-S), conditions as in Figure 6.

	Migration time (min)		
Compounds	20% ACN	10% ACN + 10% 2-PrOH	percent change
Quinidine	3.65	4.46	+ 22.2%
Procainamide	4.11	5.05	+ 22.9%
Phenylpropanolamine	4.42	5.44	+ 23.1%
Chloramphenicol	4.92	6.01	+ 22.2%
Lidocaine	5.22	6.54	+ 25.3%
Imipramine	5.35	6.76	+ 26.4%
Trimethoprim	5.73	7.21	+ 25.8%

was studied by Thornton et al. [30]. Their results show a slow decrease in the mobilities with the higher ESA concentration. This behavior could be explained by an weak ion-pairing formation, which would reduce the concentration of free cations and thus reduce the positive charges, therefore slow their migration.

The effect of replacing the 20% acetonitrile solvent with an aqueous solution containing 10% acetonitrile + 10% 2-propanol was investigated. The results in Table 2 show an increase in migration times but the percentage increases are similar for the drugs studied.

Addition of 30 mM Brij-S to the electrolyte changes the migration behavior of protonated cations drastically. Now the direction of the applied potential must be reversed; net movement is toward the positive electrode. This could only occur if the base cations are strongly associated with negatively charged Brij-S^-. Any free analyte cations would have a migration vector towards the negative electrode and thereby slow the rate of net migration.

The separation of drugs at pH 2.4 with 30 mM Brij-S as the additive is shown in Figure 7. The elution order has changed for some of the analytes. The migration times, apparent mobilities and electrophoretic mobilities with- and without Brij-S additive are given in Table 3. The table clearly shows that Brij-S gives different relative migration times due to a complexation effect that is superimposed on electrophoretic migration.

This separation mode is not limited to basic compounds though, it can be applied to both ionic and nonionic compounds as well. The interactions between these compounds and surfactant include hydrophobic interaction, hydrogen bonding and ion-pairing. Figure 8 shows a separation of 16 pharmaceutical compounds using 30 mM Brij-S and 20% ACN. A different electropherogram was obtained when 20% acetonitrile was switched to 10%

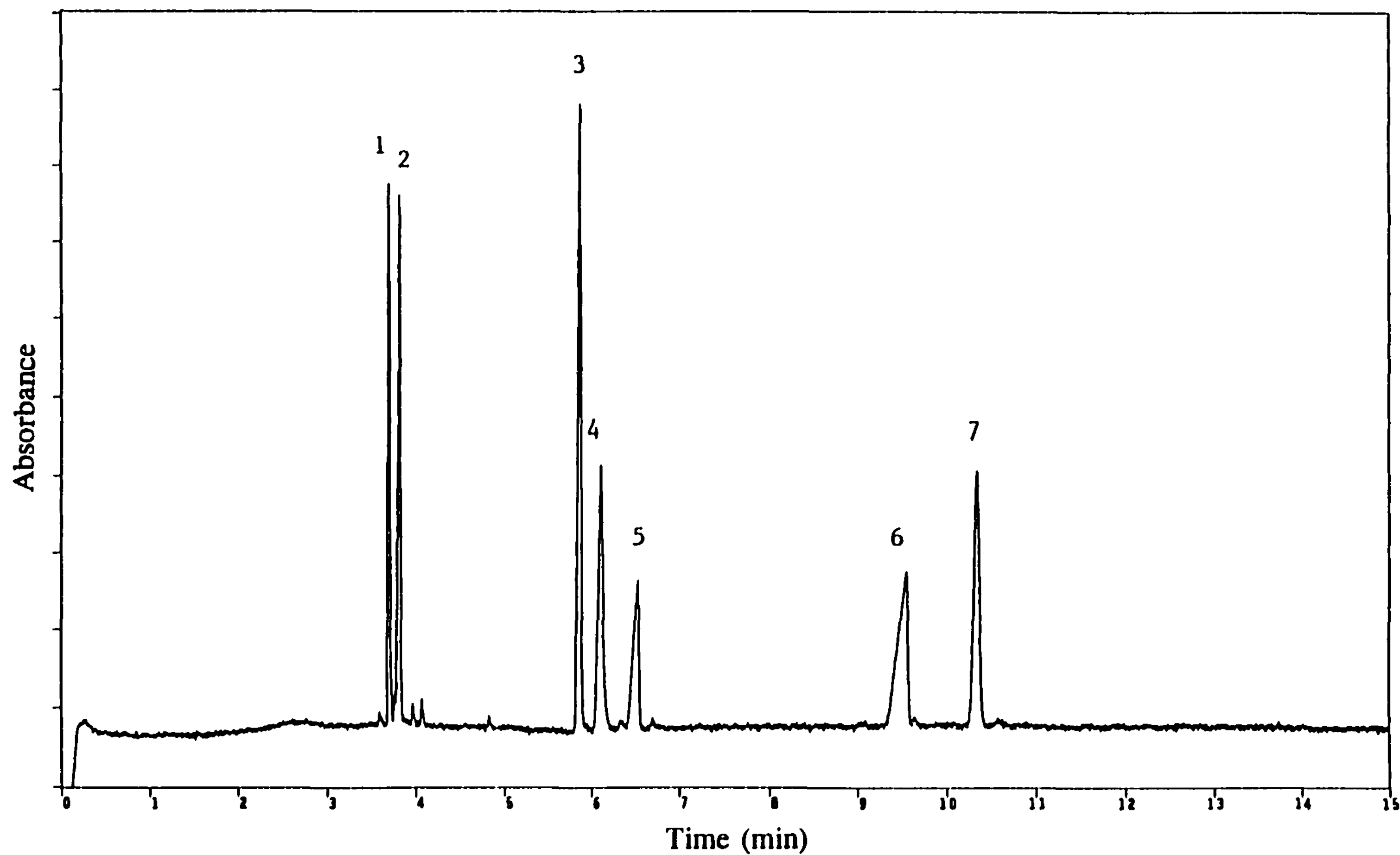

Figure 7. Separation of drugs with Brij-S. Electrolyte, 30 mM Brij-S, 20% acetonitrile, pH 2.40; injection time, 6 s; detection, 214 nm; applied voltage, 20 kV. Peaks: 1= imipramine; 2= quinidine; 3= trimethoprim; 4= lidocaine; 5= procainamide; 6= phenylpropanolamine; 7= chloramphenicol.

Table 3. Effect of Brij-S on separation of drugs in 20% acetonitrile at pH 2.4

Compound	No mM Brij-S, +20 kV $\mu_{eof}=0.22\times10^{-4}$ $cm^2/V\cdot s$			30 mM Brij-S, -23 kV $\mu_{eof}=0.44\times10^{-4}$ $cm^2/V\cdot s$		
	t_m (min)	μ_{app} (x10^4 $cm^2/V\cdot s$)	μ_{eph} ($\times10^4$ $cm^2/V\cdot s$)	t_m (min)	μ_{app} ($\times10^4$ $cm^2/V\cdot s$)	μ_{eph} ($\times10^4$ $cm^2/V\cdot s$)
Quinidine	3.65	3.85	3.63	3.10	3.94	8.00
Procainamide	4.11	3.42	3.20	5.63	2.17	5.80
Phenylpropanolamine	4.42	3.18	2.96	8.48	1.44	4.84
Chloramphenicol	4.92	2.80	2.64	8.72	1.40	4.48
Lidocaine	5.22	2.69	2.47	5.12	2.39	5.30
Imipramine	5.35	2.63	2.37	3.02	4.05	6.85
Trimethoprim	5.73	2.45	2.23	5.03	2.43	5.10

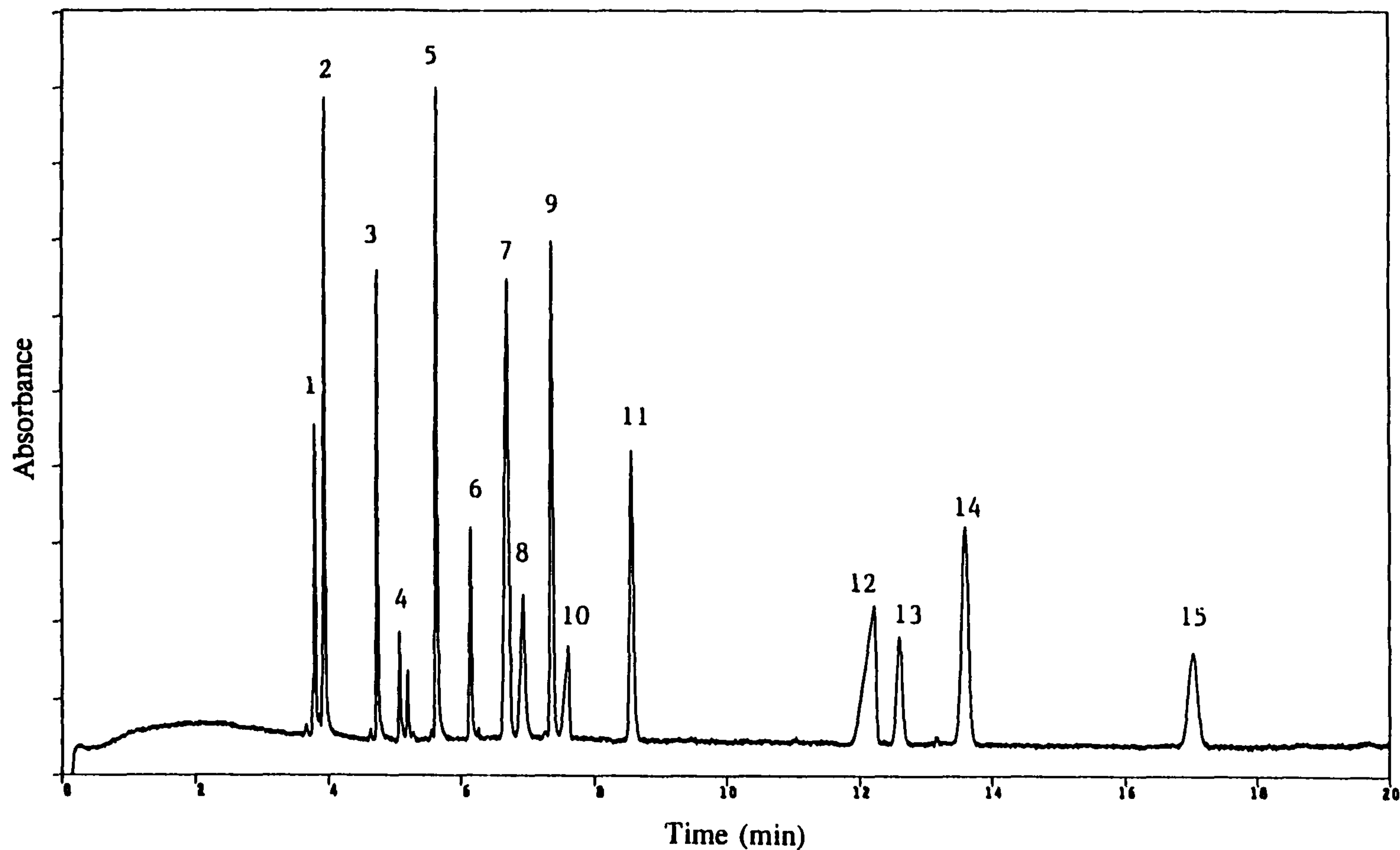

Figure 8. Electropherogram of 16 pharmaceutical compounds. Electrolyte, 30 mM Brij-S, 20% ACN, 10 mM HCl, 20% ACN, pH 2.40; applied voltage, -20 kV; detection, 214 nm; injection time, 6 s; capillary, 45 cm length and 50 μm i.d. Peaks: 1= imipramine; 2= quinidine, indomethacin; 3= naproxen; 4= nifedipine; 5= ß-naphthoxyacetic acid; 6= furosemide; 7= trimethoprim; 8= lidocaine; 9= carbamazepine; 10= procainamide; 11= salicylic acid; 12= phenylpropanolamine; 13= chloramphenicol; 14= sulfamethoxazole; 15= acetylsalicylic acid.

acetonitrile and 10% isopropanol (Figure 9). Quinidine and indomethacin were separated this time. This method also showed excellent linearity over a broad range. Both peak height and area over analyte concentration, for example salicylic acid, had a 0.997 and 0.999 coefficient, respectively (Figure 10 and 11).

There is a possibility that a dynamic equilibrium exists in which Brij-S coats the surface of the silica capillary, giving it a negative charge. Experiments were run in which the EOF was measured (formamide as a marker) with 30 mM Brij-S. Then the capillary electrolyte was replaced with electrolyte containing no Brij-S and the EOF measurement was repeated. New electrolyte containing no Brij-S was added several more times to the capillary and the EOF measured after each replacement. If Brij-S is on the surface, the EOF should gradually change but be different from a run with no Brij-S on a clean, uncoated capillary. In 10% acetonitrile the EOF shows only a slight decrease after the first non-Brij-S electrolyte replacement (see Figure 12). After additional electrolyte replacements, the EOF drops and levels off, showing that the majority of negative Brij-S layer has been washed off. However, compared to the EOF measured for uncoated capillary, which is represented as a solid line in Figure 12, it was a little higher. This indicates that there was still a notable amount of negatively charged Brij-S stuck on the capillary. This was further confirmed by a mass spectrometry experiment. Peaks for Brij-S continued to appear in the screen even after washing the capillary, which was formerly filled with an electrolyte containing Brij-S, with sodium hydroxide solution for 20 min. A similar pattern was obtained for 20% acetonitrile, but the decrease in EOF was smaller. In 40% acetonitrile the EOF decreases and levels off after the first replacement of capillary electrolyte. This behavior indicates that there is

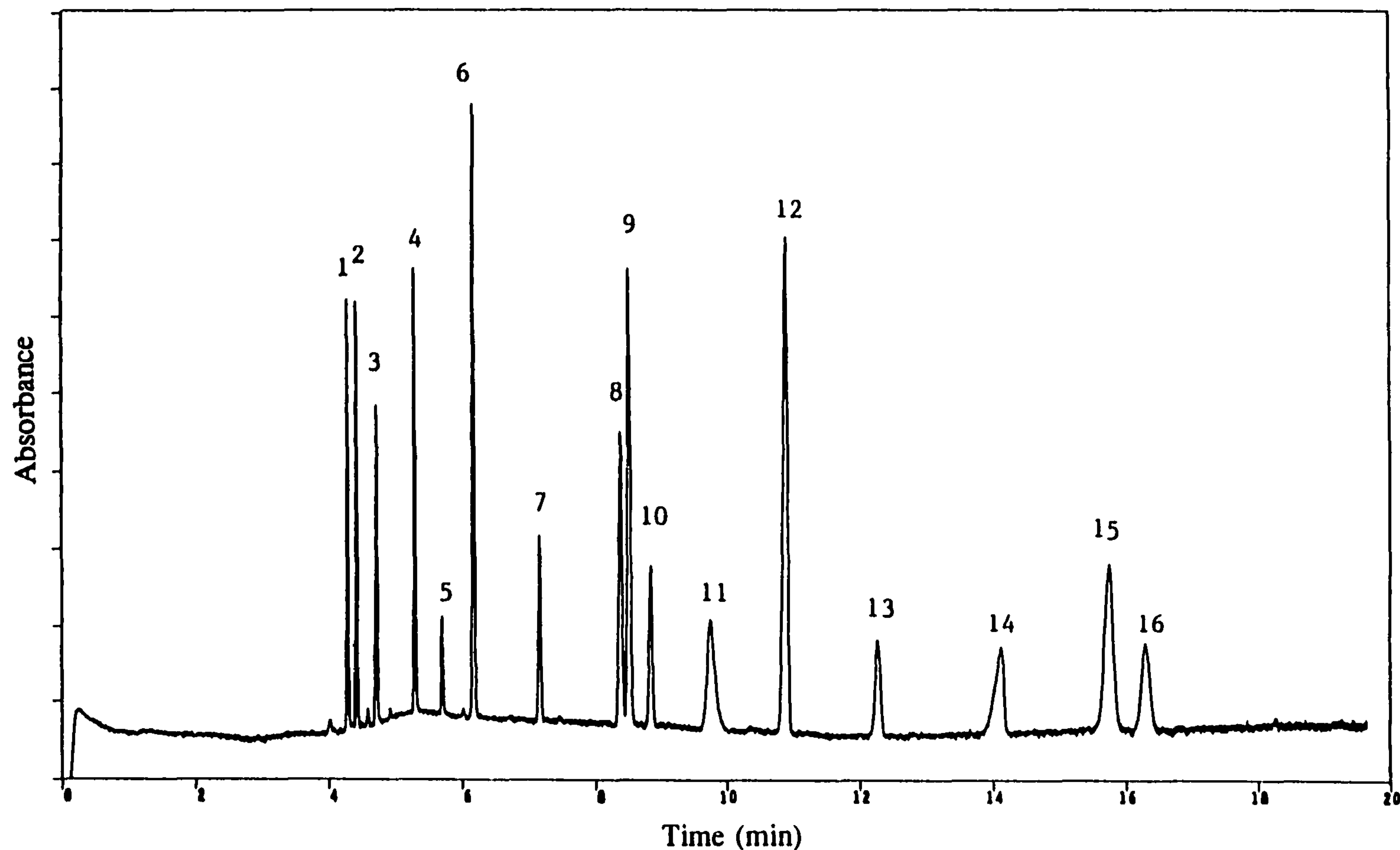

Figure 9. Electropherogram of 16 drugs. Conditions: see Figure 8 except 10% ACN and 10% 2-propanol instead of 20% ACN. Peaks: 1 = quinidine; 2 = indomethacin; 3 = imipramine; 4 = naproxen; 5 = nifedipine; 6 = ß-naphthoxyacetic acid; 7 = furosemide; 8 = salicylic acid; 9 = trimethoprim; 10 = lidocaine; 11 = procainamide; 12 = carbamazepine; 13 = chloramphenicol; 14 = phenylpropanolamine; 15 = sulfamethoxazoe; 16 = acetylsalicylic acid.

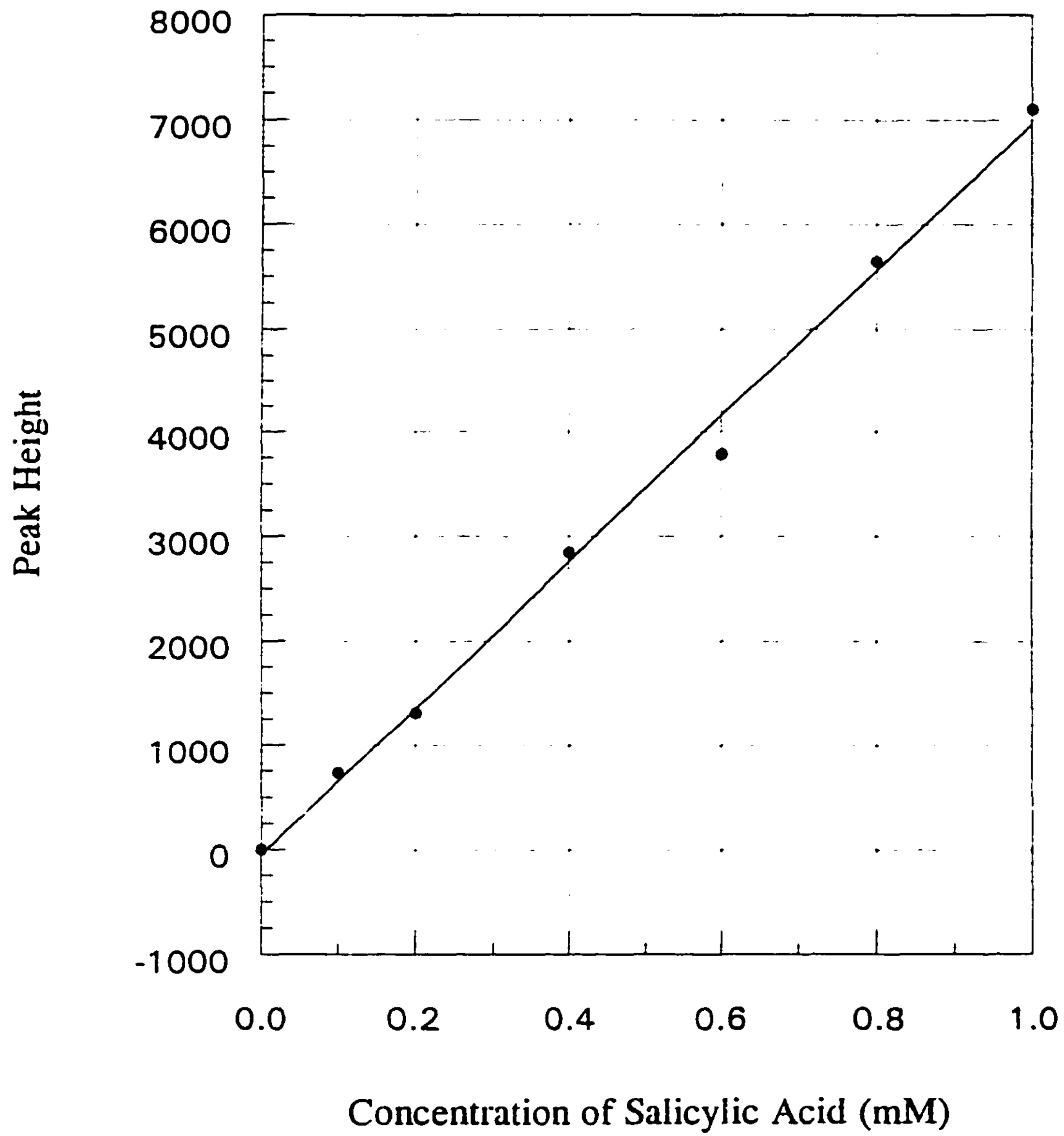

Figure 10. Plot of peak height vs. concentration.

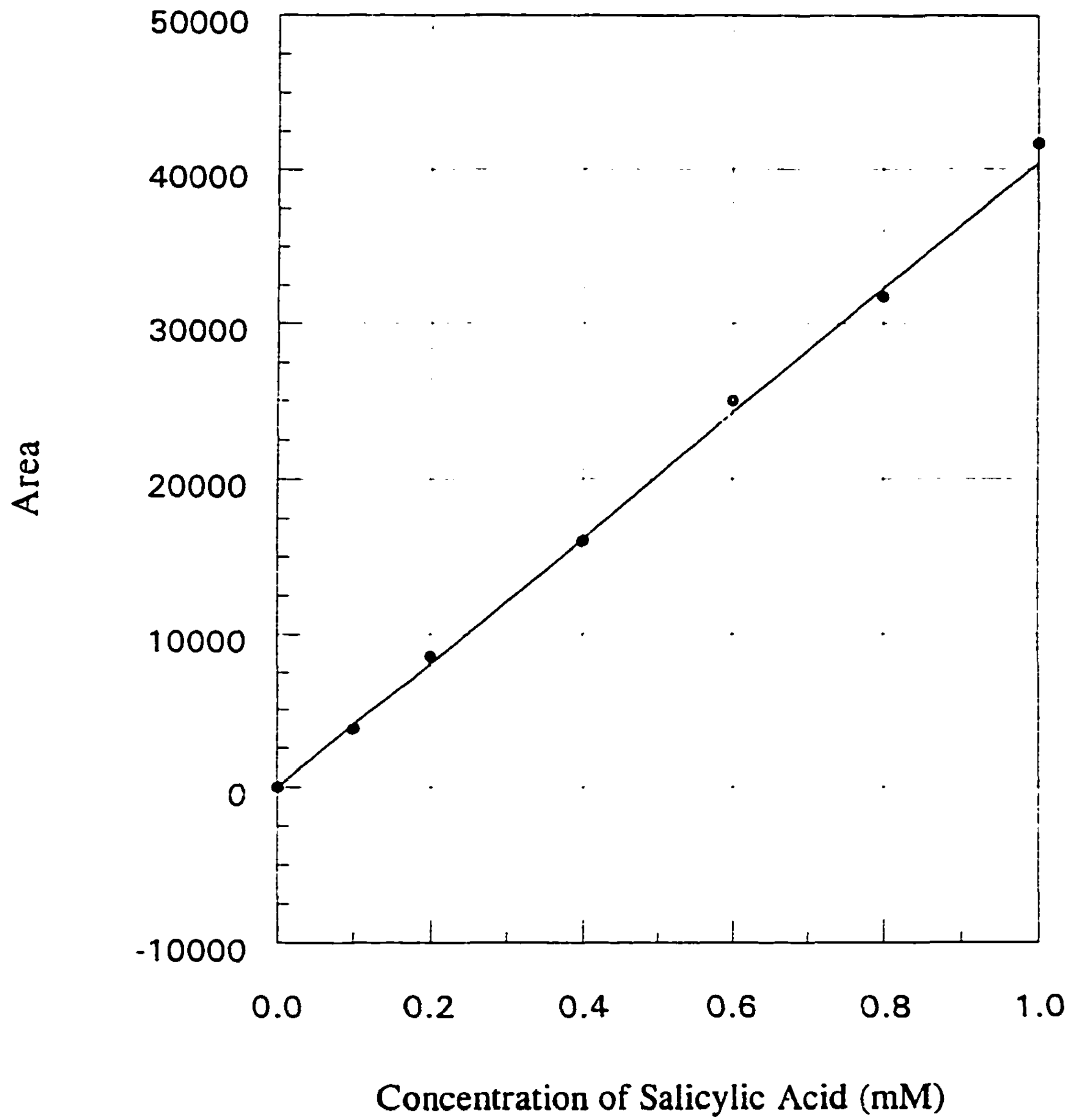

Figure 11. Plot of peak area vs. concentration.

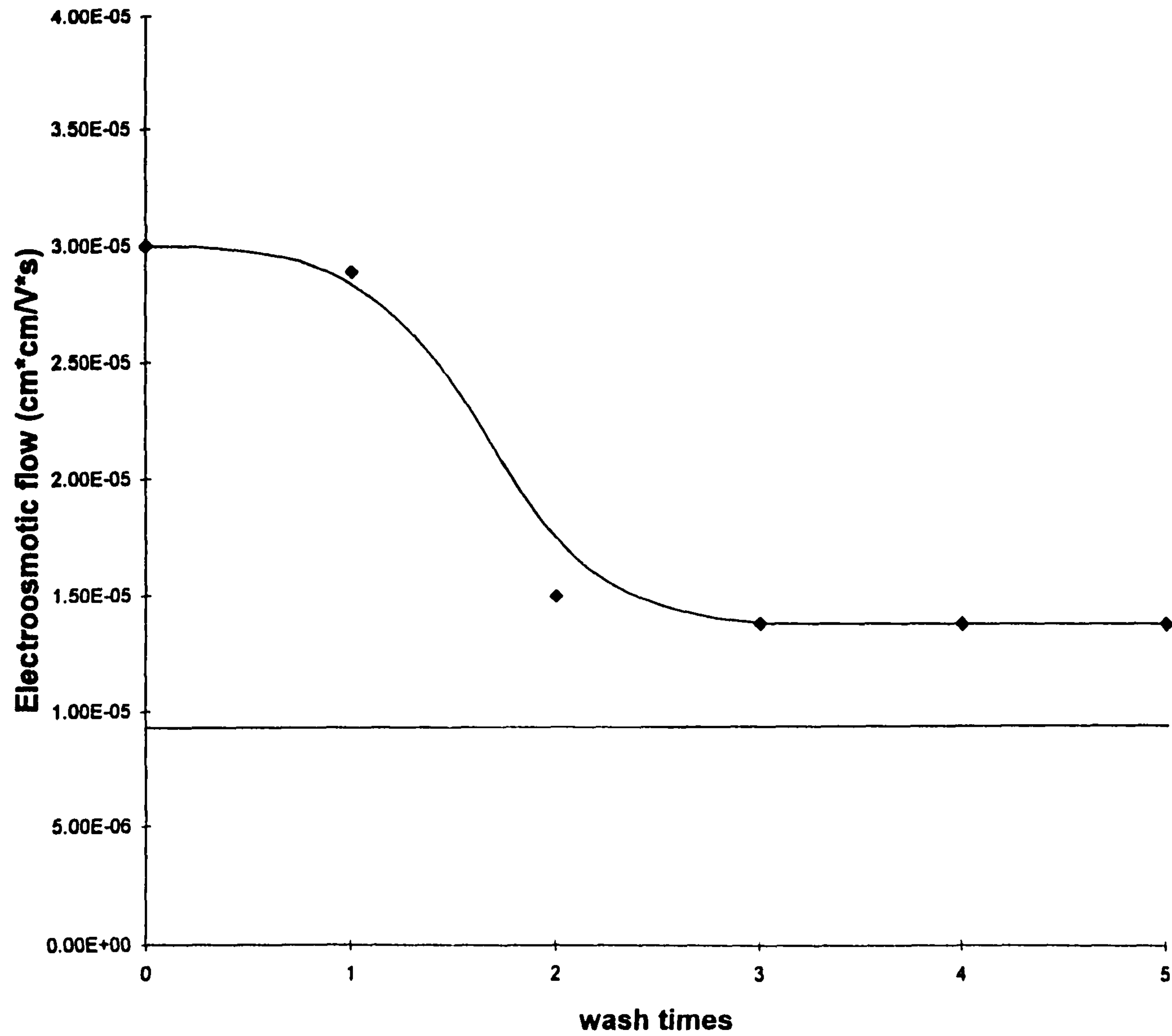

Figure 12. Change of electroosmotic flow with wash times.

essentially little, if any surface layer of Brij-S owing to the high concentration of acetonitrile.

