NEW APPROACHES IN ANALYSIS OF BIOMOLECULES USING CAPILLARY ELECTROPHORESIS – METHODOLOGICAL STUDIES

Ph.D. Thesis

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2001

DOCUMENTS DISCUSSED

This dissertation is a summary of the following papers, which will be referred to in text by their Roman numerals.

I. **Á. Végvári, M.G. Schmid, F. Kilár, G. Gübitz**
Chiral Separation of α-Amino Acids by Ligand-Exchange Capillary Electrophoresis Using N-(2-hydroxy-octyl)-L-4-hydroxyproline as a Selector
Electrophoresis **1998**, 19, 2109-2111.

II. **M.G. Schmid, N. Grobuschek, C. Tuscher, G. Gübitz, Á. Végvári, E. Machtejevas, A. Maruška, S. Hjertén**
Chiral Separation of Amino Acids by Ligand-Exchange Capillary Electrochromatography Using Continuous Beds
Electrophoresis **2000,** 21, 3141-3144.

III. **S. Hjertén, Á. Végvári, T. Srichaiyo, H.-X. Zhang, C. Ericson, D. Eaker**
An Approach to Ideal Separation Media for (Electro)chromatography
J. Cap. Elec. **1998**, 5(1&2), 13-26.

IV. **F. Kilár, Á. Végvári, A. Mód**
New Set-up for Capillary Isoelectric Focusing in Uncoated Capillaries
J. Chromatogr. A **1998**, 813, 349-360.

ABSTRACTS PRESENTED AT INTERNATIONAL SCIENTIFIC MEETINGS AND RELATED TO THE THESIS

Á. Végvári, A. Mód, M. Szécsényi, F. Kilár. New Setup for cIEF of Hemoglobin Variants in Uncoated Capillaries**, 3rd Symposium on Instrumental Analysis**. (1995) Pécs, Hungary.

Á. Végvári, A. Mód, F. Kilár. New Setup for Capillary Isoelectric Focusing in Uncoated Capillaries, **HPCE'96, 10th International Symposium on High Performance Capillary Electrophoresis and Related Microscale Techniques,** (1996) Orlando, FL, USA.

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Á. Végvári, M.G. Schmid, G. Gübitz, F. Kilár. Determination of Hemoglobin Variants with Capillary Isoelectric Focusing. **4th Symposium on Instrumental Analysis,** (1997) Graz, Austria.

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S. Hjertén, C. Ericson, T. Srichaiyo, **Á. Végvári**. Capillary Electrochromatography in Homogeneous Media. **Analytica'98,** (1998) Munich, Germany.

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S. Hjertén, **Á. Végvári,** H.-X. Zhang, C. Ericson. The Interplay Between Theory and Practice to Approach the Perfect CEC Experiment. **HPCE'99, 12th International Symposium on High Performance Capillary Electrophoresis & Related Microscale Techniques,** (1999) Palm Springs, CA, USA.

Á. Végvári, H.-X. Zhang, C. Ericson, X. Xu, J.-P. Gao, D. Tong, S. Hjertén. Development of the Second Generation of the Continuous Beds in Pursuit of the Perfect Electrochromatographic Medium. **Symposium on Frontiers of Bioorganic Chemistry, Dedicated to the 50th Birthday of Professor Jyoti Chattopadhyaya,** (1999) Uppsala, Sweden.

S. Hjertén, C. Ericson, O. Kochegarova, V. Kudirkaite, A. Maruška, **Á. Végvári**, H.-X. Zhang. In Pursuit of the Perfect Electrochromatographic Medium. **ICES'99, Electrophoresis '99, Meeting of the International Council of Electrophoresis Societies,** (1999) Tokyo, Japan.

