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DEVELOPMENT OF CAPILLARY ELECTROPHORETIC TECHNIQUES FOR BIOLOGICAL ANALYSIS

by

CHAO-CHENG WANG

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2001

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ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

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Name of the Candidate Chao-Cheng Wang

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Title Development of Capillary Electrophoretic Techniques for Biological Analysis

In this dissertation, two areas of capillary electrophoretic techniques, capillary gel electrophoresis and capillary zone electrophoresis, were explored for biochemical and biomedical applications.

A means for casting step gradients in linear polyacrylamide gel concentration for capillary electrophoresis (CE) was reported in the first manuscript. A UV-Vis whole-column detector is used to profile the gel gradients cast in the capillary, while detection of fluorescein-labeled proteins is accomplished with an epi-illumination laser-induced fluorescence whole-column detection system. The potential of gel gradients in CE for the analysis of wide molecular mass range protein or peptide samples is demonstrated.

In the second manuscript, an on-column isotachophoretic (ITP)-CE system capable of pre-concentrating polyhydroxyl species was described to fullfill the need of high-sensitivity micro-scale analytical methods for the analysis of plasma adenosine. Borate buffer was used as the CE running buffer to charge the neutral diol for separation. It also functioned as the terminating electrolyte for ITP sample pre-concentration prior to the subsequent CE separation. Detection sensitivity of polyhydroxyl species, such as catechols and ribonucleosides, was enhanced due to the increase of sample loadibility of the CE system. Adenosine was derivatized with chloroacetaldehyde to form a fluorescent derivative, and

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laser-induced fluorescence (LIF) detection was used to further improve the detection sensitivity. Quantitative measurement of adenosine as low as 10^{-9} *M*, the levels expected in plasma, was reported.

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DEDICATION

This dissertation is dedicated to my parents,

Ching-Dar and Jiin Chiou Wang,

and to my wife

Wan-Ting

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ACKNOWLEDGMENTS

Special thanks to my committee chair, Dr. William Nonidez, and all the members of my graduate advisory committee, Drs. Donald Muccio, William McCann, and Thomas Nordlund, for all their assistance during my years in graduate school and their guidance, suggestions and help in the preparation of this dissertation.

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LIST OF ABBREVIATIONS

CE	capillary electrophoresis
ITP	isotachophoresis
LIF	laser induced fluorescence
HPLC	high performance liquid chromatography
PAGE	polyacrylamide gel electrophoresis
M	molar concentration
I.D.	inner diameter
O.D.	outer diameter
CZE	capillary zone electrophoresis
CGE	capillary gel electrophoresis
EOF	electroosmotic flow
MEKC	micellar electrokinetic capillary chromatography
CIEF	capillary isoelectric focusing
CITP	capillary isotachophoresis
CMC	critical micellar concentration
PCR	polymerase chain reaction
SDS	sodium dodecylsufate
pI	isoelectric point
OPL	optical path length

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S/N	signal-to-noise ratio
FTH	fluorescein isohydantoin
CBQCA	3-(4-carboxybenzoyl)-2-quinoline-carboxaldehyde
NBD	4-clair-7-nitro benzofuran
OPA	O-phthaladehyde
FITC	fluorescein isothioiyanate
NDA	naphthalenedialdehyde
MS	mass spectrometry
GC	gas chromatography
CF-FAB	continuous-flow fast atom bombardment
ESI	electrospray ionization
EDTA	ethylene diamine -N,N,N',N'- tetraacetic acid
EGTA	ethylene glyco bis(β-aminoethyl ether)-N,N,N',N'- tetraacetic acid
MC	methyl cellulose
%T	grams acrylamide per 100 ml of solution
%C	grams Bis per 100 ml of solution
TEMED	N,N,N',N'-tetramethylethylenediamine
Ins	insulin
CytC	cytochrome *c*
LYZ	lysozyme
MYO	horse heart myoglobin
TPI	soybean trypsin inhibitor

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CTPG	chymotrypsinogen
CA	bovine carbonic anhydrase
APS	ammonium persulphate
BSA	bovine serum albumin
Tris	Tris(hydroxymethyl)aminomethane
CAA	chloroacetaldehyde
ADO	adenosine
TMS	trimethylchlorosilane
HEC	hydroxyethyl-cellulose
ε-ADO	etheno-adenosine

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INTRODUCTION

Separation technology plays a significant role in the development of current biological and biomedical research. The most powerful separation techniques used today are chromatography and electrophoresis. These two categories of techniques have been used for decades as analytical tools to facilitate process monitoring and quality control as well as quantitative determination. With different, but complementary, separation mechanisms, these two methods have provided the capability of analyzing a wide variety of sample species.

The analysis of minute quantities of micro- and macro- molecules is an important problem in bioanalytical research and poses a challenge to biological science researchers. Traditional analytical separation methods, such as high performance liquid chromatography (HPLC) and gel electrophoresis, typically require samples with volumes in the micro-liter range and hardly reach subpico-mole sensitivities. Due to the recent revolution in life science research, the need for high-resolution and high-sensitivity analysis has lead the advance of bioanalytical techniques to a new level. One of the main trends in recent development of separation techniques is to achieve the theoretical promise of high efficiency by reducing the dimensions of the separating column. The use of micro-meter-sized capillary tubes offers the potential for high separation efficiencies as well as the capability of working with nanoliter sample volume [1].

Capillary electrophoresis (CE) is a relatively new separation technology which combines aspects of both electrophoresis and HPLC in a micro-column format. Like

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electrophoresis in which the separation depends on differential migration in an electrical field, CE techniques are analogous to most conventional electrophoresis methods, such as zone electrophoresis, isoelectric focusing, and sieving separations. Like HPLC in which the detection is accomplished on-line as the separation progresses, CE techniques are well adapted to the on-line detection format, such as UV-Vis detection or fluorescence detection methods. Therefore, the staining and destaining steps which are required in conventional gel electrophoresis methods to visualize the separations, are eliminated in the CE system. This allows CE to be automated. With the features of high speed, high separation efficiency, minimal sample volume and ease of automation, CE techniques have been well accepted as a highly promising bioanalytical technique and applied in analysis of amino acids, peptides, proteins, DNA, and drugs, among many other compounds [2-8].

Historical Background

Separation by electrophoresis, which was first introduced by Tiselius in 1937 [9], is based on differential migration of solutions in an electric field. The conventional electrophoresis is employed in a slab-gel format, and the gel staining, destaining, and drying steps are required to visualize the separations. Several kinds of gel media, such as starch, agar, and polyacrylamide, are used to prevent the convection caused by Joule heat resulting from the passage of current through the buffer solution. These gel media also provide a sieving matrix that achieves separation primarily based on molecular size and mass in addition to molecular charge. Biopolymers such as DNA fragments, which possess the same charge density but differ only in molecular mass, are routinely separated by electrophoresis. As a result, electrophoresis, in particular polyacrylamide gel electrophoresis (PAGE), became a

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powerful tool for biopolymer analysis. However, conventional gel electrophoresis as practiced today is still limited by Joule heating and tends to be a slow and labor-intensive technique that is prone to relatively poor reproducibility. Also, the detection aspect of slab gels limits its quantitative capability, and it is not easily amenable to full automation.

Performing electrophoresis in small tubes or capillaries is an effective way to minimize the effects of Joule heating. The feasibility of performing electrophoresis in narrow-bore tubes was first suggested by Hjerten [10] in 1967. He performed free solution zone electrophoresis in 3000-μm I.D. glass tubes using a relatively complex UV detector to specify the separation zones. In 1974, Virtanen [11] extended this work with 200~500-μm I.D. glass tubes using potentiometric detection. His work dealt with zone electrophoresis and addressed many of the unique advantages of using small-diameter tubes. The efficiency obtained in these studies was not high, but the work was carried out at relatively low voltage (5000 V/meter). In 1980, Mikkers and co-workers [12] refined the technique to Teflon tubes of 200-μm I.D. and obtained separation of 16 organic acids by zone electrophoresis with both UV and conductivity detection. The resulting output showed detector response with time and was termed as electropherogram by analogy with the term chromatogram. The small-diameter tubes reduced diffusion due to convection. Plates heights less than 10 μm were achieved. Although the separations obtained by Mikkers were excellent, the peak shape was poor due to the limitation of high sample loading required by the equipment.

The landmark of current development capillary zone electrophoresis (CZE) was largely estabilished by Jorgenson and Lukacs [2-4], who advanced the technique by using 75-μm I.D. fused silica capillary with on-column detection in the early 1980s. The significant heat dissipation offered by the small-diameter capillaries permitted the use of high voltages

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up to 30 kV, resulting in both high separation efficiency and rapid analyses. In this work, excellent separation of fluorescent derivatives of amino acids and peptides was demonstrated with theoretical plates in excess of 400 000 and plate height of only few micro-meters. Since then, CE techniques have been developing rapidly. Several other techniques related to CZE were developed, such as micellar electrokinetic chromatography introduced by Terabe et al. [13] for non-ionic sample analysis and capillary gel electrophoresis (CGE) introduced by Hjerten [14] for size-based separation. Also, many improvements with respect to the detection systems, such as laser-induced fluorescent (LIF) detection [15,16] for higher detection sensitivity as well as capillary modification techniques [17,18] for better performance have been achieved.

Basic Principles of Capillary Electrophoretic Techniques

A general schematic diagram of a capillary electrophoresis system is shown in Fig. 1. A buffer-filled fused silica capillary is placed between two buffer reservoirs, and a high voltage power supply is used to apply an electric field across the capillary. The inner diameter of the fused silica capillaries commonly used in current CE systems is in a range of 10-100 μm. The small diameter capillary allows for efficient heat dissipation through the capillary wall. High potentials, up to 500 V/cm, can be used in CE without causing convection in the capillary from Joule heating, which allows for rapid separations.

The sample is introduced into the capillary by replacing one of the buffer reservoirs with a sample reservoir then applying either an electrical field (electrokinetic injection) or an external pressure (hydrodynamic injection). Application of a voltage across the capillary causes electrophoretic and electroosmotic movements resulting in the migration of the sample

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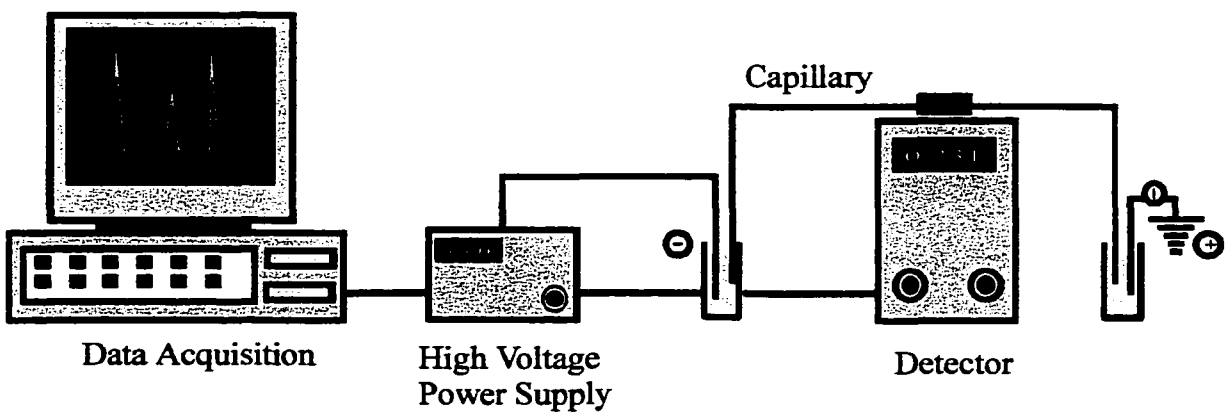

Fig 1. Schematic diagram of CE instrumentation.

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species along the capillary and passing through the on-line detector. Generally, the system operation and data acquisition are controlled by a computer and can be easily automated.

Separation by CE is based on the migration velocity differences of charged solutes in an applied electric field [2-4]. The electrophoretic migration velocity of charged solutes, υ_{ep}, is related to their electrophoretic mobilities and the magnitude of the applied electric field strength, described as

$$\upsilon_{ep} = \mu_{ep} \times E = \mu_{ep} \times (V / L) \quad (1)$$

where μ_{ep} is the electrophoretic mobility, E is the applied electric field, and V is the applied voltage across the length, L, of the capillary. The electrophoretic mobility, for a given ion and medium, is a constant which is related to the charge/mass ratio of the solute and is given by

$$\mu_{ep} = q / (6 \pi \eta r) \quad (2)$$

where q is the number of charges, η is the solution viscosity, and r is the hydrodynamic radius of the ion [9]. From this equation it is evident that small, highly charged species have high mobilities, whereas large, minimally charged species have low mobilities.

Another fundamental constituent of CE is electroosmosis. It can be described as a relative motion of a liquid to a fixed charged surface which originated from the double layer at the surface under an applied electric field [19]. The bulk flow of solvent under an applied electrical field is called electroosmotic flow (EOF). In a CE fused-silica capillary, the solid-liquid interface is surrounded by solvent and solute molecules that are not oriented as in the bulk of solution. Under normal aqueous conditions with small binary electrolytes, the solid surface of the capillary wall has an excess of anionic charge resulting from the ionization of acidic surface silanol groups. The negatively charged silanoate groups then attract positively

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charged counterions from the buffer solution and form the diffusion double layer of cations, including an inner fixed layer and an outer mobile layer. When an electric field is applied, the mobile, outer layer of cations migrates toward the cathode. Because these ions are solvated, they drag the bulk buffer solution with them, thus causing EOF. The magnitude of EOF can be expressed in terms of velocity or mobility as given in the following equation [20]:

$$\upsilon_{eof} = (\varepsilon\zeta / 4\pi\eta) \times E \qquad (3)$$

or

$$\mu_{eof} = \varepsilon\zeta / 4\pi\eta \qquad (4)$$

where υ_{eof} is the velocity, μ_{eof} is the electroosmotic mobility, ε is the dielectric constant of the buffer solution, and ζ is the zeta potential created across the diffusion double layer. The zeta potential is essentially determined by the surface charges that are related to the nature of the solid and the pH of the buffer solution. It is also dependent on the ionic strength of the solution [21]. For fused silica at high pH where the silanol groups are predominantly deprotonated, the EOF is significantly greater than at low pH where they become protonated. Also, solutions with high ionic strength result in double layer compression, decreased zeta potential, and reduced EOF.

The EOF of the bulk solution is superimposed on the electrophoretic migration of the analytes. The net velocity of the analytes is the vectorial sum of electroosmotic and electrophoretic velocities and can be given as

$$\upsilon_{net} = (\mu_{ep} + \mu_{eof}) \times V / L \qquad (5)$$

The time, t, for a solute to migrate from the injection point to the detection point, or the

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effective column length, l, can be expressed as

$$t = l / \upsilon_{net} = l L / (\mu_{ep} + \mu_{eof}) V \quad (6)$$

Generally, EOF can be sufficiently stronger than the electrophoretic migration at neutral to alkaline pH such that all species are swept toward the cathodic end of the capillary. Therefore, the presence of EOF can permit the simultaneous analysis of cations, anions, and neutral species. However, neutral species will not be separated from each other since they all migrate at the same velocity of EOF.

A unique feature of EOF in the capillary is its flat, plug-like, flow profile. In a pressure-driven system such as HPLC, the frictional forces at the liquid-solid interface result in substantial pressure drops [22]. In open tubes, the frictional forces are severe enough to result in laminar or parabolic flow profiles even at low flow rates. The flow in this system is fast in the center of the tube and gradually decreases to zero at the surface capillary. This flow gradient can result in substantial band broadening. In the electrically driven system, the driving force of EOF is uniformly distributed along the capillary. There is no pressure drop within the capillary, and the flow is nearly uniform throughout. This results in a flat profile that contributes very little to band broadening during migration. A comparison of the flow profiles is shown in Fig. 2.

In the CE separation system, the lack of a stationary phase and the flat flow profile result in longitudinal diffusion as the major source of band broadening. The variance of the migration zone, σ^2, can be described by the Einstein's law of diffusion as

$$\sigma^2 = 2Dt = 2Dl L / (\mu_{ep} + \mu_{eof}) V \quad (7)$$

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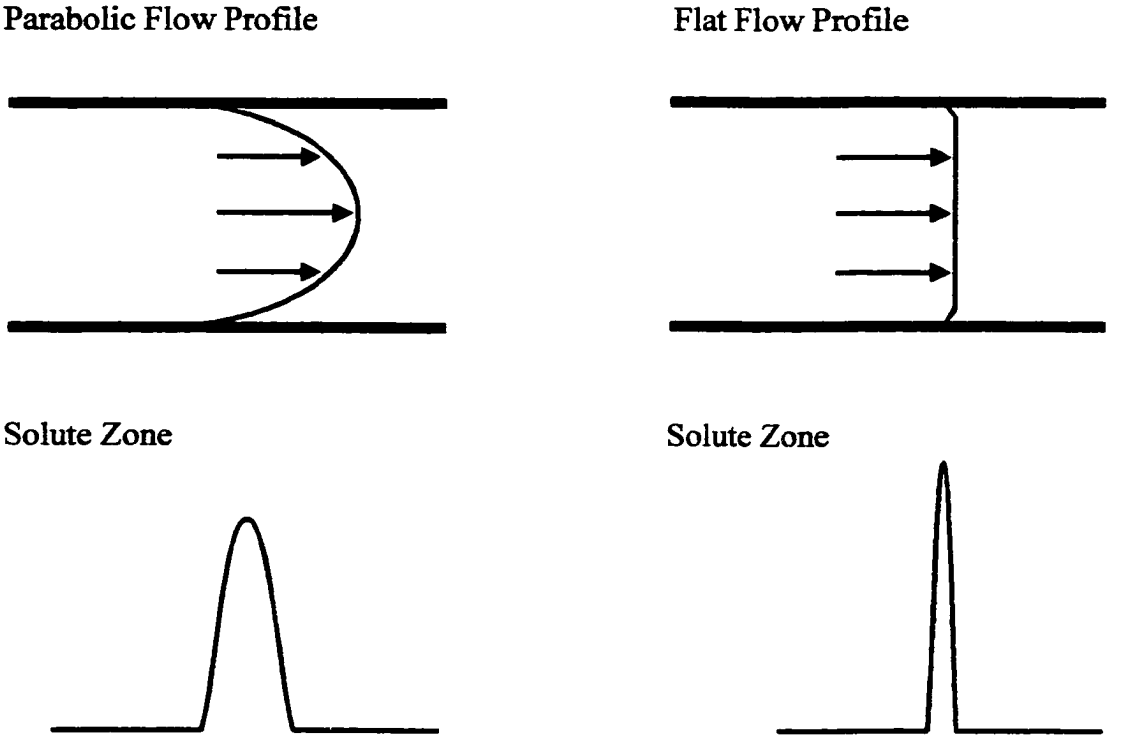

Fig. 2. Cross-sectional flow profiles and zone broadening effects of laminar flow and EOF.