4. Conclusions

Separations by CE are conveniently carried out in an aqueous-organic solution at pH 2.4 where the EOF is very small compared to electrophoretic migration. Addition of negatively-charged Brij-S to the solution permits the separation of neutral molecules such as PAH compounds and phenols. Brij-S also has a major effect on the CE behavior of protonated cations of basic analytes. The positively charged cations are complexed so strongly by the negatively-charged Brij-S that the net migration direction of the analytes is reversed.

Changes in the type of organic solvent as well as the total organic composition is shown to have a major effect on the net migration in the systems studied. While the effect of solvent type on migration of free solution cations was almost the same for the ions studied (Table 2), migration of various analytes was affected to a much different extent when Brij-S was present. The degree of complexation of both neutral and positively-charged analytes by Brij-S apparently is affected both by the type and total concentration of organic solvent in the electrolyte solution. Both the type and concentration of surfactant additive also have a profound effect on analyte migration. Manipulation of all of these variables is a powerful way to control and optimize separations of organic compounds by CE.

Acknowledgements

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CHAPTER 4. SEPARATION OF BASIC PROTEINS, PEPTIDES, AND PHARMACEUTICAL COMPOUNDS BY CAPILLARY ELECTROPHORESIS USING A CATIONIC SURFACTANT

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Abstract

Incorporation of a low concentration of cetyltrimethylammonium bromide (CTAB) in the running electrolyte is shown to dynamically coat the silica capillary and to reverse the direction of electroosmotic flow. The CTAB coating prevented interaction of proteins with the capillary surface and enabled sharp peaks to be obtained in the electropherograms. A systematic study of experimental parameters demonstrated the importance of selecting a suitable buffer electrolyte and an appropriate pH. Excellent separations were obtained for five proteins, three enkephalins and six dipeptides with an efficiency of approximately 500,000 theoretical plates per meter. The method developed is very simple to perform and was found to give excellent reproducibility.

1. Introduction

Capillary electrophoresis (CE) is a powerful technique for protein and peptide analysis [1]. However, nonspecific interactions between proteins and silanol groups on the capillary surface can cause a lot problems, such as peak broadening and tailing, nonreproducible migration times, low mass recovery, and irreversible adsorption. Several strategies have been proposed to address these problems. One approach used electrolyte solutions at very acidic pH values to suppress the silanol dissociation [2]. Another study used pH values higher than the protein pI to obtain negatively changed proteins [3]. However, pH extremes tend to denature proteins and compromise recovery by reducing solubility. Electrolyte solutions with high concentrations of alkali salts [4,5] or zwitterions [6,7] to suppress electrostatic interactions have been used, but the high current from these high ionic strengths limits the high voltage can be used, causing long analysis time and low efficiency. Another approach is to coat the capillary surface with a polymeric layer, either by covalent bonding or adsorption. A larger variety polymers has been used, including polyacrylamide [8-11], poly(ethylene glycol) (PEG) [12-15], polyethyleneimine [4,16], poly(vinyl alcohol) (PVA) [17], poly(vinylpyrrolidinone) [2], epoxy polymer [18,19], and poly(ethylene oxide) [20]. Capillaries coated with C_{18} moieties have also been used. These coatings, however, show a limited life-time; the coating process usually consists of multistep reactions, which are quite laborious and time-consuming.

It has also shown that a suitable additive in the running electrolyte can alleviate protein capillary wall interactions. Several compounds have been proposed as electrolyte additives, including cationic surfactants [21-23], primary alkyldiamines [24,25], tertiary alkylamines

[25-27], quaternary ammonium alkanes [28], amino sugars [27], chitosan [29], tertiary alkyldiamines [30,31], and CTAB [23,32,33]. Many of these additives reverse the direction of the electroosmotic flow. A recent study reviewed the literature and proposed the use cetyltrimethylammonium bromide (CTAB) as the best for the CE separation of proteins[23]. However, their method gave reasonable separations only at sub-ambient temperatures (20 °C and preferably 10°C).

In the present research CTAB was used to dynamically coat the silica capillary but the concentration was much lower and other conditions are significantly different from those used by Cifuentes et al. [23] We were able to obtain excellent separations of proteins and peptides at room temperature with excellent efficiency: > 500,000 theoretical plates per meter. The method employed was shown to give excellent reproducibility.

2. Experimental

2.1 Instrumentation

Experiments were performed on a Quanta 4000E CE system (Waters, Milford, MA, USA). The fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) used for CE were 50 μm i.d. and 50 cm in length (42.5 cm to detector). Direct UV absorbance detection was performed at 214 nm. A negative power supply was used. Separations were done at 25°C. Electropherograms were collected at speed of 25 points/s and plotted by Chromperfect data acquisition system (Justice Innovations, Mountain View, CA, USA). Prior to use capillaries were rinsed with 1.0 N NaOH for 10 min, 0.1 N NaOH for 5 min and deionized water for 5 min, then filled with fresh buffer and left to stand for 20 min to

equilibrate the column. In order to increase migration time reproducibility and eliminate protein build up on the capillary surface, the column was rinsed with 0.1 N NaOH and running buffer for 3 min each between injections. The capillary was kept in deionized water over night and was rinsed with 0.1 N NaOH and deionized water every morning. By using this cleaning method capillaries could be used for at least two weeks.

2.2 Samples and chemicals

All proteins and peptides were purchased from Sigma (St.Louis, MO, USA) and used as received. These standards were stored at -4 °C when not in use. The other chemicals were supplied by Aldrich (Milwaukee, WI, USA).

All samples and buffer solution were prepared with 18.2 MΩ deionized water from a Barnstead Nanopure II system (Syboron Barnstead, Boston, MA, USA). The mixture of standard proteins contained approximately 0.1 mg/ml of each individual protein; the standard peptides were about 0.05 mg/ml each. Tris(hydroxymethyl)aminomethane (Tris) was used in all the running buffers. Buffers were prepared by dissolving the weighed amount of Tris in deionized water and adding 0.3 ml of 0.1 mol/L CTAB, 0.1 ml of 1.0 mol/L NaCl, and 0.1 mol/L hydrochloric acid (HCl) to obtain the desired pH, and diluted to 50.0 ml.

3. Results and Discussion

3.1 Conditions for separation

The approach used was to use a running electrolyte sufficiently acidic to protonate the sample compounds so that they could be separated as cations. As might be expected, our

first experiments with proteins gave very broad peaks with very little separation. The large protein molecules were apparently interacting with the surface of the silica capillary. Addition of a low concentration of CTAB to the electrolyte resulted in a dramatic change. The capillary surface became coated with CTAB by a dynamic equilibrium. The positive charge of the quaternary ammonium group of the CTAB resulted in a strong electroosmotic flow toward the anode. The coated surface with its positive charge repelled the protein cations and enabled sharp peaks to be obtained in the electropherogram.

Preliminary experiments showed that reasonably good separations of proteins could be obtained at pH 3.45 with low concentrations of Tris and CTAB in the running buffer. An applied voltage of -15 kV was used. Using these conditions, the effect of CTAB concentration was studied. At 0.4 mM CTAB we did not observe reversed electroosmotic flow. At 0.6 mM CTAB, reversed EOF was observed (toward the anode) and peak shape was quite good. Similar results were obtained at 1.5 mM CTAB. Increases in CTAB concentration up to 23 mM showed no significant improvement in peak shape, so we decided to use 0.6 mM CTAB. This concentration is below the critical micelle concentration, which has been listed as 0.94 mM [34].

As an alternative to Tris-HCl buffer, the use of phosphate, formate, ethanesulfonate, CHES and trifluoroacetate as a buffer was also studied. Of these, only Tris-HCl, phosphate and formate gave electropherograms with narrow peak widths. However, phosphate and formate buffers caused some of the protein peaks to be fronted. For this reason Tris-HCl was selected even though it has rather low buffering capacity at the acidic end of the pH range. However at pH values around 2.5-3.5 the relatively high concentration of HCl

provides a buffering effect. Several concentrations of Tris-HCl buffer (5-, 10-, 20-, 50-, 80-, and 100 mM) at pH 3.45 were tried with ribonuclease A and lysozyme as the sample proteins. The peaks started to broaden at 80 mM Tris-HCl, which may have due to the high current generated. A medium concentration of 15 mM was selected for use.

It was found that addition of a low concentration of 2 mM NaCl or KCl to the electrolyte helped in obtaining narrow peaks. The effect of methanol was investigated briefly. Addition of 2% methanol to the buffer slowed the electroosmotic flow and broadened the peaks without improving the separation.

3.2 Effect of pH

In acidic solution the degree to which proteins and peptides are protonated will vary with pH. Because of this, their electrophoretic mobilities will change with pH. It is extremely important to choose the right pH for any particular separation. We studied five standard proteins: α-chymotrypsinogen A, ribonuclease A, lysozyme, cytochrome c, and myoglobin. Their apparent electromobilities, as well as the electroosmotic mobility, are plotted as a function of pH in Figure 1.

The direction of electroosmotic flow was reversed because of the positively charged layer of CTAB on the capillary surface. We found that this reversed flow also depended on pH, and the electroosmotic mobility increased with higher pH. This can be explained by the fact that ionization of silanol groups increases with rising pH. The $-SiO^-$ groups attract $CTAB^+$ more strongly to the surface. Additional $CTAB^+$ molecules are attracted hydrophobically to the first CTAB layer, but the second layer has the $-N^+$ pointing out into

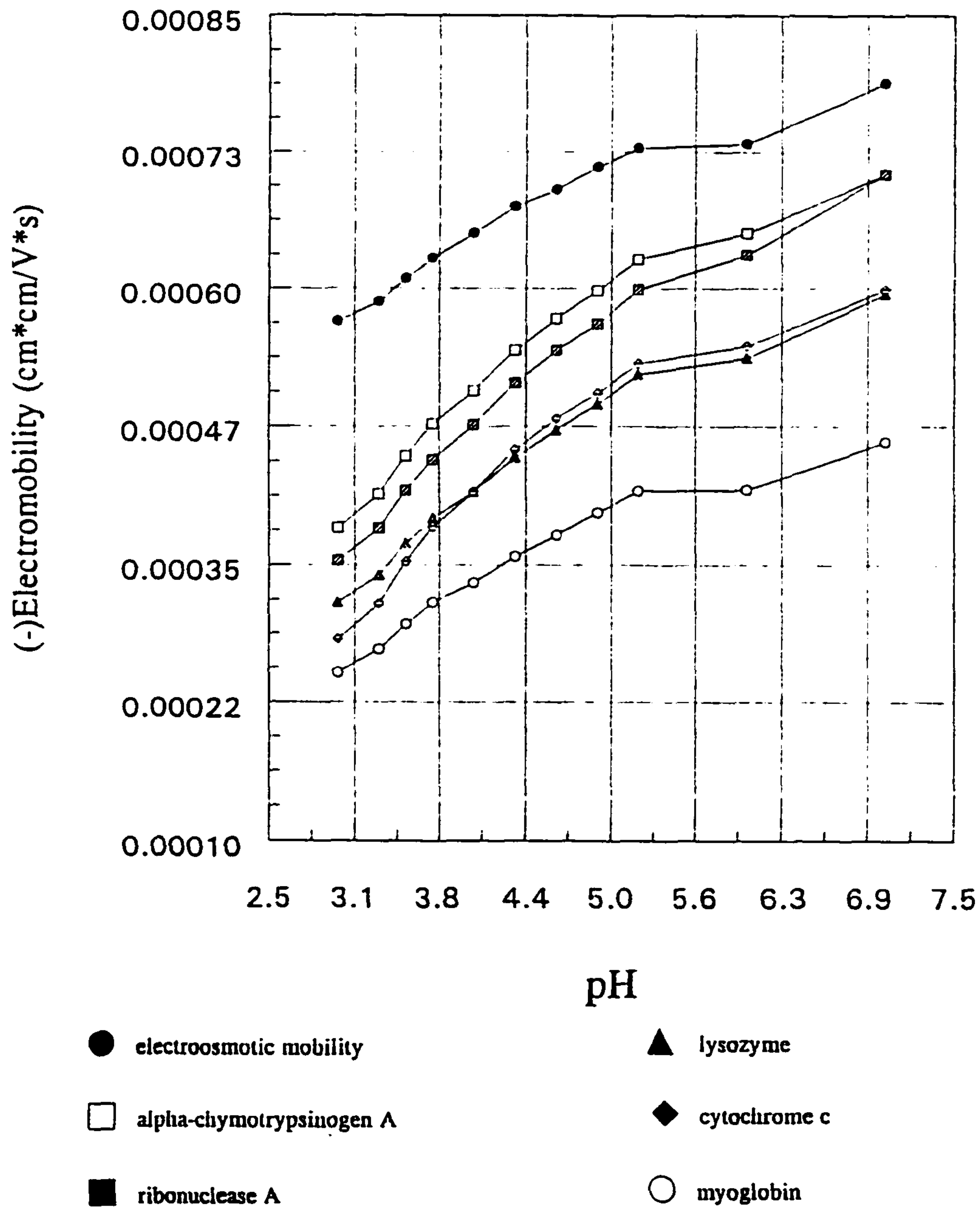

Figure 1. Apparent electromobilities of proteins and electroosmotic mobility vs. pH.

the solution, thus giving the surface a positive charge. A thicker $CTAB^+$ layer at higher pH means more positive charges on the surface and a greater electroosmotic mobility.

The electrophoretic mobilities of the sample proteins were obtained by subtraction of the apparent electromobilities from the electroosmotic mobility. Note that a counter migration mode is used and the electroosmotic and electrophoretic vectors are in the opposite direction. Electrophoretic mobilities are plotted as a function of pH in Figure 2. The electrophoretic mobility of myoglobin does not change with pH very much. The electrophoretic mobilities of the other four proteins decrease with rinsing pH but with different slopes. This decrease in electrophoretic mobility can be attributed to decreased protonation, and thus a lower positive charge, as the pH becomes less acidic.

It is clear from the plots in Figures 1 and 2 that pH has a very important effect on separations. At pH 7.0 lysozyme and cytochrome c had almost the same mobilities and the mobilities of chymotrypsinogen A and ribonuclease A were identical. There is a crossover in the mobilities of cytochrome c and lysozyme at pH 4.0. An excellent separation of all five proteins was obtained at pH 4.60 (Figure 3). At pH 3.45 the separation took a little longer but the peaks were resolved even better (Figure 4). At pH 3.45 even the impurities in the proteins were resolved.

The separation of three enkephalins was also dependant on pH. Figure 5 shows that tyr-gly-gly and tyr-gly-gly-phe-met had the same apparent mobility at pH 3.26. At pH 2.46 tyr-gly-gly-phe-met and tyr-gly-gly-phe had identical mobilities and could not be separated. The three enkephalins were well separated at pH 2.8 (Figure 6).

We also studied six dipeptides and found that pH 2.45 was a good choice (Figure 7).

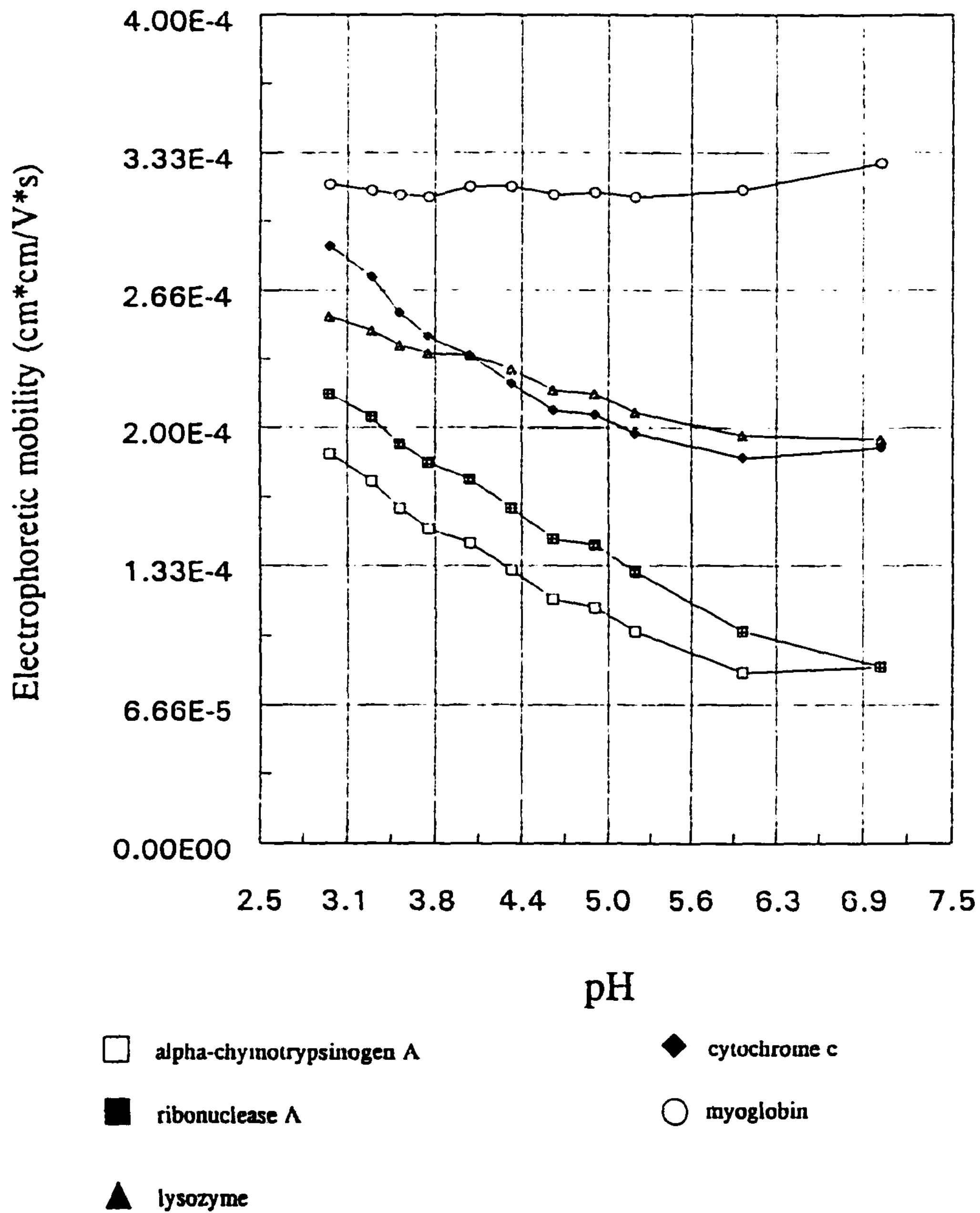

Figure 2. Electrophoretic mobilities of the five proteins vs. pH.

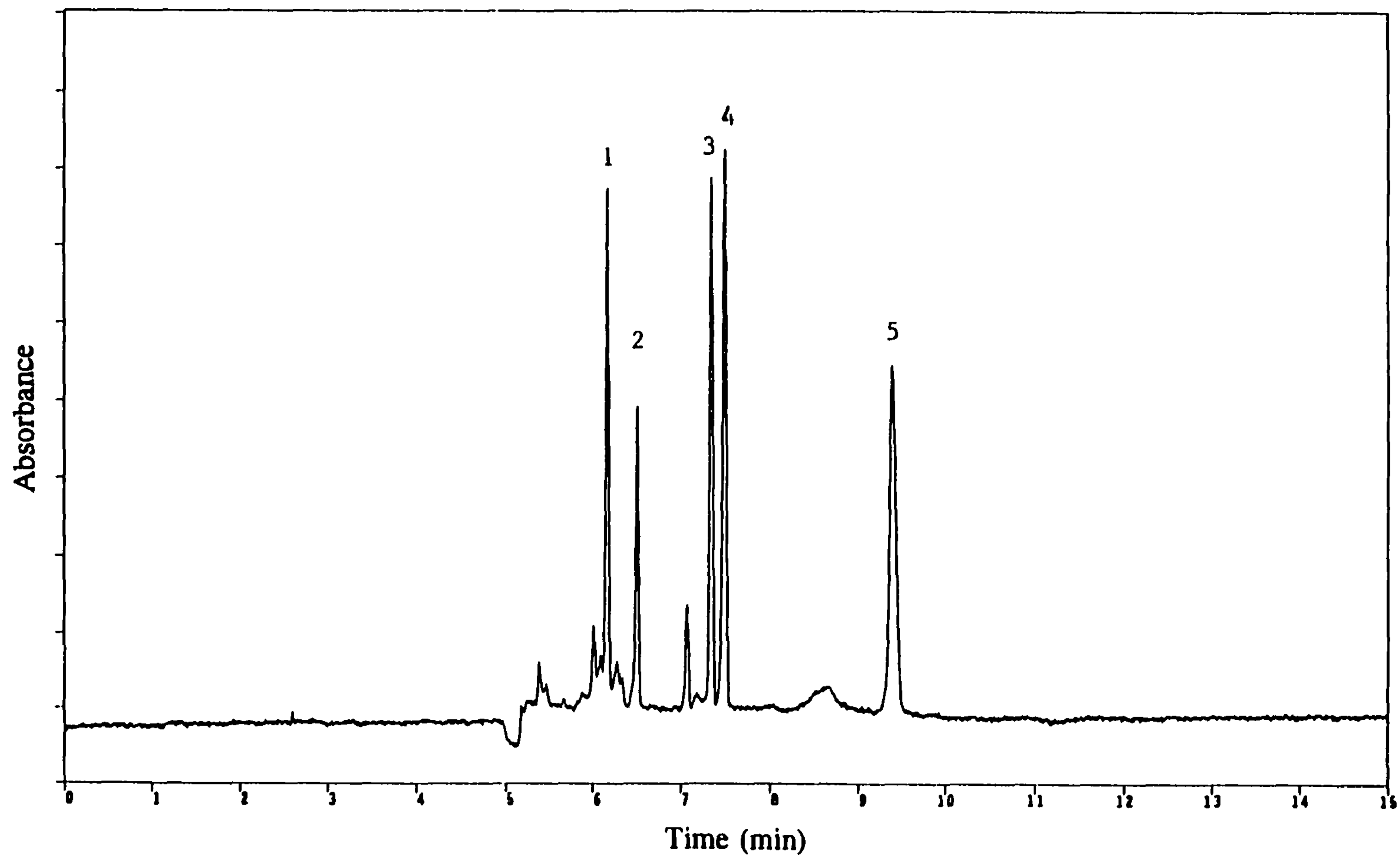

Figure 3. Separation of five proteins at pH 4.60. Electrolyte, 15 mM Tris-HCl, 0.6 mM CTAB, 2 mM NaCl, pH 4.60; capillary, 50cm*50μm; injection, 40 seconds; applied voltage, -10 kV; detection, 214 nm. Peaks: 1= α-chymotrypsinogen A; 2= ribonuclease A; 3= cytochrome c; 4= lysozyme; 5= myoglobin.

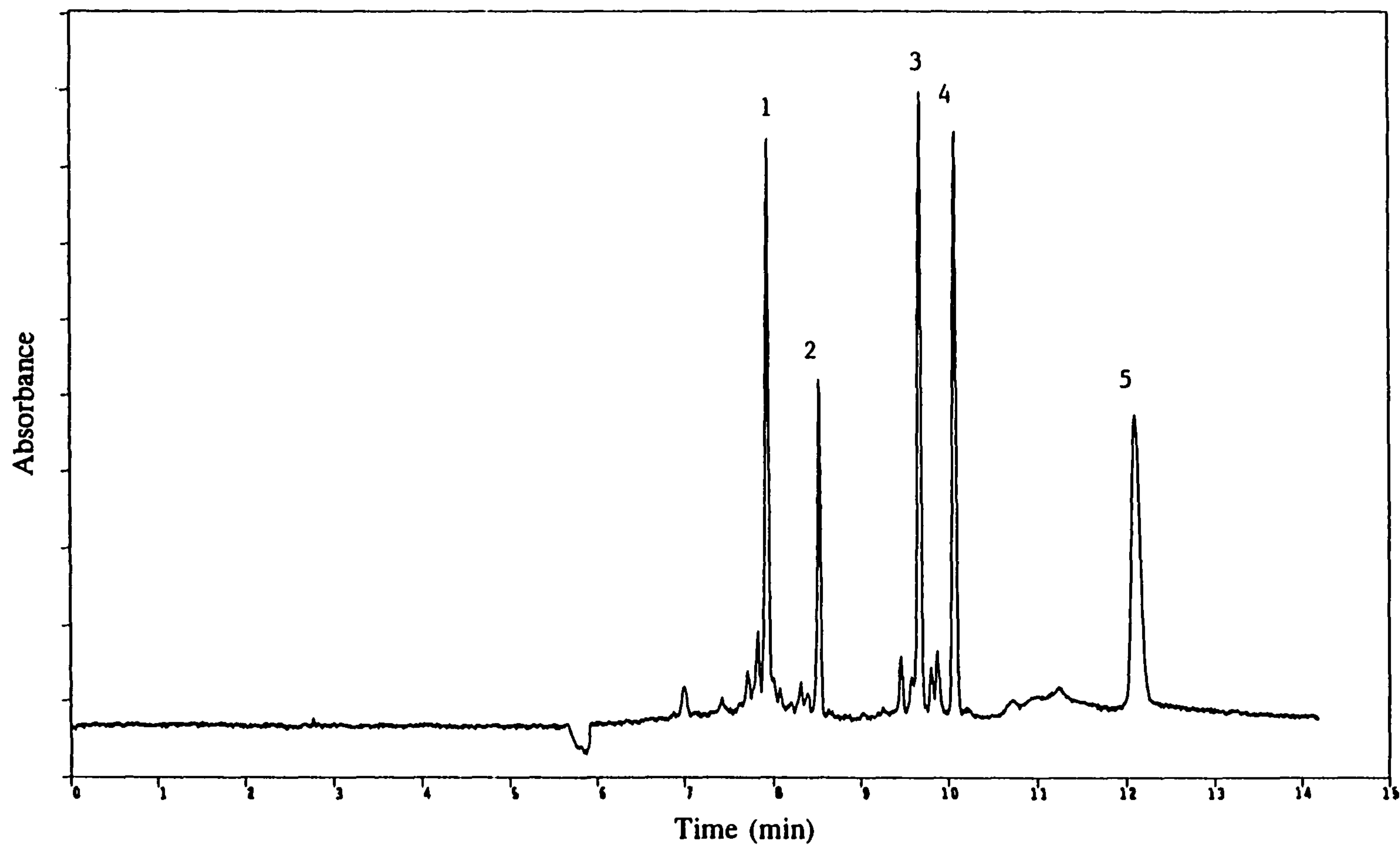

Figure 4. Separation of five proteins at pH 3.45. Electrolyte, 15 mM Tris-HCl, 0.6 mM CTAB, 2 mM NaCl, pH 3.45; capillary, 50cm*50μm; injection, 40 seconds; applied voltage, -10 kV; detection, 214 nm. Peaks: 1 = α-chymotrypsinogen A; 2 = ribonuclease A; 3 = lysozyme; 4 = cytochrome c; 5 = myoglobin.