Á. Végvári, H.-X. Zhang, C. Ericson, X. Xu, J.-P. Gao, D. Tong, S. Hjertén. Advantages of Completely Homogeneous Continuous Beds in CEC Applications. **Analysdagarna**, (1999) Uppsala, Sweden

Á. Végvári, H.-X. Zhang, C. Ericson, X. Xu, J.-P. Gao, D. Tong, S. Hjertén. Properties and Applications of Homogeneous Continuous Beds in CEC. **Analysdagarna**, (1999) Uppsala, Sweden

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Á. Végvári, C. Ericson S. Hjertén, Completely Homogeneous Continuous Beds in CEC. **HPCE2000, 13th International Symposium on High Performance Capillary Electrophoresis & Related Techniques**, (2000) Saarbrücken, Germany

Á. Végvári. Completely Homogeneous Gels in Capillary Electrochromatography - An Extremely High Resolving Medium. **Uppsala University, Department of Biochemistry, Thursday Seminar Series**, (2000) Uppsala, Sweden

TABLE OF CONTENTS

ABBREVIATIONS

Aa	Amino acid
AA	Acrylic acid
APB	Acryloyl-phenylboronic acid
BGE	Background electrolyte
CB	Continuous bed
CE	Capillary electrophoresis
CIEF	Capillary isoelectric focusing
CEC	Capillary electrochro-matography
CSP	Chiral stationary phase
EOF	Electroendosmotic flow
HAP-L-Hypro	N-(2-Hydroxy-3-allylpro-pyl)-L-4-hydroxyproline
Hb	Hemoglobin
HO-L-Hypro	*N*-(2-hydroxyoctyl)-L-4-hydroxyproline
HPCE	High Performace Capillary Electrophoresis
HPLC	High-performance liquid chromatography
I.D.	Inner diameter
LC	Liquid chromatography
LECE	Ligand-exchange capillary electrophoresis
L-Hypro	L-4-hydroxyproline
LOD	Limit of detection
MOR	4-morpholinyl
MPIPE	1-(4-methylpiperazinyl)
PIP	1-piperidyl
UV	Ultraviolet light
Vis	Visible light
D	Diffusion coefficient
e	Elementary charge
E	Electric field
ε_0	Permittivity of vacuum
ε_r	Relative dielectric constant
H	Plate height
η	Viscosity
I	Ionic strength
k	Capacity factor
κ	Conductivity
l	Effective length of capillary
L	Total length of capillary
μ	Mobility
N	Plate number
r_i	Radius of ion
r_c	Radius of capillary
t	Migration time
T	Absolute temperature
v	Velocity
V	Applied voltage
w	Width of peak
z	Charge
ζ	Zeta potential

INTRODUCTION

Organic compounds are composed particularly of four chemical elements (carbon, hydrogen, oxygen and nitrogen), building up all living organisms and taking place in their metabolism, energy transport and movement. These biomolecules present in living matter occur in extraordinary varieties, and most of them are extremely complex. According to some estimations all living organisms, ranging in complexity from *E. coli* to the human organism, may contain somewhere between 10^{10} and 10^{12} different kinds of protein molecules and about 10^{10} different kinds of nucleic acids. If we compare these figures with the total number of all organic compounds that have been synthesized to date, which is only about 1 million (or 10^6) it is clear that we know the precise structure of only an extreme small fraction of all the organic molecules that are believed to exist in living matter. Therefore, to isolate, identify, and synthesize all different organic molecules present in living matter would appear to be a hopeless undertaking.

Fortunately, the physico-chemical properties of biomolecules are characteristically different from each other, which can be utilized in the analyses of biologically important molecules in order to identify and obtain them in high purity. In the development of new separation techniques the final goal is to design general methods that can be adapted to special analyses with convenience and speed using simple, automated instrumentation.

Among modern separation techniques, capillary electrophoresis (CE) has become popular due to several important features. CE can be applied to analyses of small inorganic/organic ions, peptides, biopolymers such as proteins and DNA, viruses and cells.

This work is based on methodological studies of CE, using biomolecules to exemplify the usefulness of the technique.

HISTORICAL BACKGROUND

Electrophoresis is the migration of electrically charged species in an electric field [Kohlrausch, 1897; Longsworth, 1959; Vesterberg, 1989]. Since the 1920's electrophoresis has been used for separation of large molecules, such as proteins and nucleic acids and small molecules like peptides. It can be conducted in different modes and in different media, such as carrier-free electrolyte solutions and agarose or polyacrylamide gels, the latter having not only a stabilizing effect against convection but also often a size-sieving function. A basic theoretical foundation was laid in the late nineteenth century, particularly by *F. Kohlrausch* who introduced the so-called regulating function [Kohlrausch, 1897] that has the same numerical value in all moving phases.