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The separation efficiency of the CE system can be expressed in terms of the number of theoretical plates, N, as

$$N = l^2 / \sigma^2 = (\mu_{ep} + \mu_{eof}) V / 2 D_m \quad (8)$$

where D_m is the diffusion coefficient of solute in the buffer system [2,3,23]. Since the number of theoretical plates is directly proportional to the applied voltage, the highest voltage possible is suggested to obtain high plate numbers. The higher the applied voltage, the less time the solute spends in the capillary and therefore the higher will be the number of the heoretical plates. Thus, higher applied voltage yields faster separation with higher efficiency. However, the practical voltage used in CE today is generally kept below 30 kV to prevent band broadening caused by high Joule heating.

Since the separation by CE is based on the difference in migration velocity or mobility, the resolution, Rs, of two zones in CE can be given by the equation

$$Rs = 0.25 \sqrt{N} (\Delta \upsilon / \upsilon) \quad (9)$$

where N is the average number of theoretical plates, $\Delta\upsilon$ the difference in zone velocity, and υ is the average zone velocity [23]. Substituting Equation 5 and Equation 7 into Equation 8, the resolution can be expressed as

$$Rs = \frac{1}{4\sqrt{2}} (\mu_{ep,1} + \mu_{ep,2}) \sqrt{\frac{V}{D_m (\mu_{ep,ave} + \mu_{eof})}} \quad (10)$$

where $\mu_{ep,1}$ and $\mu_{ep,2}$ are the electrophoretic mobilities for the two solutes, and $\mu_{ep,ave}$ is the average electrophoretic mobility [1,2].

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Separation Modes of CE

CE comprises a family of techniques that have dramatically different operative and separation characteristics. The fundamental feature for each electrophoretic separation is based on the nature of the electrophoretic buffer systems. Either continuous or discontinuous buffer systems can be used in CE. In the continuous buffer systems, the composition of the electrolyte is constant along the migration path, and the electric potential and the electrophoretic mobilities of the analytes remain constant. CZE, CGE, and micellar electrokinetic capillary chromatography (MEKC) are examples for this type of electrophoretic methods. In discontinuous systems, the composition of the electrolyte changes along the migration path, the electric potential, and the electrophoretic mobilities of the analytes are changing until they reach a steady-state process. Capillary isoelectric focusing (CIEF) and capillary isotachophoresis (CITP) belong to this type of electrophoretic method.

Capillary zone electrophoresis (CZE). CZE, also known as free solution capillary electrophoresis, is the most basic but important technique in CE because of its simplicity and separation power. The principles of CZE have been described above. Separation in CZE is solely based on the electrophoretic mobility differences of analytes. A prerequisite to CZE application is that the analytes, which will migrate under an applied electric field, must contain electrical charges. Electrically neutral compounds will co-elute with EOF without mutual separation. Separation selectivity in CZE can be altered simply through changes in running buffer pH, which will influence the electrophoretic mobility by modifying the mass/charge ratio of the analytes. CZE has been applied to the analysis of a very wide variety of samples

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including inorganic ions [24,25], organic acids and bases [26-28], amino acids [2,29,30], peptides [6,31,32], and proteins [6,33-35].

Micellar electrokinetic capillary chromatography (MEKC). MEKC was first introduced by Terabe et al. in 1984 [13], and has become one of the most widely used CE techniques. It is distinguished because it is the only electrophoretic technique that can accomodate the separation of electrically neutral molecules as well as charged ones while retaining the advantages of the capillary electrophoresis format.

MEKC is a hybrid of electrophoresis and chromatography. The key difference between MEKC and normal CE is the addition of a surfactant into the running buffer. When the concentration of surfactant is above its critical micelle concentration (CMC), aggregates of surfactant molecules form a micellar phase, which acts as a pseudo-stationary phase. All classes of surfactants, including nonionic, ionic, and zwitterionic surfactants, can be used in MEKC; however, generally, ionic surfactants are more useful. Similar to the retention mechanism in reverse-phase chromatography, retention of nonionic solutes in this system is based on hydrophobicity and partitioning into the micelles. While hydrophobic solutes interact strongly with the micelle phase and are therefore retained longer, hydrophilic solutes are carried through the capillary at the rate of the EOF and are the first to elute. All neutral solutes will elute in the time intervals that are given by the migration time of the EOF (t_{EO}) and the micelles (t_{MC}), which represent the retention time of a solute totally insoluble and soluble in the micelle, respectively. Separation of neutral solutes is due to the differences in their distribution between the micelles and the aqueous buffer solution. Ionic molecules may be separated based on both the differences in electrophoretic mobilities and the interaction

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with the micelles. MEKC has been used to separate charged solutes with identical mobilities that could not be separated by CZE [36].

Since the separation mechanism of neutral solutes in MEKC is essentially chromatographic, the retention of the neutral solutes is determined by the capacity factor, k', which is the ratio of the total moles of solute in the micelle to those in the mobile phase. The factor is defined as [37]

$$k' = K\left(\frac{V_{MC}}{V_{MP}}\right) = \frac{(t_r - t_{EO})}{t_{EO}\left(1 - \frac{t_r}{t_{MC}}\right)} \qquad (11)$$

where V_{MC} and V_{MP} are the volumes of the micellar phase and the mobile phase, respectively, K is the partition coefficient, t_r is the migration time of the solute, and t_{EO} and t_{MC} are the migration time of the solute that is totally insoluble or soluble in the micelles, respectively. The selectivity of solutes in MEKC can easily be manipulated by changing the k' value, which is depend on the physical nature of the micelle, such as the type of surfactants and its concentration. Modifiers such as urea or organic solvents also can be used to manipulate solute-micelle interaction and change the selectivity in MEKC. The use of organic modifier may also reduce EOF; therefore, the overall peak capacity will increase due to the extension of the separation window between the t_{EO} and t_{MC}. MECC is suitable for small hydrophobic compounds. A wide range of applications, including pharmaceutical [38,39], aromatic hydrocarbons [40], and explosive constituents [41], have been reported.

Capillary gel electrophoresis (CGE). Gel electrophoresis has principally been used in biological sciences for the size-based separation of macromolecules, such as proteins and

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nucleic acids. Gels were initially used in conventional electrophoresis as anti-convective media with additional function of sieving matrix. Separation is based on the sieving effect, i.e., the differences in the abilities of different-sized analytes to migrate through the gel matrix. As charged solutes migrate through the gel matrix they become hindered, with larger solutes hindered more than smaller ones. With the successful development of the CE techniques, utilization of polymer gels as separation media has transferred well from conventional slab-gel electrophoresis to the instrumental CE system. The CE format can offer several advantages over traditional slab-gel electrophoresis, such as the use of 10-100 times higher electric field, resulting in faster separation than in conventional slab-gel format, on-column detection, and instrumental automation. Resolution and efficiency in CGE are comparable to those obtained in CZE with efficiency of 10^{-6}-10^{-7} theoretical plates per meter [42,43].

Early work with CGE involved cross-linked gels [44] with a fixed pore structure, such as cross-linked polyacrylamide, which is one of the most commonly used gel matrix in the slab-gel system. However, the routine preparation of homogeneous stress-free gels in capillaries is difficult due to polymerization-induced shrinkage and appearance of air bubbles inside the capillaries [45]. Several approaches have been tried to overcome this problem, such as performing gel polymerization under high pressure to compensate for the shrinkage and avoid air bubble formation [46], or polymerizing the monomer solution gradually along the capillary by electrophoretically migrating the catalyst and initiator into the capillary, allowing the gel to adjust to the volume change during the reaction without forming air bubbles [47]. Recently, linear (non-cross-linked) polymers have been applied in CGE as macromolecular sieving media. The physical entanglement of the long polymer chains provides dynamic pore structure for sized-based separation. Similar to those in cross-linked gels, the pore size of

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linear gels is dependent on the polymer concentrations. The linear gel columns generally experience less bubble formation problems and are relatively easy to be prepared [48]. Some linear gel columns can be refreshed by introducing new gel into the capillary due to the relatively low viscosity of the linear gel; therefore, elimate the contamination of gel matrix and allow better reproducibility of the separation [49-50]. Several non-cross-linked polymers have been used as the sieving matrix in CGE, such as linear polyacrylamide [48,51-53], polyethylene glycol [54], polyvinyl alcohol [55], and various derivatives of cellulose [45,56,57].

In CGE, solutes are separated on the basis of size, so the technique is well suited for the analysis of charged molecules that vary in size, but not their charge-to-mass ratios, such as oligonucleotides [44,50,51], Polymerase Chain Reaction (PCR) products [58,59], and DNA restriction fragments [60,61]. Addition of a nucleotide to these biopolymers changes their charge and size proportionally, so their charge-to-mass ratios remain the same. The separation of proteins in CGE is commonly performed under denaturated conditions in the presence of sodium dodecylsulfate (SDS) [50,52,62,63]. SDS disrupts the tertiary structure of proteins and binds to proteins at the same weight ratio, resulting in all SDS-protein complexes having nearly the same charge-to-mass ratio with similar rod-shaped conformation. Generally, a calibration plot of the log molecular mass of the protein against migration time (mobility) can be employed to estimate the molecular mass of the proteins.

Capillary isotachophoresis (CITP). Isotachophoresis (ITP) is another electrophoretic technique closely related to CZE. Similar to CZE, separation in CITP is based on the differences in electrophoretic mobilities, but all sample zones migrate with the same velocity

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as the steady state is established. This technique allows higher sample loading and is known to be an efficient means of sample concentration [64-65].

Generally, CITP is performed in a discontinuous buffer system. Sample solution is injected into the capillary between a leading electrolyte and a terminating electrolyte, which are chosen to have higher and lower mobility than all sample molecules, respectively. Under an applied constant electric current, the leading electrolyte moves rapidly toward the electrode, thus reducing the ion concentration at the interface of the sample zone. As a result, the electric field strength increases according to the Ohm's law with decreasing mobility or conductivity of consecutive zones. Separation is achieved due to the different velocities of the analytes in the mixing zone. Each analyte will condense into discrete zones until the steady-state velocity is reached. The focused zones are in immediate contact with each other in order of decreasing mobility and migrate down the capillary with the same velocity. Concentration of each analyte in its ITP zone is dependent on the composition and concentration of the leading electrolyte and can be described by the Kohlrausch equation [66]:

$$C_x = C_L \frac{\mu_X (\mu_L + \mu_R)}{\mu_L (\mu_X + \mu_R)} \frac{Z_L}{Z_X} \qquad (12)$$

where C_L and C_X are the concentrations of the leading electrolyte and sample ion, and Z_L and Z_X are the charges of the leading electrolyte and sample ion, respectively. Since the focused sample zones are in immediate contact with each other, it is difficult to resolve individual analytes in CITP with an universal UV detector, unless some non-UV-absorbing spacers are used to form discriminating zones between UV-absorbing analytes [67]. Conductivity detection is usually used for CITP to display a stepwise isotachopherogram. Each of the plateaus from the conductivity detection represents one of the focused zones [68,69].

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For dilute samples CITP leads to a concentration of the analytes, and it has been used as a pre-concentration technique for CZE. Generally, two instrumental arrangements for coupling ITP with CZE (ITP-CZE) have been described. A coupled-column arrangement allows large sample loading and provides a sensitivity improvement up to 1000-fold, but requires a sophisticated experimental design with ITP in one column and CZE in another separation column [70-74]. In the second arrangement ITP is directly coupled, on-column, to CE with automatic transition to a CE separation mechanism, providing concentration enhancements of 50-100 fold without any modification of the CE instrumentation [74-76].

Capillary isoelectric focusing (CIEF). Isoelectric focusing (IEF) is a unique technique among the electrophoretic modes. This technique involves separation of amphoteric sample components in a pH gradient. In contrast to other electrophoretic techniques where the separation is based on differences in electrophoretic mobilities, in IEF analytes are separated on the basis of their isoelectric point (pI) [19]. In CIEF, the capillary is filled with sample and the carrier ampholytes. The ampholytes contain a series of zwitterionic compounds with various pI values, which in addition display a high buffering capacity at their pI. Under the effect of applied electric field with OH^- and H^+ as catholyte and anolyte, the ampholytes align themselves along the capillary according to their pI, thus forming a steady, buffered pH gradient. Meanwhile, the charged sample molecules, usually proteins or peptides, migrate toward the attracting electrode and focus at a pH region corresponding to their pI, where they become electrically neutral and stop migrating. The status of the focusing process is indicated by the current. Once complete, a steady state is reached and current no longer flows. A mobilization step is required to drive the focused

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sample bands toward the detector, which is configured in the on-column format as in other CE techniques. Two mobilization techniques are commonly used, pressure elution [77] and electrophoretic elution [78,79], which is accomplished by changing the pH or adding salt to one of the buffer reservoirs.

Since the sample is filled into the whole capillary, the sample loadability is significantly higher than in most CE modes, resulting in high detection sensitivity. Resolution with respect to the pI difference is given by [80]:

$$\Delta pI = 3\sqrt{\frac{D\frac{dpH}{dx}}{E\frac{-d\mu}{dpH}}} \qquad (13)$$

where D is diffusion coefficient, dpH/dx is the pH gradient in the zone, and dμ/dpH is the mobility slope at the pI which depends on the charge of the sample near its pI. CIEF processes the potential for resolving power and can be used to separate proteins that differ by 0.02 pI units or less [81,82]. CIEF has been used successfully to measure protein pI [81,82] and separate protein isoforms, such as hemoglobin [83] and transferrin [84].

Detection Methods

Detection is one of the most demanding aspects in the current state of CE development. The small capillary dimensions encountered in CE and the concomitant minuscule probe volumes create a system in which sensitive detection of solute zones without introducing zone dispersion is a major challenge. To avoid the loss of resolution from band broadening resulting from the joints, fittings, and connectors used in conventional off-column detection systems, detection in an on-column configuration is more suitable for CE and easily

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implemented [85]. Optional detection principles, such as UV-Vis and fluorescence, are well adapted from conventional detection systems and commonly used for CE separation. Recent advances have allowed the use of several non-optical detection methods, including electrochemical detectors [86,87] and mass spectrometry [88,89].

UV-Visible (UV-Vis) detection. UV-Vis detection is applicable to a wide spectrum of compounds. Because of its relatively universal nature, UV-Vis detection is the most common detection system of CE separation. Most commercial instruments available today rely on this mode of detection. Although the use of narrow diameter separation columns in CE provides several advantages, such as higher mass sensitivity than HPLC due to the nanoliter injection volume, CE with UV detection suffers from low concentration detection limits due to the short optical path length (OPL) which is limited by the diameter of the capillary. The usual concentration detection limit obtained with this detection mode is 10^{-4}-10^{-6} molar, which is 10-100 times higher than in HPLC [90].

Several approaches have been developed to enhance the sensitivity through increasing the detection OPL in CE. The design of Z-shaped [91,92] or bubble-shape flow cells [93,94], rectangular capillary [95], and multi-reflection detection cell [96] aims to enhance detectability by increasing the optical length. Sensitivity increases of 10-40-fold are typically claimed with these designs.

On-column concentration is another approach to improve the detection in CE by increasing the sample loadability in CE. Sample stacking [65,97] or field amplification [98-100] is based on the difference of ionic strength between the sample matrix and the running buffer, while sample focusing [101,102] is dependent on the pH difference between sample

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and running buffer. These methods can provide a 10-100-fold sensitivity improvement. Isotachophoretic [71-76] or chromatographic [103] sample enrichment also have been developed for sample pre-concentration for CE by focusing a much larger sample volume into a narrow zone. Sensitivity enhancement on the order of 10-1000-fold can be obtained.

Fluorescence detection. Fluorescence detection is also easily adapted to CE in an on-column configuration. Unlike the OPL limited UV-absorption, fluorescence intensity primarily depends on the intensity of the light source, the optical match of the excitation source to the fluorophore, and the quantum efficiency of the fluorophores. Also, detection of fluorescence is performed at a 90 degree angle relative to the excitation light source, resulting in nearly zero background and high sensitivity. Typically, fluorescence detection can provide detection limits 1-3 orders of magnitude better than those obtained with UV detection [90,104]. The successful use of lasers as excitation light sources LIF an extremely high-sensitivity detection method for CE. Lasers are superior excitation light sources which provide the advantages of high intensity, better focusing capability which allows effective energy transfer to very small sample volumes, and better monochromicity which reduces stray light levels and increases signal-to-noise (S/N) ratio. Generally, LIF detection for CE can provide impressive concentration detection limits at nanomolar to picomolar ranges [105-107], which results in mass sensitivities at attomole to zeptomole range (10^{-18}-10^{-21} mol or hundreds of molecules) [16].

While on-column fluorescence detection can provide excellent detection limits, this technique is less versatile than UV detection because most analytes are not intrinsically fluorescent and must be derivatized with a fluorophore with high fluorescent character.

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Several fluorescent tags, including fluorescein isohydantoin (FTH), 3-(4-carboxybenzoyl)-2-quinoline-carboxaldehyde (CBQCA) [107], dansyl chloride [108], fluorescamine [109-110], 4-clair-7-nitro benzofuran (NBD) [111], O-phthaladehyde (OPA) [112,113], fluorescein isothioiyanate (FITC) [5,15,114], and naphthalenedialdehyde (NDA) [115,116] are commonly used.