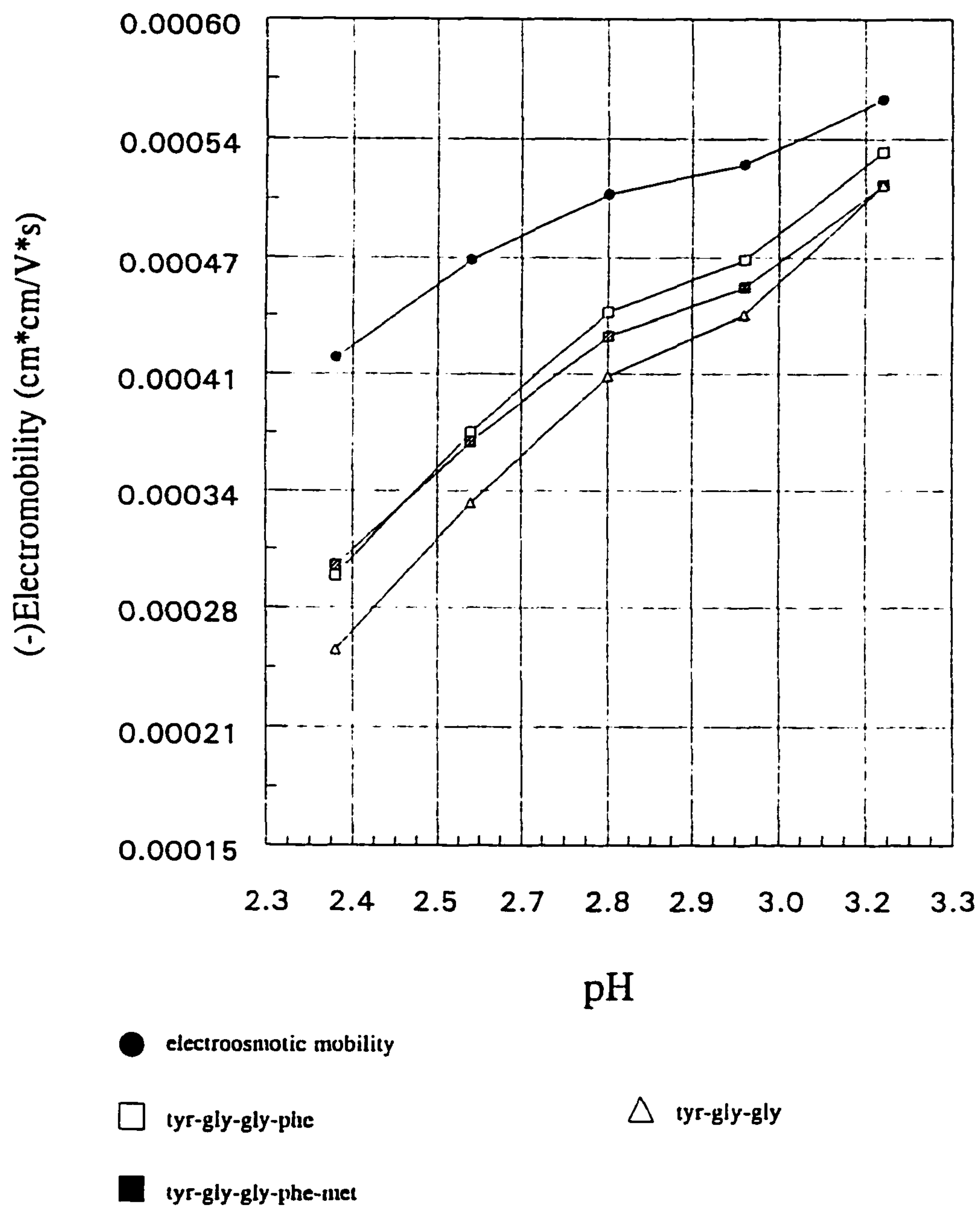

Figure 5. Apparent electromobilities of three enkephalins vs. pH.

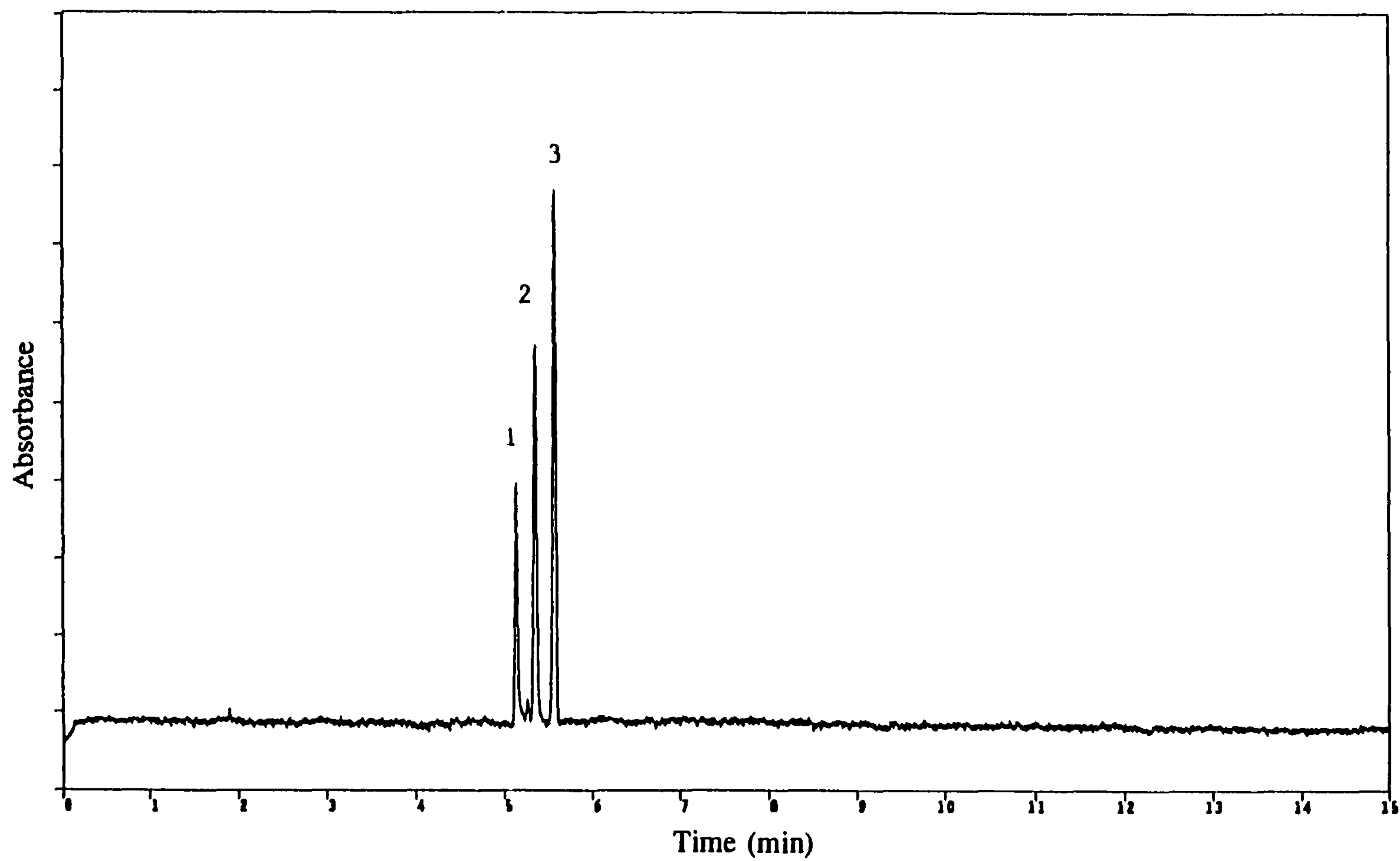

Figure 6. Separation of three enkephalins. Electrolyte, 15 mM Tris-HCl, 0.6 mM CTAB, 2 mM KCl, pH 2.80; capillary, 50cm*50μm; injection, 40 seconds; applied voltage, -15 kV; detection, 214 nm. Peaks: 1= tyr-gly-gly-phe; 2= tyr-gly-gly-phe-met; 3= tyr-gly-gly.

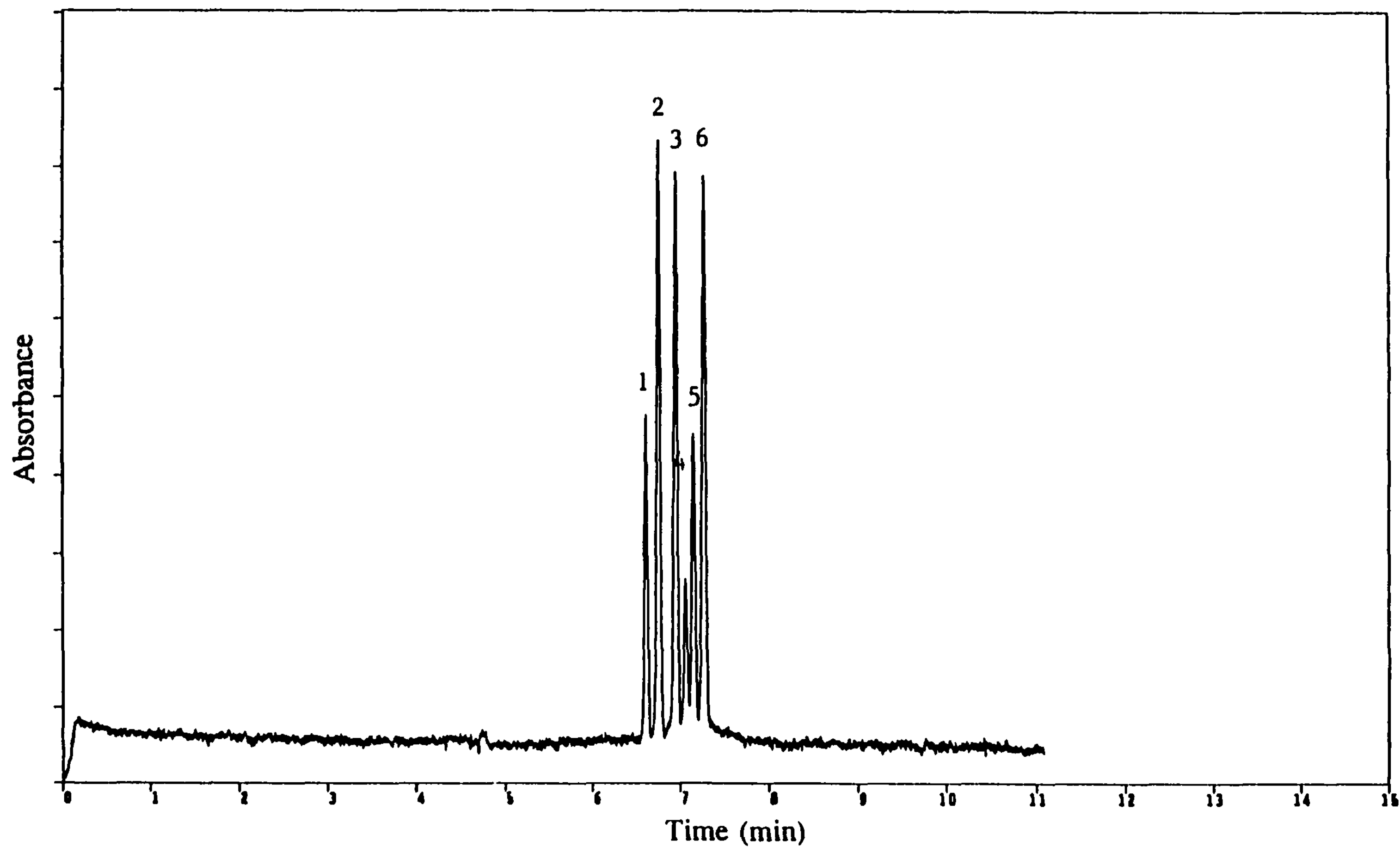

Figure 7. Separation of six dipeptides. Electrolyte, 30 mM Tris-HCl, 0.6 mM CTAB, 2 mM KCl, pH 2.45; Capillary, 50cm*50μm; injection, 15 seconds; applied voltage, -15 kV; detection, 214 nm. Peaks: 1= phe-tyr; 2= phe-met; 3= phe-leu; 4= phe-val; 5= phe-gly; 6= phe-ala.

Even at this low pH protonation of the dipeptides to form a cation is apt to be incomplete (that is, the average charge may be less than 1+). This being the case, the migration order would be related to pK_{a1} to some degree. The elution order for six dipeptides at pH 2.45 was: phe-tyr, phe-met, phe-leu, phe-val, phe-gly and phe-ala. The pK_{a1} values for tyr, met, leu, val, gly and ala are: 2.20, 2.28, 2.36, 2.32, 2.35 and 2.34, which is similar to the elution order of the dipeptides. Those with higher pK_{a1} values would be more completely protonated and, because of counter migration, would have longer net migration times.

3.3 PEG additive effect

Poly(ethylene glycol) (PEG) is often used as an additive in CE. We studied the effect of adding PEG of different molecular weights to the buffer, ranging from 300 to 1500. In each case the PEG was added to the buffer to make a 1% solution. The electroosmotic mobility and the apparent electromobilities of several proteins at pH 3.45 are plotted against the PEG molecular weight in Figure 8. The electroosmotic mobility decreased as the molecular weight of the PEG became higher. This effect could be due to increased viscosity. The apparent electromobilities for the four proteins decreased at the same degree as the electroosmotic mobility. There was no improvement in the separation; it merely took longer with the PEG additive.

3.4 n-Butanol additive effect

In the CE separation of inorganic anions and small organic anions, Benz and Fritz [35] found that reversal of the direction of electroosmotic flow by a quaternary ammonium salt

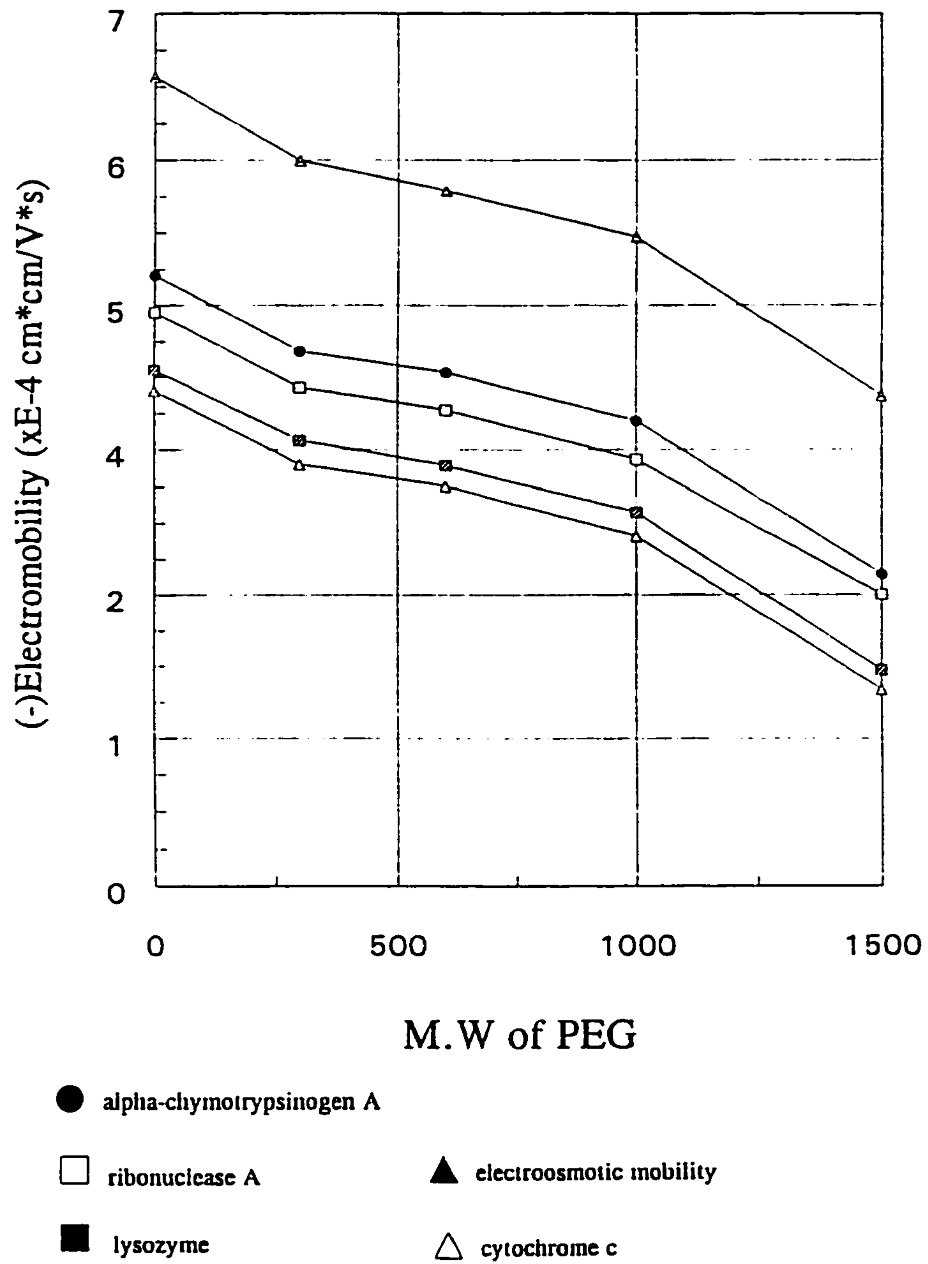

Figure 8. Effect of PEG on separation of four proteins.

(Q^+) similar to CTAB could be achieved at much lower concentrations (<0.1 mM) if about 5% n-butanol was added to the electrolyte. They explained that use of such a low concentration of Q^+ was possible because both butanol and Q^+ were adsorbed on the capillary surface. The adsorbed butanol shifted the dynamic equilibrium so that more Q^+ would be drawn to the surface even though a very low concentration was added to the buffer.

Instead of the usual 0.6 mM CTAB in 15 mM Tris-HCl buffer, we added only 0.06 mM CTAB plus 4% v/v of n-butanol. Three enkephalins were well separated under these conditions (Figure 9) but the peaks were broader than with 0.6 mM CTAB and no butanol.

3.5 Sample solutions

Most of the samples were prepared each day in deionized water. No attempt was made to acidify the samples prior to injection. Under these conditions an electrostacking effect would be expected which would result in sharper peaks in the electropherograms.

In the separation of enkephalins it was found that 10 mM Tris in the sample solution gave sharper peaks than when the sample was in water alone. Sample peaks were still sharper with 30 mM Tris in the sample. We did not observe such peak sharpening when Tris was added to protein samples.

3.6 Simultaneously separation of cationic and anionic pharmaceutical compounds

By using a concentration of a cationic surfactant such as CTAB or CTAC just below its critical micellar concentration (cmc), electroosmotic flow (EOF) can be reversed whereas

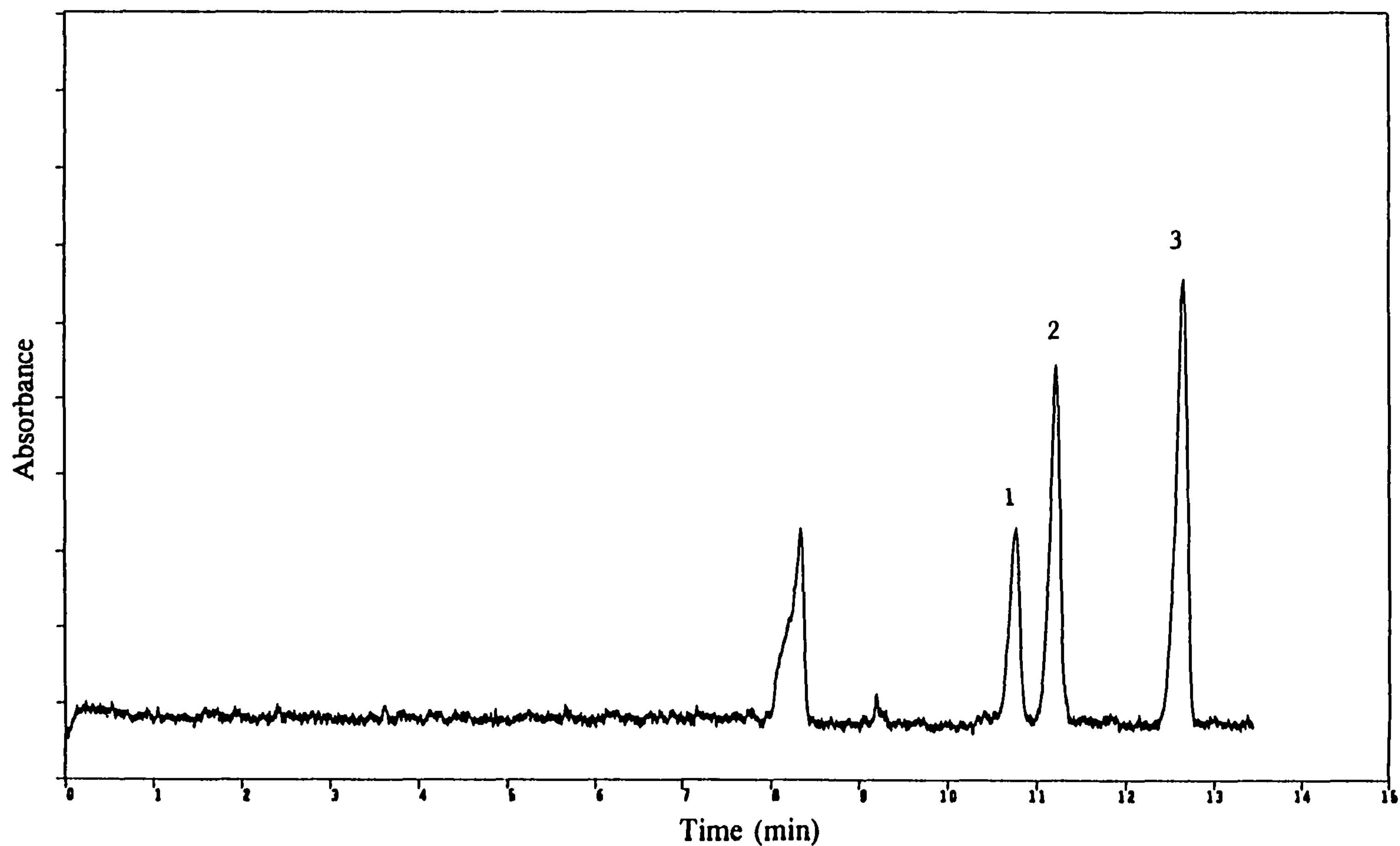

Figure 9. Separation of three enkephalins in 4% BuOH. Electrolyte, 15 mM Tris-HCl, 0.06 mM CTAB, 2 mM KCl, 4% BuOH, pH 2.80; capillary, 50cm*50μm; injection time, 40 seconds; applied voltage, -15 kV; detection, 214 nm. Peaks: 1= tyr-gly-gly-phe; 2= tyr-gly-gly-phe-met; 3= tyr-gly-gly.

no micelle is formed in the electrolyte. Neutral compounds are moving at the same speed as the EOF while anionic compounds are moving faster than the EOF and cationic analytes slower than the EOF provided a reversed power supply is used. It is possible to use this mode for individual and simultaneous class separations of cationic and anionic compounds.

Shamsi and Danielson [36] demonstrated that cationic and anionic surfactants can be separated simultaneously using regular CE conditions. Optimization results indicated a pH of 6-7 with at least 50% (v/v) methanol is necessary for separations of long-chain cationic (tetrahexylammonium, didodecyl dimethylammonium) or anionic (C_{14}-$C_{18}SO_3^-$ or C_{14}-$C_{18}SO_4^-$) compounds. The addition of methanol in the running buffer improved peak resolution but at the sacrifice of short separation times. Peak capacity is found higher for the anionic surfactants because these compounds countermigrated with the EOF.

In this study, cetyltrimethylammonium chloride (CTAC) was employed as an electroosmotic flow modifier since it has a similar structure as CTAB except the counterion, and it has not been well used in the literature. The difference in the counterions gives rise to a small increase in cmc for CTAC (1.3 mM) [34].

After several preliminary experiments, a low concentration of 1 mM of CTAC was found to be good enough to reverse EOF while maintaining high separation efficiency. Figure 10 clearly shows how the increment in CTAC concentration changed the separation mode. At 0.6 mM CTAC, there was no micelle in the solution, thus analyte (acetophenone) had no interaction with CTAC and it migrated at the same speed as solvent (water). There is only one peak in Figure 10a. At 1.2 mM CTAC, which is a bit above its cmc, micelle started to form in the solution. Acetophenone had a small association with CTAC micelles.

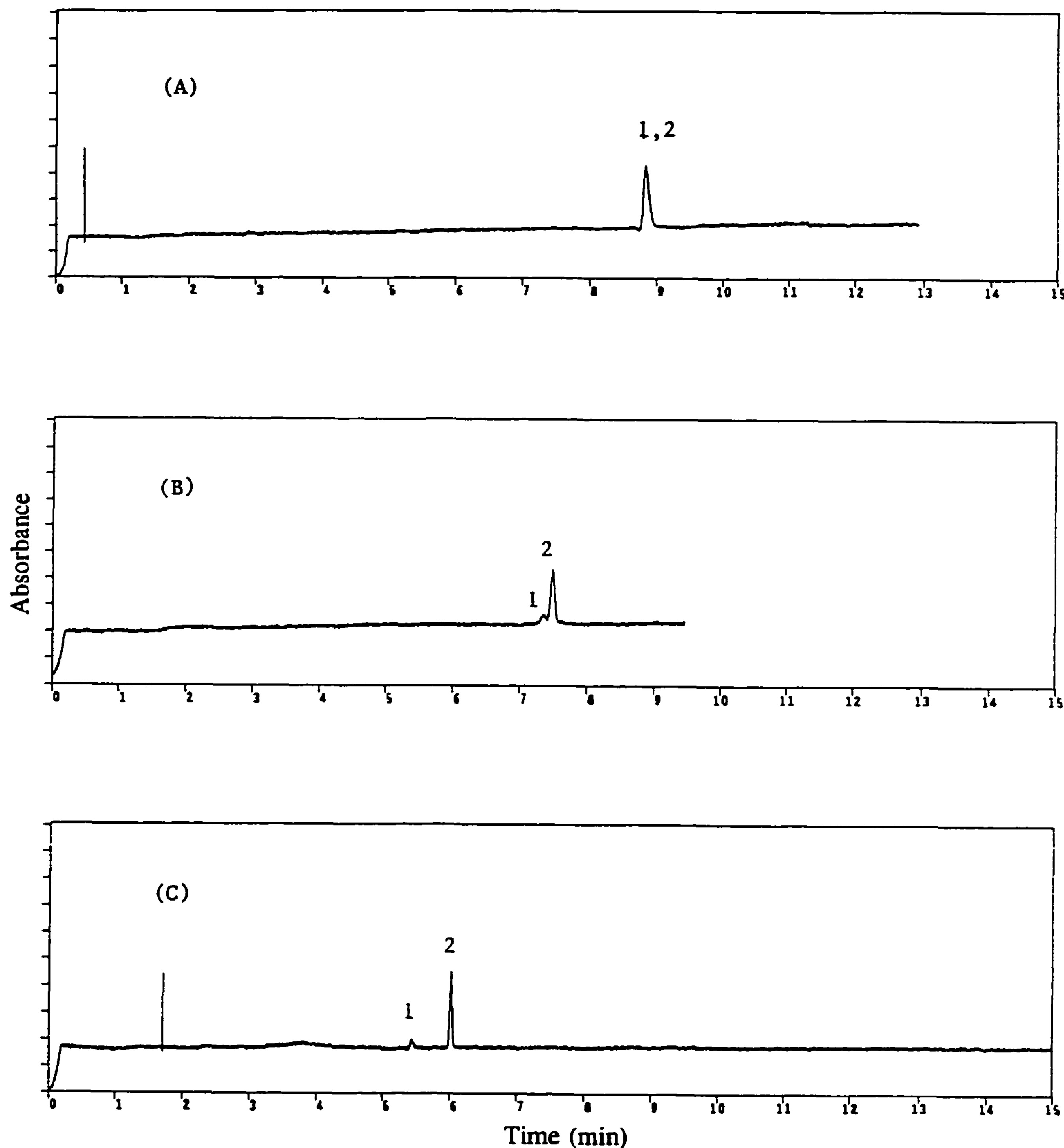

Figure 10. Effect of different concentration of CTAC on separation: (A) 0.6 mM; (B) 1.2 mM; (C) 4.5 mM. Conditions: Electrolyte, 100 mM phosphoric acid, 10% ACN, pH 2.6; capillary, 50 cm length, 50 μm i.d.; injection time, 10 s; applied voltage, -20 kV; detection, 214 nm. Peaks: 1, water; 2, acetophenone.

Figure 10b shows acetophenone moved a little behind the solvent peak. When CTAC concentration was well above cmc, as in the case of Figure 10c, acetophenone was dragged by micelles moving in the opposite direction, resulting in two well separated peaks (one for solvent and the other for acetophenone).

Figure 11 shows an excellent separation of fifteen pharmaceutical compounds including both acidic and basic classes. Phenol was added as an neutral marker which separates acidic compounds from basic compounds. Acidic compounds co-migrated with the EOF, thus came out before neutral marker. On the contrary, basic compounds counter-migrated with the EOF, therefore a better peak capacity was achieved for basic compounds. The whole separation was fast and took only 6 minutes. Changing pH from 4.25 to 3.80 (Figure 12) gave rise to an even better separation. Imipramine and lidocaine was baseline resolved this time and the separation took about 7 minutes.

The buffer constitution is worth mentioning. One of the ultimate goal of this study is to analyze drugs in biological fluids such as serum. Since the majority of the proteins in serum has a low pI, they exist as negative ions in most pH. In order to eliminate these proteins interact with cationic surfactant on the capillary wall, which will cause peak broadening, an acidic buffer was utilized. A high phosphate concentration was found to help prevent protein precipitation in acidic condition and in organic solvent. Addition of 8 mM ethanesulfonic acid in the buffer was also found to largely decrease peak width for acidic compounds. Ethanesulfonic acid will have an strong ion-pairing effect with CTAC so that it shields the ion-exchange sites on the capillary surface upon which acidic compounds can interact.