As a new technique for the study of the physico-chemical properties of proteins, *A. Tiselius* developed the moving boundary method [Tiselius, 1930; 1937], which led to the Nobel Prize in 1948. This method revealed the heterogeneity of serum: four moving boundaries corresponding to albumin, α-, β- and γ-globulin. *S. Hjertén* improved the device by reducing the cross-sectional area of the U-tube (increasing the surface area relative to the volume), which required elimination of electroendosmosis and the introduction of a UV-scanning method to obtain high resolution and sensitivity [Hjertén, 1967]. A weakness of the moving boundary method is that only partial separations can be accomplished, i.e., the sample constituents cannot be fully resolved from each other. Therefore, *Hjertén*, in the 1960's, developed an electrophoresis apparatus where the different components in a sample mixture could completely be resolved into distinct zones; so-called free zone electrophoresis [Hjertén, 1967]. The device also permits the zones to be withdrawn for preparative purposes. He used tubes of 1 to 3 mm internal diameters (I.D.) made of quartz rotating around their longitudinal axis in order to suppress convection due to the gravitational field. The tubes were treated (coated) with methylcellulose to prevent electroendosmosis. A UV-scanning detector was employed for monitoring the separated constituents. By the use of these relatively narrow-bore tubes in combination with efficient cooling the advantage of increasing the field strength could be realized thereby increasing the separation efficiency. This apparatus laid the foundation for what today is called high-performance capillary electrophoresis (HPCE). *Hjertén* emphasized that there should be no need to stabilize the sample zones by rotation or a stabilizing media if tubes of sufficiently narrow bore were

employed. Moreover, if tubes with a narrow bore were employed in combination with thin tube walls, even higher field strengths could be used to decrease the analysis time and increase the resolution, since the heat development during electrophoresis could then be dissipated more efficiently, thereby avoiding disturbing thermal effects. However, in those days the technology to realize the theoretical know-how was not available, especially regarding sensitive absorption detectors.

The advantage of performing the separation in capillaries were recognized early and the first attempts to develop capillary electrophoresis as a microanalytical separation tool were performed in the mid-60's to the late 70's by *S. Hjertén* [1967], *R. Virtanen* [1974] and *F.E.P. Mikkers and co-workers* [1979]. *R. Virtanen*, in the early 1970's was the first to exploit the anti-convective properties of stationary narrow bore tubes. He used glass capillaries with an I.D. of 0.2 mm employing a potentiometric detector [Virtanen, 1974]. Earlier, *F.M. Everaerts* used tubes made of teflon with I.D. 0.6 mm in an isotachophoretic instrument equipped with a thermometric detector [Everaerts and Hoving-Keulemans, 1970]. In the early 1980's, *J.W. Jorgenson and K.D. Lukacs* employed glass capillaries with an I.D. of 0.075 mm and a sensitive fluorescence detector [Jorgenson and Lukacs, 1981]. They also clarified the basic relationships between operational parameters and separation quality [Jorgenson and Lukacs, 1981, 1983; Lukacs and Jorgenson, 1985].

Today, fused silica is the material most commonly employed to manufacture capillaries. Fused silica tubing was introduced by *Bente and co-workers* [1981] for gas chromatography. This invention was a prerequisite for HPCE to become a widely used analysis method. It has excellent thermal, optical and electrical properties and an outer coating of polyimide makes the fused silica flexible and mechanically stable. Together with a sensitive detector, normally a versatile UV-detector that is relatively inexpensive, the efficiency of narrow-bore tubes for high-efficient separations is approaching what is theoretically predictable.

THEORETICAL BACKGROUND

Electrophoresis

Electrophoresis is the migration of charged particles in an electric field. When an electric field is applied, the positively and negatively charged ions are forced to migrate towards the cathode and anode, respectively. The migration of the components is differential due to different properties of the analytes, such as charge and mass.