Electrochemical detection. Electrochemical detection is based on monitoring the steady state change on the surface of electrodes caused by the oxidizing reaction of electro-active compounds. The measurement is independent of path length, allowing very narrow capillaries to be used. Generally, both conductivity and amperometric detection offer significant sensitivity enhancement over that possible with absorbance techniques. In addition, the components required to fabricate the electrochemical cell are inexpensive relative to LIF detection. However, in order to perform electrochemical detection, the high voltage (10-30 kV) employed in the separation must be isolated from the small electrochemical potentials (~ 1V) used to control analyte oxidation and reduction processes [86].

Several electrochemical detection schemes have been developed to solve this problem. In general, isolation can be performed by introducing a small fracture at the point of capillary and electrode coupling and covering with porous glass [117], nafion [118], or cellulose acetate-coated polymer [119] as a decoupler. Electrochemical detection also can be performed by using smaller internal diameter capillaries with microelectrodes to be manipulated up to the end of the column [120-122]. It appears that the potential field caused by the separation voltage decays very rapidly at the end of a narrow capillary (with i.e. < 25 μm), and no coupler is needed in this configuration [120,121].

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Although, electrochemical detection can provide excellent sensitivity for CE separation, the major drawback is the inherent selectivity of the technique, which generally limits analysis to easily oxidized or reduced species. Conductivity detection has been used to detect metal ions and amino acid [123] and anions [124] with detection limits at 1-10 μM range.

Amperometric detection is the most commonly used electrochemical detector due to its simplicity of implementation and high sensitivity. The application of amperometric detection to catecholamines [125-127], carbohydrates [117,128], and thiols [129] has been reported with detection limits of 10^{-7} -10^{-8} M range and mass sensitivity on the attomole level. Also, due to its high sensitivity and ability to couple with extremely small sampling volumes, Ewing and co-worker have demonstrated the analysis of catecholamines from a single neuron cell [130,131].

Mass spectrometry (MS) detection. MS detection plays an important role in the analytical and structural characterization of biological substances. The use of MS as a detector for separation techniques provides several unique advantages over other detection modes including the universal nature of detection, high sensitivity and selectivity, and the capability of providing structural information. On-line coupling MS with separation techniques, such as gas chromatography (GC) and HPLC, has been well established [132-136]. Recently, coupling of CE with MS detection is rapidly gaining interest in order to combine the high separation efficiency in CE and the identification potential in MS [88,89].

The key to on-line coupling of CE with MS is to have an interface that permits the introduction of the CE-fractionated sample components into the MS. The two most common

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ionization techniques used in combination with CE are continuous-flow fast atom bombardment (CF-FAB) [137-140] and electrospray ionization (ESI) [141-144]. These techniques can provide mild ionization conditions that ensure molecule weight determination. The ESI method also can produce a series of multiple-charges molecules which extend its applicability to higher molecular weight compounds. Interfacing of CE with MS is more complicated than HPLC-MS interfacing. An electrical contact must be made with the CE column to provide a circuit for both the CE current flow and the electrospray current flow. Also, because the flow rate in CE is commonly too low for reproducible operation of most ions sources, an additional make-up flow is needed to provide a flow rate of about 0.5-1.0 $\mu l\ min^{-1}$ [145]. Several interfaces, including coaxial sheath-flow [137,141,142] and liquid-junction [138,143], have been developed to couple CE with MS detection. Another approach to interface CE with MS without a make-up flow is by a sheathless interface [139,146]. The sheathless interface requires a sharpened CE outlet coated with metal for completing the CE and ESI electrical paths. Without the sample dilution from the make-up flow, the sheathless interface provides better sensitivity than other interfaces; however, it is not as popular as the sheath-flow system because of the need for specialized CE capillaries and ESI hardware [147].

CE/MS has been applied successfully to a wide range of biologically important compounds, such as proteins and peptides [148,149], metabolites [146], sulfonates and amino acids [150], and amine and ammonium salts [139]. Detection sensitivity from CE/MS is generally reported at femtomole levels for a conventional quadrupole mass spectrometer [151,152]. Currently, several types of MS, such as ion-trapping [153], Fourier transform ion cyclotron resonance [154] and orthogonal time-of-flight [155] mass spectrometer, have been

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developed to reach higher sensitivity by increasing the overall ion sampling and transmission efficiency of the ionization interface and the ion utilization efficiency.

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OVERVIEW AND RATIONALE OF RESEARCH PROJECTS

The general goal of this research is focused on the development of capillary-scale separation techniques and other micro-scale analytical methods for biochemical and biomedical applications. Two research projects are presented in this dissertation: development of gel density gradients for capillary gel electrophoresis to extend the separation potential of CGE to wide molecular mass samples and development of alternative micro-scale methods for improved analysis of trace amounts of plasma adenosine by capillary electrophoretic techniques.

Development of Capillary Gel Gradient Electrophoresis

CGE, as described above, is a rapidly developing method for biopolymer separation. The use of polymer gels as separation media has transferred well from conventional slab-gel electrophoresis to the instrumental capillary system. CGE provides the advantages of fast analysis and high efficiency over conventional slab-gel electrophoresis, and it has been demonstrated as a powerful tool for size-based analysis of biomacromolecules, such as oligonucleotides and proteins.

Slab gels cast with gradients in gel density, i.e., pore structure, along the separation axis have been widely used in biomedical research for high-solution separation of wide molecular mass range samples and particularly for glycoprotein analysis [156]. Gradient gels have two considerable advantages over uniform concentration gels in the analysis of complex protein mixtures. First, the variation of gel density with distance

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along the separation bed permits separation of a wider molecular mass range of solutes than is possible with single-concentration gels, without compromising resolution of closely-sized species. Furthermore, the gradient in pore size causes significant sharpening of protein bands due to the velocity decrease while migrating into higher gel densities. Although CGE has been explored for more than a decade, there have been only few reports dealing with gel gradients CE [157-160]. The critical reason is the difficulty in preparing gel gradients in micro-liter total volume and detection. In Manuscript 1, a unique method for preparing gel step gradients for CE is described. The potential of gel gradients in CE will be evaluated by the separation of protein samples with wide molecule mass range.

Development of Improved Methods for Analysis of Trace Plasma Adenosine by CE Techniques

Adenosine is a purine nucleoside comprised of adenine and ribose joined by a glycosidic bond. It is both a precursor and a metabolite of adenine nucleotides. It has been shown that adenosine has widespread effects on several physiological systems, such as coronary and cerebral circulation [161]; control of blood flow [162,163]; prevention of cardiac arrhythmias [164]; and nerve-tissue functions, such as the inhibition of neurotransmitter release and the modulation of adenylate cyclase activity [165]. It has been suggested that these physiological actions are due to the interactions of adenosine with specific receptors, which are mediated by the changes of interstitial adenosine concentration [161]. A large amount of literature exists concerning plasma adenosine concentrations in the study of the role of this endogenous substance in cardiovascular physiology and the pharmacology of drugs which alter adenosine physiology or mimic it.

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However, the reported adenosine level in normal human plasma ranges from a few nanomolar up to micromolar levels. Even within an individual study there may be a very large range of values observed. These are features to be expected when the analytical methods employed are subject to uncontrolled artifacts.

Conventional methods for analyzing plasma adenosine usually involved blood drawing and plasma sample preparation. Artificial adenosine can be formed as well as lost due to the adenosine metabolism by the blood cells and enzymes in blood during these processes. Due to the huge concentration (about 2 m*M* level) of adenine nucleotides in blood cells [166], even if only 1% of red cells were hemolyzed during the drawing of blood with a hematocrit of 45%, the total concentration of adenine nucleotides in the plasma could rapidly exceed 16 000 n*M*. Given the very rapid conversion of these nucleotides to adenosine by ectonucleotidases [167], adenosine production in the plasma is large even in the face of concomitant rapid transport into cells followed by degradation of adenosine by adenosine deaminase. "Stopping solutions," which contain a variety of inhibitors and chelators, are usually used to mix with the blood sample at the point of sampling to prevent the artificial adenosine production or metabolism.

In 1990, data presented by McCann and Katholi [168] showed that enormous differences in observed plasma adenosine concentrations could occur in blood drawn at 15-s intervals from the arm vein of the same human subject. The values in four such samples ranged from over 800 n*M* to less than 20 n*M*, depending on the type of stopping solution into which the blood was collected. The lowest level was obtained when the stopping solution contained not only heparin and inhibitors of the nucleoside transporter and of adenosine deaminase but also the chelators (ethylene diamine -N,N,N',N'-

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tetraacetic acid (EDTA) and ethylene glyco bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)) of calcium and magnesium to inhibit phosphatases and 5'-nucleotidases. It indicated that in resting human arm vein blood the plasma adenosine concentrations could be in the nanomolar range. This concept of low plasma adenosine concentrations in man is solidly in line with the finding that the half-life of radioactive adenosine added to human blood is a matter of about 1 sec [169]. This short half-life is highly compatible with published data on the kinetics of cellular uptake of adenosine by the nucleoside transporter in human blood cells [169,170].

It is difficult to define an ideal stopping solution to effectively and completely inhibit the adenosine artificial during plasma preparation. Also, most of the plasma adenosine methods require relatively large blood volumes (usually > 1 mL) and cumbersome sample preparation. Futhermore, the drawing of large blood samples may interrupt the in vivo equilibrium, particularly in small animals.

To overcome these problems for plasma adenosine analysis, a micro-scale method with high detection sensitivity is essential. One alternative technique that will permit the use smaller blood samples or in situ studies in small animals is micro-dialysis. Micro-dialysis is a technique that has been successfully used for sampling many interstitial substances from the brain and other tissues in anesthetized or conscious animals [171,172]. During dialysis, a perfusion medium is constantly pumped through a membrane probe position in the target tissue of study at micro-liter per min flow rate or lower. The interstitial substances that diffuse across the dialysis membrane into the perfusate are carried away and sampled at the outlet of the dialysis probe. The substance

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of interest recovered in the perfusate is then measured with an appropriate analytical method.

The micro-dialysis sampling technique offers a degree of selectivity since only molecules below the size cut-off of the membrane can cross the barrier. This is extremely relevant to the studies of plasma adenosine since the red blood cells and enzymes that cause the artifact formation and loss of plasma adenosine will not be co-sampled with the smaller ribonucleosides. Also, it is possible to sample plasma adenosine continuously without inducing the stress responses to the animal caused by blood drawing.

Micro-dialysis has been applied successfully to sample adenosine for HPLC analysis from the cortex of the rat kidney [173], in rat hearts during ischemia [174], and in the myocardium of anethesized dogs [175]. However, in each these cases the adenosine levels are in the micromolar range, substantially higher than expected levels in plasma. Also, relatively long sampling time is needed to collect adequate samples for each HPLC analysis, resulting in a loss of temporal resolution for continuous studies of model animals. To explore the potential of microdialysis for the analysis of plasma adenosine or other biologically important substances, a micro-scale analytical method with high detection sensitivity is essential.

In Manuscript 2, we describe a novel on-column transient ITP-CZE with borate buffer for the analysis of micro-liter volumes of the neutral adenosine and polyol sample species. Along with the use of LIF detector, analysis of nanomolar levels of adenosine is demonstrated.

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PREPARATION OF LINEAR POLYACRYLAMIDE GEL STEP GRADIENTS FOR CAPILLARY ELECTROPHORESIS

by

CHAO-CHENG WANG, STEPHEN C. BEALE

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Abstract

A means for casting step-gradients in linear polyacrylamide gel concentration for capillary electrophoresis is presented. A UV-Vis whole-column detector is used to profile the gel gradients cast in the capillary, while detection of fluorescein-labeled proteins is accomplished with an epi-illumination laser-induced fluorescence whole-column detection system. Fluorescence images of the capillary during the separation indicate a sharpening of the zones as they traverse the interface between gel concentrations. The potential of gel gradients in capillary electrophoresis for the analysis of wide molecular mass range protein or peptide samples is demonstrated. A mixture of proteins that range in molecular weight from $6 \cdot 10^3$ to $9.7 \cdot 10^3$ is separated in less than 15 min with baseline resolution of closely sized proteins that were not be resolved in a single concentration gel. Finally, analysis of several capillary images acquired during solute migration through the gel gradient permits the generation of the Ferguson plot from a single electrophoretic run.

1. Introduction

Capillary electrophoresis (CE) has become a powerful analytical tool for separating and quantitating samples of biomedical and biological origin. The capillary format permits rapid separations, sensitive detection and is compatible with different separation media. In particular, utilization of polymer gels as separation media has transferred well from conventional slab-gel electrophoresis to the instrumental capillary systems. Capillary gel electrophoresis (CGE) has proven to be very powerful for high resolution DNA sequencing (for example see references [1-3] and forensic DNA analysis [4,5]). CGE has also been applied to the size-based separation of proteins and polypeptides [6-11]. Early work with gel-

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filled capillaries [2] involved cross-linked gels with a fixed pore structure, however fabrication problems and limited lifetime have hindered their widespread acceptance. More recently, linear gels (polymer networks) have been used with considerable success for DNA applications [1,3-5] and for size-based separations of proteins [7,11].

An area that holds some promise for CE applications is the implementation of gel-density gradients as the separation medium. Slab gels cast with gradients in density along the separation axis have been widely used in biomedical research for high-resolution separations of wide molecular mass range samples and particularly for glycoprotein analysis [12]. The variation of gel density with distance along the separation bed permits separation of a wider molecular mass range of solutes than is possible with single-concentration gels, without compromising resolution of closely sized species. Furthermore, if the density gradient is continuous, a migrating zone will eventually reach a point along the gradient when it can no longer penetrate the pore structure. The net result is a focusing effect as the electric field drives the trailing edge of the zone against the gel; leading to more sensitive detection of individual zones and greater peak capacity.

To date, there have been few reports dealing with gel gradients in capillary electrophoresis [13-15]. Sepaniak [14,15] described the preparation of a two-step gradient in methyl cellulose (MC) concentrations, used in conjunction with a spatial-scanning laser-induced fluorence detection (LIF) system to optimize resolution per unit time for separation of DNA fragments [14]. Effective column length was altered by positioning the capillary on a translational stage and re-positioning the detection point between runs [14]. In a subsequent report [15] refinements to the scanning detector and its application to fundamental studies of band dispersion and other electrophoretic phenomena were described. The

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scanning detection system was used to probe the effects on peak width upon migration across an interface between two different MC concentrations. The results indicated an unexpected broadening of the zone upon crossing the interface. It was suggested that hydrodynamic introduction of the different density polymer solutions into the capillary (and the concomitant parabolic flow profile) resulted in a severe radial viscosity gradient at the interface region [15].

Chen and co-workers [13, 16] reported a method to prepare highly condensed (30% T + 5% C), cross-linked polyacrylamide gel-filled capillaries and establish gradients in gel concentration along the length of the capillary [13]. The highly condensed gels appear to be critical for high-resolution of oligosaccharides and polyamino acid polymers [13, 16-17]. The described procedure for preparing the dense gels included application of slight pressure during polymerization, control of the polymerization direction and careful control of initiators and catalysts [13,16]. With this procedure cross-linked gels with concentrations as high as 30% T + 5% C [16] as well as step gradients [13] over a range of gel concentrations (10% T + 5% C to 15% T + 5% C) were prepared. Using the step gradient a decrease in separation time (fixed point detection) for a polyaspartate homopolymer was demonstrated.

In this paper, a simple method for casting step gradients along the capillary is demonstrated. A micro-chamber device that allows for changing the gel solution reservoirs without removing the capillary tip from solution is described. In this manner, air bubbles are not introduced into the interface region between gel steps. Gels are profiled using a whole-column UV-Vis absorbance detector, operating at 214 nm. The fluorescein isothiocyanate (FITC)-labeled solutes are detected as they migrate through the separation capillary by a whole-column LIF detector (argon ion laser at 488 nm), that can be operated in either a fixed

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detection point mode or scanning mode to image the entire capillary during the separation [19]. Focusing of solutes as they traverse the interfaces between gel concentrations is observed. The application of a gel gradient to the separation of a wide molecular mass range sample of FITC-labeled proteins is shown. Furthermore, if the gel profile is known, a single electrophoretic analysis provides sufficient data to generate a Ferguson Plot.

2. Experimental

2.1. Materials

Electrophoretic grade acrylamide and N,N,N',N'-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Labs. (Richmond, CA, USA,). The bi-functional reagent γ-methacryloxypropyltrimethoxysilane was purchased from HULS America. (Bristol, PA, USA). Fluorescein isothiocyanate isomer I was purchased from Aldrich (Milwaukee, WI, USA). The protein samples utilized in these studies, FITC-labeled insulin (Ins, molecular mass: 6000), cytochrome *c* (CytC, 12 400 dal), lysozyme (LYZ, 14 400), horse heart myoglobin (MYO, 17 000 dal), soybean trypsin inhibitor (TPI, 21 500 dal), chymotrypsinogen (CTPG, 25 000), bovine carbonic anhydrase (CA, 29 000), bovine serum albumin (BSA, 66 000) and phosphorylase *b* (97 400) and ammonium persulphate (APS), Tris(hydroxymethyl)aminomethane (Tris), boric acid, sodium dodecyl sulphate (SDS) were reagent grade from Sigma (St. Louis, MO, USA). Fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA).

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2.2. Preparation of FITC labeled proteins

All proteins, except insulin (which was purchased as the FITC conjugate), myoglobin and phosphorylase *b*, were dissolved in 50 m*M* sodium carbonate buffer pH 9.5. Myoglobin was dissolved in 50 m*M* potassium phosphate buffer pH 6.8 while phosphorylase *b* was dissolved in 50 m*M* sodium carbonate buffer pH 9.5 containing 15% glycerol, 1% 2-mercapoethanol and 1% SDS. The conjugation with FITC (dissolved in acetone) was carried out with a 2.5:1 mol ratio of FITC to protein for all samples except myoglobin (1:1, FITC to protein) and phosphorylase *b* (10:1, FITC to protein). The FITC-protein mixtures were allowed to react in dark at 25°C for 3.5 h, and then dialyzed extensively against 5 m*M* phosphate or carbonate buffer to remove unreacted FITC and low molecular mass reaction by-products. All FITC-labeled proteins were kept in the freezer before use.