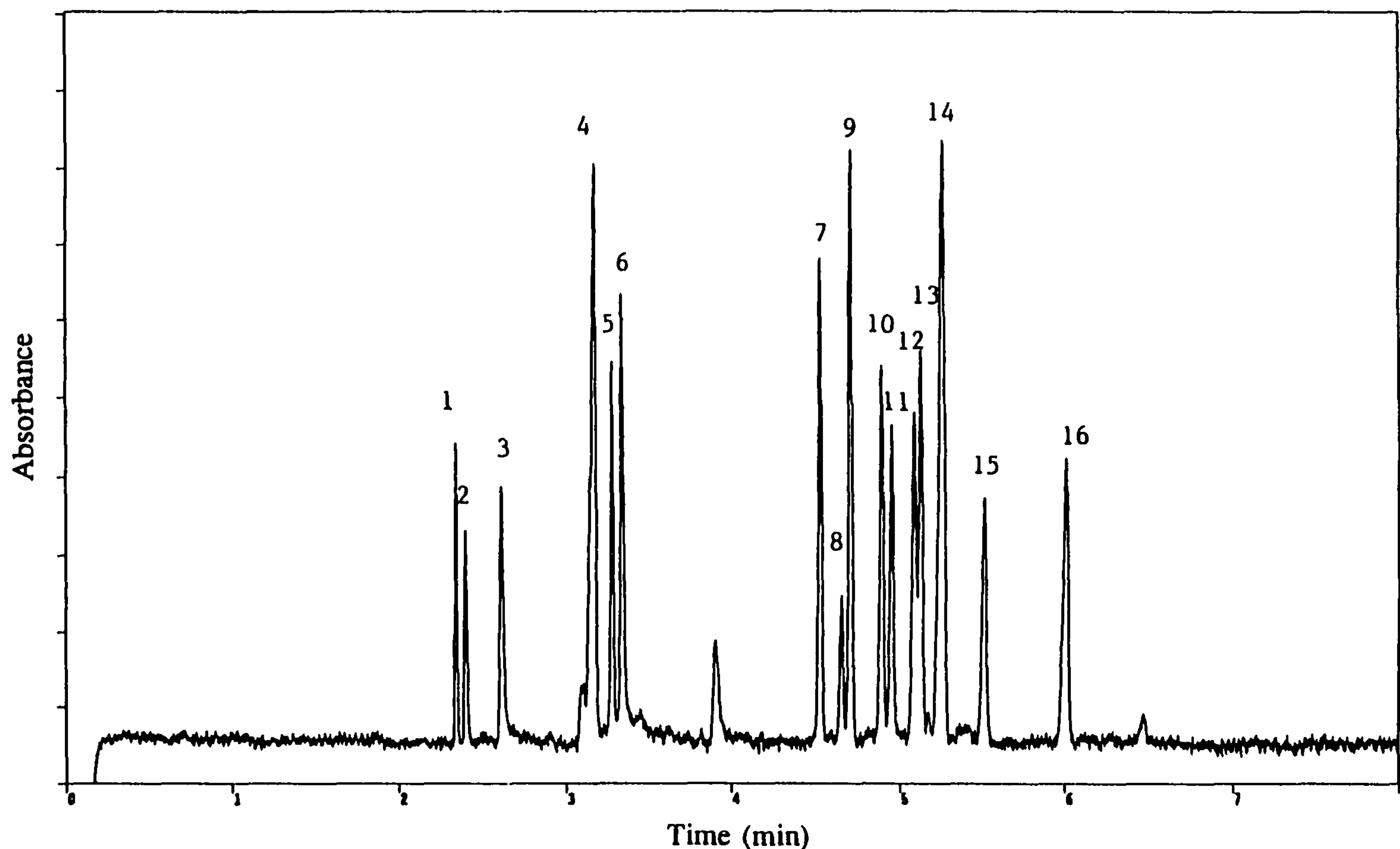

Figure 11. Electropherogram of fifteen acidic and basic pharmaceutical compounds in a single run. Conditions: Electrolyte, 100 mM phosphoric acid, 1 mM CTAC, 8 mM ethanesulfonic acid, 5% ACN, 5% 2-propanol, pH 4.35; capillary, 45 cm length, 50 μm i.d.; injection time, 4 s; applied voltage, -20 kV; detection, 214 nm. Peaks: 1= hippuric acid; 2= acetylsalicylic acid; 3= o-hydroxyhippuric acid; 4= phenol; 5= carbamazepine; 6= 5-(p-hydroxyphenyl)-5-phenylhydantoin; 7= laudanosine; 8= metoprolol; 9= trimethoprime; 10= propranolol; 11= pindolol; 12= imipramine; 13= lidocaine; 14= nefopam; 15= ketamine; 16= (1R,2S)-(-)-phenylpropanolamine.

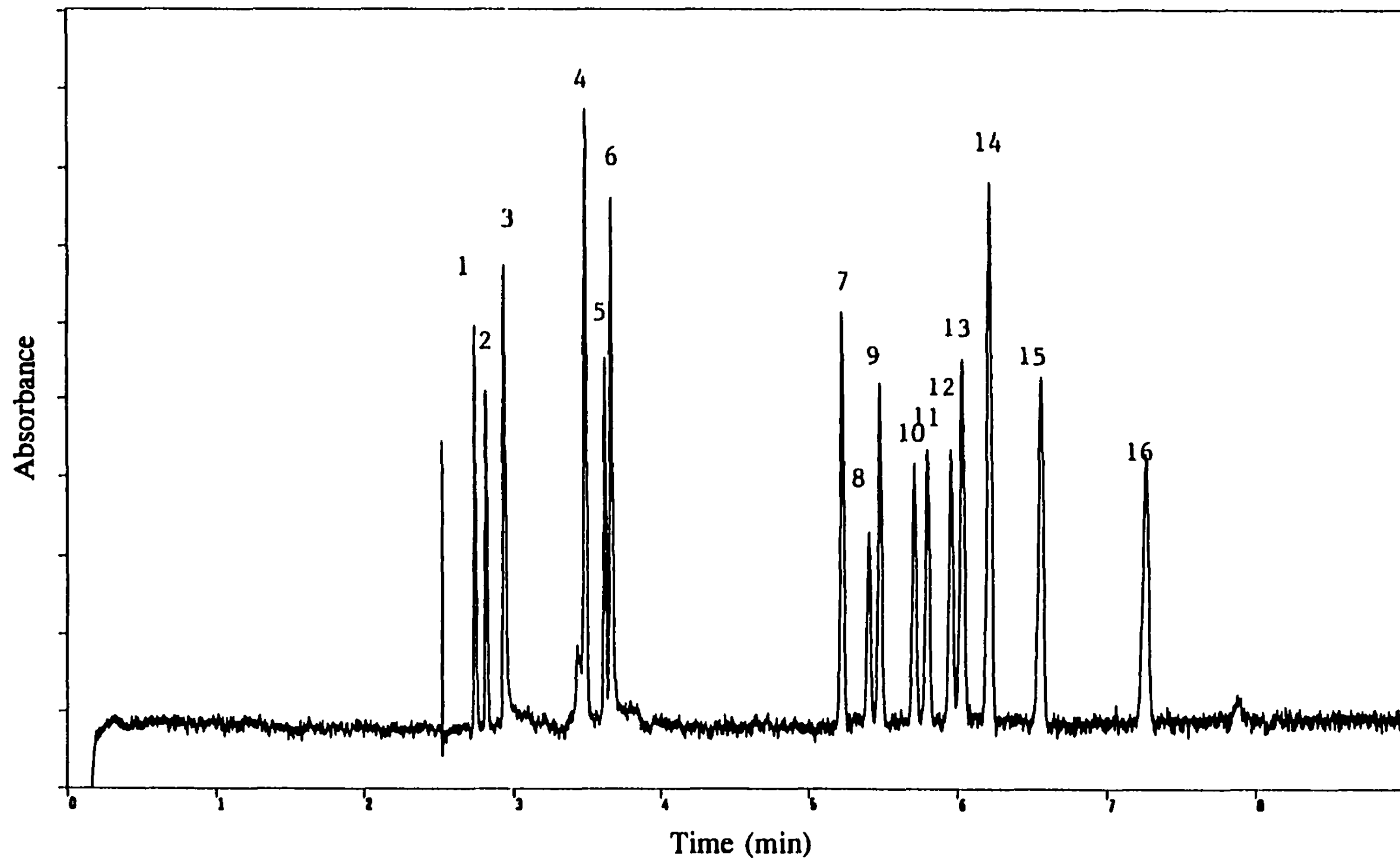

Figure 12. Electropherogram of fifteen drugs at pH 3.80. Conditions and peaks identities: see Figure 11.

Direct analysis of drugs in serum was attempted using CTAC and low pH buffer. In order to eliminate interference from serum, it is necessary to separate serum from drugs as far as possible. This can be achieved by increasing CTAC concentration to drag serum far behind of drug peaks. Figure 13 shows that four drugs, including acetylsalicylic acid, sulfamethoxazole, carbamazepine and phenylhydantoin, were easily analyzed in serum sample using 4 mM CTAC.

3.7 Reproducibility

While reproducibility is always an important consideration, it is of particular interest in the present system. In the dynamic coating system used, will there be a gradual buildup of CTAB on the surface from run to run that would affect migration times? Or would a surface layer of proteins form on the surface that would have a major effect on later sample runs?

These questions were answered by running a sample containing ribonuclease and lysozyme on the same capillary 10 times in succession, rinsing the capillary between each run for 3 min with 0.1 M NaOH and 3 min with buffer. The average migration time for 10 runs was 6.165 min for ribonuclease with a R.S.D. of ±1.0%. The average migration time for lysozyme was 6.930 min with a R.S.D. of ±1.1%. There was no particular trend in the results.

Based on peak height the R.S.D. (10 runs) was ±4.6% for ribonuclease and ±2.7% for lysozyme. This was significantly better than R.S.D. based on peak area, which was ±6.5% and ±8.7% for the two proteins.

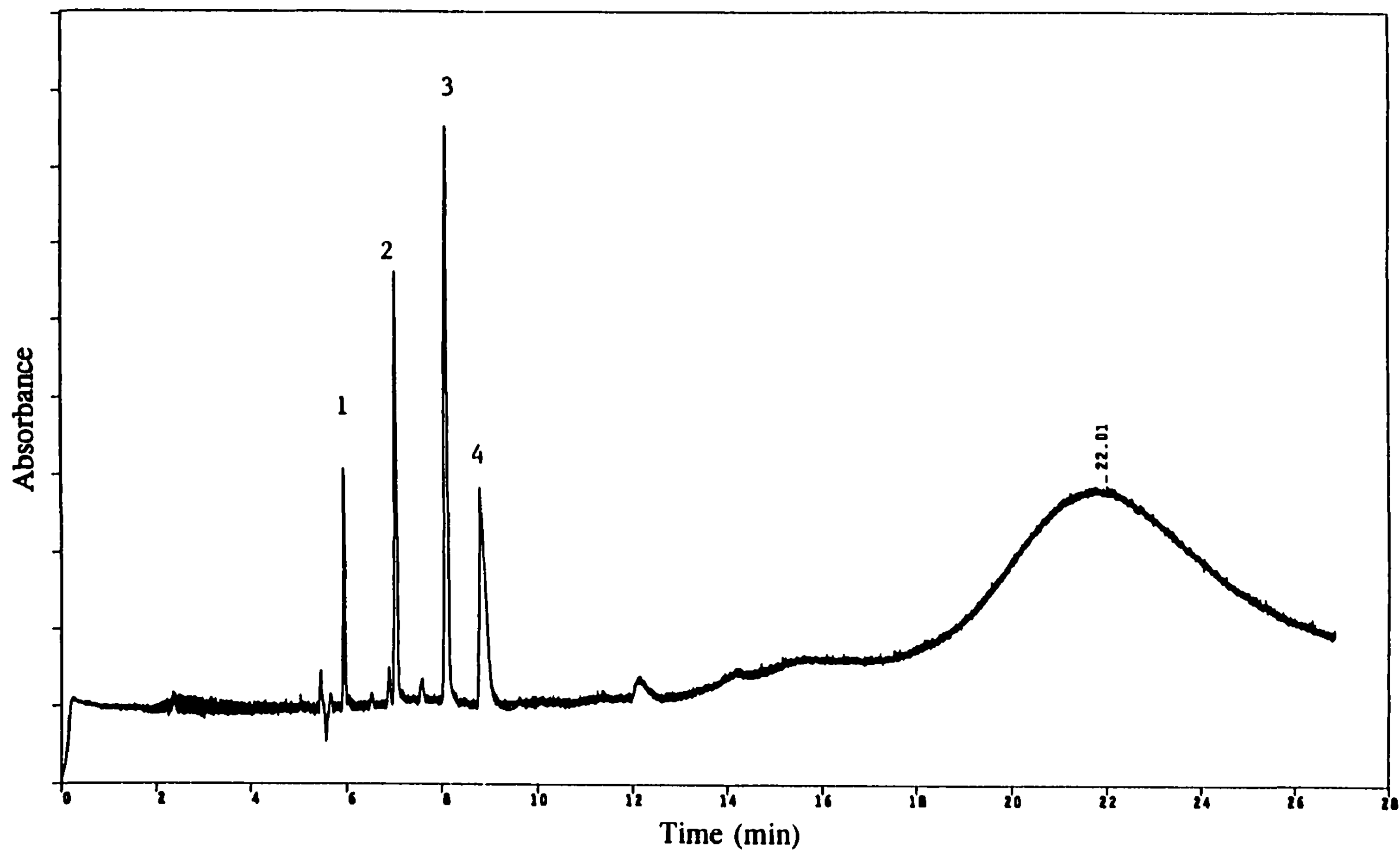

Figure 13. Direct analysis drugs in serum. Conditions: the same condition as in Figure 11 except 4 mM CTAC was used and pH was 2.8. Peaks: 1= acetylsalicylic acid; 2= sulfamethoxazole; 3= carbamazepine; 4= phenylhydantoin.

A more severe test of reproducibility was obtained by separating the protein samples 10 times in succession on the same capillary with no treatment whatsoever between runs. In this case there was a gradual increase in migration times of both proteins. After 5 runs the increase in migration time was +2.8% for ribonuclease and +2.7% for lysozyme. After 10 runs the increase was 7.6% and 7.2%, respectively.

These results demonstrate that the present CE system is rugged and dependable.

4. Conclusions

It is now well known that some kind of coating or other surface treatment is needed to prevent interaction of large biomolecules with silica capillary walls in CE. Many coating procedures also substantially eliminate electroosmotic flow, which is generally considered to be an advantage. But this is not always true. Some of the very best separations in CE are obtained when the electrophoretic and electroosmotic migration vectors are in opposing directions. The separations obtained in this research were obtained with such a counter-migration mode. The positive charge of the dynamic surface coating (CTAB) reverses the normal direction of electroosmotic flow and also repels the sample cations from the capillary surface.

The dynamic surface coating procedure described in this work is the simplest and one of the most effective for the CE separation of large molecules. Excellent reproducibility was obtained with only minimal rinsing between runs. Migration times of protein peaks increased only moderately (and in tandem with one another) when no treatment at all was used between runs.

pH was shown to be a very important experimental variable. It is somewhat surprising that some authors have paid only cursory attention to the effect of pH. If a satisfactory separation of protein or peptide cations is not obtained under the conditions first used, a quick but systematic investigation of the effect of pH should be undertaken.

Acknowledgements:

We wish to thank Waters Co. for their gift of the Waters Quanta 4000E CE system and associated supplies. This work was performed in the Ames Laboratory which is operated for the U.S. Department of Energy under Contract No. W-7405-Eng. 82. This work was supported by the Director of Energy Research, Office of basic Energy Sciences.

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CHAPTER 5. STUDY OF CHIRAL SELECTOR STRUCTURE AND MIXED SELECTORS SYSTEM ON CHIRAL SEPARATION IN CAPILLARY ELECTROPHORESIS

A paper to be submitted to the *Journal of Chromatography A*

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Abstract

Chiral resolution in capillary electrophoresis is studied with several novel synthetic surfactants, which are synthesized from amino acids (L-leucine, L-valine, L-isoleucine, and L-serine) and alkyl chloroformate with a chain length of C4 to C12. Seven chiral drugs, including atenolol, benzoin, laudanosine, propranolol, ketamine, hydrobenzoin, and nefopam are used as test compounds. It was found that resolution can be readily manipulated by varying the chain length, amino acids, and surfactant concentration. Sulfonated ß-cyclodextrin (ß-CD) was also studied and compared with these novel surfactants. A different selectivity was found for sulfonated ß-CD due to its distinct structure, however, neither of these surfactants nor this ß-CD gave a satisfactory chiral resolution for all the seven drugs

that we tested. The use of novel mixtures of sulfonated ß-CD and these surfactants was further examined and found suitable for the analysis of all the seven drugs. Chiral selectivity is largely enhanced by using this combination. This duo-chiral-selector system was also applied in a separation of eight DL-dansyl amino acids. A mechanism of chiral recognition by this system is discussed.

1. Introduction

The importance of enantiomeric differences in biological interactions and reactions has been well acknowledged [1]. The analytical techniques for the chiral resolution of optically active molecules have been developed rapidly in the past decade, which includes high performance liquid chromatography (HPLC), gas chromatography (GC), thin-layer chromatography (TLC), and capillary electrophoresis (CE). However, chiral separations by HPLC often suffer from poor efficiency, and GC is limited to volatile compounds.

The application of CE for chiral separation has undergone an enormous development over recent years and offers many advantages over conventional analytical methods [2-5], such as fast speed, high efficiency, low cost, and small sample volume requirement. Chiral resolution in CE is based on the differential complexation between enantiomers and a chiral selector added in the buffer. The addition of chiral selectors results in the formation of transient noncovalently bound diastereoisomers, which can be easily separated based on their different physical properties.

Cyclodextrins (CDs) including native and derivatized CDs are among the most widely used chiral additives in CE. Nielen [6] studied chiral separations of basic drugs using

cyclodextrins. 9 out of 10 drugs investigated showed resolutions higher than 1.4, which is far more superior than liquid chromatographic methods. He also found that the resolution is strongly dependent on the applied field strength.

Quang and Khaledi [7] improved chiral separation of basic compounds using ß-CD and tetraalkylammonium salts. Short-chain tetralkylammonium cations were found more effective in the regard of controlling the electroosmotic flow and improving resolution of the cationic enantiomers. This is because the short-chain reagents provide better capillary wall coverage, and the short-chain tetraalkylammonium cations are less likely to occupy the hydrophobic cavity of ß-CD than the long-chain cationic surfactants, leaving the enantioselective interaction sites more available for the analytes.

Penn et al. [8] utilized a systematic approach to optimize enantiomeric separations using cyclodextrins selectors. They found that maximum electrophoretic mobility difference between the enantiomers occurs when the concentration of free selector is equal to the reciprocal of the average binding constant.

In the aspect of separation time frame, resolving power and solubilities, derivatized CDs has been demonstrated to be better than the parent CDs. The modifications influence the overall hydrophobic character of the CDs, resulting in changes in the shape and size of their cavities and their hydrogen-bonding ability.

Heptakis (2,6-di-O-methyl)-ß-CD (DM-ß-CD) is a typical example of alkylated cyclodextrins. Yoshinaga and Tanaka [9] applied DM-ß-CD in chiral separation of dansylamino acids. Valko and co-workers [10] investigated effect of the degree of substitution of (2-hydroxy)propyl-ß-CD on the enantioseparation of organic acids.

Charged CDs, in which hydroxyl groups have been either carboxyalkylated [11], sulfonated [12], sulfoalkylated [13], or aminoalkylated [14], present a distinct advantage over their neutral relatives due to a significant electrophoretic mobility which may be in opposition to electroosmotic flow. Consequently, a greater separation window may be available, resulting from a better interaction between analytes and CDs.

Mixtures of neutral, heptakis(2,6-di-O-methyl)-ß-CD, and anionic cyclodextrin, sulfobutyl ether ß-CD, were used for cationic drugs by Lurie et al. [15] The resolution and migration speed can be readily adjusted by varying the ration of the two added CDs provided the anionic CD acted as a countermigrating complexing reagent. Similarly, a dual system of CDs consisting of a cationic mono(6-amino-6-deoxy)-ß-CD and a neutral CD (trimethyl-ß-CD or dimethyl-ß-CD) was studied by Lelievre and co-workers [16] for separation of arylpropionic acid enantiomers. CD-modified micellar electrokinetic chromatography (CD-MEKC), implementing CDs and a achiral surfactant such as sodium dodecyl sulfate (SDS), were explored by other authors [17,18].

Other chiral selectors such as vancomycin [19], ristocetin A [20], crown ethers [21], bovine serum albumin [22], maltooligosaccharides [23] have been investigated by capillary electrophoresis.

The use of chiral surfactants in micellar electrokinetic chromatography (MEKC) is another important separation mode for optically active compounds. The chiral surfactants include naturally occurring compounds, for instance, bile salts [24], and synthetic surfactants derived from simple chiral molecules such as amino acids [25], or glucose [26].

N-dodecanoyl-L-valinate [25] combined with SDS is one of the earliest example of

using synthetic surfactant to resolve enantiomers. Other amino acid ester derivatives, e.g., L-threonine [27] and L-alanine [28] have also been used. Mazzeo et al. [29] introduced (R)- and (S)-N-dodecoxycarbonyl-valines to resolve a range of twelve pharmaceutical drugs including beta-blockers, bupivacaine, and homatrophine. Dodecyl-ß-D-glucopyranosyl derivatives is another novel class of chiral surfactant introduced by Tickle and co-workers [26].

One of the advantages of using synthetic surfactants is that the structure can be readily varied. In our study, a series of chiral surfactants were derivatized from a range of amino acids including L-valine, L-leucine, L-isoleucine, and L-serine, with butyl-, hexyl-, octyl-, decyl- or dodecyl chloroformate, and were applied to seven model compounds. Since chiral discrimination using surfactant is still unclear, this study may provide some indication on how chiral analytes interact with the surfactant.

The results obtained from these surfactants were further compared with those acquired from using ß-cyclodextrin sulfate as a chiral additive. Moreover, a novel system combining the two was further investigated.

2. Experimental

2.1 Apparatus

CE experiments were performed with a Waters Quanta 4000E capillary electrophoresis system (Waters Corporation, Milford, MA, USA). The uncoated fused-silica capillaries (Polymicro Technology, Phoenix, AZ, USA) were 50 μm i.d. and 60 cm in length with the detection window place 52.5 cm from the injector end. Hydrostatic sampling mode was used

with a sampling time of 6 s and height of 10 cm. The positive power supply with the detection window on the cathode end was used at 18 kV for each experiment unless indicated. Direct UV detection was employed at either 254 nm or 214 nm. Separations were performed at 25°C. Electropherograms were collected and plotted by the data acquisition system Chromperfect Direct (Justice Innovations, Mountain View, CA, USA).

The capillary was initially rinsed with 0.1 M NaOH for one hour followed by a one hour rinse with deionized (DI) water, which was subsequently replaced with the running electrolyte. Between each run, the capillary was rinsed with 0.1 M NaOH for 2 min, DI water for 3 min, and the electrolyte for 3 min.

2.2 Reagents

2.2.1 Chemicals

All standards and electrolytes were prepared with analytical-reagent grade chemicals and 18 MΩ deionized waters obtained from a Barnstead Nanopure II system (Sybron Barnstead, Boston, MA, USA). The running electrolyte consisted of 0.025 M borate and 0.025 M phosphate buffer adjusted to pH 8.8 with 0.1 M sodium hydroxide and filtered through a 0.45 μm membrane filter (Costar, Cambridge, MA, USA).

Sodium borate, sodium phosphate, sodium hydroxide, calcium chloride, acetonitrile, pyridine, methylene chloride, ether were obtained from Fisher Scientific (Fairlawn, NJ, USA). Butyl chloroformate, hexyl chloroformate, octyl chloroformate, decyl alcohol, 1-dodecanol, 1-tetradecanol, triphosgene, sulfated ß-cyclodextrin were purchased form Aldrich (Milwaukee, WI, USA). L-valine, L-leucine, L-isoleucine, L-methionine, L-alanine, L-

serine, L-aspartic acid, and L-asparagine were obtained from sigma (St. Louis, MO, USA).

The stock solutions of atenolol, benzoin, laudanosine, propranolol, ketamine, hydrobenzoin, nefopam (Sigma, St. Louis, MO, USA) were prepared at a concentration of 10,000 mg/L (ppm) in methanol, and diluted to 100 ppm in running electrolyte before analysis.

2.2.2 Synthesis of decyl chloroformate [30]

A solution of 3.16 g of decyl alcohol (0.02 mole), 1.98 g of triphosgene (0.0067 mole) and 20 mL methylene chloride was stirred and cooled to 10-15°C. 1.6 g of pyridine (0.02 mole) was added dropwise over one hour period. The reaction mixture was stirred for an additional one hour, then heated in a water bath at 65°C for 15 min till all methylene chloride evaporated. The residue was washed three times with cold water, and dried over calcium chloride (4-20 mesh), giving 3.66 g (83% yield) of decyl chloroformate ($CH_3(CH_2)_9OOCCl$). Other alkyl chloroformate can be synthesized in a similar method.

2.2.3 Synthesis of (S)-(+)-N-decoxycarbonyl-leucine [31]

A solution of 1.31 g (0.01 mole) of L-leucine in 5 mL of 2 N sodium hydroxide is placed in a 50-mL three-necked flask fitted with two dropping funnels. The solution is stirred and cooled in an ice bath, and 2.2 g (0.01 mole) of decyl chloroformate and 5 mL of 2 N sodium hydroxide are added alternatively to the vigorously stirred solution over 1 h, such that the pH of the mixture is kept at 9-10. The mixture is stirred for an additional 1 h at room temperature. The aqueous solution is acidified with concentrated hydrochloric

acid to pH 1-2. 4 mL ether is added to help extract product from the aqueous layer. The upper ether layer is separated, washed with 0.1 M hydrochloric acid, and dried overnight, giving 2.4 g (76% yield) of (S)-(+)-N-decoxycarbonyl-leucine. Other surfactants can be synthesized in a similar manner.

3. Results and Discussion

3.1 Effect of surfactant concentration

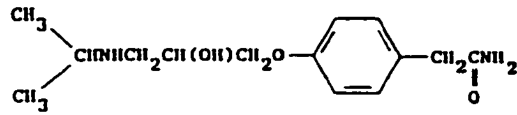
In cyclodextrin-based chiral separation, the mechanism is quite clear. The chiral nature of the glucose units inside the cyclodextrin cavity permits the possibility of stereoselective interaction with analytes. Enantiomers which have appropriate structural geometry will fit favorably into cyclodextrin cavity, resulting in different migration times. Usually, a higher enantiomeric resolution can be obtained by increasing the concentration of cyclodextrin, due to the formation of stronger inclusion complexes. However, a lower concentration of cyclodextrin may be needed to obtain a faster and better separation, as Wren [32] indicated that the optimum cyclodextrin concentration is related to the affinity of the analyte fitting into the cyclodextrin cavity.

The chiral surfactants all have a long hydrocarbon tail, and a chiral functional group which is very close to the charged head. When they form micelles, the chiral functional headgroups stick out on the micelle surface, making direct contact with aqueous solution and analytes. The mechanism of chiral discrimination using surfactants is still under investigation. However, it is widely accepted that there may be two steps during the process. First step involves stereoselective interactions between optically active sites on the

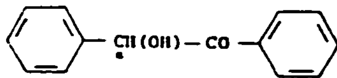
micelle surface and the chiral groups of analytes. This is followed by inclusion of the preferred enantiomers into the interior hydrophobic core of the micelle. On the contrary to cyclodextrin where chiral interfaces are inside the cavity, chiral surfactants interact with analytes from outside. It is reasonable to assume that some theories may be applied well in cyclodextrin-case but found inapplicable for chiral surfactants. Nevertheless, these two distinct mechanism may be complemental to each other. For instance, analytes which are too large or too small to fit into the cyclodextrin cavity may be very well suited for chiral surfactants.

The structures of seven text compounds are shown in Figure 1. These compounds were chosen because they represent some typical chiral compound structures. Their chiral carbons either on a hydrocarbon chain, such as atenolol, propranolol, or within a cycloalkyl ring, for instance, ketamine, nefopam. This structural variation may result in different stereoselectivity.

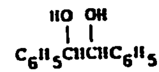
The surfactant concentration plays a very important role in chiral discrimination. When the surfactant concentration below its critical micelle concentration (cmc), there is little or no separation for enantiomers. This may be due to a less interaction between the surfactant monomer and analytes. More detailed study was done using (S)-N-octoxycarbonylvaline as a chiral selector. Since the hydrocarbon chain in (S)-N-octoxycarbonylvaline comprises 12 atoms, which is similar to sodium dodecyl sulfate (SDS), the cmc for this chiral surfactant is estimated to be close to 10 mM provided the cmc for SDS is 8.3 mM [33]. Capillary electrophoresis was done at three different concentrations of this surfactant, e.g, 25-, 50-, and 100 mM, which are well above its cmc. The results are summarized in Table 1. (S)-



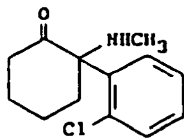
Atenolol



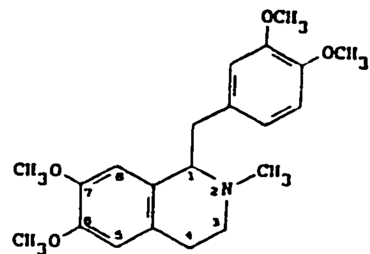
Benzoin



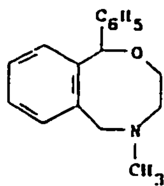
Hydrobenzoin



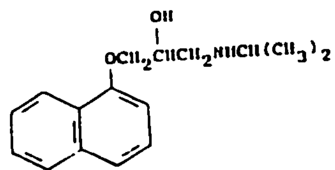
Ketamine



Laudanosine



Nefopam



Propranolol

Figure 1. Structures of seven drugs.