The linear velocity (v_i) of a migrating ion (i) in an electrolyte solution is given by:

$$v_i = \mu_i \cdot E \qquad (1)$$

where E is the electric field applied and μ_i is the electrophoretic mobility of the ion i. Thus, an increase in field strength increases the velocity. One of the major advantages of capillary electrophoresis is the ability to employ high field strengths and thereby to obtain short analysis times and high-efficiency separations, although care must be taken to avoid disruptive thermal effects (see *Zone broadening effects* below). When an ion has been accelerated to constant velocity in a constant electric field, the electric force on the particle is equal to frictional force, which gives the relation:

$$v_i = \frac{z_i \cdot e \cdot E}{6 \cdot \pi \cdot r_i \cdot \eta} \qquad (2)$$

where z_i is the charge, r_i is the solvated radius of the ion, e is the charge of an electron and η is the viscosity of the fluid.

The mobility is a characteristic constant for a species that is strongly dependent on the properties of the solution. The mobility is determined by the following expression:

$$\mu_i = C \cdot \frac{\varepsilon_0 \cdot \varepsilon_r \cdot \zeta}{\eta} \qquad (3)$$

where C is a constant, ε_0 is the permittivity of vacuum, ε_r is the relative dielectric constant of the solution, ζ is the zeta potential close to the capillary wall (i.e., the electrostatic potential at the plane of shear) and η is the viscosity of the solution. Since the magnitude of the ζ potential is affected by the solution properties so is the mobility. Especially important is the pH, which strongly influences the net surface charge density.

The mobility is determined from electrophoresis experiments. By replacing v_i with l/t and E with V/L in equation 1, the following formula is obtained:

$$\mu_{eff} = \frac{l \cdot L}{t \cdot V} \qquad (4)$$

where l is the length to the detector, L is the total capillary length, t is the migration time and V is the applied voltage. An absolute mobility can be defined at some limiting conditions, for instance as the mobility when the ionic strength approaches zero.

When a charged ion is dissolved in an electrolyte ions of opposite charge accumulate around the ion by electrostatic forces [Ross and Morrison, 1988]. A thin region around the counter ion is termed the electrical double layer. When an electric field is applied the ions in this layer begin to move. The counter ions migrate in an opposite direction leading to a retardation of the charged sample ions. The thickness of the electrical double layer is given by the Debye length ($1/\kappa$), where κ is defined as:

$$\kappa = F \cdot \left(\frac{2 \cdot I}{\varepsilon_0 \cdot \varepsilon_r \cdot \mathrm{R} \cdot T} \right)^{1/2} \qquad (5)$$

F is the Faraday constant (96,500 $Cmol^{-1}$), ε_0 is the permittivity of vacuum ($8.85 \cdot 10^{-12}$ $C^2N^{-1}m^{-2}$), ε_r is the dielectric constant of the fluid medium, R is the gas constant, I is the ionic strength and T is the absolute temperature.

Electroendosmosis

Electroendosmosis occurs when an electric field is applied parallel to the axis of a capillary tube and refers to the motion of the liquid at a constant speed induced by the field [Helmholtz, 1879; Hunter, 1981].

The flow is generated by the presence of charge excess near the surface of the oppositely charged wall (the electroneutrality equation is not valid). The applied electric field forces the charge excess to move towards one of the electrodes and the motion of these ions will draw the bulk liquid along with them, creating a flow in which shear forces are prominent only within a very short distance (a few Å) between the surface and the region of maximal flow velocity. When the radius of the capillary is large compared to the electrical double layer the bulk velocity of the liquid is given by the Smoluchowski equation:

$$\mu_{eo} = \frac{\varepsilon_0 \cdot \varepsilon_r \cdot \zeta}{\eta} \qquad (6)$$

where μ_{eo} is the electroosmotic mobility.

When charged particles with an affinity for the capillary surface are dissolved in the BGE they adsorb to the wall surface thereby causing a change in the surface potential (Ψ_0) and, consequently, the ζ-potential.