2.3. Preparation of polyacrylamide gel-filled capillaries

Single-concentration linear polyacrylamide gels were prepared by dissolving 0.2 g of acrylamide in 5 ml of TB buffer (250 m*M* Tris and 100 m*M* borate, 0.1% SDS, pH 8.2) followed by degassing for 25 min under sonication and vacuum. Upon degassing, polymerization of gel solutions was initiated by adding 20:1 TEMED (10%) and 20:1 APS (10%). The capillaries were filled with the gelling solutions by applying vacuum at the distal end. Gel-filled capillaries were kept at room temperature for at least 10 h to ensure complete polymerization.

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2.4. Preparation of step-gradient polyacrylamide gel-filled capillaries

The micro-chamber system utilized for casting gel step gradients is shown in Fig. 1. The caps and bottoms of microcentrifuge vials (0.6 ml, Fisher Scientific, Pittsburgh, PA, USA), were cut off for use as the gel solution chambers. Each chamber was separated by parafilm to avoid mixing of gel solutions in the chambers. Fused-silica capillaries, 75 μm I.D., were pre-coated as described by Hjerten [19] using γ- methacryloxypropyltrimethoxysilane and 4% linear polyacrylamide. All solutions were thoroughly mixed and de-gassed, then chilled in an ice bath prior to initiation of polymerization. Immediately after addition of the polymerization agents, each micro-chamber reservoir was filled with the appropriate gelling solution. Gradients of at least six steps can be easily cast with this device.

The capillary, with polyimide outer coating removed [18], was mounted on a clear, flat plexiglass platform. To cast the gel, one end of the capillary was inserted into the first chamber and the first gelling solution was drawn into the capillary by applying light vacuum from an aspirator using a two-arm glass 'T' with PTFE stopcocks in each arm to act as a splitter device. The applied vacuum (controlled by slightly opening the side-arm stopcock) was just enough to draw the gelling solution through the capillary. The flow of gelling solution in the capillary can be monitored easily with the unaided eye or under the microscope for precise control. As each gelling solution filled the capillary to desired length, measured by a mark made on the platform adjacent to the capillary, the capillary was pushed through the Parafilm barrier into the next gelling solution chamber. The next gelling solution could then be pulled into the capillary without introducing an air pocket between the gels. The gelling solutions of various concentrations were gradually pulled into the capillary until the front of the first solution reached the tip of the capillary. In this manner it is possible to cast

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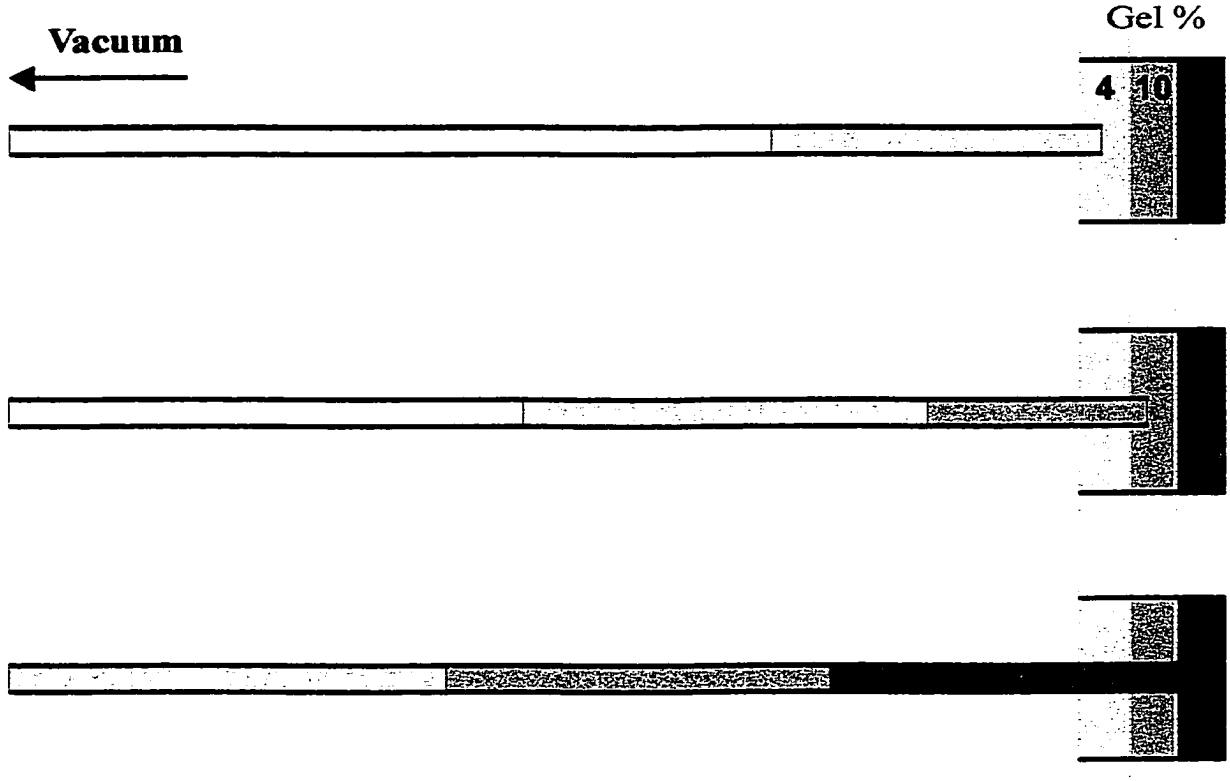

Fig. 1. Illustration of the micro-reservoir system used to cast step gradients in the capillary. Each reservoir chamber was separated by parafilm and filled with gelling solutions containing the different monomer concentrations. To change the composition of the solution drawn into the capillary, the parafilm was pierced and the capillary inserted into the next chamber.

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a gel step gradient with precise control over the length of each step. The capillary was kept flat and undisturbed overnight at room temperature. Columns prepared in this fashion have a useful lifetime of approximately 2 weeks.

2.5. Step-gradient gel profile

The step-gradient gel was profiled using a scanning UV-Vis detection system [20,21] available in our laboratory. In this system, the column is moved along its axis between a fiber optic and photodiode connected to a Linear Instruments Model UV-200 (Spectraphysics, Piscataway, NJ, USA) UV-Vis absorption detector to monitor absorbance along the length of the capillary. Schematic diagrams and a description of the UV-Vis scanner are availabile to anyone requesting this information from the corresponding author.

2.6. Capillary gel electrophoresis

The gel electrophoresis was performed using an in-house constructed CE system consisting of a Spellman 0-30 kV power supply (Plainview, NY, USA). Laser-induced fluorescence detection was accomplished with a spatial-scanning, whole-column detection system developed in our laboratory [18]. This detector can be operated using a fixed detection point or images of the entire capillary can be generated during the migration of solutes through the capillary by translating the column through the probe beam.

The operating buffer for the gel electrophoresis was the same as that used for preparing gel solutions. All gel capillaries were pre-run by applying 5 kV for 25 min to pre-condition the column. The FITC-labeled proteins were diluted in water containing 0.1% SDS followed by heating at 90°C for 10 min. After cooling to room temperature, protein samples

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were injected into the gel column from the cathode end. All separations of protein samples were performed at 5 kV, and the current through the gel capillaries did not exceed 20 μA.

3. Results and Discussion

In conventional slab-gel electrophoresis the density gradients commonly employed is cross-linked polyacrylamide in which the concentration (pore size) of the gel varies continuously along the separation path [12]. The gels usually range from a very loose pore structure (i.e. 5% T + 3% C) at the sample injection end of the separation bed to highly condensed, tight gel structures (30% T + 3% C). The gradients can be cast with very precise control over the final gradient profile (linear or exponential) using a simple device consisting of two plastic syringes [22].

The situation in CE is somewhat different. The small column volume and stringent restrictions on external mixing volumes, make casting continuous gradients along the length of the capillary more difficult. Preparing gel gradients in which the distal end of the capillary consists of highly condensed, cross-linked gels is further complicated by the well-known difficulties associated with preparing dense, cross-linked gels [16,17].

The gels reported herein consist of step-gradients in linear polyacrylamide concentration. It was our experience that linear acrylamide in concentrations up to 20% T can be routinely cast without significant air bubble formation within the gel. The most significant problem encountered in this work was bubble or void formation at the interface between steps, even when the time to transfer the capillary between the different gel vials was short [13] and only light vacuum applied. Utilization of the micro chamber system eliminated

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bubble formation at the interface region of the capillary since the capillary is never removed from the gel solution.

To evaluate the shape of the gel gradients, UV-Vis absorption profiles of the entire column were examined. Capillary images were generated by scanning the length of the column with a modified UV-Vis detector at 214 nm. Subsequent to polymerization of the gel (at least 10 h after the initiation of polymerization), the absorbance and refractive index differences of the different gel concentrations allow for visualization of each interface. A typical whole-column absorbance profile of a 3-step gel, formed using the micro-chamber reservoir device, is shown in Fig. 2. The peak 21 cm from the distal end of the capillary is a reference mark made on the outside wall of the capillary with a permanent marker. The shaded area shown below the position axis represents the expected gel profile, based on the length of each solution drawn into the capillary. With the micro-chamber reservoir the transition interface between gels was never more than 2 cm. This is in direct contrast to the profiles of gels formed using full vacuum from the aspirator to rapidly draw gel solutions from individual reservoirs into the capillary. In this cast the transition interface region between gel steps was never less than 3 cm.

The performance of a gel concentration step-gradient is illustrated in Fig. 3, a series of five fluorescence images of the capillary acquired as FITC-BSA migrates through a two-step gradient. The gel cast in this capillary was a two-step gradient at the extremes of gel concentration, 4%T - 20%T, the expected profile is shown in the shaded area above the position axis. As the solute (FITC-BSA) migrates through the first 12 cm of capillary (4%T) it encounters the transition interface between the gels (center image, acquired 10 min after injection), indicated by a sharpening of the band and a decrease of migration velocity. This

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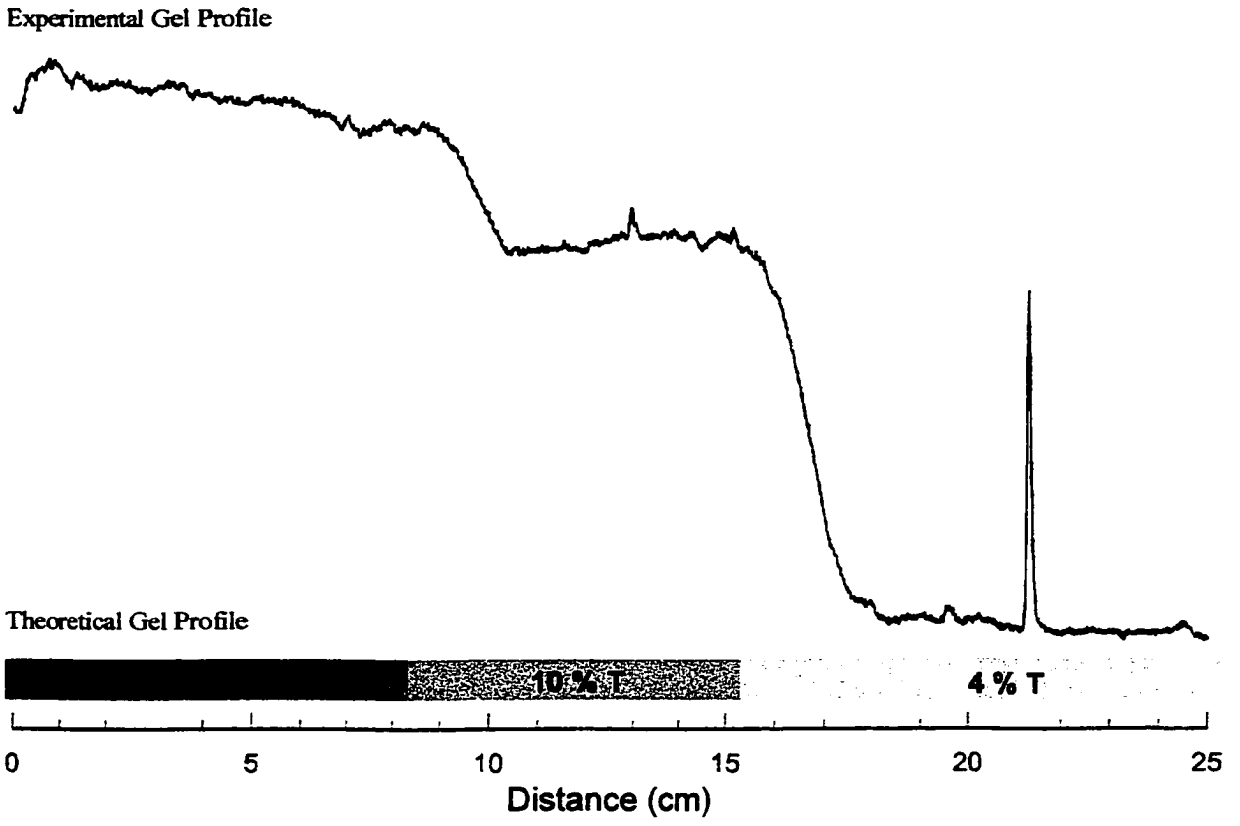

Fig. 2. UV-Vis absorption profile of a three-step denaturing gel gradient cast in a 75 μm I.D. capillary. The column was positioned in the UV-Vis scanner and a mark was made on the capillary with a permanent marker 21 cm from the distal end of the capillary. The expected gel profile, based on observation of the meniscus position during the filling process, is shown in the shaded area above the position axis.

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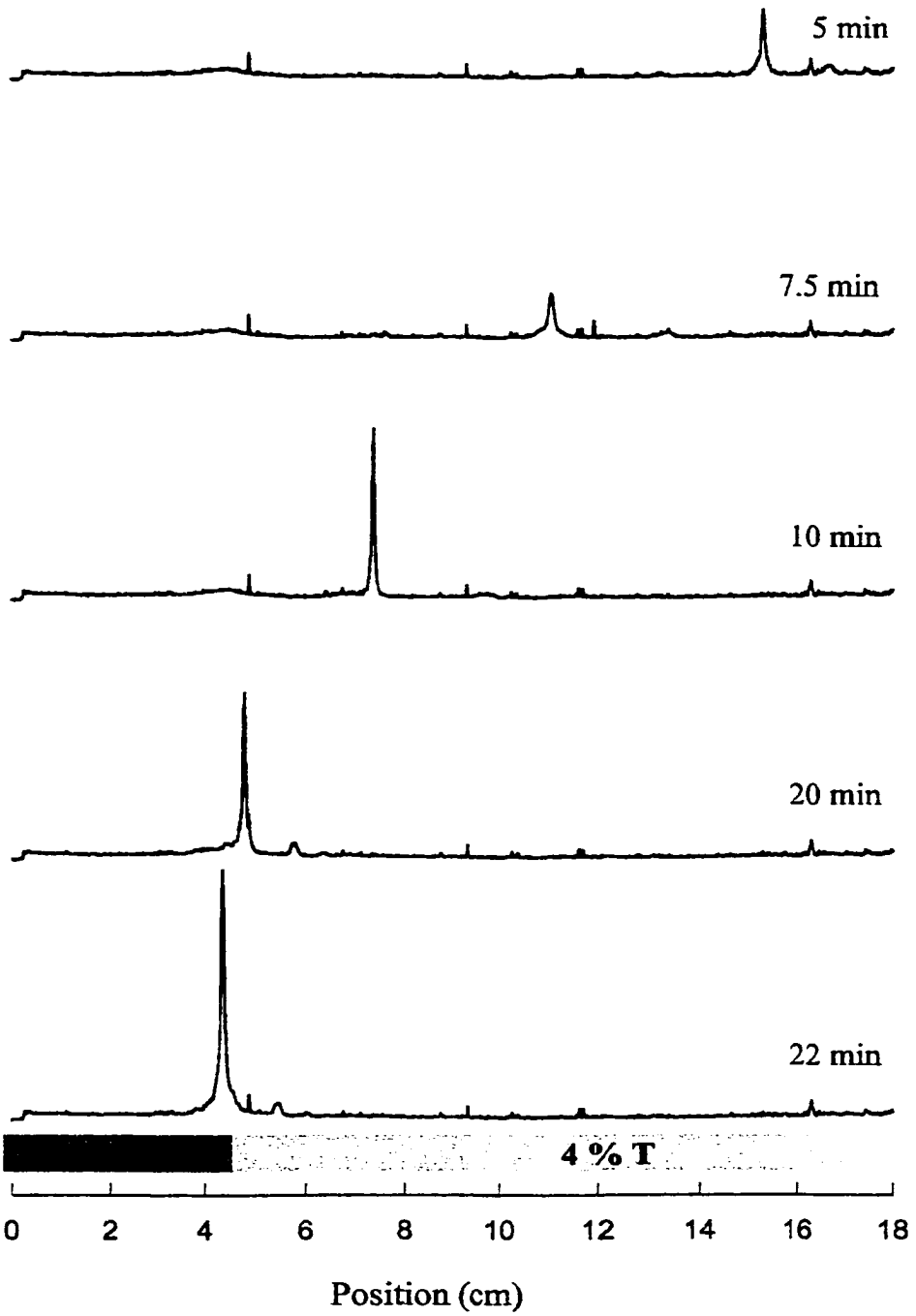

Fig. 3. Five fluorescence images acquired during the migration of FITC-BSA through a capillary in which a 4-20% T two-step gradient was cast. Capillary, 30 cm × 75 μm I.D.; applied voltage, 8 kV; injection, 2 kV for 1 sec; buffer composition and other operating conditions are given in Section 2. The expected gel profile is shown in the shaded area just above the position axis. The capillary was scanned at 1.5 cm/sec with a pixel width of 50 μm to generate each image.

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particular gel was not cast with the micro-chamber system and as such a much broader interface region is expected as there is less control over the gel profile. In fact, analysis of several scans acquired during solute migration through this gel indicated the apparent interface between the gels occupies about 4 cm of the capillary, starting 8 cm from the anode. The expected interface should be 4.5 cm from the anode.

Once BSA migrates completely into the more dense gel it has focused into a narrow zone and its velocity has decreased considerably. In fact, migration entirely through the remaining 4 cm of the 20%T gel portion of the capillary requires an additional 30 min. Inspection of the last two images indicates a small peak that begins to appear about 5.5 cm from the anode. This may be due to a dimer or trimer of BSA that is detectable only after focusing in the dense gel. This higher molecular weight species is observed when using short effective column lengths in single-concentration gels, but the signal from this species is rapidly lost during migration through longer columns.