Table 1. Effect of surfactant concentration.

Compounds	Resolution (R_s)		
	25 mM C_8-Valine	50 mM C_8-Valine	100 mM C_8-Valine
Atenolol	0.80	1.77	2.93
Benzoin	0.52	1.65	1.95
Laudanosine	1.06	1.13	1.03
Propranolol	0.97	1.51	1.15
Ketamine	0.0	0.0	0.0
Hydrobenzoin	0.0	0.0	0.0
Nefopam	0.0	0.0	0.0

oriented surfactant incline to interact with (R)- optically active enantiomers, therefore, (S)-isomer will migrate faster that (R)- isomer under this condition. Resolution (R_s) of two peaks (R- and S- enantiomers) is calculated according to the following equation:

$$R_s = \frac{\Delta t}{\frac{w_1 + w_2}{2}} \qquad (2)$$

where Δt is the difference in migration times, w_1 and w_2 are the peak width for peak 1 and peak 2, respectively. Atenolol, benzoin, laudanosine, and propranolol all showed increment in chiral resolution when (S)-N-octoxycarbonylvaline concentration increased from 25 mM to 50 mM (see Figure 2). Atenolol and benzoin displayed greater enhancement in resolution with an almost 100% and 200% increment, respectively. Laudanosine increased by only 7%. Nonetheless, increasing surfactant concentration from 50 mM to 100 mM, we did not see a large improvement in resolution except for atenolol. Instead, laudanosine and propranolol showed decreases in resolution. These two compounds are among the most hydrophobic molecules in this group. A higher surfactant concentration inevitably gives rise to a higher micelle concentration. Highly hydrophobic compounds do not need chiral recognition to participate into the hydrophobic interior of the micelle, resulting in a decrease in chiral resolution.

Ketamine, hydrobenzoin and nefopam showed no chiral recognition with (S)-N-octoxycarbonylvaline. This can be explained by hydrophobicity of these three compounds as we discussed earlier, moreover, their rigid structures may play a factor here. The chiral carbons are shielded by benzene rings, and it may be difficult for the chiral functional groups of the surfactants accessing these sites, resulting in no chiral discrimination.

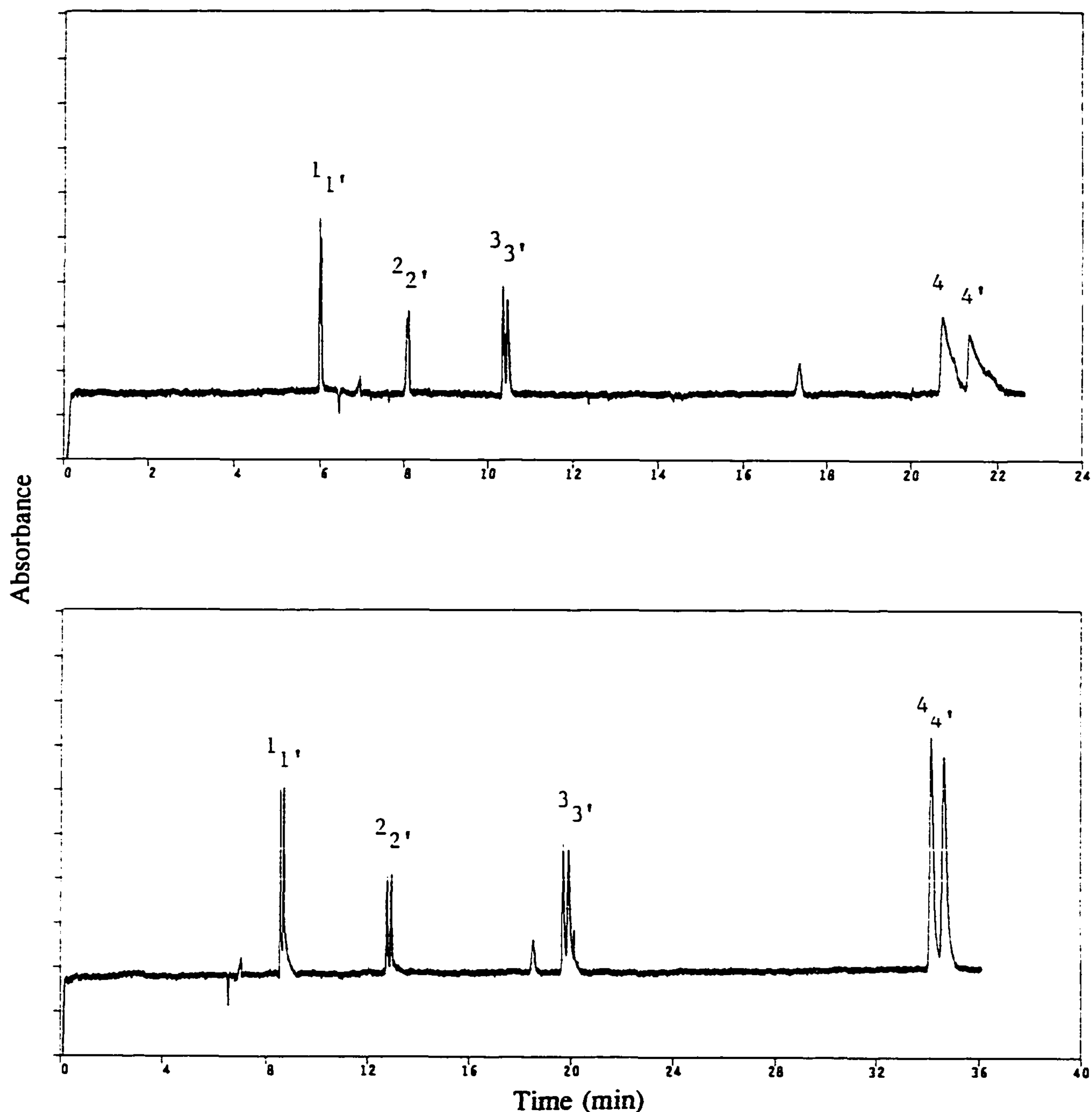


Figure 2. Enantiomeric separation of four drugs at different surfactant concentration: (A) 25 mM; (B) 50 mM (S)-(+)-N-octoxycarbonylvaline. Electrolyte: 25 mM borate, 25 mM phosphate, pH 8.8; capillary, 60 cm length and 50 μm i.d.; applied voltage, 18 kV; injection time, 6 s. Peaks: 1, (S)-atenolol; 1', (R)-atenolol; 2, (S)-benzoin; 2', (R)-benzoin; 3, (S)-laudanosine; 3', (R)-laudanosine; 4, (S)-propranolol; 4', (R)-propranolol.

3.2 Effect of amino acids on the surfactant structure

A variety of amino acids were reacted with octyl chloroformate to make chiral surfactants, including L-valine, L-leucine, L-isoleucine, L-alanine, L-aspartic acid, L-asparagine, L-methionine, and L-serine. Only (S)-N-octoxycarbonylvaline, (S)-N-octoxycarbonylleucine, and (S)-N-octoxycarbonylisoleucine are readily soluble in aqueous solution. The other products either have difficulties in dissolving in water, or producing bad baseline in CE. Therefore, these three surfactants were selected in further study.

Figure 3, 4 and 5 show separations of atenolol, benzoin, landanosine, and propranolol using different chiral surfactants. The calculated resolutions (R_s) are shown in Table 2. It is obvious that the variation in surfactant structures bring a change in selectivity, even though there is only one carbon difference. For example, resolution for atenolol is 1.77 using 50 mM (S)-N-octoxycarbonylvaline, while 2.17 using 50 mM (S)-N-octoxycarbonylleucine. There is no chiral discrimination for ketamine and nefopam when either 50 mM (S)-N-octoxycarbonylisoleucine or 50 mM (S)-N-octoxycarbonylvaline was added in the electrolyte, but these two compounds can be enantiomerically separated by 50 mM (S)-N-octoxycarbonylleucine. The extra methyl group in leucine may change the chiral functional group geometric structure on the micelle surface, resulting in a more appropriate position on which ketamine and nefopam can interact.

3.3 Effect of surfactant chain length

Chain length of the surfactants was varied by reacting L-leucine with alkyl chloroformates ranging from butyl chloroformate to tetradecyl chloroformate. Same

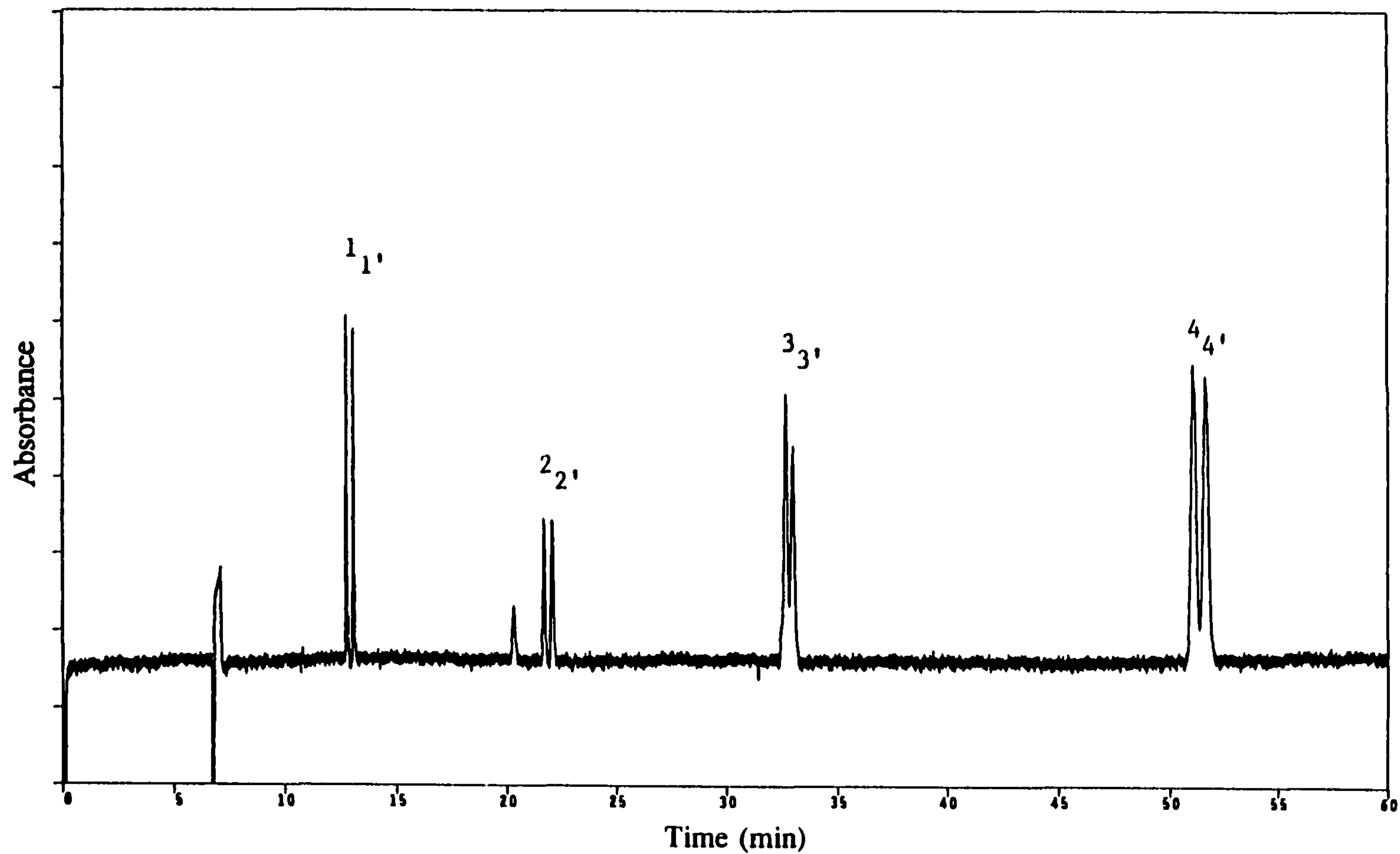


Figure 3. Enantiomeric separation of drugs using 100 mM (S)-(+)-N-octoxycarbonylvaline. Conditions and peak identities: see Figure 2.

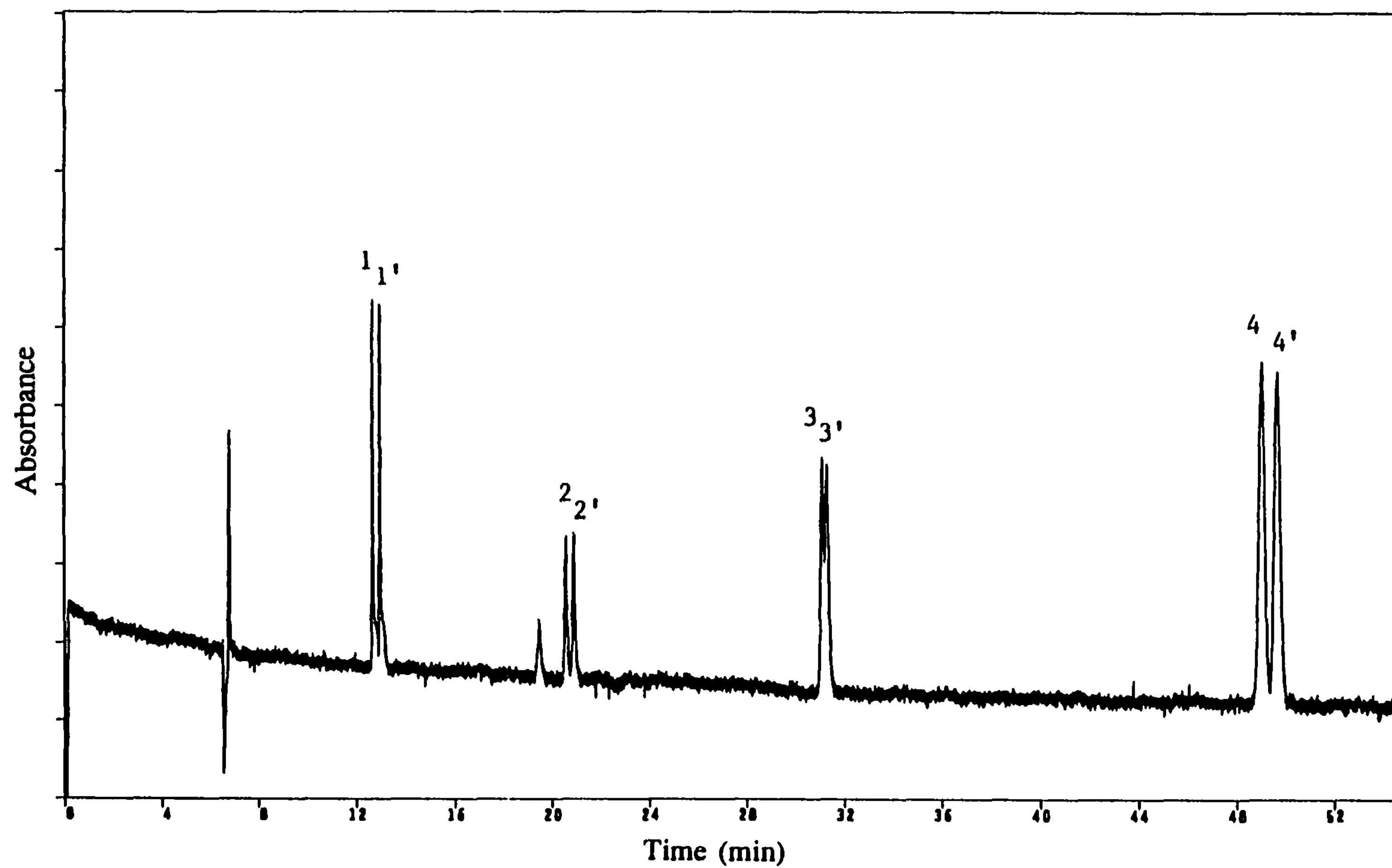


Figure 4. Enantiomeric separation of drugs using 100 mM (S)-(+)-N-octoxycarbonylleucine. Conditions and peak identities: see Figure 2.

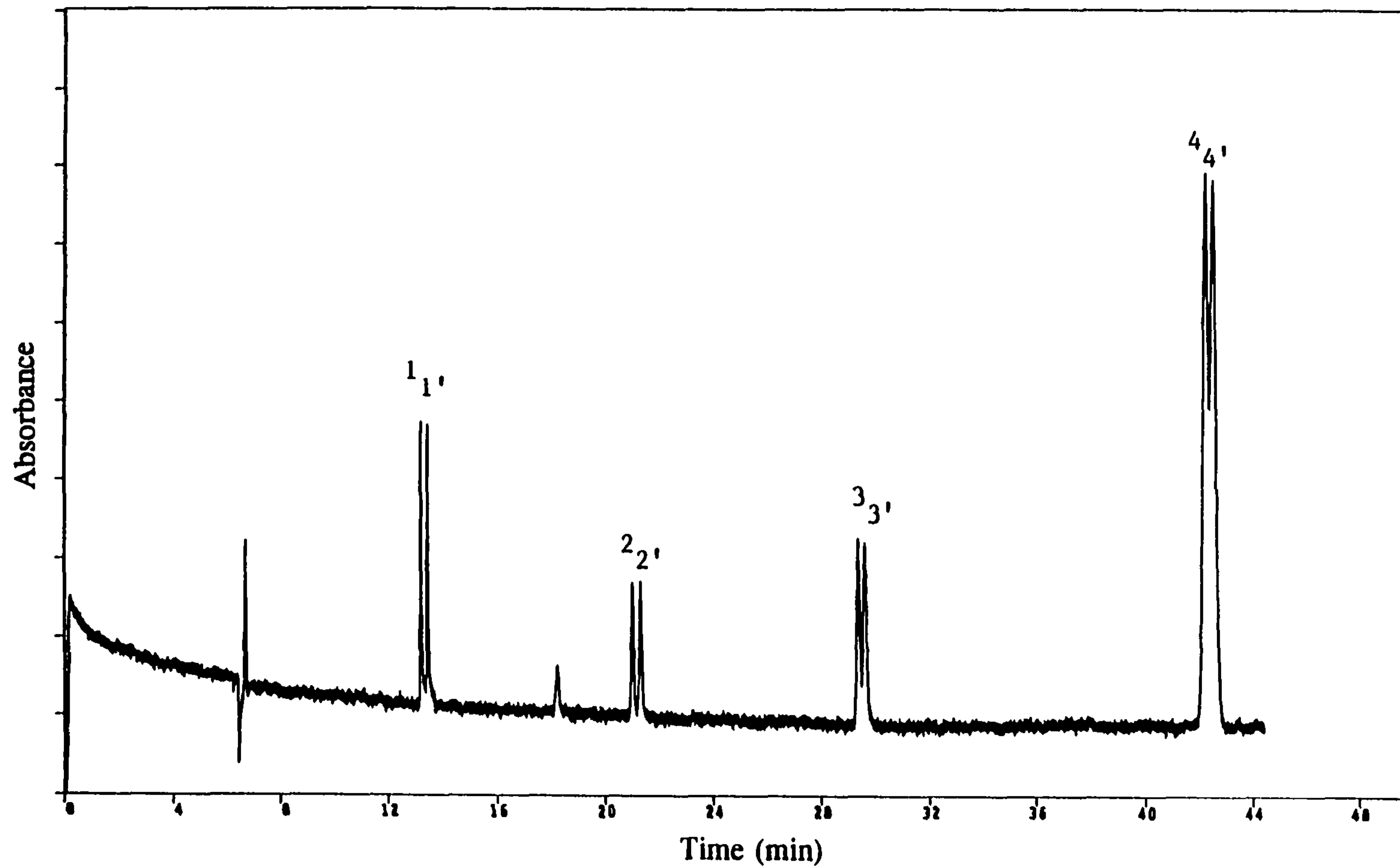


Figure 5. Enantiomeric separation of drugs using 100 mM (S)-(+)-N-octoxycarbonylisoleucine. Conditions and peak identities: see Figure 2.

Table 2. Effect of amino acid structure.

Compounds	Resolution (R_s)		
	50 mM C_8-Valine	50 mM C_8-Leucine	50 mM C_8-Isoleucine
Atenolol	1.77	2.17	1.93
Benzoin	1.65	1.56	1.59
Laudanosine	1.13	0.85	1.79
Propranolol	1.51	1.55	0.95
Ketamine	0.0	0.71	0.0
Hydrobenzoin	0.0	0.0	0.0
Nefopam	0.0	0.79	0.0

concentration (50 mM) of surfactants was used, and results are shown in Table 3. Short chain surfactants, e.g., (S)-N-butoxycarbonylleucine or hexoxycarbonylleucine, are not very effect in enantioseparation. Micelle provides the sites upon which optically active molecules interact, micelle existence in the electrolyte is therefore critical in chiral recognition. Critical micelle concentration for short chain surfactants is normally in the range of a couple hundred millimole [34]. Under the condition which we investigated (50 mM), (S)-N-butoxycarbonylleucine or hexoxycarbonylleucine existed as monomers. This can explain why there is no chiral resolution in these two surfactants.

(S)-N-tetradecoxycarbonylleucine has low a solubility in water, and its data is not shown in Table 3. There is a decrease in resolution from (S)-N-decoxycarbonylleucine to dodecoxycarbonylleucine. The hydrophobic interaction between micelle and analyte becomes stronger in longer chain surfactants, and it competes with chiral interaction between analytes and chiral functional headgroups, resulting in a decrease in chiral recognition.

3.4 A mixed system containing sulfated ß-CD and chiral surfactant

Sulfated ß-CD was first used by Stalcup and co-workers [12] to separate enantiomers at acidic conditions in CE. We attempted to use this chiral selector in basic conditions (see Figure 6), and the results were compared with those obtained from using 100 mM (S)-N-octoxycarbonylleucine (see Table 4). Since the ß-CD cavity is well suited for a benzene ring, it is not surprising to see benzoin and hydrobenzoin getting a large increase in resolution. Apparently, (S)-N-octoxycarbonylleucine is good for atenolol and ketamine. Each chiral selector seems to have its own specialty in chiral separation area, and

Table 3. Effect of surfactant chain length on chiral resolution.

Compounds	Resolution (R_s)			
	50 mM C_6-Leucine	50 mM C_8-Leucine	50 mM C_{10}-Leucine	50 mM C_{12}-Leucine
Atenolol	0.0	2.17	2.36	1.80
Benzoin	0.0	1.56	1.75	1.04
Laudanosine	0.0	0.85	0.59	0.0
Propranolol	0.0	1.55	0.39	0.0
Ketamine	0.0	0.71	1.33	0.92
Hydrobenzoin	0.0	0.0	0.86	1.00
Nefopam	0.0	0.79	0.29	0.10

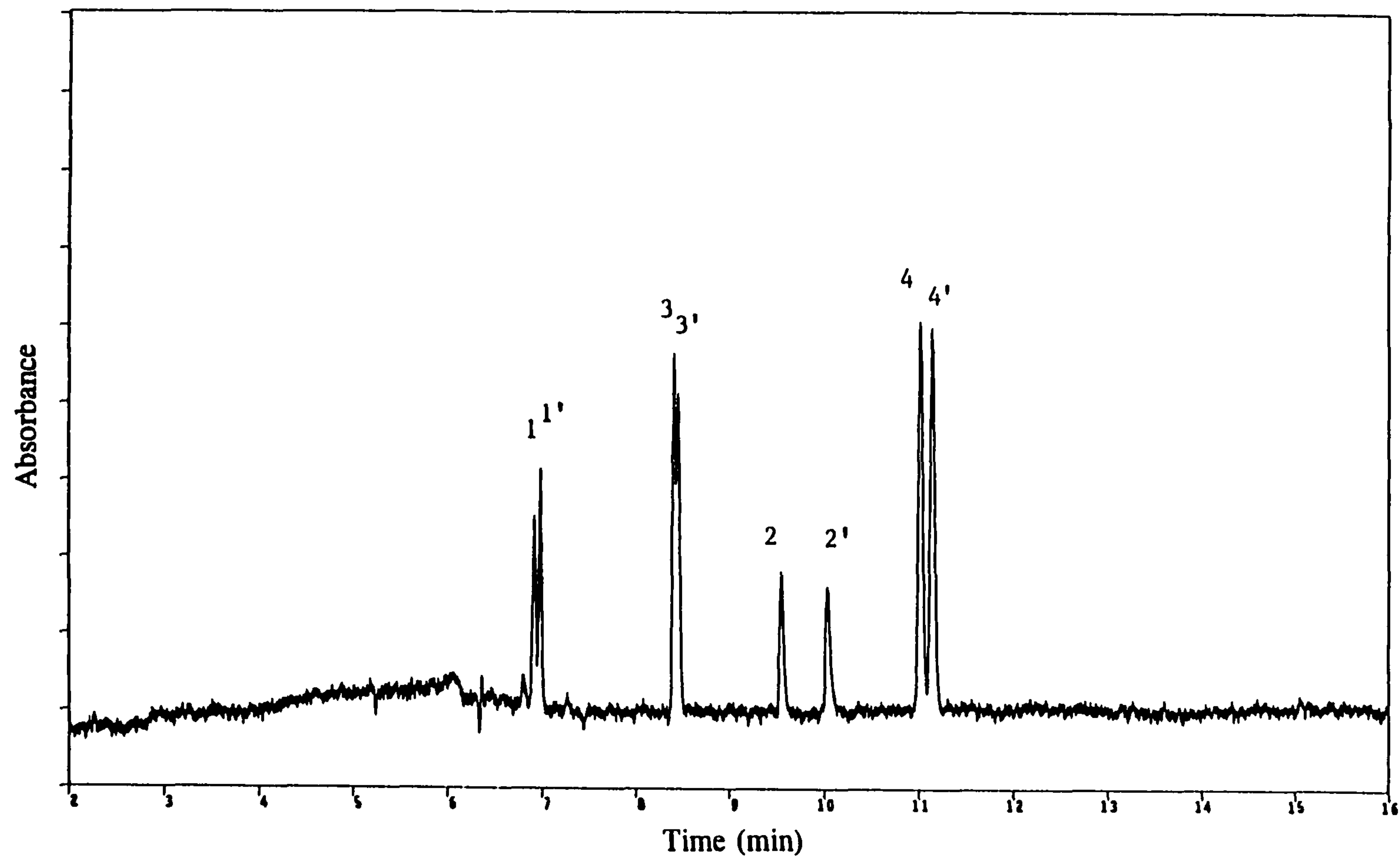


Figure 6. Enantiomeric separation of drugs using 25 mM ß-CD sulfate. Conditions and peak identities: see Figure 2.

Table 4. Comparison single surfactant with mixed surfactants.

Compounds	Resolution (R_s)		
	100 mM C_8-Leucine	25 mM ß-CD sulfate	10 mM ß-CD sulfate with 50 mM C_8-Leucine
Atenolol	2.93	0.92	2.34
Benzoin	1.75	5.21	2.61
Laudanosine	0.59	0.45	0.53
Propranolol	1.35	1.31	3.05
Ketamine	0.43	0.0	0.81
Hydrobenzoin	0.91	2.75	2.05
Nefopam	0.58	NA	8.03

unfortunately, it is still impossible to predict which chiral compound can be separated by which chiral selector, and this remains a problem for analytical chemists. In order to achieve an enantiomeric separation, many chiral selectors are tried one by one. It is a very time consuming process. We get the idea from the experiments result using sulfated ß-CD and (S)-N-octoxycarbonylleucine which have apparently very different structures and separation mechanism. Instead of using one selector each single time, why not put them all together in a same buffer to let them acting on their own, hopefully generating a far more better "chiral selector" than each one alone.

The results of using sulfated ß-CD with (S)-N-octoxycarbonylleucine are shown in Table 4. Because of high current encountered, lower concentration of sulfated ß-CD and (S)-N-octoxycarbonylleucine was utilized. However, it is enough to illustrate the idea. Chiral resolutions are greatly improved by using this mixture of selectors, and most of them are even better than just simple adding-effect. The enhancement in chiral resolution and enantioselectivity can be explained by the prolongation of the analyte interaction time with anionic CD and chiral micelles. Enantioselectivity is boosted by multiple stereoselective interactions with both CDs and chiral micellar surface.