The application of a multi-step gel gradient to the separation of a wide molecular weight range sample is shown in Fig. 4 and Fig. 5. Fig. 4 depicts an electropherogram of nine FITC labeled proteins (detection is fixed point LIF with an effective column length of 22 cm) separated in a single concentration (4%T) denaturing linear acrylamide gel. The proteins range in molecular mass from 6000 to $9.7 \cdot 10^3$ (peak identities are given in the figure caption). In this gel, two of the observed peaks are actually composed of co-migrating solutes (lysozyme and myoglobin, peak no. 3,4 and chymotrypsinogen and carbonic anhydrase, peak no. 6,7). The individual solutes in these zones can be resolved using higher concentration gels, but at the expense of analysis time for the BSA and phosphorylase B (about 50 min for 15% T gel).

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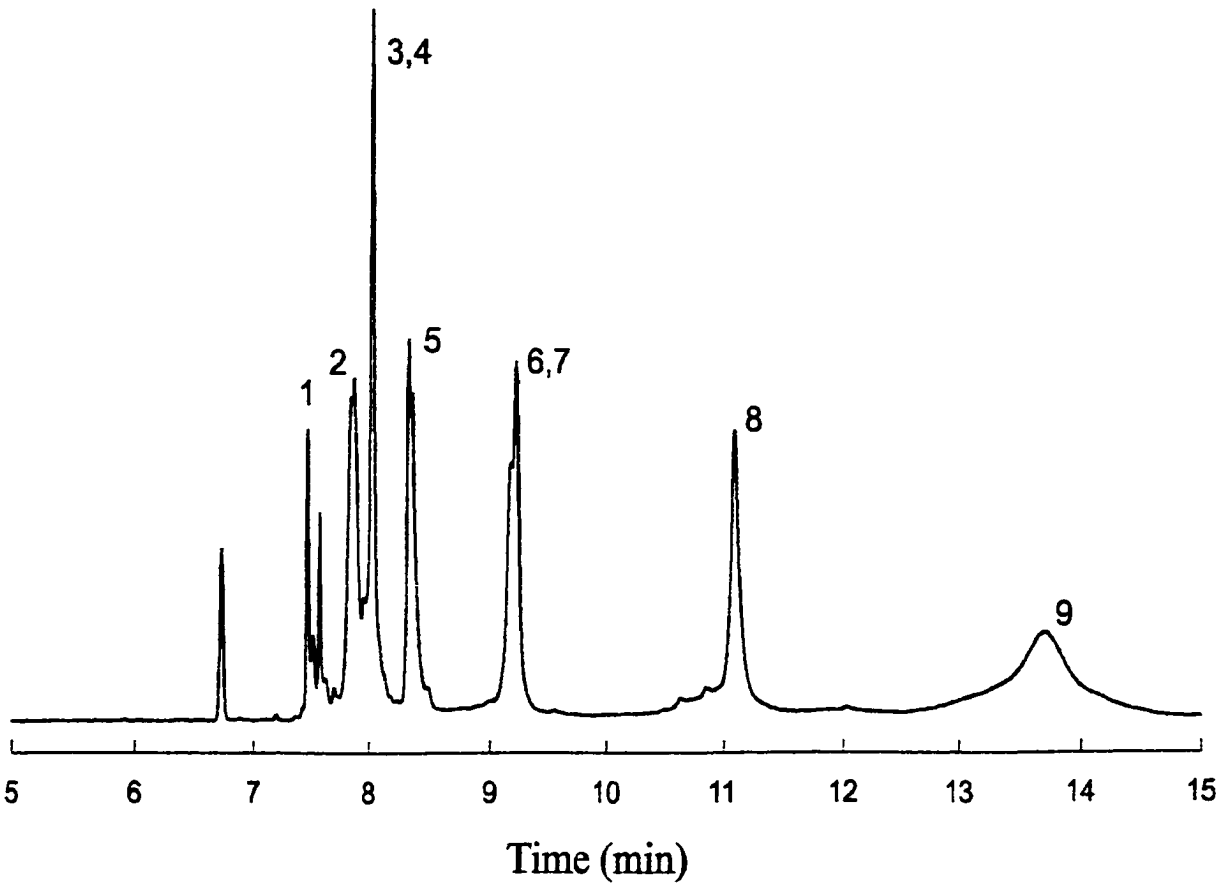

Fig. 4. Separation of FITC-labeled proteins in a denaturing 4%T linear acrylamide, single concentration gel with LIF detection. The detection point is fixed 22 cm from the cathodic end of the capillary. Peak identifications are as follows: 1) insulin; 2) cytochrome c; 3) lysozyme; 4) myoglobin; 5) soybean trypsin inhibitor; 6) chymotrypsinogen; 7) carbonic anhydrase; 8) bovine serum albumin and 9) phosphorylase b. Capillary, 30 cm × 75 μm I.D.; applied voltage, 8 kV; current, 20 μA; injection 2 kV for 1 sec.

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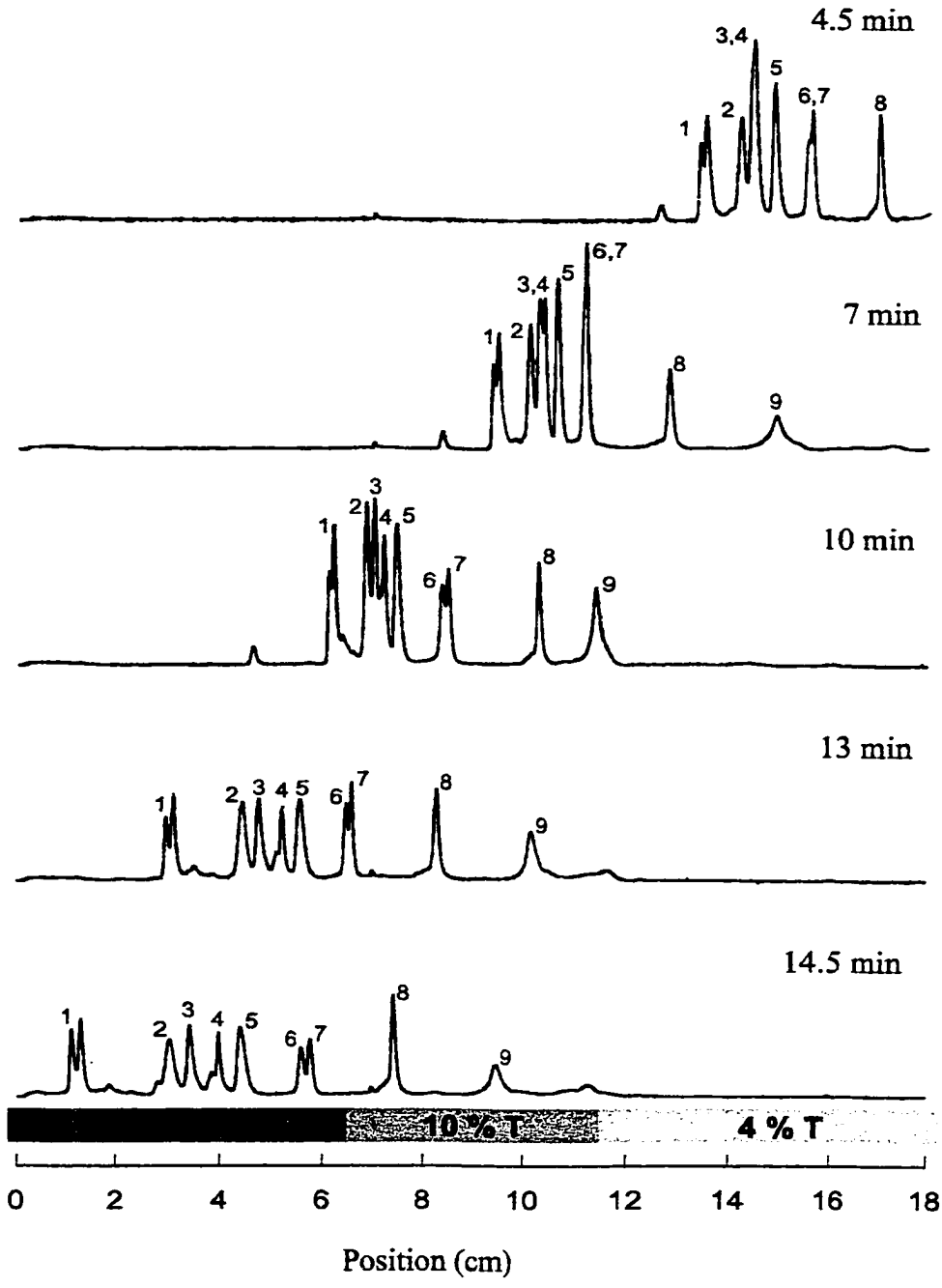

Fig. 5. Five fluorescence images of the separation capillary during the migration of 9 FITC-proteins through a three-step gradient (4%T - 10%T - 16%T). The peak identifications are as in Fig. 4. Capillary, 30 cm × 75 μm; applied voltage, 8 kV, current 15 μA; injection, 2 kV for 1 sec; operating conditions are given in the experimental. The capillary was scanned at 1.5 cm/sec with a pixel width, 50 μm; the position axis is distance from the anodic end of the capillary.

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Separation of the same nine FITC-labeled proteins in a capillary in which a gel gradient has been cast is illustrated in the series of fluorescence images shown in Fig. 5. A three-step gradient, cast with the micro-chamber reservoir system, consisting of 4%T - 10%T - 16%T was established in the capillary. After sample injection, a series of images was acquired at 1-minute intervals. In less than 5 min all the zones are separated to approximately the same extent as seen after migration through the entire single-concentration gel (Fig. 4), phosphorylase b has not yet migrated into the detection window. As the solutes migrate further through the gel, focusing is evident at each interface. Within 15 min there is baseline resolution between those zones that could not be adequately resolved in the 4% T single-concentration gel (cytochrome c, lysozyme and myoglobin).

Step-gradients in gel concentration, combined with whole-column detection, permit the facile construction of Ferguson Plots. By acquiring capillary images at sufficient intervals to measure solute position at two points in each gel, the apparent mobility of each solute in each gel can be calculated. If the separation mechanism is size-based, plots of log [mobility] vs. gel composition will yield a straight line (different slope for each sample component) with a common y-intercept, equal to the free-solution mobility. All the mobility data for the Ferguson Plot shown in Fig. 6 was generated in a single electrophoretic run on the 3-step gradient column used for the separation shown in Fig. 6.

In this work no special efforts were made to control the FITC labeling reaction [23,24] and limit the number or position of the attached FITC labels. The reaction of FITC with myoglobin was carried out at a lower pH than the other reactions because the myoglobin derivative resulting from the higher pH reaction (9.5) featured a very broad electrophoretic

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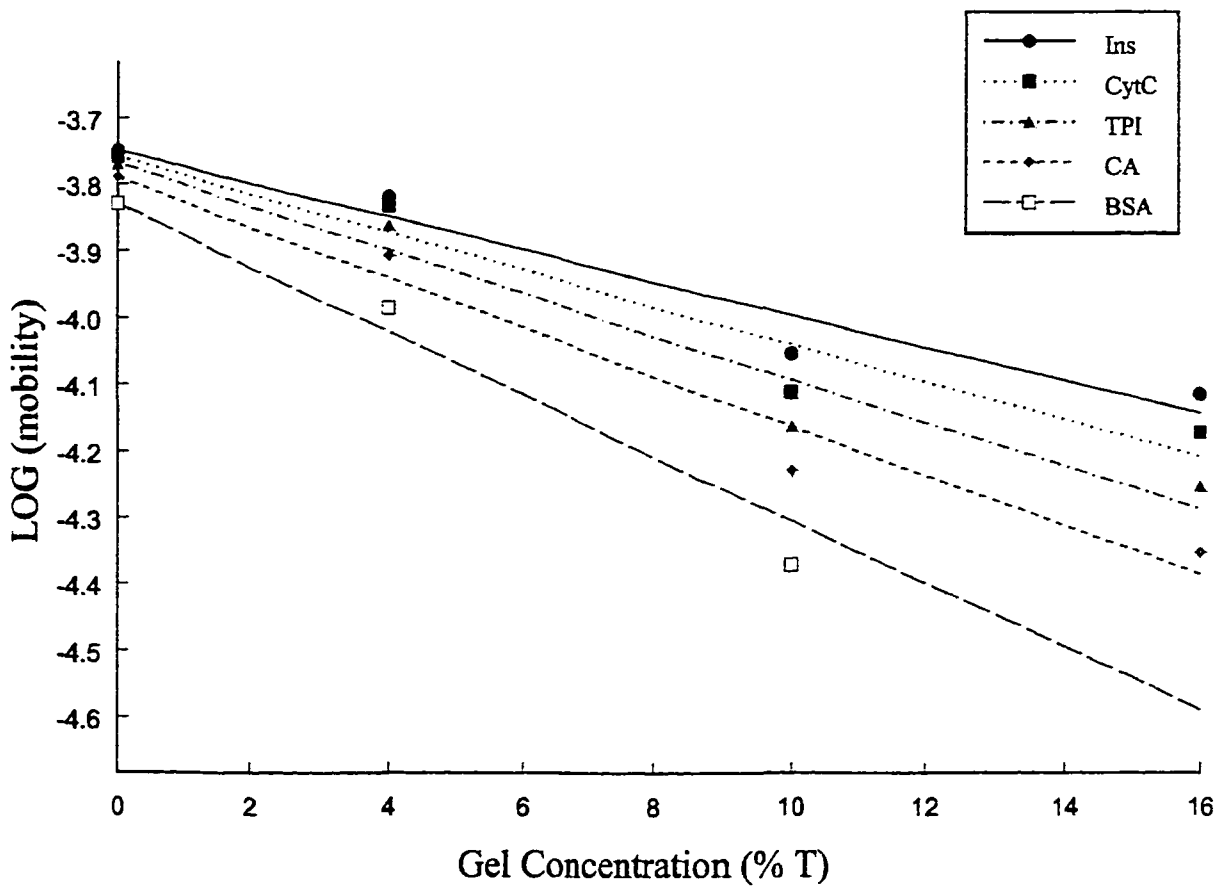

Fig. 6. Ferguson plot for 5 FITC-labeled proteins (insulin, cytochrome c, soybean trypsin inhibitor, carbonic anhydrase and bovine serum albumin). The mobility data for this curve was generated in a single electrophoretic run. Operating conditions and column (three-step gradient) are the same as given in Fig. 5.

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zone. Lowering the pH most likely restricted the number of attached labels. Quantitation of FITC-labeled proteins by size-sieving should pose no problems since the molecular weight differences due to the incorporation of more than one FITC label are generally not sufficient to produce multiple peaks. This is supported by the fact that all the solutes investigated for this work migrated as a single zone, with the exception of the smallest component, insulin.

4. Conclusions

We have described a simple method to cast step gradients in linear polyacrylamide gel concentration for capillary electrophoresis. The length of each step is controlled by filling the capillary with the gelling solutions using gentle vacuum and the microscope to monitor the filling process. Such gradients have been used in conjunction with LIF whole-column detection to demonstrate their potential as an alternative to single concentration gels for the separation of wide molecular mass range samples. Whole-column detection permits visualization of the expected sharpening of the zones and slowing of their migration velocity at the interface region between steps. When using the linear acrylamide gels it was not possible to completely inhibit migration through the gel. Presumably the dynamic nature of entangled polymer matrices will not afford such focusing. The more rigid pore structure of cross-linked gels may be required to impede migration along the capillary to achieve narrow focusing of the solute zones.

Acknowledgments

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the capillary stage. This work was supported, in part, by the National Science Foundation under Grant No. CHE9624884. We also acknowledge the UAB Department of Chemistry and UAB Graduate School for additional financial support.

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MEASUREMENT OF ADENOSINE BY CAPILLARY ZONE ELECTROPHORESIS WITH ON-COLUMN ISOTACHOPHORETIC PRECONCENTRATION

by

CHAO-CHENG WANG, WILLIAM P. MCCANN, STEPHEN C. BEALE

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Abstract

An on-column isotachophoretic (ITP)-capillary electrophoresis (CE) system capable of preconcentrating polyhydroxyl species is reported. The ITP-CE system utilizes borate complexation of the neutral diol species to form anionic compounds that can be directly separated by CE. Borate buffer functions as both the terminating electrolyte for the ITP preconcentration and the operating buffer for the subsequent CE separation. Isotachophoretic preconcentration allows injection volumes as large as 50% of the column volume, without compromising separation integrity, to yield detection limits about 70-fold lower than direct CE separation (with borate operating buffer). In this paper we also present an application of the ITP-CE system, with laser-induced fluorescence (LIF) detection, to the quantitative analysis of adenosine from urine. Nanomolar concentration levels of adenosine are successfully derivatized with chloroacetaldehyde (CAA) to form a fluorescent derivative whose spectral characteristics match the He-Cd laser. The technique is shown to be capable of quantitative measurement of adenosine as low as 10^{-9} *M*, the levels expected in plasma and urine.

1. Introduction

Adenosine has widespread effects on coronary and cerebral circulation [1], control of blood flow [2-3], prevention of cardiac arrhythmias [4] and nerve-tissue functions such as the inhibition of neurotransmitter release and the modulation of adenylate cyclase activity [5]. It has been suggested that these physiological actions are due to the interactions of adenosine with specific receptors, which are mediated by the changes of interstitial adenosine concentration [1]. To understand the involvement of adenosine in these systems, reliable

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analytical methods must be available to determine adenosine concentrations in plasma, tissue extracts, or urine.

Several methods, including HPLC with UV [5-7] or fluorescence detection [8-12] and radioimmunoassay [15,16], have been developed to quantitate plasma and urinary levels of adenosine with reported results varying between 10-100 n*M* [4-14]. Radioimmunoassay can provide very sensitive, quantitative results from nanomolar concentration levels, however, the inconvenience of using radioactive materials limits its applicability. The HPLC methodologies tend to be cumbersome with borderline sensitivity and subject to artifacts and interferences. HPLC with UV detection lacks the sensitivity required to accurately quantitate plasma levels of adenosine. Although fluorescence detection can provide suitable sensitivity for adenosine analysis by HPLC, the use of large blood samples required for HPLC may interrupt the in vivo equilibrium, particularly in small animals.

Capillary electrophoresis is a powerful tool for analyzing ionic species based on their electrophoretic mobility differences. The use of capillary-scale columns provides several advantages over conventional-scale separation methods that have been set-out elsewhere [17,18]. The minimal separation volumes result in mass detection limits at the femtomole level, with appropriate detection [19,20]. However, due to volumetric restrictions, the corresponding concentration detection limits are not as impressive.

Concentration detection limits of CE can be improved by increasing the sample capacity of the system. Sample stacking, in which samples are dissolved in a low electrical conductivity solvent, has been successfully used to improve the sensitivity of CE [21-23]. The effectiveness of stacking is limited by the trade-off of resolution and injection plug length [24]. Chromatographic concentration is another method for on-line CE detection limit

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enhancement involving large sample loading. Although this technique can improve sensitivity up to 1000-fold [25], the cost and difficulties in reproducibility of manufacture of on-line systems restrict its applications [24].

Isotachophoresis allows for larger sample loading than free-solution CE and has been successfully utilized on-line with CE to improve concentration detection limits [26-31]. By selecting proper leading and terminating electrolytes, large volumes of dilute sample can be focused into narrow bands. The narrow concentration pulses then serve as injection plugs for the free-zone CE separation. Two instrumental arrangements for ITP-CE have been described [30]. A coupled-column arrangement allows large sample loading and provides a sensitivity improvement up to 1000-fold, but requires a sophisticated experimental design [26-30]. In the second arrangement ITP is directly coupled, on-column, to CE with automatic transition to a CE separation mechanism, providing 50-100 fold concentration enhancements without any modification of the CE instrumentation [30,31].

Since only charged species can be directly analyzed by CE, neutral species will require an alternative means for separation. Micellar electrokinetic chromatography (MEKC) has been demonstrated for separation of neutral ribonucleosides [32,33]. The MEKC technique provides similar advantages as CE, such as high separation efficiency [34] and minimal sample requirements [35], but moderate concentration detection limits restrict the applications for trace samples. Furthermore, surfactants in the buffer solution may affect the reproducibility. Another option, for certain polyhydroxyl compounds, is the use of borate additives to the operating buffer. Borate will complex with polyhydroxyl compounds containing the *cis*-diol moiety to form anionic complexes which have a higher electrophoretic mobility than free boric acid [36,37]. With these properties, borate buffer has been used as an operating buffer to

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separate ribonucleosides in gel electrophoresis [38] and carbohydrates by CE [39-41]. Also, mobility differences between boric acid and the complexes makes it an attractive terminating electrolyte for the ITP separation of polyols [36,37].

Here we will describe an on-column transient ITP-CE system for analysis of borate complexes and applications to adenosine analysis from biological samples. In this system, borate functions as both operating buffer in which charged complexes from the neutral adenosine are formed, and as a suitable terminating electrolyte for the ITP preconcentration step. Laser-induced fluorescence detection is used to spectroscopically differentiate adenosine from other ribonucleosides that represent possible interferences, as their $1,N^6$-etheno derivatives, and improve concentration detection limits to the sub-nanomolar range without complicated, user-intensive operation or instrumental modification.

2. Experimental

2.1 Chemicals

Adenosine (ADO), cytidine, guanosine, uridine, and chloroacetaldehyde (CAA) were purchased from Aldrich (Milwaukee, WI, USA). The sugar and catechol samples were purchased from Sigma (St. Louis, MO, USA). The commercial preparation of CAA was found by titration [42] to have a concentration of 7.5 *M*. All dilutions are based on this concentration. Trimethylchlorosilane (TMS) was obtained from HULS (Bristol, PA, USA) and hydroxyethyl-cellulose (HEC) was from Fluka (Ronkonkoma, NY, USA). Electrophoretic grade Tris (hydroxymethyl) aminomethane (Tris) was purchased from Sigma and other analytical grade reagents were from Fisher Scientific (Pittsburgh, PA, USA). Calf intestinal adenosine deaminase, in 50% glycerol, was purchased from Sigma.

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2.2. Apparatus

A home-built CE system was used in this work. Briefly, a Bertan (Hicksville, NY, USA) ± 30 kV high-voltage power supply was used to provide the driving voltage across a 65-cm long, fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA), either 25 or 50 μm inner diameter. To eliminate the electroosmotic flow, the fused-silica capillaries were treated with TMS (procedure described below) and 0.1% HEC was incorporated in all operating buffers. The high voltage end of the capillary was housed in a plexiglass box to prevent accidental electrical discharge to the operator. Prior to TMS treatment, a small portion of the fused silica coating was removed with a flame to form a detection window 15 cm from the end of the capillary.

Detection was accomplished with an UV detector (Linear Instruments Model UVis200, Reno, NE, USA) or a home-built LIF system [43]. The LIF system consisted of a He-Cd laser (Liconix, Santa Clara, CA, USA) to provide about 6 mW of excitation power at 326-nm. The 415-nm emission fluorescence was isolated with spectral filters (Corion Corp. Vermont, MA, USA) and focused onto a photomultiplier tube. Optical mounts were from Newport Corp. (Irvine, CA, USA) and other optical components were from Melles Griot (Irvine, CA, USA). A low-noise current amplifier (Model SR750 Stanford Research Systems, Sunnyvale, CA, USA) was used to amplify and filter the signal. Data were collected and stored on an IBM PC clone microcomputer.

2.3. Capillary Coating

The coating procedure was modified from the method described by Yao and Regnier [44]. Fused-silica capillaries were first treated with 1*M* KOH for 15 min and washed with

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deionized water followed by methanol for 20 min each. The residual methanol was then evaporated by pulling air through the capillaries for 30 min. Trimethylchlorosilane (50%) in toluene was continuously drawn through the capillary by vacuum over an eight-hour period. The capillary was then rinsed with methanol to remove residual TMS solution. The capillaries were washed with deionized water for 20 min and flushed with the operating buffer containing 0.1% HEC for 20 min to complete the coating and column equilibration procedure.

2.4. Sample Derivatization

Aqueous ribonucleoside stock solutions were diluted to the appropriate concentration with 10 m*M* boric buffer. In 1.5 ml sample vials, 100 µl of 2 *M* CAA aqueous solution was added to 1 ml of nucleoside solutions. The sample vials were heated at 90°C for 50 min [9], and then stored in an ice bath.

2.5. Urine Sample Preparation

Urine samples were obtained from a healthy subject (Asian male, age 30). Freshly voided urine was collected into a chilled bottle containing sodium azide so the final azide concentration was 0.03% (w/v). After centrifugation to remove insoluble materials and particulates, 25 ml of the supernatant was mixed with 25 ml of 0.1 *M* phosphate buffer (pH 7.5), and put on ice.

To isolate adenosine, a boronic acid affinity column consisting of 0.8 ml immobilized boronic acid gel (Pierce, Affipak) was built in a 3-ml disposable syringe (Fisher Scientific). After saturating the column with 0.1 *M* carbonate buffer (pH 10), 10 ml of the buffered urine was loaded onto the column. The column was washed with 2 ml of 25 m*M* carbonate buffer

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(pH 10) and adenosine was then eluted with 9 ml of 0.1 *M* HCl. The eluent fraction containing adenosine was adjusted to approximately pH 7 with 0.5 *M* NaOH and applied onto a Sep-Pak C_{18} cartridge which had been prewashed with 5 ml of methanol followed by 5 ml of distilled water. The Sep-Pak was then washed with 1 ml of water, and the adenosine eluted with 1.5 ml of 25% methanol in 50 m*M* HCl (v/v). The aliquot was adjusted to a pH 6 with concentrated NaOH, and lyophilized. The dried sample was re-suspended in 1 ml of water and mixed with CAA solution for derivatization and further analysis.

A second 10 ml portion of buffered urine was incubated with adenosine deaminase (Sigma Chemical), 0.06 unit/ml for 50 min at 25°C to eliminate adenosine, and carried through the pretreatment procedure described above.

2.6. CE and ITP-CE

For CE analysis, samples were diluted in the operating buffer and about 7 nl was injected hydrodynamically (20 cm elevation for 25 sec) at the cathodic end of capillary. For ITP-CE analysis, samples were diluted with 50% of 10 m*M* borate buffer (pH 10) in the leading electrolyte solution. After filling the capillary and both reservoirs with 80 m*M* borate buffer (pH 10), sample was injected from cathodic end of capillary by hydrodynamic pressure. The calculation of injection volume is based on the measured time for the sample to flow from the injection to the detection end of the capillary under constant pressure. All analyses were performed at 15 kV for 30 min. The current during CE operations was constant at 28 μA. In ITP-CE, the current started at 25 μA for 50% column volume injection, dropped to 10 μA, then returned to about 20 μA.

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The leading electrolyte was prepared by titrating 180 m*M* HCl with Tris (1 *M*) to pH 7.5. The terminating electrolyte and operating buffer were 80 m*M* borate pH 10. Also, 10 m*M* borate pH 10 was used in sample preparation. All buffer and sample solutions contained 0.1% HEC and were filtered with 0.22-μm membrane filter (MSI, Westboro, MA, USA).

3. Results

To test the general performance of the ITP-CE system we examined the separation of various neutral polyol species. The direct CE separation (200 m*M* borate buffer) of amino-pyridine labeled sugars (peak identifications are given in the figure legend) with detection by UV absorbance at 240 nm is shown in Fig. 1a. Fig. 1b is the same mixture of sugars diluted 50-fold and preconcentrated on-line from a 50-fold larger injection volume (87 nl vs. 1.7 nl) by ITP. The detector sensitivity setting was identical in both runs. At this point, we do not have a clear understanding why xylose and ribose co-migrate in the ITP-CE arrangement. It may be due to inefficient de-stacking of solute zones as detailed by Hjerten [23].

Similar results were obtained for 6 neutral catechols (peak identifications are given in the figure legend). Fig. 2a is the direct separation of the catechols by CE using 200 m*M* borate buffer. Fig. 2b depicts the ITP preconcentration of the same mixture, again diluted 50-fold from the sample in Fig. 2a. The injection volume was 50 times greater for the ITP-CE system. With both the sugars and catechols injections of up to 50% of the column volume can be made without sacrificing column performance. This leads to concentration detection limit enhancements of ca. 70-fold over free-solution CE.

Since our interests lie in the quantitation of adenosine, we investigated the ITP-CE system for the analysis of ribonucleosides. In Fig. 3a the free-solution CE separation profile

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of adenosine, uridine, guanosine and cytidine at the 10^{-5} *M* level in 80 m*M* borate buffer is shown. Thymidine does not have a favorable diol arrangement to form an anionic borate complex and therefore does not migrate in this system (data not shown). Fig. 3b demonstrates the on-line ITP preconcentration capability for the four ribonucleosides in Fig. 3a. Comparison of the peak areas and variances from the two electropherograms indicates a 70-fold enhancement in concentration sensitivity for the ITP-CE, due to larger sample loading (injection volume about 490 nl) tolerated by the ITP-CE system. Calibration data for the ITP-CE system indicate concentration detection limits of $6 \cdot 10^{-7}$ *M* ($r^2 = 0.999$) with UV detection. This level of detection is still inadequate for expected adenosine levels in plasma or urine. Furthermore, cytidine co-migration with adenosine interferes with adenosine quantitation.

Our approach to simultaneously improve concentration sensitivity and resolve the adenosine/cytidine co-migration problem is LIF detection. The mass sensitivity enhancements associated with LIF detection are well known [19, 45-47]. Furthermore, by appropriate selection of fluorogenic reagent, excitation and emission wavelengths it is possible to spectroscopically separate the adenosine signal from the cytidine response.

Since the primary amine moiety present on the base functionality of adenosine, cytidine and guanosine is deactivated by the ring system, conventional fluorogenic amine reagents are unsuitable for ribonucleoside analysis. Chloroacetaldehyde (CAA) is commonly used to form 1,N^6-etheno-derivatives of adenosine and cytidine for fluorometric analysis [10, 48-50]. The structures of the etheno-derivatives of cytidine and adenosine are shown in Fig. 4.

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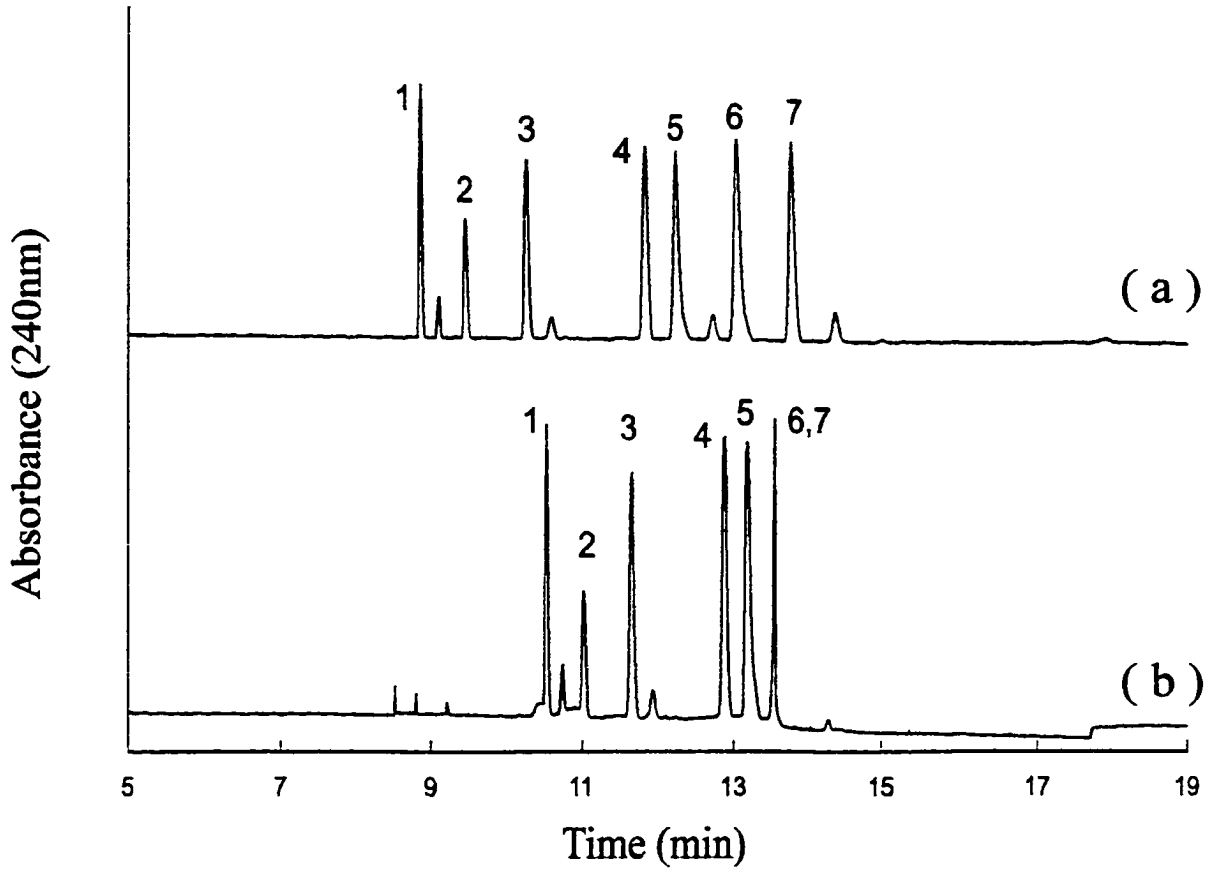

Fig. 1. (a) Direct CE (borate buffer) separation of amino-pyridine labeled reducing monosaccharides, peak identifications are as follows: 1) glucurone, 2) *trans*-cinnamic acid, 3) galactose, 4) arabinose, 5) glucose, 6) ribose, 7) xylose. All samples are $8.3 \cdot 10^{-4}$ *M* (injection volume 1.7 nl, electrokinetic). (b) The same sample diluted 50-fold and preconcentrated by on-line ITP-CE (injection volume 87 nl, hydrodynamic). Detection is UV at 240 nm.