This concept was further demonstrated in the next application. Figure 7 shows a separation of eight DL-dansyl amino acids by 10 mM sulfate ß-CD. It is hard to see any enantiomeric separation in this electropherogram. Same amino acids were tried using 50 mM (S)-N-octoxycarbonylleucine, shown in Figure 8. Some were partially separated enantiomerically, however, the result was still unsatisfactory. Combining these two selectors was then attempted, and a much better separation was obtained (Figure 9). The migration

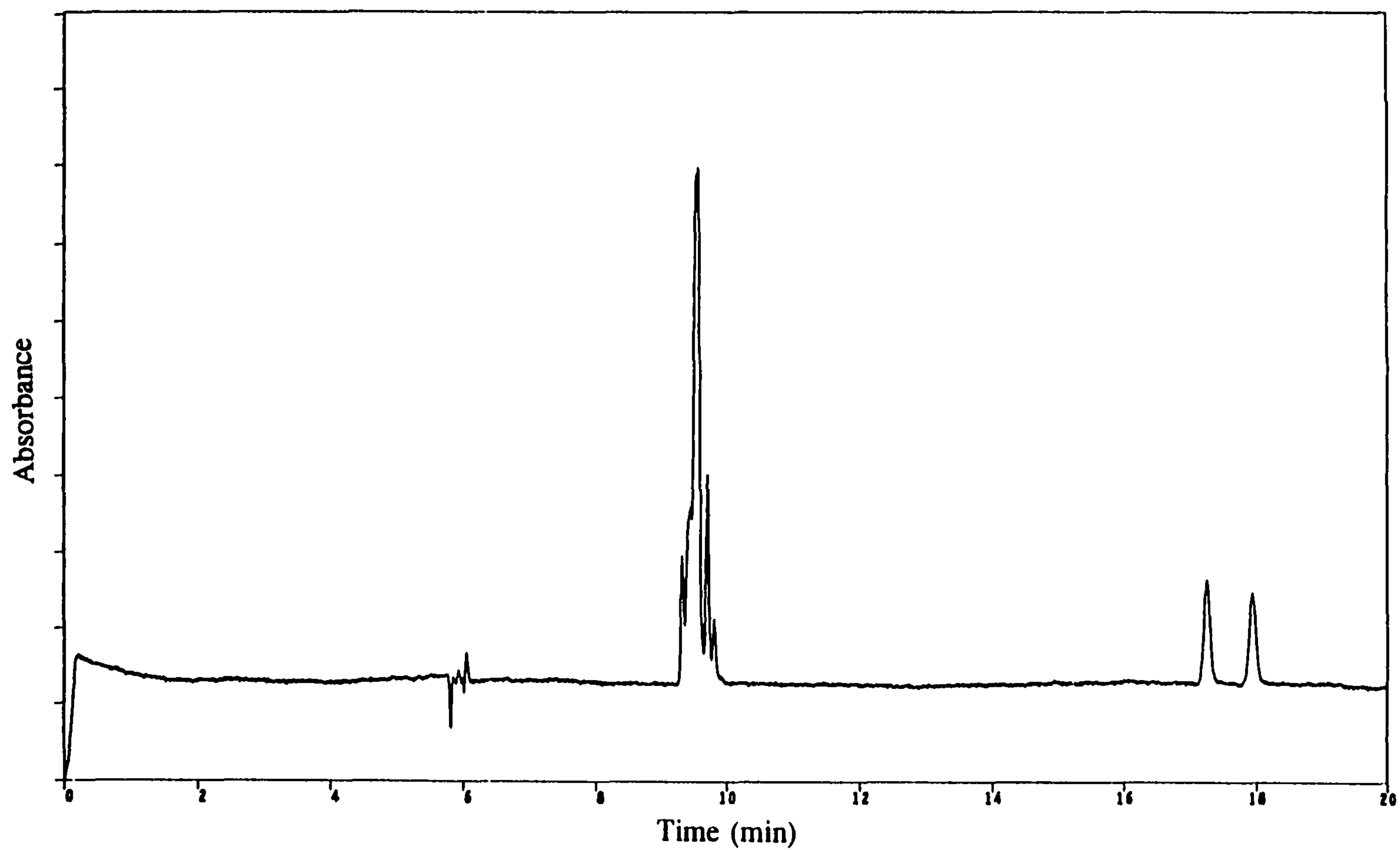


Figure 7. Electropherograms of dansyl amino acids by 10 mM ß-CD sulfate. Conditions: see Figure 2.

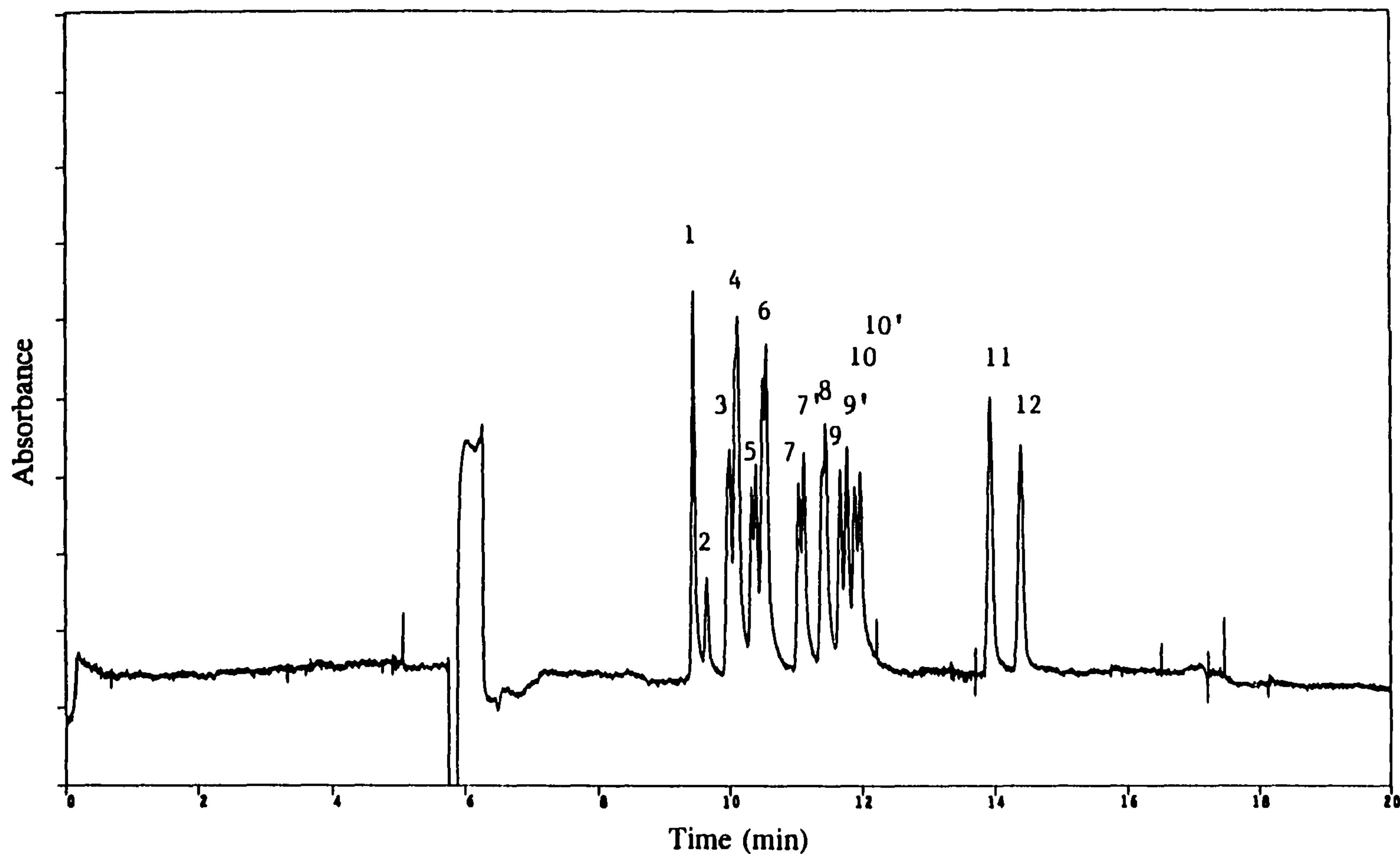


Figure 8. Electropherogram of dansyl amino acids using 50 mM (S)-(+)-N-octoxycarbonylleucine. Conditions: see Figure 2. Peaks: 1= threonine; 2= serine; 3= α-amino-n-butyric acid; 4= valine; 5= methionine; 6= norvaline; 7= leucine; 8= tryptophan; 9= phenylalanine; 10= norleucine; 11= glutamic acid; 12= aspartic acid.

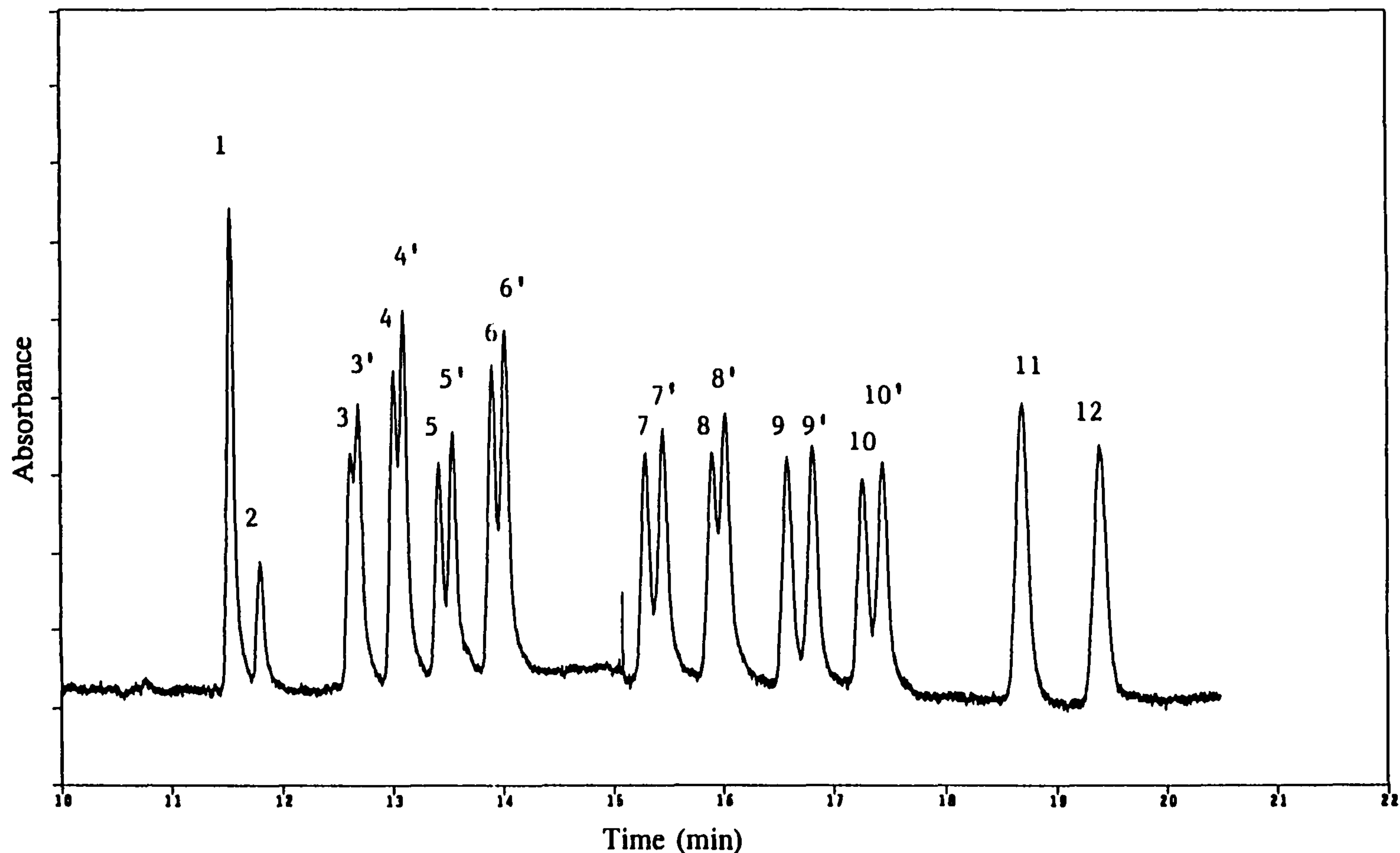


Figure 9. Electropherogram of dansyl amino acids using 10 mM ß-CD sulfate and 50 mM (S)-(+)-N-octoxycarbonylleucine. Conditions: see Figure 2. Peaks: 1= threonine; 2= serine; 3= (S)-α-amino-n-butyric acid; 3'= (R)-α-amino-n-butyric acid; 4= (S)-valine; 4'= (R)-valine; 5= (S)-methionine; 5'= (R)-methionine; 6= (S)-norvaline; 6'= (R)-norvaline; 7= (S)-leucine; 7'= (R)-leucine; 8= (S)-tryptophan; 8'= (R)-tryptophan, 9= (S)-phenylalanine; 9'= (R)-phenylalanine; 10= (S)-norleucine; 10'= (R)-norleucine; 11= glutamic acid; 12= aspartic acid.

times are longer than using just surfactant alone, proving that a prolonged interaction time between analytes and selectors. Increasing (S)-N-octoxycarbonylleucine concentration 50 mM to 100 mM, these eight amino acids were enantiomerically baseline resolved, though the total analysis time took even longer time (Figure 10).

4. Conclusions

It is found that chiral recognition is depend on surfactant structure in micellar electrokinetic chromatography (MEKC). Changing chain length in the surfactant, varying the amino acids attached are among the effective ways to manipulate the chiral selectivity. Increasing surfactant concentration can largely improve chiral resolution, however, the resolution reaches a maximum after a certain concentration owing to hydrophobic interaction competing with chiral recognition. Short chain surfactant, e.g., butoxycarbonylleucine, or hexoxycarbonylleucine, proved to have no chiral interaction with enantiomers due to its high critical micelle concentration (cmc). A too-long-chain surfactant such as tetradecoxycarbonylleucine has a low solubility in aqueous solution, and is therefore not very useful as an additive. A chain length between C8- to C10 is demonstrated to be the most effective in achieving the best chiral resolution. Changing amino acids attached to the surfactants give rise to a difference in chiral functional group, which results in a difference in chiral recognition. From the seven drugs we studied, L-leucine derivatives were more effective additives than L-valine or L-isoleucine derivatives.

Sulfated ß-CD is compared with these synthetic chiral additives, and it showed a distinct chiral selectivity resulting from its unique chiral recognition mechanism. A

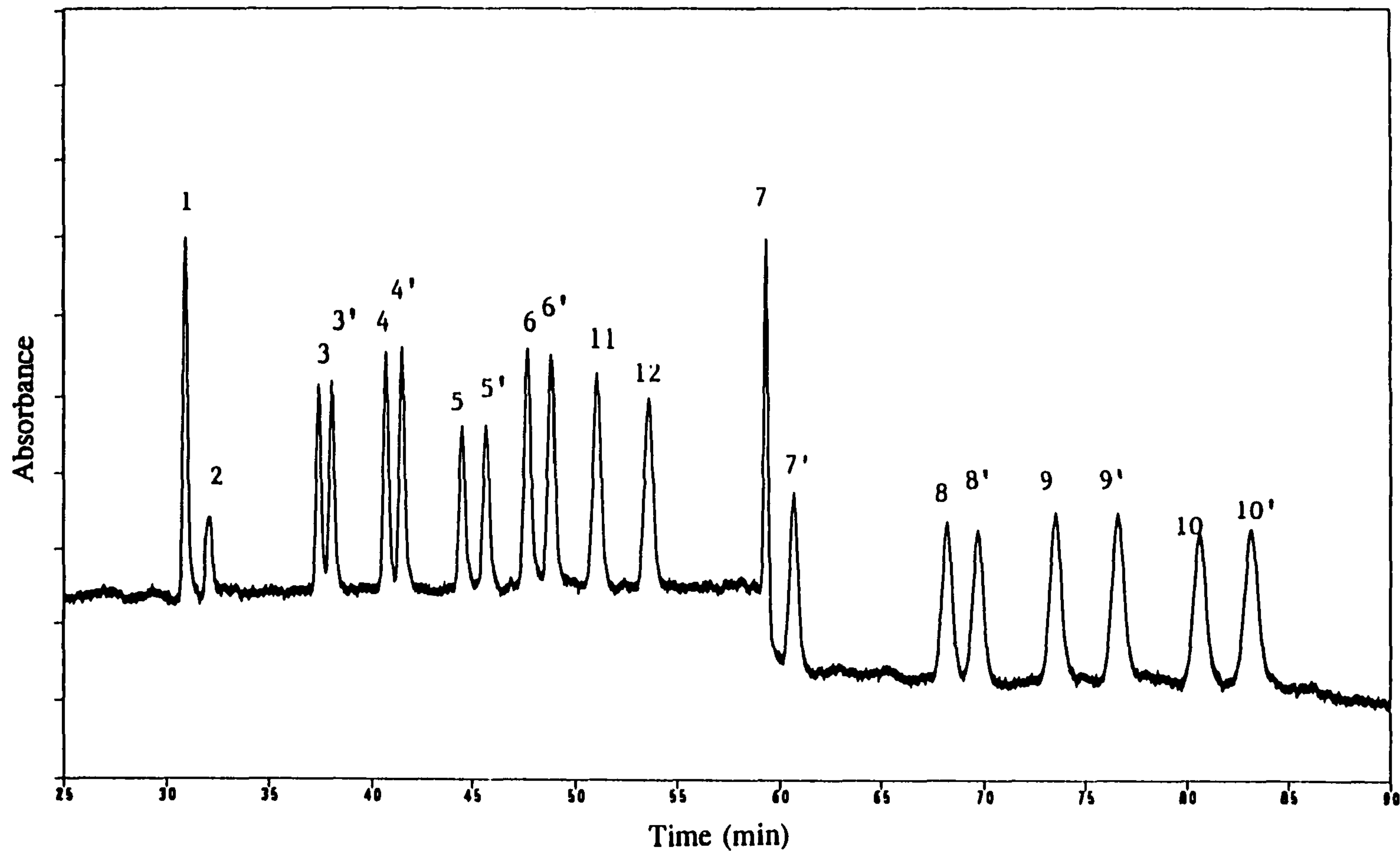


Figure 10. Electropherogram of dansyl amino acids using 100 mM (S)-(+)-N-octoxycarbonylleucine and 10 mM ß-CD sulfate. Conditions: see Figure 2 except applied voltage is 12 kV. Peaks identifications: see Figure 9.

combination of these two different additives was applied to enantiomeric compounds, including the seven test drugs and dansyl amino acids. Better results were obtained using this duo-chiral system than either one alone, and chiral resolution in most cases was largely enhances. This new system provides a fast and effective way in chiral method development and preliminary studies.

Acknowledgements:

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CHAPTER 6. CAPILLARY ELECTROPHORESIS OF ANIONS AT HIGH SALT CONCENTRATIONS

A paper submitted to *Electrophoresis*

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Abstract

It is commonly thought that even a moderately high ionic concentration in the background electrolyte would lead to Joule heating and serious peak distortion. However, we obtained very satisfactory separations of both inorganic and organic anions in electrolyte solutions as high as 5 M sodium chloride using direct photometric detection. Samples containing a 0.5 M concentration of a salt can be analyzed directly by making the BGE concentration of the same salt even higher to obtain electrostacking. The temperature in the center of the capillary was calculated to be 49°C when the current is at its maximum of 280 μA. The effect of various salts on electrophoretic and electroosmotic mobility is discussed. Several examples are given of CE separations under high-salt conditions.

1. Introduction

Capillary electrophoresis (CE) is a powerful separation technique for both ions [1-6] and neutral compounds [7-10]. Almost all of the work has been done on samples containing a relatively low total ion concentration. Little has been done on samples with high salt content, such as seawater. Such samples normally would require either dilution of the sample [11] or removal of salts in the sample by some sample cleanup method [12,13]. Direct injection of high-salt samples is rarely used without some pretreatment. High-salt sample matrices compete with the analyte ions for migration into the capillary, resulting in irreproducible injections and migration times [14,15].

In a few cases salts have been added to the background electrolyte (BGE) to achieve some specific effects. Alkali metal salts [16-18], dodecasodium salt of phytic acid [19], or zwitterionic salts [20] have been added to the BGE to suppress ion exchange at anionic sites on fused silica, and prevent adsorption of proteins or oligonucleotides on fused silica. However, the authors all pointed out that the presence of a moderate to high concentration of salt can generate excess of Joule heating. Joule heating is believed to negatively affect the quality of the separation and can cause peak distortion. Several approaches can be taken to minimize the effect of Joule heating. The applied voltage can be lowered, the column length can be increased, or the inner diameter of the capillary can be reduced. One obvious disadvantage of these approaches is the relatively long analysis time.

In this study we report that CE separations can be carried out successfully in solutions of high salt content. Separation of anions in samples containing 0.5 M sodium chloride or several other salts is possible provided the salt content of the buffer electrolyte is at least

three times higher. Electroosmotic flow is greatly reduced in electrolytes containing a high salt concentration, thus eliminating the need for a flow modifier.

2. Materials and Methods

2.1 Chemicals

All electrolytes and standards were prepared with analytical-reagent grade chemicals and 18 MΩ deionized waters obtained from a Barnstead Nanopure II system (Sybron Barnstead, Boston, MA, USA). Seawater and lithium sulfate were purchased from Sigma (St. Louis, MO, USA). Sodium chloride, sodium hydroxide, boric acid, potassium chloride and sodium sulfate were obtained from Fisher Scientific (Pittsburgh, PA, USA). The other chemicals were obtained from Aldrich (Milwaukee, WI, USA).

2.2 Instrumentation

A Waters Quanta 4000 capillary electrophoresis system (Millipore Waters, Milford, MA, USA), equipped with a negative power supply was used. The separation was performed on a polyamide-coated, 33 cm × 50 μm (or 75 μm) i.d. fused silica capillary (Polymicro Technology, Phoenix, AZ, USA), applying a constant voltage of -10 kV. The distance from the point of injection to the window of on-column detection was 25.5 cm. Under these conditions a current of 280 μA was encountered. The time of hydrostatic injection was 40 s. On-column detection was at 214 nm. Electropherograms were collected and plotted by the data acquisition system Chrom Perfect Direct (Justice Innovations, Mountain View, CA, USA). Polyethylene sample vials were used as containers

for the carrier electrolyte and for all of the standards and samples. Electrolytes were filtered through a 0.22 μm pore size filter (Costor, Cambridge, MA, USA).

New capillaries were preconditioned by rinsing with 0.1 M NaOH for 1 h followed by a 30 min rinse with deionized water, and a 30 min rinse with the electrolyte. Prior to each run, the capillary was rinsed with the electrolyte for 2 min.

3. Results and Discussion

3.1 Analysis of high-salt samples

Previous work had demonstrated the feasibility of separating various inorganic anions at pH values sufficiently acidic to almost eliminate electroosmotic flow [6]. The first experiments on the effect of high sodium chloride concentrations in analytical samples were run at pH 2.4 using a negative power supply (-10 kV) and a 75 μm fused-silica capillary. Both the sample and the background electrolyte (BGE) contained 0.5 M sodium chloride. The results for several inorganic anions under these conditions with direct photometric detection were very poor. The peaks were badly shaped and there was almost no resolution of individual peaks. However, peak shape and resolution improved dramatically with increasing sodium chloride concentration in the BGE. At 1.5 M sodium chloride in the BGE, excellent separation was obtained for samples containing 0.5 M sodium chloride and low ppm concentrations of five inorganic anions (Figure 1). Other experiments showed that salt concentrations as high as 5 M in the BGE could be handled. The salt content of the BGE needed to be at least three times that of the sample in order to provide sufficient peak focusing (electrostacking) during the sample introduction. These experiments demonstrated

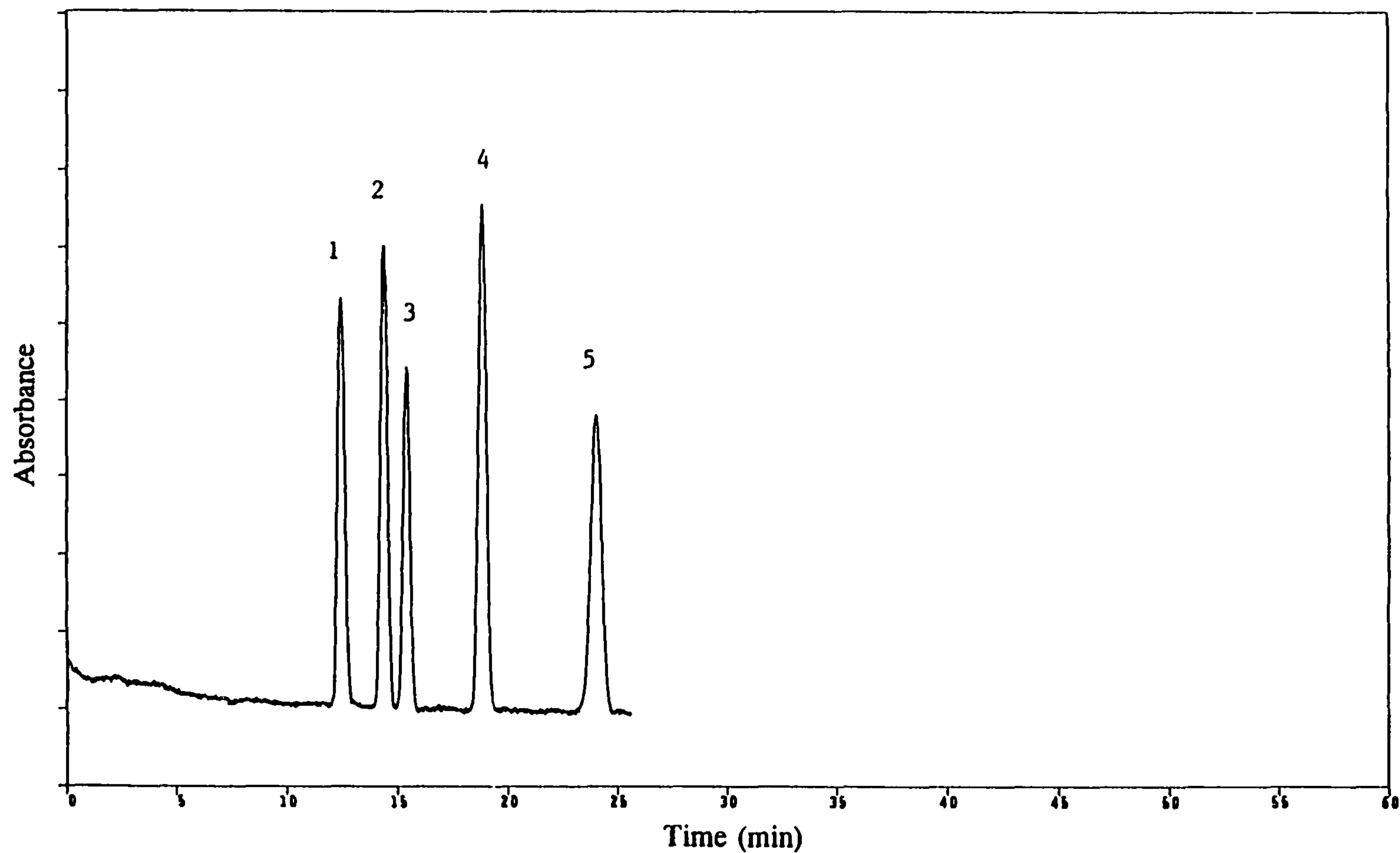

Figure 1. Electropherogram of five anions in 0.5 M NaCl. Capillary, fused silica (75 μm i.d., 35 cm length); carrier, 1.5 M NaCl with pH 2.4 adjusted by HCl; sampling time, 30 s; voltage, -10 kV; current, 280 μA; wavelength for detection, 214 nm; peak assignment, (1) I^-, (2) SCN^-, (3) NO_3^-, (4) IO_4^-, (5) IO_3^-.

that the limits of salt concentration of both the sample and the BGE are much higher than had been expected.

The effect of capillary length was investigated briefly on the migration times of I^-, SCN^-, NO_3^-, and IO_3^- using a 30-, 35-, 40-, 45-, and 50 cm length capillary. It was found that the migration times were proportional to the length of the capillary. Even though IO_3^- and NO_3^- were better separated using a 50 cm capillary, a decent separation efficiency still could be obtained by using a 30 cm capillary, where the migration time for IO_3^- was largely decreased from 48 min to 21 min.