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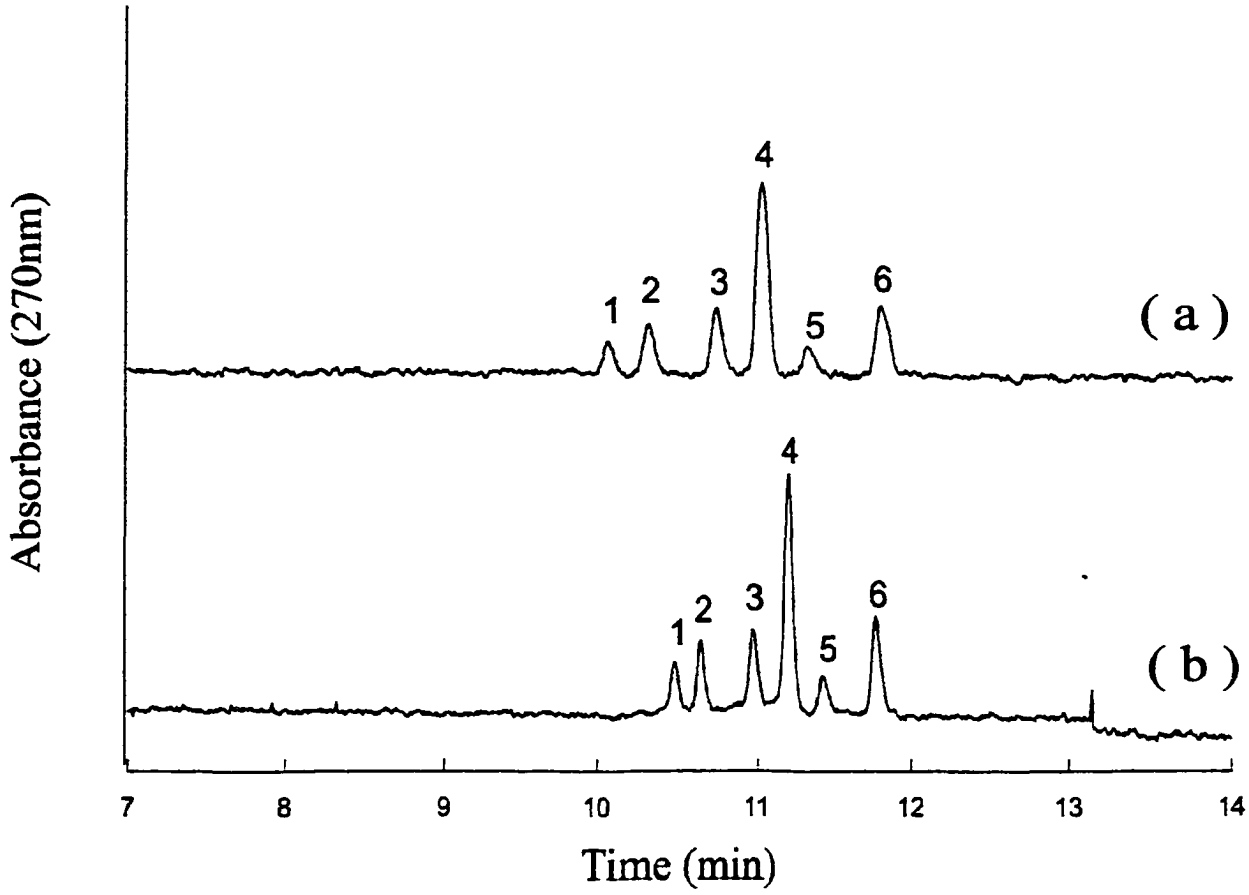

Fig. 2. (a) Separation of some catechols by direct CE in borate buffer. Peaks: 1 = pyrogallol, 2 = pyrocatechol, 3 = 3,4-dihydroxybenzaldehyde, 4 = 2,3-dihydoxybenzaldehyde, 5 = methyl catechol, 6 = 2,3-dihydroxynaphthalene at $6 \cdot 10^{-4}$ *M*. (injection volume 1.7 nl, electrokinetic). (b) The same sample as Fig. 2a, diluted 50-fold and preconcentrated by on-line ITP-CE (injection volume 87 nl, hydrodynamic). Detection is UV at 270 nm.

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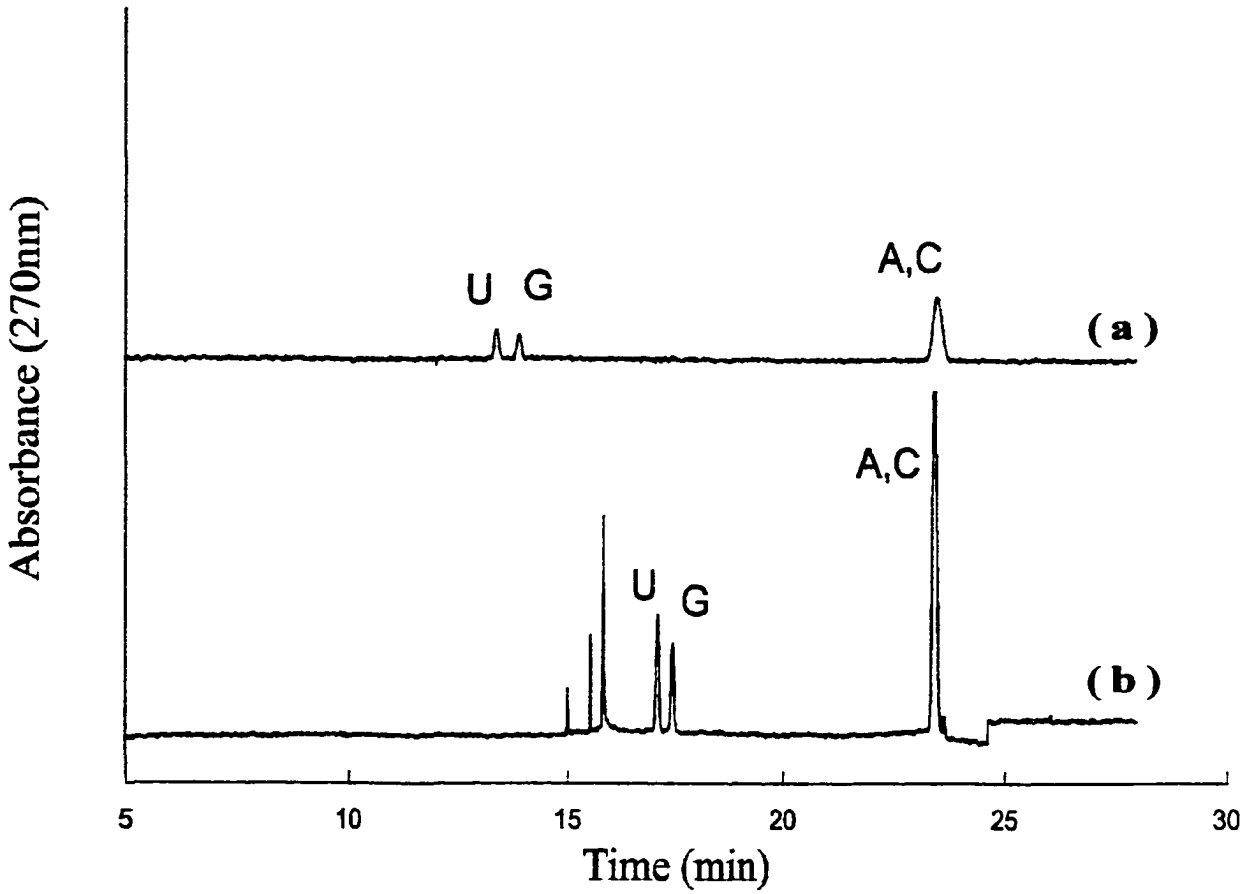

Fig. 3. (a) CE separation of ribonucleosides. The ribonucleosides were dissolved in operating buffer (80 m*M* borate, pH 10, 0.1% HEC) with the following concentrations: A = adenosine ($3.1 \cdot 10^{-5}$ *M*); U = uridine ($4.1 \cdot 10^{-5}$ *M*); G = guanosine ($2.18 \cdot 10^{-5}$ *M*); C = cytidine ($4.36 \cdot 10^{-5}$ *M*). Injection: 7 nl. UV detection at 270 nm. (b) On-column transient ITP-CE separation of the ribonucleosides. The sample was diluted with 50% 10 m*M* borate (pH 10) in leading electrolyte (180 m*M* HCl, pH 7.5) to the following concentrations: A = adenosine ($1.24 \cdot 10^{-6}$ *M*); U = uridine ($1.64 \cdot 10^{-6}$ *M*); G = guanosine ($1.74 \cdot 10^{-6}$ *M*); C = cytidine ($1.74 \cdot 10^{-6}$ *M*). Injection: 490 nl. UV detection at 270 nm.

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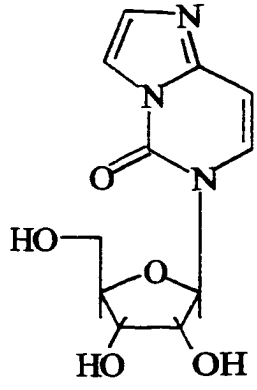
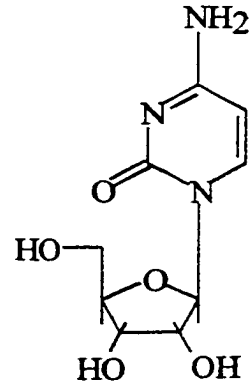


1,N^6-ethenoadenosine



1,N^6-ethenocytidine

Fig. 4. Structure of the etheno-adenosine and etheno-cytidine compounds.

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The etheno-derivatives all feature an excitation maximum at 270 nm and emission maximum at 415 nm. However, the similarities in the excitation spectra of the various derivatives diverge with increasing excitation wavelength. At 326-nm, the output wavelength of the He-Cd laser, etheno-adenosine (ε-ADO) still has sufficient fluorescence emission for sensitive analysis [51]. Cytidine, on the other hand, has no detectable quantum yield when excited at 326 nm, as shown in Fig. 5. It is thus possible to selectively excite ε-ADO fluorescence in the presence of the co-migrating ε-cytidine.

To evaluate the selectivity of the LIF system for ε-ADO, two free-zonal electropherograms (no ITP preconcentration), in 80 m*M* borate buffer were generated. Peak areas of ε-ADO were measured for 2 reaction mixtures, the first containing only adenosine and the second containing adenosine and a large excess of the other ribonucleosides. The adenosine peak areas for each reaction mixture were identical, within experimental error, indicating that any contribution to the fluorescence signal from other etheno-ribonucleoside derivatives is negligible. Furthermore, there is no observable signal from any other ribonucleoside present in the reaction mixture. Therefore quantitation of adenosine with LIF detection can be carried out selectively in the presence other ribonucleosides.

Fig. 6 depicts the ITP-CE analysis of $1.6 \cdot 10^{-9}$ *M* ε-ADO with LIF detection. The adenosine was derivatized at the 10^{-4} *M* level and diluted. The separation of ε-ADO derivatives by free-zonal CE analysis gave an efficiency of 8 000 plates; while the ITP preconcentration provides separation efficiency up to 400 000 plates. Adenosine laser-induced fluorescence calibration curves feature a dynamic range of 5 orders of magnitude (r^2 = 0.999) with concentration detection limits of $5.4 \cdot 10^{-10}$ *M*.

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To evaluate the CAA derivatization efficiency at the concentration levels expected from biological samples, the reaction was carried out on adenosine present at nanomolar concentrations. Fig. 7 depicts the electropherogram form the ITP-CE analysis of the ε-ADO produced by reaction of $5.4 \cdot 10^{-9}$ *M* ADO with 0.1 *M* CAA. The reaction mixture was diluted by 50% with leading electrolyte for the ITP-CE quantitation of ε-ADO. Even at a reaction concentration of $1.7 \cdot 10^{-9}$ *M* ADO, the ε-ADO signal is still observed with an average signal-to-noise (S/N) ratio of 7 ($n = 3$). Concentration detection limits for ITP-CE analysis of ε-ADO derivatized in this manner (real analysis situation) are $9.8 \cdot 10^{-10}$ *M*.

To demonstrate the capabilities of the ITP-CE system in a realistic biological system, we measured adenosine levels in urine. The urine sample was collected and centrifuged immediately to any remove cells and insoluble material. The supernatant was separated into two fractions, one was eluted through a boronic acid gel column followed by a Sep-Pak C_{18} prior to derivatization, as detailed in Section 2. Recovery of ε-ADO through the Sep-Pak column was 40%. The second fraction was treated for 50 min with adenosine deaminase to remove adenosine, then passed through the boronic gel and Sep-Pak columns and treated exactly as the first fraction. The ITP-CE runs for both fractions are shown in Fig. 8, the adenosine deaminase treated fraction is overlaid on the fresh urine sample. The ε-ADO peak was identified by comparison of migration time with authentic standards and by spiking experiments. Confirmation of the peak identity is indicated by the elimination of that peak upon treatment with adenosine deaminase (Fig. 8, solid line). Quantitation based on adenosine recovery through the pretreatment steps and calibration curves indicate that the concentration of adenosine in this urine sample was 1.3 μ*M*, which falls within the expected range of 0.3 - 7 μ*M* for urinary adenosine levels in humans.

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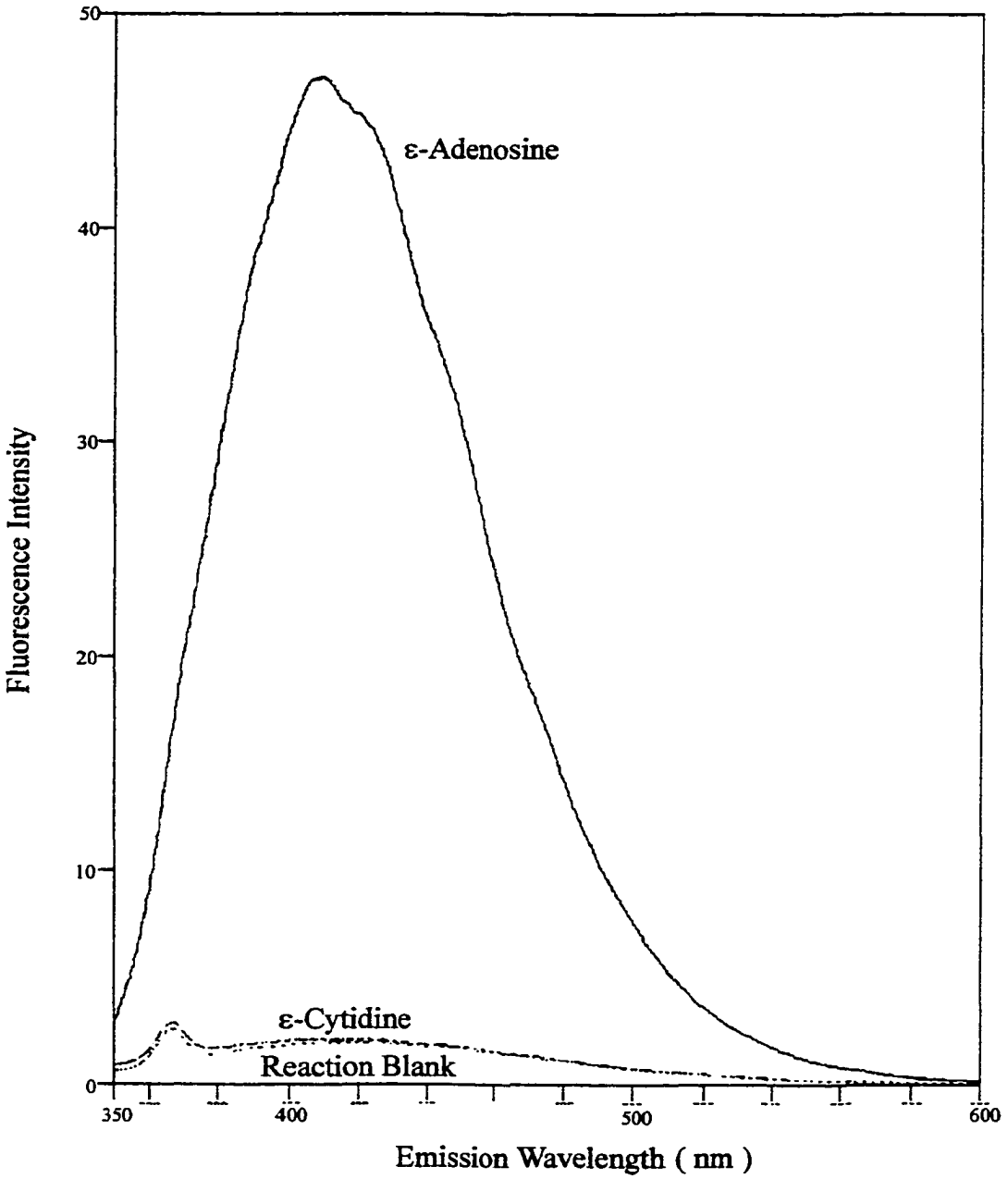

Fig. 5. Fluorescence emission spectra (excitation at 326 nm) of the etheno-derivatives of adenosine, cytidine, and a reaction blank. Samples were derivatized at 10^{-4} *M*, then diluted with 80 m*M* boric acid (pH 10) to concentrations: [ε-adenosine] = $4.6 \cdot 10^{-6}$ *M*; [ε-cytidine] = $3.6 \cdot 10^{-5}$ *M*.

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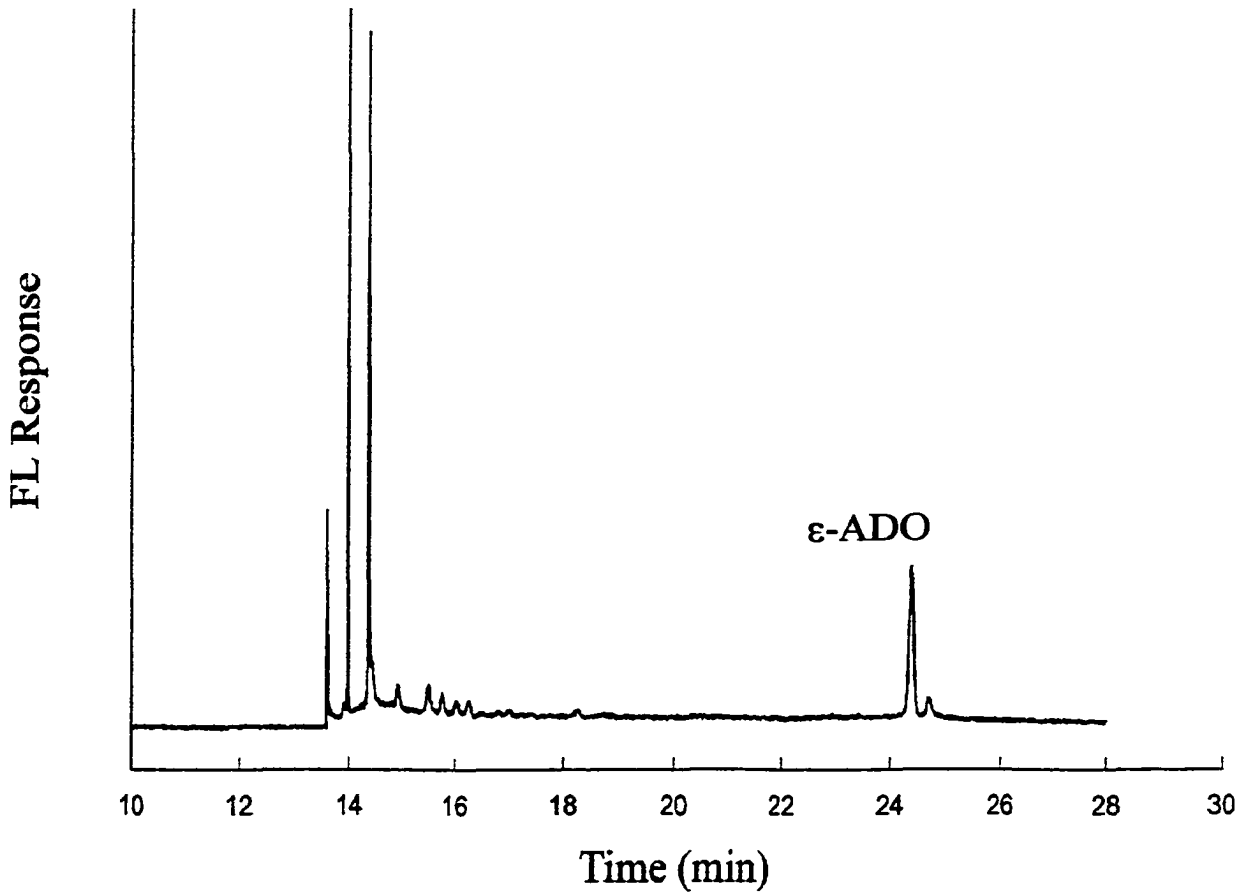

Fig. 6. On-column transient ITP-CE analysis of $1.6 \cdot 10^{-9}$ *M* etheno-adenosine with LIF detection. The sample was diluted from 10^{-4} *M* reaction stock with 50% 10 m*M* boric acid (pH 10) in leading electrolyte (180 m*M* HCl, pH 7.5). Injection: 490 nl.