A nearly linear increase in migration times with increasing sodium chloride concentration was also observed between 1 M and 5 M sodium chloride in the BGE. Electrophoretic mobilities of ions are directly related to the salt concentration in the BGE [23]. Thus,

$$\mu = \frac{q_{eff}}{6\pi\eta R} \qquad (3)$$

where μ is the electrophoretic mobility, q_{eff} is the effective charge on the ion, η is the viscosity of the surrounding medium and R is the apparent hydrodynamic radius of the sample ion. At higher salt concentration μ is smaller because the effective charge on an analyte ion (q_{eff}) decreases due to greater shielding,

$$q_{eff} = q\ (\frac{1}{1 + \kappa R}) \qquad (4)$$

In this equation q is the ion net charge and κ is an important parameter related to the charge-shielding properties of an electrolyte solution, and the thickness of the electrical double layer

adjacent to a charged surface is given by κ^{-1},

$$\kappa^{-1} = \frac{1}{\sqrt{\frac{e^2}{\varepsilon kT} \sum_i z_i^2 n_{i0}}} \tag{5}$$

where e is the electronic charge, ϵ is the permittivity of the medium, which is given by the expression $\epsilon = \epsilon_0 \epsilon_r$ where ϵ_0 is the permittivity of a vacuum, and ϵ_r is the dielectric constant of the medium, k is the Boltzmann constant, T is the absolute temperature, z_i is the valence of the ions, n_{i_0} is the number concentration of ions in the electrolyte solution, and the sum is taken over all ions in the solution. It is apparent that high concentration of salts in the electrolyte decreases the thickness of the double layer, q_{eff}, thus μ.

A short study was conducted on pH effect over a broad range from pH 3.0 to pH 12.0. The sodium chloride concentration was 1.5 M in all buffers. There were no observed differences in either migration times or peak shapes for I^-, SCN^-, NO_3^- and IO_3^- between pH 3.0, 7.0, and 12.0. This effect strongly indicates that the electroosmotic flow is greatly suppressed, and the ionized silanol groups at the capillary surface is effectively shielded by the high concentration of cations, M^+, in the buffer solution [19],

$$Q = \frac{Q_0}{1 + K_{wall}[M^+]} \tag{6}$$

where Q is the surface charge per unit area, Q_0 is the number of ionized silanol groups per unit area, K_{wall} is the equilibrium constant for the adsorption mechanism,

$$M^{+} + SiO^{-} \rightleftharpoons SiO^{-}M^{+} \quad (7)$$

The magnitude of the electroosmotic flow is expressed in the following equation [23]:

$$\mu_{os} = \frac{Q\,\kappa^{-1}}{\eta} \quad (8)$$

where κ^{-1} is the double-layer thickness (see Equation 3). It is clearly seen that an increased concentration of electrolytes will serve to reduce κ^{-1}, Q, and μ_{os}. A study of the effect of varying sodium chloride concentration on μ_{os} has been reported [24], but the concentration range was much lower than in the present study.

The effect of capillary diameter on the migration times of anions was studied using iodide and nitrate as sample ions. Migration times for both ions in the 50 μm capillary were about one half of those in the 75 μm capillary. The peaks were also sharper in the capillary of smaller i.d.. For these reasons, 50 μm capillaries were used in all the remaining studies.

3.2 Effect of BGE salt content on CE separations

A more detailed study on the effects of salts on the separation was conducted for several anions: Br^-, CrO_4^-, NO_2^-, NO_3^-, MnO_4^-, $CrOx_3^{3-}$, and ReO_4^-. These anions were dissolved in deionized water instead of in 0.5 M sodium chloride solution. The electrolytes were 10 mM borate at pH 8.5 with different amounts of sodium chloride. As we expected, when there was only 10 mM borate in the electrolyte, the peak shapes for all the anions were very broad and fronting (Figure 2a). Migration times for the first four peaks were very long and no peaks at all were detected for MnO_4^-, $CrOx_3^{3-}$, or ReO_4^- in 60 min. At low salt concentrations in the BGE there was very little electrostacking and the peak migration was

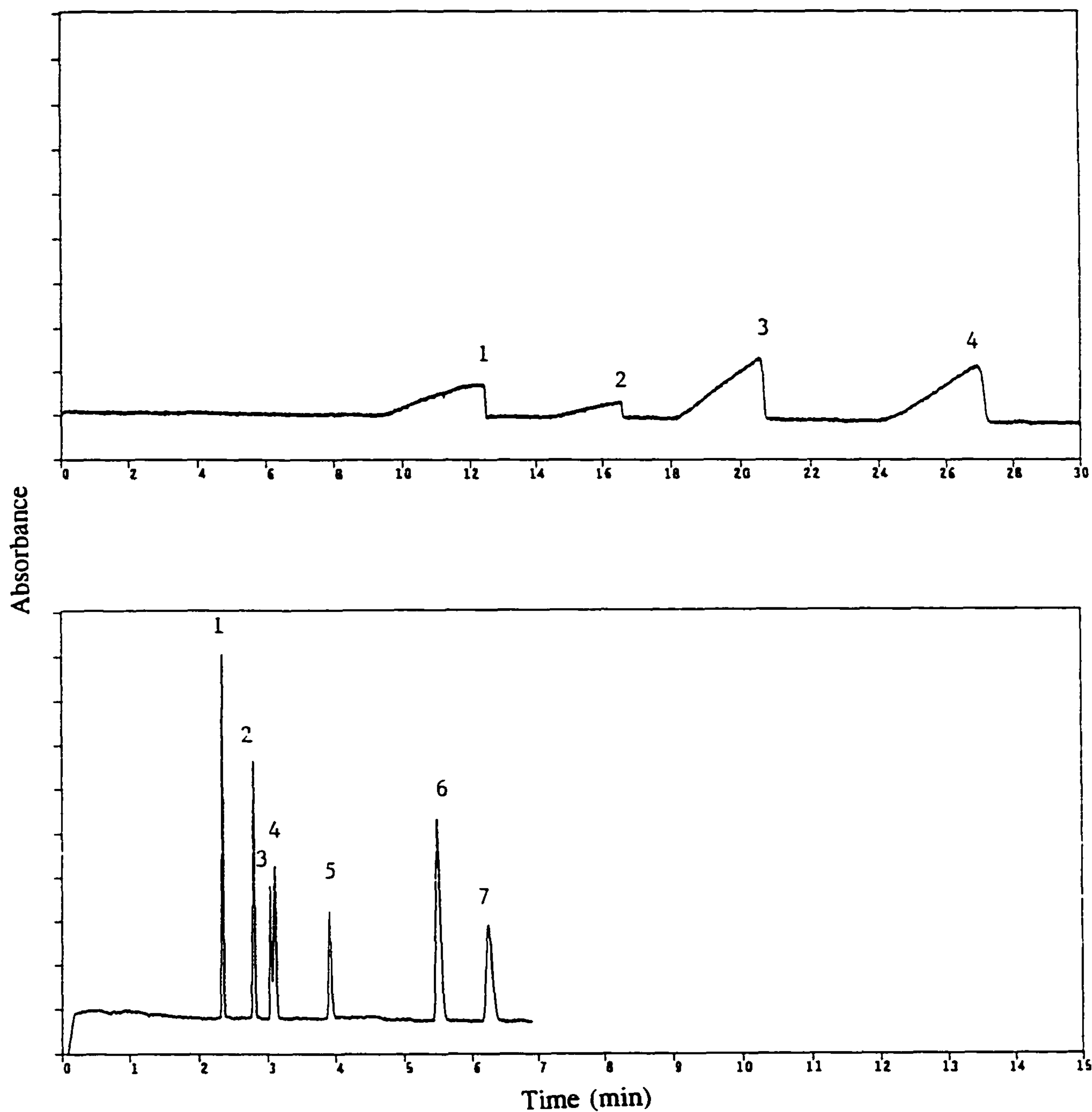

Figure 2. Effect of background salt content on CE separations. Capillary, 50 μm i.d., 33 cm length; carrier electrolyte, 10 mM borate, pH 8.5 adjusted by NaOH; sampling time, 40 s; voltage, -10 kV; wavelength for detection, 214 nm. (a) no sodium chloride was added in the electrolyte; (b) 150 mM sodium chloride was added. Peaks: (1) Br^-, (2) NO_2^-, (3) CrO_4^{2-}, (4) NO_3^-, (5) MnO_4^-, (6) $CrOx_3^{3-}$, (7) ReO_4^-.

slowed by EOF counter to the electrophoretic migration. Figure 2b shows an entirely different picture when the electrolyte contained 150 mM sodium chloride in addition to the 10 mM borate buffer. Less than 7 min was needed for the entire separation. Peaks were very sharp due to a strong electrostacking effect. The higher salt concentration greatly reduced counter EOF and thus permitted much shorter migration times.

Again using sample solutions of low ionic concentration, electropherograms were run at pH 8.5 with increasing concentrations of sodium chloride or lithium sulfate in the BGE. The plots in Figure 3 and 4 show several interesting effects. One is that the current increases very rapidly with increasing salt concentration and levels out at 280 μA around 200 mM sodium chloride or lithium sulfate. This sharp increase in current can be attributed to less electrical resistance. The maximum current that can be obtained in our instrument is set at 280 μA. In order to maintain this current, the voltage was automatically lowered as the salt concentration in the BGE continued to increase. The full power of the instrument's power supply was then being used. This was further demonstrated by the fact that migration times were the same at voltage "setting" of -10 kV, -20 kV and -30 kV at salt concentration >200 mM.

Figure 4 clearly shows that electrophoretic mobilities of the sample anions increased at the same time the current was increasing between 0 and 200 mM added sodium chloride lithium sulfate. Equation 1 predicts a decrease in electrophoretic mobilities at increasing salt concentration.

This effect can be explained by Joule heating. By applying the equation described by Grossman and Colburn in their book [21], we were able to calculate the total temperature

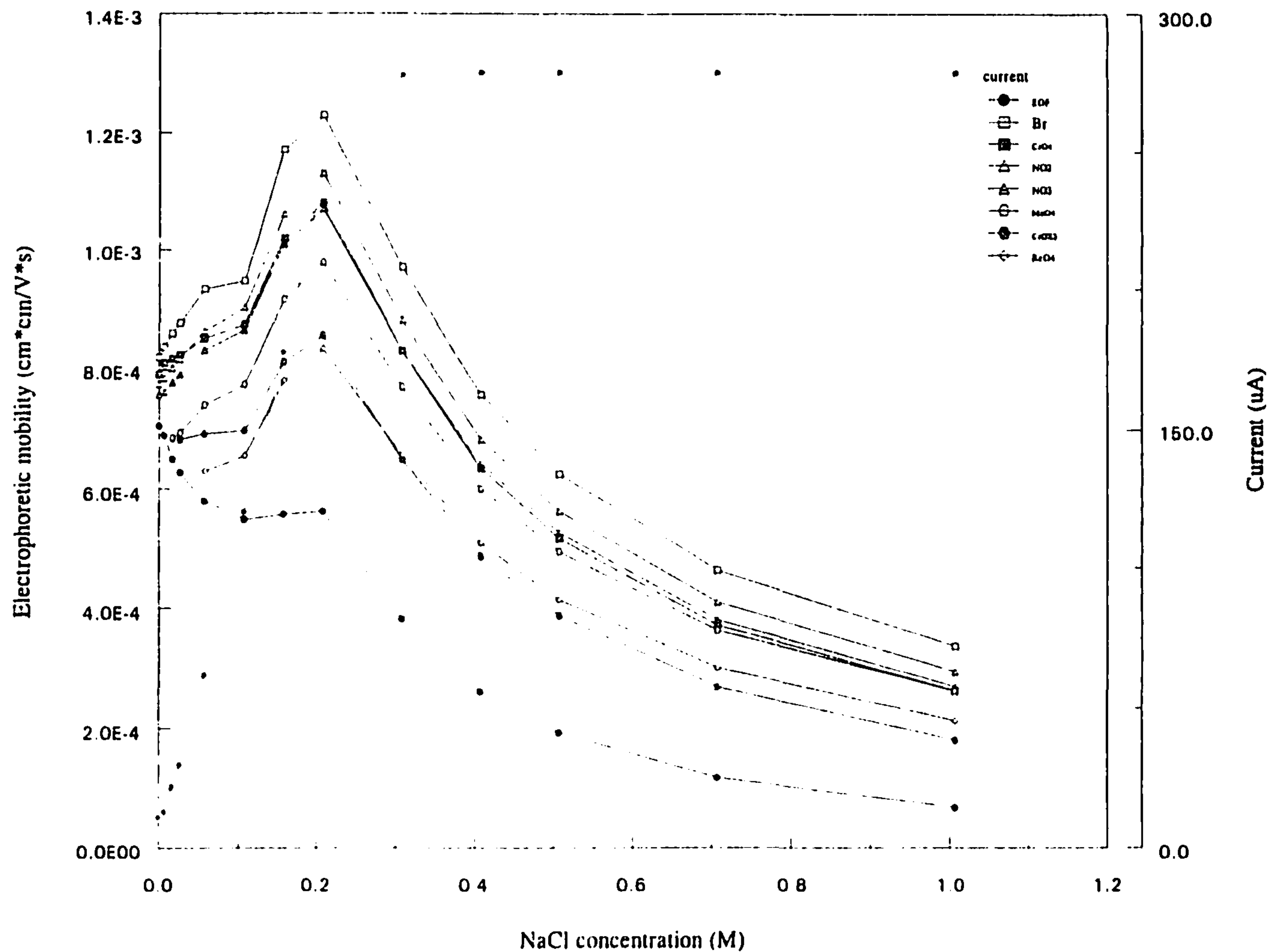

Figure 3. Plots of electrophoretic mobility and current against the concentration of sodium chloride. The experimental conditions were the same as those described in Fig. 2.

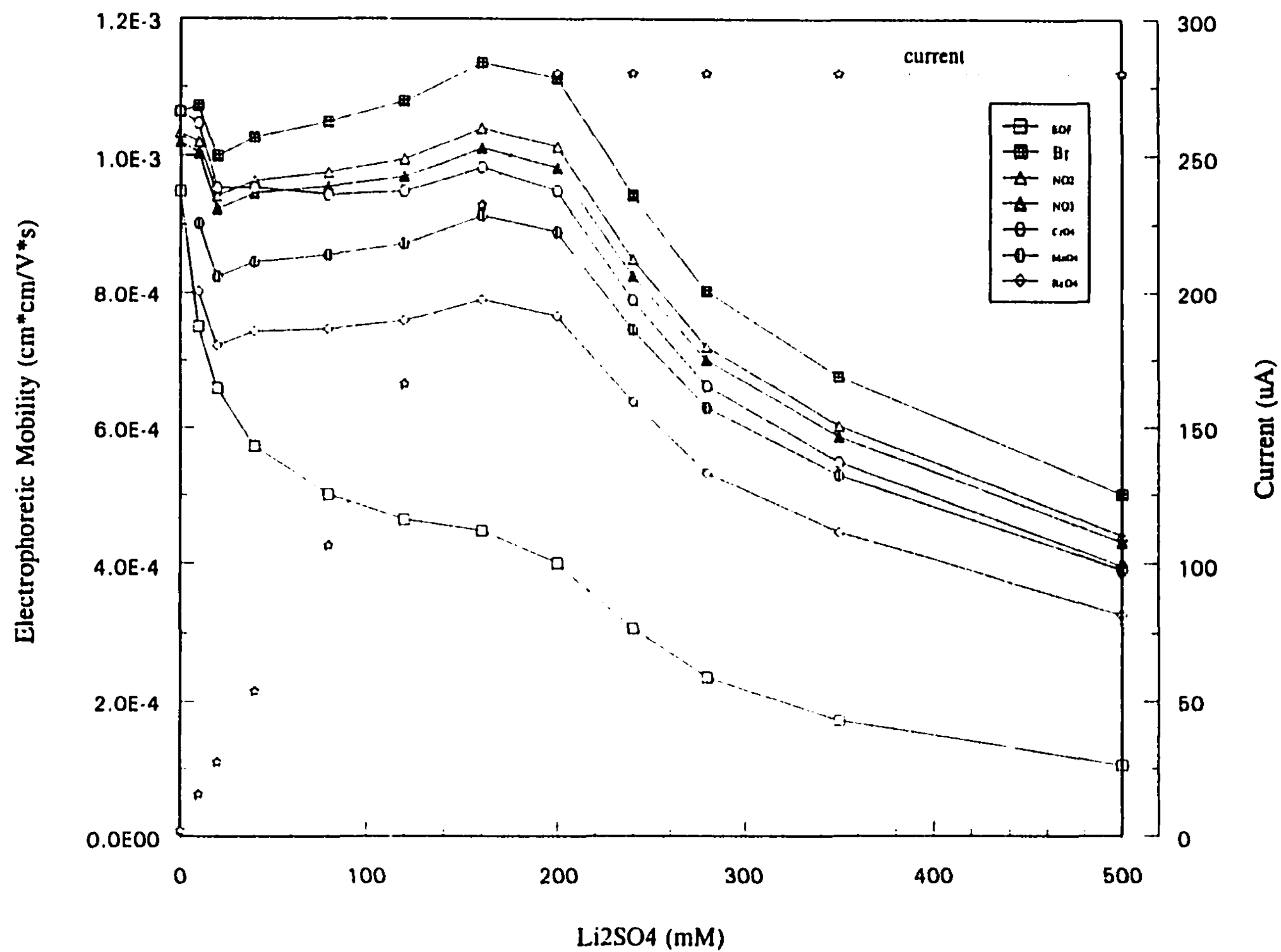

Figure 4. Plots of electrophoretic mobility and current against the concentration of lithium sulfate. The experimental conditions were the same as those described in Fig. 2.

rise, T_0 - T_a, where T_0 is the temperature at the capillary center-line and T_a is the ambient temperature of the surrounding medium, which could be expressed by,

$$T_0 - T_a = \frac{iVR_1^2}{2SL_{tot}} \left(\frac{1}{2k_b} + \frac{1}{k_s} \ln(\frac{R_2}{R_1}) + \frac{1}{k_p} \ln(\frac{R_3}{R_2}) + \frac{1}{R_3 h} \right) \tag{9}$$

where i is the current, 280 μA, V is the applied voltage, 10,000 volts, S is the cross-sectional area of the capillary, L_{tot} is the total length of the capillary, 33 cm, R_1, R_2, and R_3 are the radii of the open channel, 50 μm, the silica wall, 360 μm, and the polymer coating, ~360 μm, respectively, k_b, k_s, and k_p are the thermal conductivities of the buffer, the silica and the polymer coating respectively, and h is the capillary-air heat transfer coefficient, 190 W/m^{2}K, with an air flow of 210 feet^3/min. The value of T_0 - T_a is computed to be 24°C, thus T_0, the temperature inside the capillary, is ~49°C, regarding that the surrounding air temperature is 25°C. The full power of the power supply was used at ~200 mM sodium chloride and above so that essentially no further increase in capillary temperature would be expected at higher salt concentrations.

Electropherograms such as Figure 2b and those run at still higher salt content showed that this moderate amount of Joule heating is not enough to destroy a separation. On the contrary, some heating may be helpful. Ions migrate faster at higher temperatures and interact less with the capillary wall. As a result faster separations and sharper peaks are expected.

After reaching a maximum at 200 mM sodium chloride or lithium sulfate, further increases in salt concentration result in decreased mobility of the sample ions (Figure 3, 4). This would be a result of a lower effective charge on the solute anions owing to greater

shielding from the electrostatic field by the higher concentration of counter ions in the surrounding environment (see Equation 2). The dependence of κ on the square root of BGE ionic concentration (Equation 3) predicts that q_{eff}, and hence electrophoretic mobility, should have an inverse dependence on the square root of BGE salt concentration. Replotting the electrophoretic mobilities in Figure 3 against the square root of sodium chloride concentration does give perfectly linear plots (r = -0.9998 to -1.0000) between 200- and 400 mM sodium chloride. These plots then flatten out considerably at higher salt concentrations. The electroosmotic mobility also shows a linear plot against the square root of salt concentration between 200- and 400 mM salt concentrations.

It should be noted that the electrophoretic mobilities and the electroosmotic mobilities plotted in Figure 3 and 4 are actually in opposite directions. The greatest differences, and hence the largest net mobilities are at approximately 200 mM sodium chloride or lithium sulfate. The separation of 10 anions in 220 mM sodium chloride (Figure 5) shows excellent resolution and is significantly faster than separation in 150 mM sodium chloride (Figure 2). Separation of the same anions in 500 mM sodium chloride was also quite good but a little slower than in Figure 5. Even though the electrophoretic mobilities of anions decrease significantly between 200- and 1,000 mM sodium chloride, this is partially offset by a corresponding decrease in electroosmotic mobility.

Excellent separations were also obtained in lithium sulfate electrolytes. In 200 mM lithium sulfate, 10 mM borate, pH 8.5, 33 cm capillary, six test anions were baseline resolved with the following migration times: bromide 1.97 min, nitrite 2.29 min, nitrate 2.41 min, chromate 2.56 min, permanganate 2.87 min, perrhenate 3.84 min. All peaks were

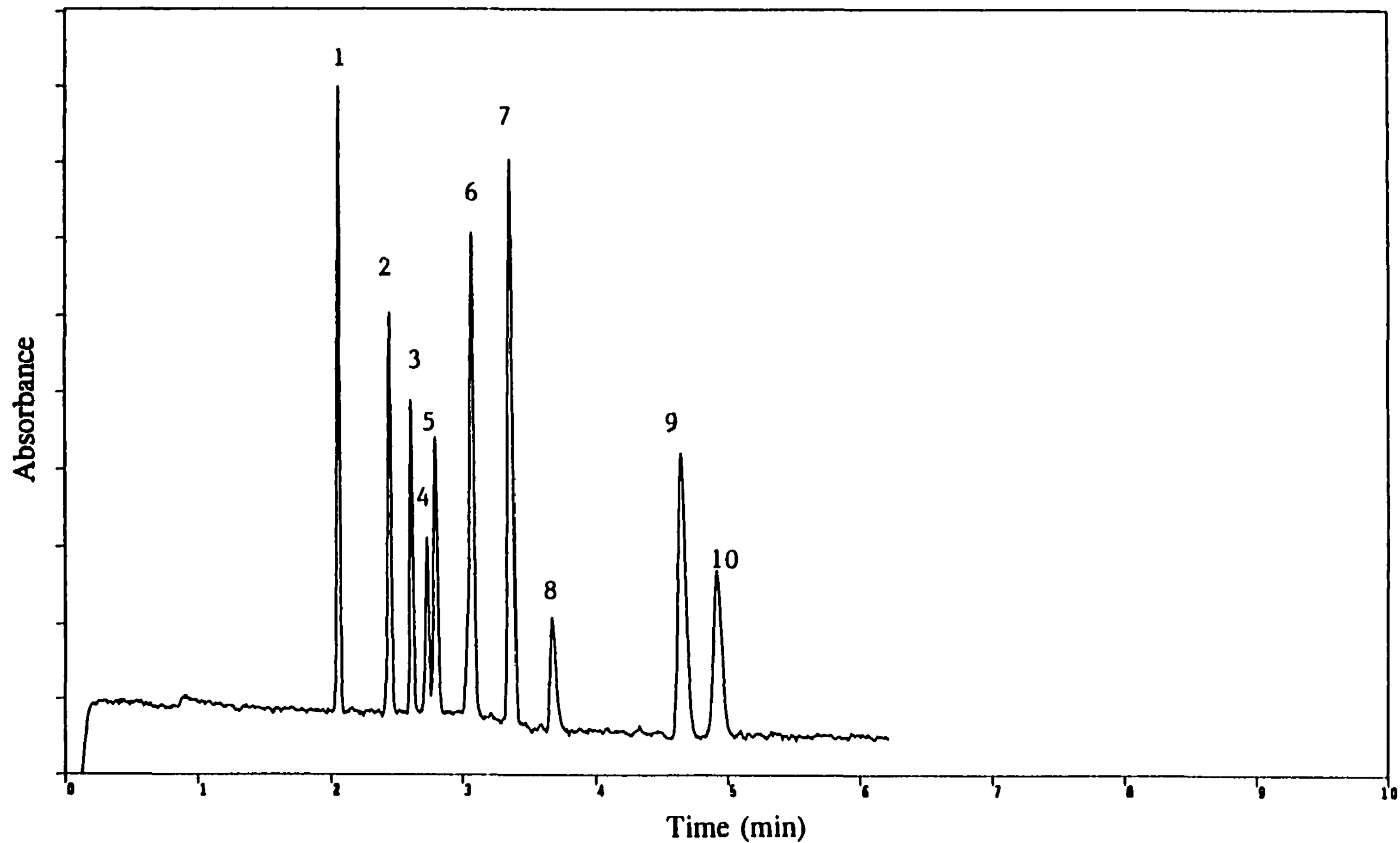

Figure 5. CE separation of ten inorganic anions. The experimental conditions were the same as those described in Fig. 2, except that 220 mM NaCl was added in the carrier electrolyte. Peaks: (1) Br^-, (2) NO_2^-, (3) $S_2O_3^-$, (4) NO_3^-, (5) N_3^-, (6) $Fe(CN)_6^{4-}$, (7) MoO_4^{2-}, (8) WO_4^{2-}, (9) $CrOx_3^{3-}$, (10) ReO_4^-.

symmetric with actual plate numbers ranging from 41,000 to 61,000, average 47,600. Plates per meter averaged 143,000.

3.3 Effect of Methanol

A short study on organic solvent effect on separation was conducted on several anions. Methanol was found to aid the separation of inorganic cations in a paper by Shi and Fritz [3]. A separation is shown in Fig. 5a for 5 anions: CrO_4^{2-}, MnO_4^-, MoO_4^{2-}, WO_4^{2-} and VO_3^-. Again, 220 mM sodium chloride was added in the electrolyte. MnO_4^- and MoO_4^{2-} were partially separated in this case. Adding 5% methanol in the electrolyte greatly improved the separation (Fig. 5b). The peak shape for VO_3^- was also improved. This change in the selectivity could be due to differences in the solvation of the anions.

3.4 Separation of organic anions

Organic anions have slower mobilities compared to most inorganic anions. We did an extensive study on the effect of different salts on the separation of 1,3-benzenedisulfonic acid and 1,2-benzenedisulfonic acid. The concentration was 220 mM for each of the salts studied. These salts included sodium chloride, sodium phosphate, sodium sulfate, sodium citrate, sodium perchlorate, sodium formate, sodium acetate, sodium propionate, sodium butyrate, sodium methanesulfonate, sodium ethanesulfonate, sodium butanesulfonate, potassium sulfate, potassium chloride, and lithium sulfate. Reasonable separations were obtained in each case but there were some differences regarding peaks sharpness and migration time. Sodium sulfate, potassium sulfate and lithium sulfate gave the best results.

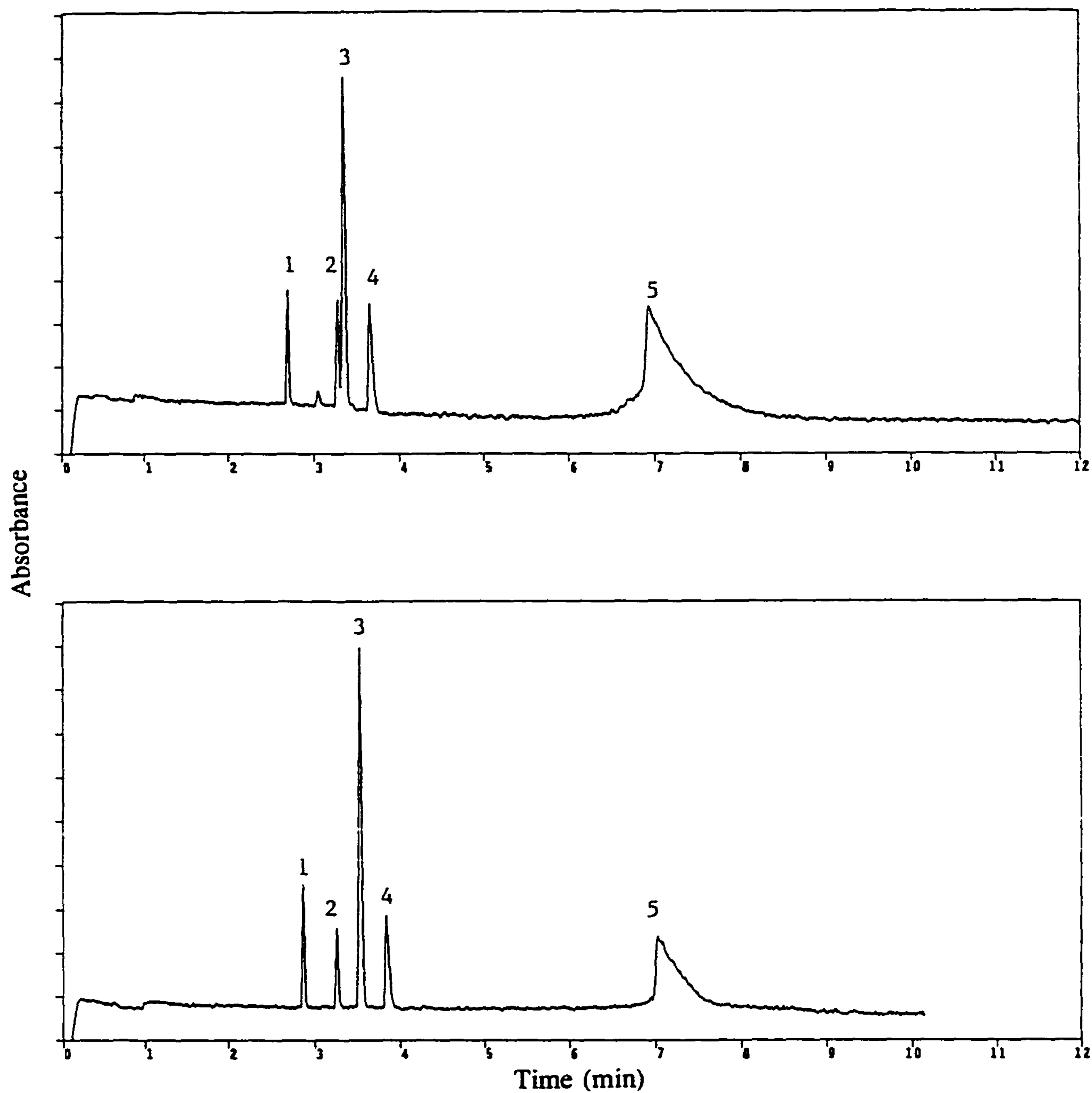

Figure 6. Effect of methanol on CE separation of anions. The experimental conditions were the same as those described in Fig. 4, except that (b) 5% methanol was added in the carrier electrolyte. Peaks: (1) CrO_4^{2-}, (2) MnO_4^-, (3) MoO_4^{2-}, (4) WO_4^{2-}, (5) VO_3^-.

The migration times for 1,3-benzenedisulfonic acid and 1,2-benzenedisulfonic acid were 5.40 min and 10.80 min in 220 mM sodium sulfate, while in 220 mM sodium butanesulfonate they were 7.39 min and 18.78 min. Thus sodium sulfate and lithium sulfate were chosen in further studies. A separation of 11 organic anions, including some positional isomers, is shown in Fig. 6 using 220 mM sodium sulfate. The concentration of each analyte was 25 mg/L. Seven of the ten peaks were detected between 5 and 7 min. Isophthalic acid and 1,2,3-benzenetricarboxylic acid co-migrated together. The last peak was benzenesulfonic acid, because it was the only single charged anion in this group. It took almost 23 min to pass through the detection window. Excellent reproducibility for migration times was obtained, with a relative standard deviation of 0.5% in 9 consecutive runs.

A much better separation was obtained by 220 mM lithium sulfate (Fig. 7). All the anions were nicely separated, including isophtalic acid and 1,2,3-benzenetricarboxylic acid, where the migration times were 9.0 min and 10.2 min. This big difference in selectivity was caused by the different polarizibility between sodium and lithium. Sodium or lithium ions move towards the cathode, just in the opposite direction of the analyte anions. Lithium has a larger ionic radius in solution than sodium because of greater solvation, so it is more polarizable and forms stronger ion-pairs with analyte anions.

It is known that bromide and iodide have almost identical mobilities, and cannot be separated by ordinary CE conditions. However, we got a partial separation for these two anions using 220 mM lithium sulfate. The large anion, iodide, migrated after bromide due to a stronger interaction with lithium.

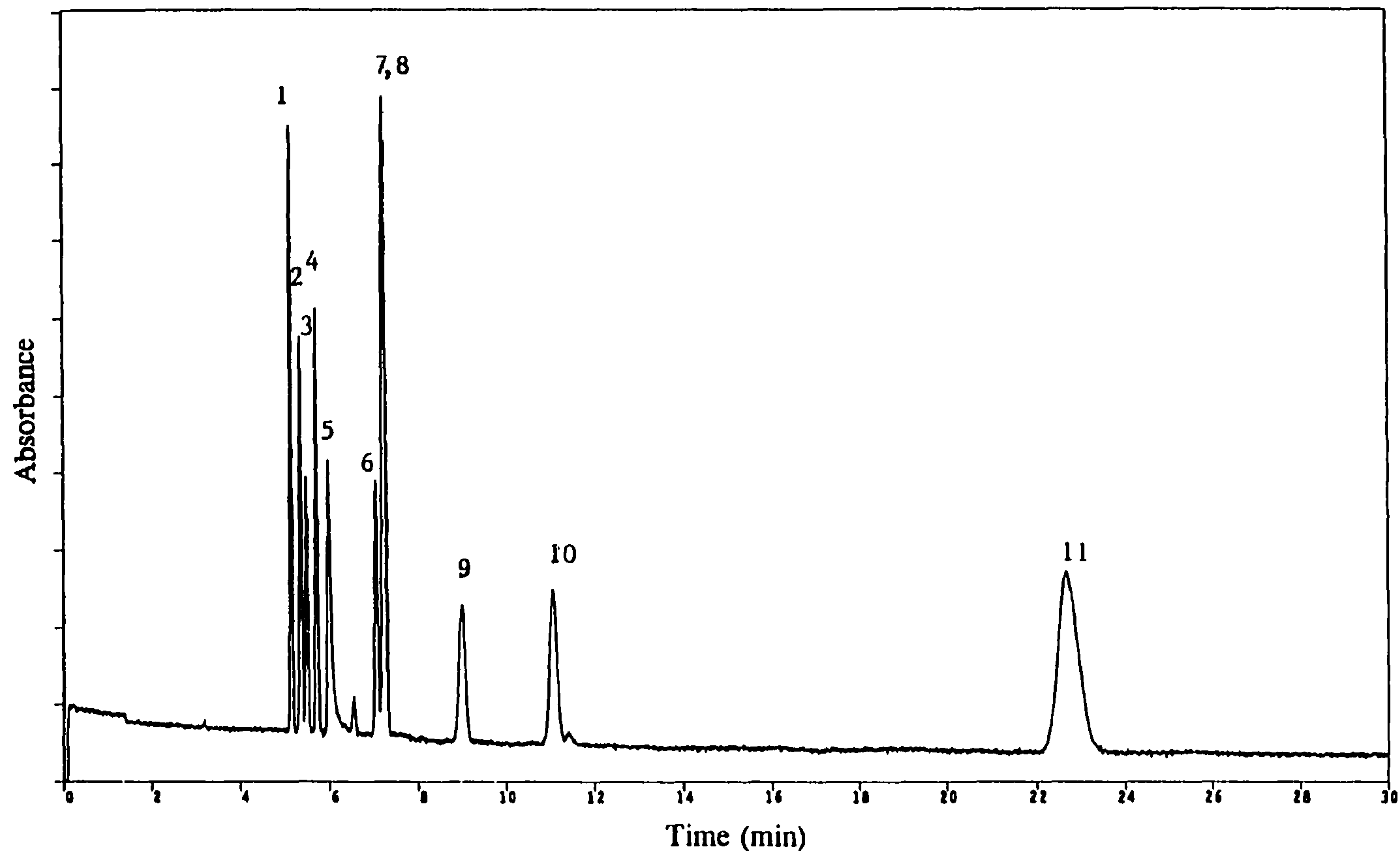

Figure 7. Electropherogram of organic anions. Capillary, 50 μm i.d., 33 cm length; carrier electrolyte, 20 mM borate, 220 mM sodium sulfate, pH 8.5; sampling time, 40 s; voltage, -10 kV; current, 280 μA; wavelength, 214 nm. Peaks: (1) 1,3,5-benzenetricarboxylic acid, (2) 1,2,4,5-benzenetetracarboxylic acid, (3) 1,3-benzenedisulfonic acid, (4) 1,2,4-benzenetricarboxylic acid, (5) 4,5-dihydroxy-1,3-benzenedisulfonic acid, (6) terephthalic acid, (7) isophthalic acid, (8) 1,2,3-benzenetricarboxylic acid, (9) phthalic acid, (10) 1,2-benzenedisulfonic acid, (11) benzenesulfonic acid.

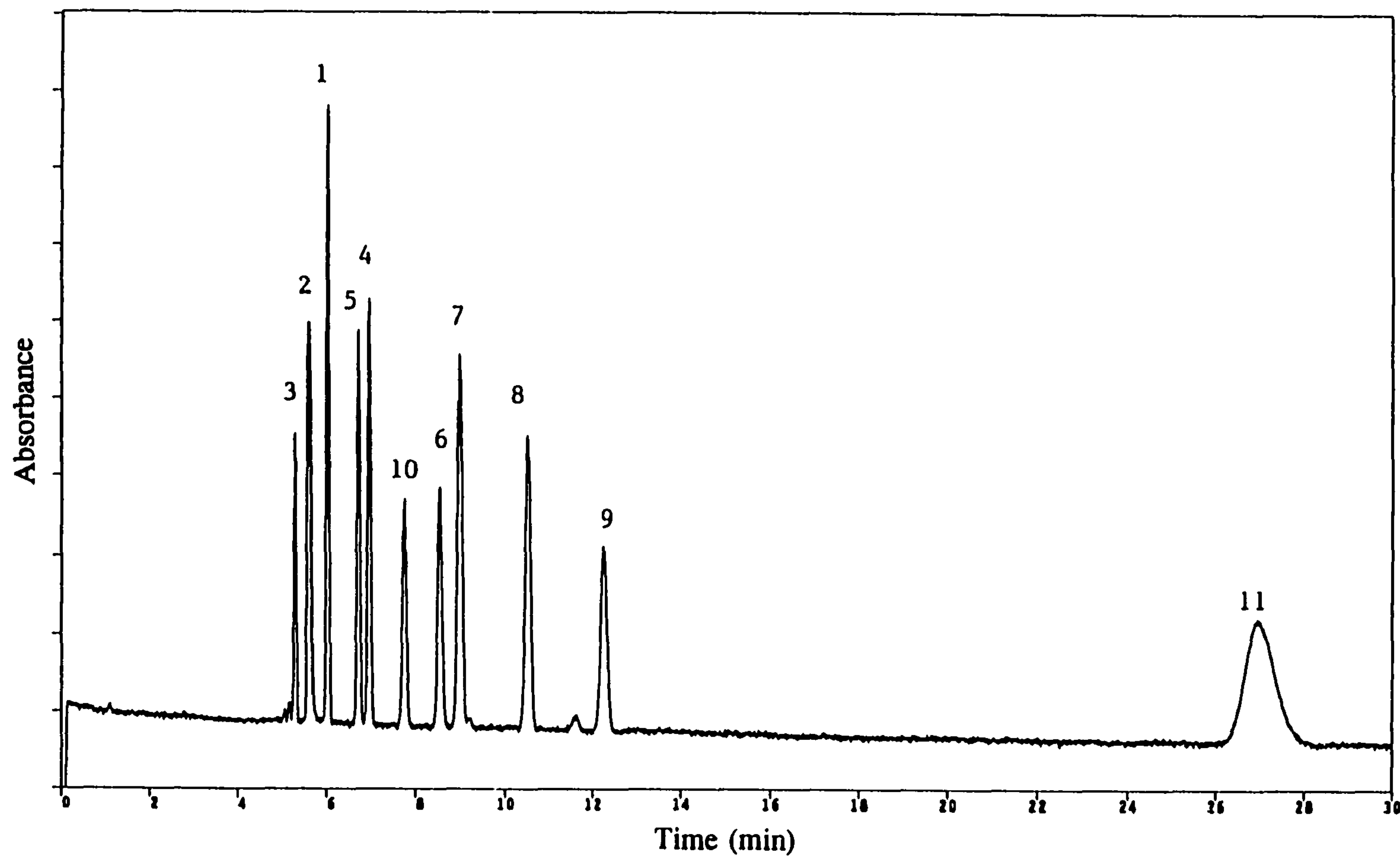

Figure 8. Electropherogram of organic anions. The experimental conditions were the same as those described in Fig. 7, except 220 mM lithium sulfate instead of sodium sulfate. Peak identifications see Fig. 7.

3.5 Practical applications

Seawater analysis is essential to oceanography, and the distribution of minor species such as iodide and bromide gives us clues for understanding the marine environment. Ion Chromatography [25-27] and electrochemical [28,29] methods have been used to determine trace iodide in seawater. Little if any work has been done on direct seawater analysis by capillary electrophoresis. Since sodium chloride concentration is seawater is ~0.5 M, we used 1.5 M sodium chloride with 20 mM borate as an electrolyte in order to get a good electrostacking. Seawater sample was injected directly without any dilution. The electropherogram is shown in Fig. 8. A nice bromide peak at 5.2 min was followed by a smaller nitrate peak. Because of low detection limits in CE we were not able to detect any iodide peak. A standard - addition method was used to determine the amount of bromide in seawater. Data from measuring from both peak area and peak height indicated the bromide concentration in this seawater sample was ~5.5 ppm. The migration time for bromide was very reproducible, with a relative standard deviation of 0.5% for 9 consecutive injections.

Anions in other high-salt samples, such as sodium sulfate and sodium perchlorate could also be analyzed directly. Figure 10a shows the electropherogram for separation of small amounts of bromide and nitrate in a 0.5 M sodium sulfate sample. The BGE contained 1.5 M sodium chloride. The nitrate peak is followed closely be a large, tailed peak due to sulfate. When the same sample was separated using 1.5 M sodium sulfate in the BGE, the only peaks obtained were bromide and nitrate (Figure 10b). The separation is slower in this case owing to the higher ionic strength of 1.5 M sodium sulfate compared to 1.5 M sodium

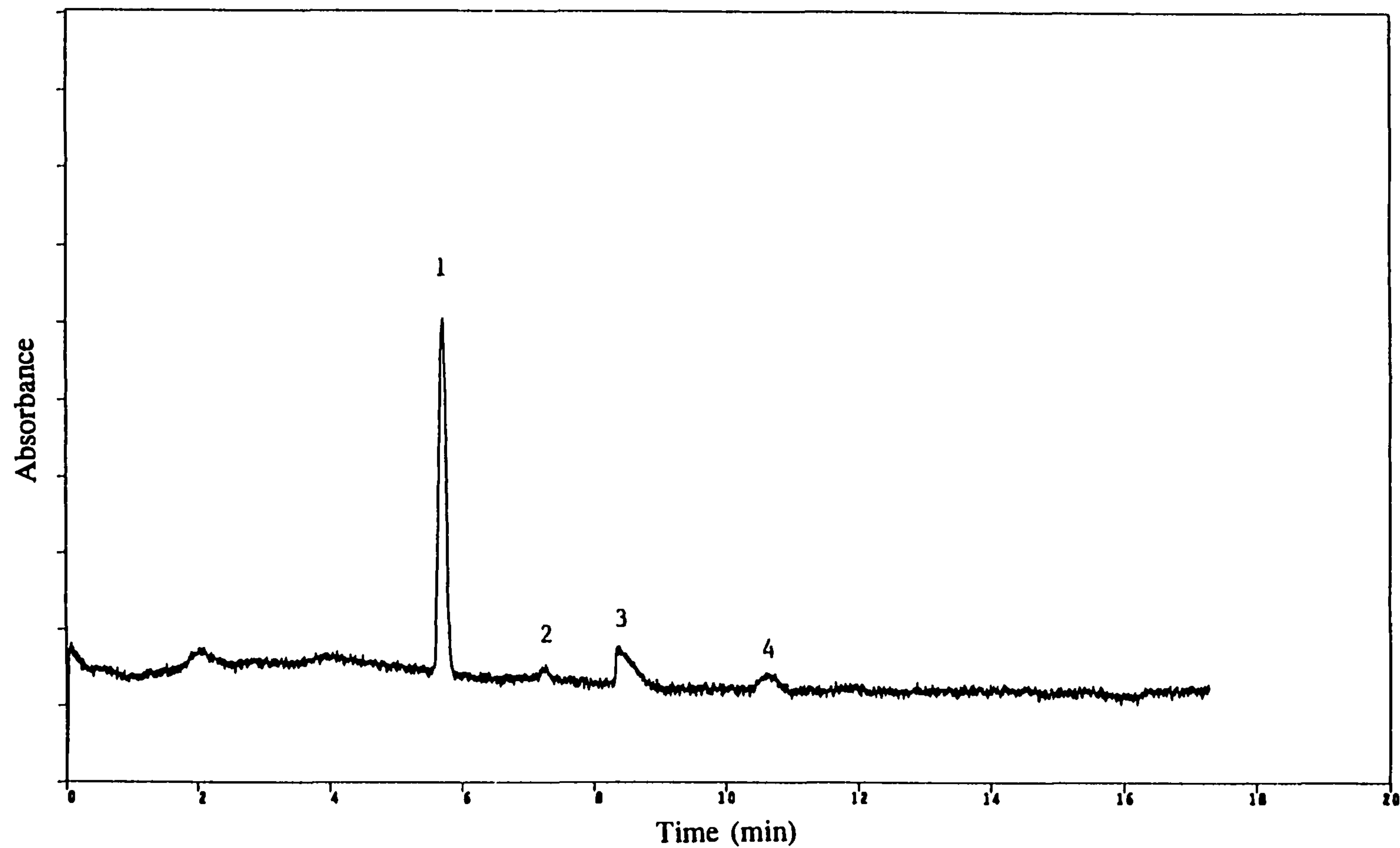

Figure 9. CE analysis of seawater sample. Capillary, 33 cm length, 50 μm i.d.; carrier, 1.5 M sodium chloride, 20 mM borate, pH 8.5; voltage, -10 kV; current, 280 μA; sampling time, 80 s; detection wavelength, 214 nm. Peaks: (1) Br^-, (2) NO_3^-, (3) unknown, (4) unknown.

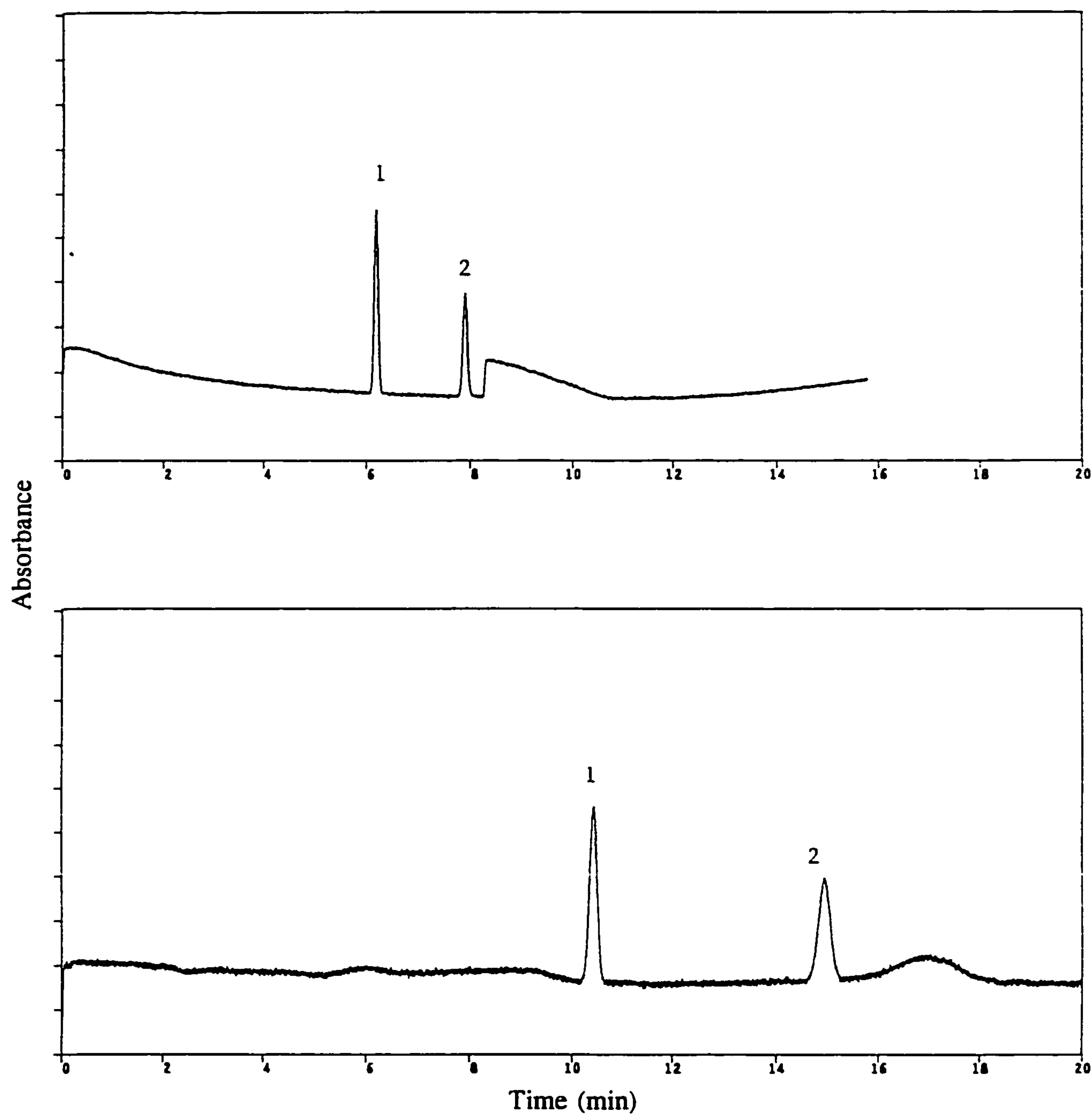

Figure 10. CE analysis of other high salt samples. Sample was in 0.5 M sodium sulfate. The experimental condition were the same as those described in Fig. 8, except (b) 1.5 M sodium sulfate was added in the electrolyte instead of sodium chloride. Peaks: (1) Br^-, (2) NO_3^-.

chloride. It was also possible to determine both bromide and nitrate in 0.5 M sodium perchlorate by using 1.5 M sodium perchlorate in the BGE. With 1.5 M sodium chloride in the BGE, perchlorate and nitrate co-eluted and only bromide could be measured.

4. Conclusions

Practioners of capillary electrophoresis have almost always kept the ionic concentration of the BGE rather low to avoid Joule heating and its associated effects such as peak distortion. It is now clear that perfectly acceptable electropherograms can be obtained, even with samples and electrolyte solutions containing a very high concentration of some salt. The key is to make the BGE salt content at least three times higher than that of the sample.

In the vast majority of publications, CE separation of anions are carried out by adding a long-chain quaternary ammonium salt as a flow modifier to coat the capillary surface and reverse the direction of electroosmotic flow. However, the use of such a flow modifier becomes unnecessary when the electrolyte contains a high concentration of salt. This is because the electroosmotic flow is reduced to a small value by a high salt concentration.

Anions in samples of high salt content such as seawater can be determined directly without dilution. Smaller amounts of anions in samples containing a salt such as sodium sulfate or sodium perchlorate can also be determined directly simply by using the same salt in the BGE. A major requirement is that the matrix salt have little, if any absorbance and that direct photometric detection of the sample anions can be used.

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CHAPTER 7. GENERAL CONCLUSIONS

A novel surfactant (lauryl polyoxyethylene sulfate) was developed to separate neutral organic compounds, especially for highly hydrophobic compounds. Its unique structure consisting of several oxyethylene groups, making it more hydrophilic than commonly used surfactant sodium dodecyl sulfate (SDS). It offers a significant advantage over SDS, such that it is suitable for a large variety compounds ranging from polyaromatic hydrocarbons (PAHs) to small polar compounds. Retention factor can be greatly increased by adding an organic solvent such as acetonitrile. A largest separation window is found where acetonitrile concentration is about 15-25%. Nonetheless, surfactant concentration is another experimental parameter that can be varied to manipulate migration times. Excellent separations are obtained for PAHs as well isomers, normal and deuterated acetophenones.

Separation of neutral compounds at acidic condition is successfully done for the first time. At pH 2.4, electroosmotic flow is virtually eliminate due to deprotonation of silanol groups on the capillary wall. This separation mode has several advantages over conventional MEKC (basic) mode. Fast migration times can be achieved because there is no counter-migration. Large, hydrophobic compounds migrate first owing to strong association with the surfactant. Second, acidic condition may add another dimension to basic compound separation owing to ion-pair interaction. The method gives excellent results for the separation of PAHs, basic compounds, alkyl substituted phenols, and acidic drugs and basic drugs. Using two organic solvents together, such as acetonitrile and isopropanol, different selectivity can be attained.

Protein or peptides adsorption on the capillary surface is effectively suppressed by incorporation of a cationic surfactant, namely cetyltrimethylammonium bromide (CTAB) in the running electrolyte. The surfactant can be employed at a low concentration below cmc so that peak broadening caused by protein-micelle interaction can be eliminated. High resolution and efficiency can be obtained after a suitable buffer and pH is carefully chosen. This method is proven to be simple and give excellent reproducibility.

Chiral selectivity in capillary electrophoresis can be manipulated easily by changing chiral surfactant structures, including in both chiral functional group and achiral hydrocarbon tail. Chiral resolution increases with the surfactant concentration before it reaches a maximum. An enhanced chiral resolution can be obtained by adding two totally different selectors in the same electrolyte. Baseline resolution of DL-dansyl amino acids enantiomers are attained using this mixture of selectors. It is concluded that combination is not confined to two selectors, more chiral additives can be used together to achieve some difficult enantiomeric separations.

It is found that good electropherograms can be obtained even with samples and electrolytes containing a very high concentration of salt, provided the electrolyte solution has a higher salt content (three times higher) than that of the sample. One advantage of the use of high salt content in electrolyte is that electroosmotic flow is reduced to a small value. A flow modifier, usually a quaternary ammonium (Q^+), is not needed in anion separations. This is particularly important for some anions that will precipitate with Q^+. Anions in seawater such as bromide and nitrate can be determined directly using the same salt (sodium chloride) in the electrolyte.

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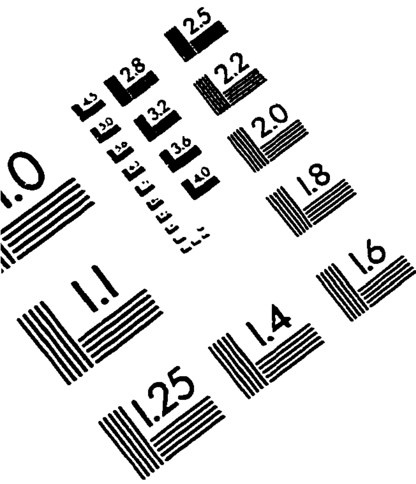
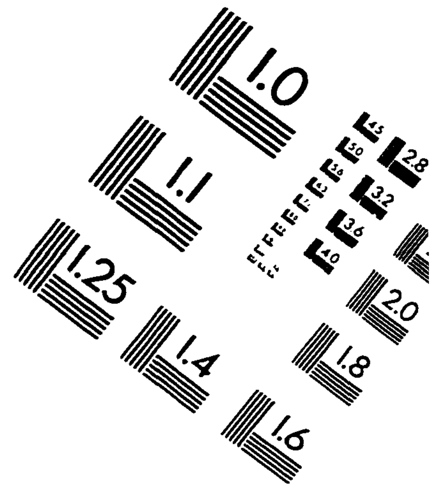
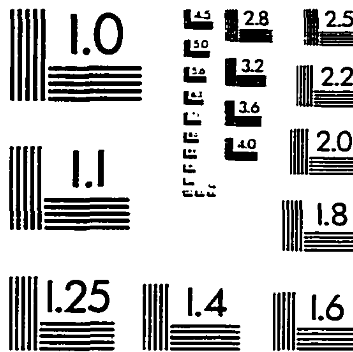
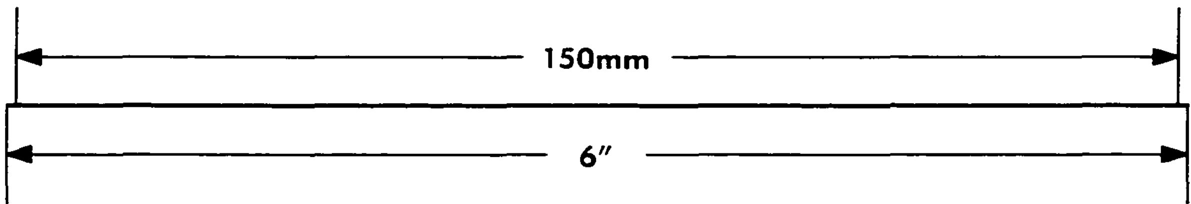

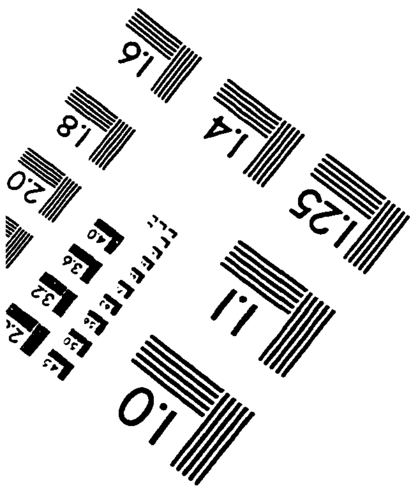
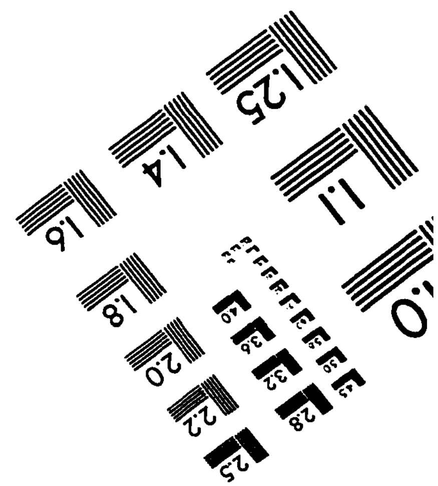

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