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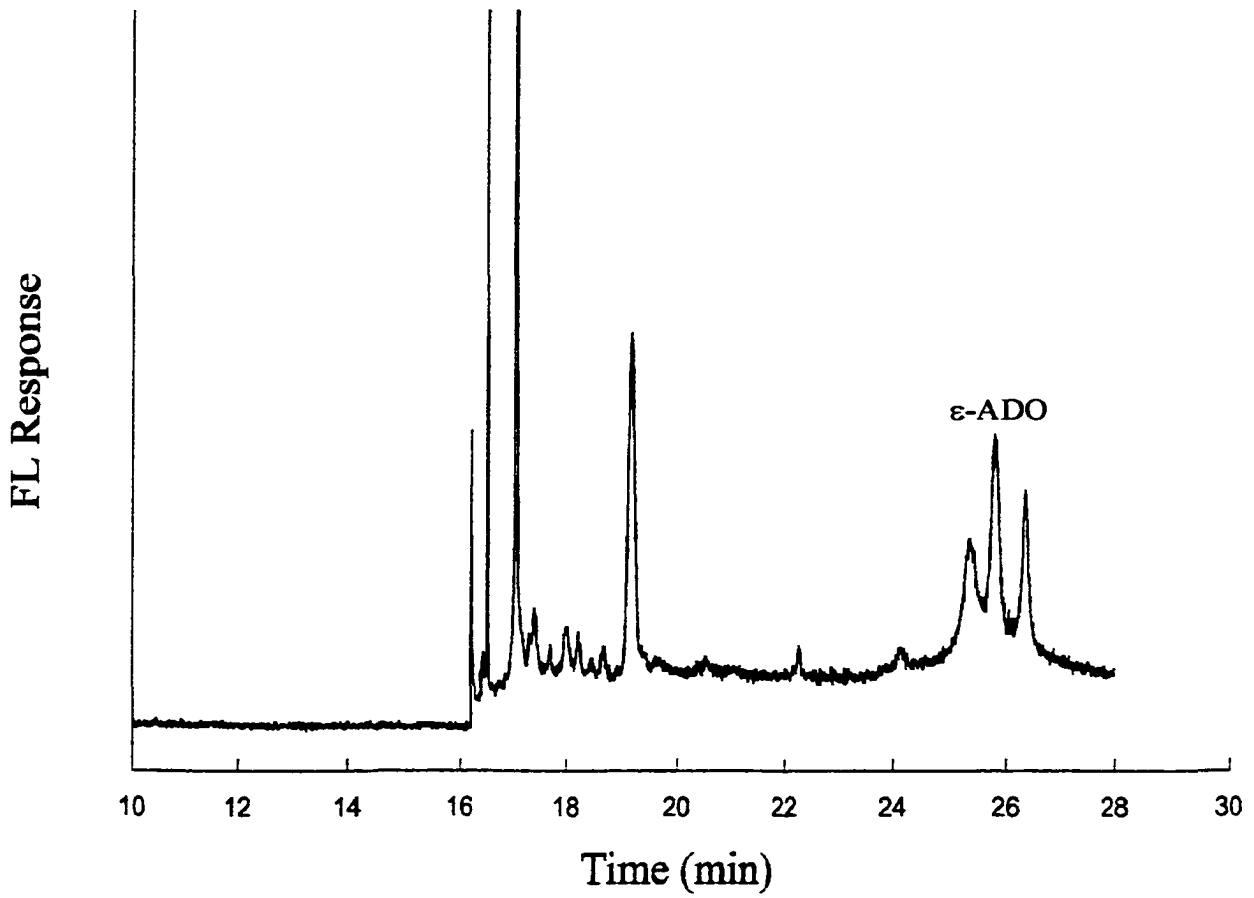

Fig. 7. On-column transient ITP-CE analysis of ethenoadenosine with LIF detection. The sample was $3 \cdot 10^{-9}$ *M* etheno-adenosine diluted from reaction of $6 \cdot 10^{-9}$ *M* ADO and 0.1 *M* CAA with leading electrolyte (180 m*M* HCl, pH 7.5). Injection: 490 nl.

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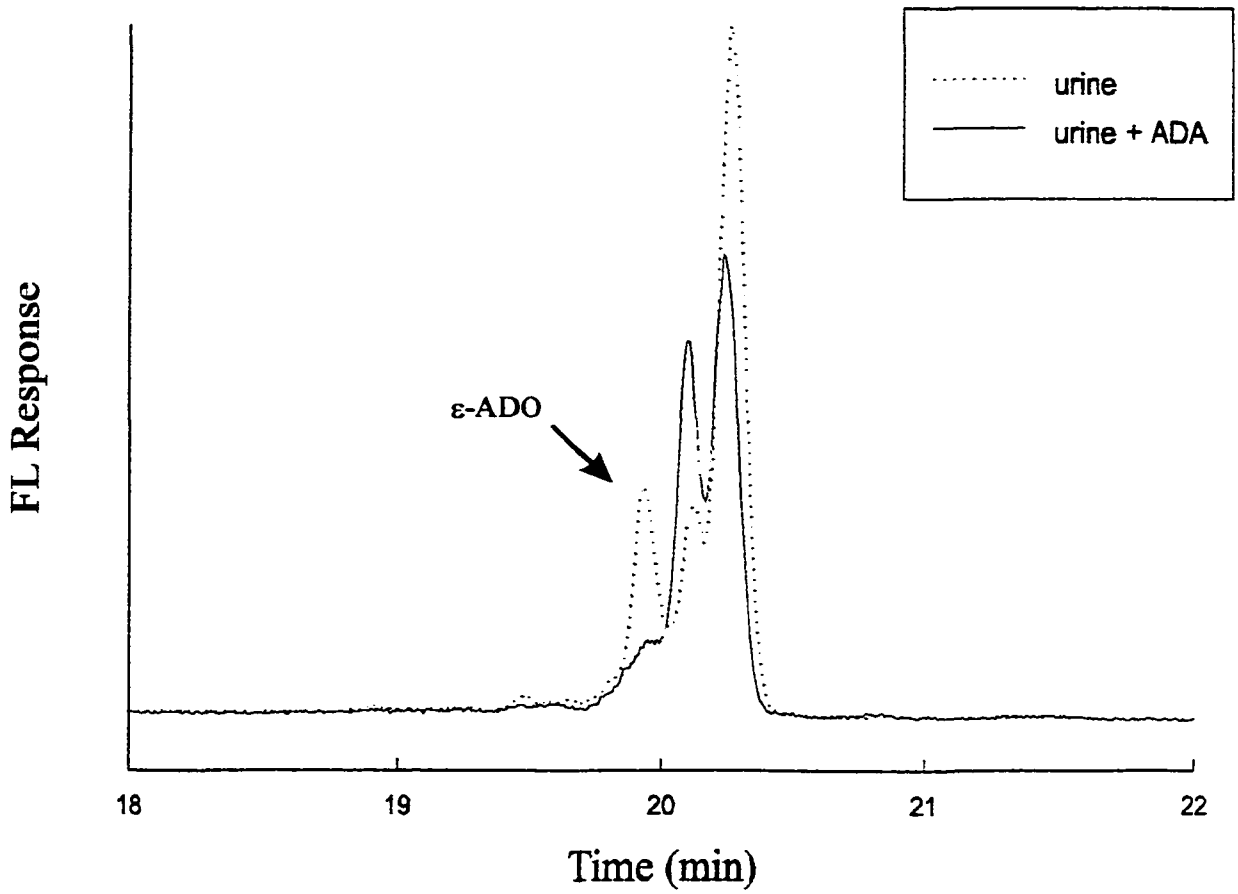

Fig. 8. Adenosine portion of the ITP-CE electropherogram of urine, pretreated as described in the Section 2. The dashed line is the urine sample. The solid line superimposed on the urine trace is the same urine sample treated with adenosine deaminase for 50 min to remove the adenosine.

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4. Discussion

We are interested in improved means to quantitate plasma or urinary levels of adenosine. Capillary electrophoresis techniques offer attractive, often complementary, alternative separation modes to the well-established HPLC methodologies, while requiring less samples and providing faster analyses. However, as mentioned earlier, poor concentration detection limits pose a substantial obstacle to widespread acceptance of CE techniques for biological problems in which solutes are present in low concentration. In addition, we believe results from current methodologies may be skewed by platelet aggregation, cell lysing and adenosine uptake during the sampling process.

Several methods of on-line preconcentration for CE have been described [21-31]. Foret, et al. [30,31] have presented two modes for on-column coupling ITP with CE. In the first, suitable leading and terminating electrolytes are used for the isotachophoretic preconcentration step. After the sample preconcentration is achieved, the terminating electrolyte is then replaced by the leading electrolyte, which also functions as operating buffer for CE separation. The second mode of ITP-CE is suitable when the CE operating buffer has a mobility lower than the sample ions, as is the case with the borate buffer system. The samples are supplemented with leading electrolyte while the column and buffer reservoirs contain the CE operating buffer (which functions as ITP terminator). In this case, isotachophoretic preconcentration of sample ions takes place at the beginning of the analysis with an automatic transition to CE mode separation as the ITP sample migrates through the column.

To our knowledge there have been no literature reports for coupling ITP and CE for polyol species. There are reports dealing with the analysis of polyol species by CE as anionic

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borate complexes using borate buffer or tetraborate buffer additives for direct free-zonal separation [39-41]. In addition, ITP with borate acting as both the terminator and complexation agent for the analysis of catechols [36] has been demonstrated. The borate complexes feature higher electrophoretic mobilities than free boric acid [36,37], thus borate is a good terminator for the ITP as well as operating buffer for the CE. We have demonstrated here a novel ITP-CE system that is useful for uncharged *cis*-diol species.

In order to separate and quantitate adenosine by CE, relatively high concentrations of borate are required to drive the complexation equilibrium to favor the charged complex. However, the ITP is most effective when the driving current is carried primarily by the sample and leading and terminating electrolytes (low background electrolyte concentration). We have found that the best results in coupling ITP and CE for separation of polyol species by borate complexation are achieved when a small amount (5 m*M*) of boric acid is incorporated in the sample solution.

For successful ITP preconcentration, electroosmotic flow must be eliminated. The popular polyacrylamide coating procedure described by Hjerten [51] is not suitable for the buffer system used in this work due to the base catalyzed hydrolysis of that coating which limits capillary lifetime to less than four runs. Our procedure for eliminating electroosmotic flow is a modified version of a report from Yao and Regnier's group [44] which involves adsorbing polymer buffer additives to alkyl-silane derivatized capillaries. We treat the capillary with TMS and incorporate 0.1% HEC into the operating buffer. In this manner, the electroosmotic flow was effectively eliminated as measured by the lack of migration of a neutral marker (acetone) after 45 min under a field of 300 V/cm. The TMS-coated capillary

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had a lifetime of over 100 working hours even with the basic buffer conditions described in this paper.

Here we have quantified adenosine in urine and demonstrated the capability of the ITP-CE technique to quantitate levels of adenosine from biological sources. The identification of the adenosine peak is based on migration time comparison with standard adenosine, spiking standard adenosine into the sample and the enzymatic metabolism of adenosine from the urine sample.

The electropherogram of the urine sample contains two unidentified components that migrate very closely with adenosine. As of yet we have not identified these two zones, but they seem to be related to adenosine. The relative peak areas of these solutes are sensitive to the presence of adenosine deaminase, they are retained by both the boronic acid gel and the C_{18} Sep-Pak and react with chloroacetaldehyde to form fluorescent derivatives with appreciable fluorescence when excited at 325 nm. An aliquot of urine treated as above, without CAA derivatization shows no peaks in this area of the electropherogram. The unidentified zones are not adenine, which does not migrate in our ITP-CE system or phosphate esters of adenosine (AMP, ADP or ATP), which are not retained by the Sep-Pak and migrate very rapidly in the ITP-CE system.

Removal of excreted cells and other particulate matter by centrifugation appears to be an important factor contributing to the relative peak area ratio for the two unidentified zones. If the sample is not immediately centrifuged their relative peak areas are very sensitive to the presence of adenosine. In data not presented here, we found that if the naturally occurring adenosine is removed by addition of adenosine deaminase to a urine sample not subjected to centrifugation, the faster migrating of these peaks increases dramatically in area.

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The addition of adenosine back to the urine sample, following inhibition of adenosine deaminase, results in the very rapid reduction in the size of that peak. Currently, we are looking further into the nature of these interactions, and the identity of these zones.

5. Conclusions

With borate buffer, on-column transient ITP-CE of neutral sugars, catechols and ribonucleosides is demonstrated. In this system, the borate serves as the complexation reagent to charge neutral species, functions as terminating electrolyte for ITP preconcentration and is the running buffer for CE separation. Without any instrumentation modification, the borate on-column ITP-CE system improves both the sample loadability and sensitivity more than an order of magnitude over CE. The LIF detection system, with 326-nm excitation not only enhances the sensitivity by about 2 orders of magnitude over UV detection, it also allows the spectroscopic separation of the adenosine signal from other ribonucleosides, such as cytidine which co-migrates with adenosine. The concentration detection limits demonstrated here are at the limits required for the analysis of plasma levels of adenosine. Furthermore, we have shown the ability to perform fluorogenic derivatization on analytes present at nanomolar concentration levels.

We have shown results from the analysis of adenosine in urine. The adenosine peak was identified by spiking with authentic standards. In addition, the identity was confirmed using adenosine deaminase to eliminate adenosine from the sample. The ITP-CE technique is shown to be a simple robust means of quantitating adenosine from urine.

The next step is to be the refinement of techniques for investigating more difficult samples, such as interstitial plasma adenosine concentrations. Production and metabolism of

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adenosine during the sampling process currently restricts adenosine analysis. We plan to address this issue through experimentation in the area of microdialysis and ultrafiltration sampling techniques, coupled with ITP-CE preconcentration and separation. Future experiments will hinge upon species differences in adenosine levels and the pharmacological role of adenosine.

Acknowledgments

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CONCLUSIONS

The general goal of this research is to extend the development of capillary electrophoretic techniques for biochemical and biomedical applications. Two research projects are involved in this dissertation: development of gel density gradients for CGE to extend the separation potential of CGE to wide molecular mass samples and development of alternative micro-scale methods for improved analysis of trace amounts of plasma adenosine by capillary electrophoretic techniques.

We have described a simple method to cast step gradients in linear polyacrylamide gel concentration for CE. The length of each step is controlled by filling the capillary with gelling solutions using gentle vacuum and the microscope to monitor the filling process. Such gradients have been used in conjunction with LIF whole-column detection to demonstrate their potential as an alternative to single concentration gels for the separation of wide molecular mass range samples. Whole-column detection permits visualization of the expected sharpening of the zones and slowing of their migration velocity at the interface region between steps. When using the linear acrylamide gels, it was not possible to completely inhibit migration through the gel. Presumably the dynamic nature of the entangled polymer matrixes will not afford such focusing. The more rigid pore structure of cross-linked gels may be required to impede migration along the capillary to achieve narrow focusing of the solute zones. With borate buffer, on-column transient ITP-CZE of neutral sugars, catechols, and ribonucleosides is demonstrated. In this system, the borate serves as the complexation reagent to charge

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neutral species, functions as terminating electrolyte for ITP preconcentration, and is the running buffer for CE separation. Without any instrumentation modification, the borate on-column ITP-CZE system improves both the sample loadibility and sensitivity more than an order of magnitude over CE. The LIF detection system, with 326-nm excitation, not only enhances the sensitivity by about 2 orders of magnitude over UV detection, but also allows the spectroscopic separation of the adenosine signal from other ribonucleosides, such as cytidine which co-migrates with adenosine. The concentration detection limits demonstrated here are at the limits required for the analysis of plasma levels of adenosine. Furthermore, we have shown the ability to perform fluorogenic derivatization on analytes present at nanomolar concentration levels.

We have shown results from the analysis of adenosine in urine. The adenosine peak was identified by spiking with authentic standards. In addition, the identity was confirmed using adenosine deaminase to eliminate adenosine from the sample. The ITP-CE technique is shown to be a simple robust means of quantitating adenosine from urine.

The next step will be to refine the techniques for investigating more difficult samples, such as interstitial plasma adenosine concentrations. Production and metabolism of adenosine during the sampling process currently restricts adenosine analysis. This issue can be addressed through experimentation in the area of microdialysis and ultrafilitration sampling techniques, coupled with ITP-CZE preconcentration and separation. Further experiments will hinge upon species differences in adenosine levels and the pharmacological role of adenosine.

In the past decade, transition of analytical techniques into nanoscale level is not only in the capillary format but also in chip-based devices. With the development of

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current micromechine and photolithographic technology, now it is possible to construct compact microfluidic channels and novel components in a single-piece of quartz, glass, or plastic. Integrated, microfluidic analytical systems have been applied to a variety of enzyme assays [176], restriction enzyme digestion and mapping of DNA [177], PCR and DNA sequencing [178,179], and immunological assays [180]. By integrating all these components into a single analytical system, the lab-on-a-chip concept can in the future possibly provide low-cost and high-throughput analyses for biomedical research and allow analytical separations to be performed in a non-laboratory environment [181,182].

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