In a fused silica capillary, silanol groups start to dissociate above pH 2. Under the influence of an electric field, bulk liquid will then be transported towards the cathode since the surface is negatively charged. If the internal diameter is large the flow profile will be almost flat (plug-like) as compared to the parabolic laminar, hydrodynamic flow generated by pumps.

The flat flow profile does not contribute (except in a thin layer close to the tube wall) to a broadening of a solute zone as does a parabolic profile, EOF only displaces the solutes.

Equation 6 shows that electroendosmosis would disappear if the ζ-potential were zero. Therefore, the inner wall of the silica capillaries can be coated with an electrically neutral thin layer formed by polymers [Hjertén, 1967, 1985] to eliminate EOF. If the absence of EOF is required for separation, like in one of the modes of capillary isoelectric focusing (CIEF), coated capillaries should be used. Stable neutral coatings can reduce effectively the adsorption of proteins onto the capillary wall.

Unfortunately, these coating procedures often involve tedious, complicated chemical treatments and tend to degrade at high pH values.

Zone broadening effects

Broadening of a solute zone is an inevitable process in most modes in CE (exception is CIEF under ideal conditions) and should be kept as small as possible to attain high resolution. CE separations are mostly carried out by introduction of a narrow sample zone at the one end of the capillary. During the migration through the capillary several dispersion phenomena will contribute to broadening of the sample zone.

The total variance (σ^2_{tot}) can be splitted into individual contributions to the dispersion, which can be expressed with respect to their variances (σ^2). The zone broadening effects that results in Gaussian peaks may be expressed as:

$$\sigma^2_{tot} = \sigma^2_{inj} + \sigma^2_{diff} + \sigma^2_J + \sigma^2_{ads} + \sigma^2_{\perp\kappa} \ldots \qquad (7)$$

We assume here that the variances are independent of each other.

The length of the sample zone injected (l_{inj}) should not exceed 1-2 % of the electrophoretic migration distance. Its variance is expressed as:

$$\sigma^2_{inj} = \frac{l^2_{inj}}{12} \qquad (8)$$

In ideal separations its contribution should be negligible. Due to the short path length of the detector cell (i.e., the diameter of the capillary) the sensitivity in CE systems is limited. Therefore, longer sample zones need to be injected and concentrated.

As the solute migrates in an electric field Brownian motion gives rise to a longitudinal diffusion of the sample plug [Giddings, 1991]. The magnitude of this motion is governed by the diffusion coefficient ($D=\mu_i \cdot R \cdot T / z_i \cdot F$ where μ_i is the mobility of a given ion, R is the gas constant, T is the absolute temperature, z_i is the charge of the ion and F is the Faraday constant). The diffusional motion of the

solutes varies with the size of the molecules. The contribution from the diffusion to σ^2_{tot} in the CE systems is depending on the migration time of the zone (t) as shown in the Einstein equation:

$$\sigma^2_{diff} = 2 \cdot D \cdot t \qquad (9)$$

The electric current that originates from the migration of the buffer ions in the capillary generates a rise in temperature. This temperature effect is called Joule heating. Inside the capillary a temperature gradient develops since the capillary is cooled by the surrounding medium. As the viscosity of water changes 2-3 % for a change in temperature of one degree [Giddings, 1991] a viscosity gradient is formed from the axis of the capillary towards the inner wall. *S. Hjertén* [1990] has derived an equation for zone broadening due to Joule heating (σ^2_J):

$$\sigma^2_J = \frac{\left(\frac{B \cdot \kappa \cdot L}{\lambda} \left(\frac{r_c \cdot E}{2 \cdot T_0} \right)^2 \right)^2}{12} \qquad (10)$$

where r_c is the radius of the capillary, λ is the thermal conductivity of the buffer, B is a constant (2400 K), κ is the electrical conductivity of the buffer, L is the capillary length to the detector and T_0 is the temperature of the coolant liquid.

Interactions between the sample ions and the capillary surface should be minimized otherwise tailing peaks may occur.

The interaction will be strong if the solute and the capillary surface have opposite charges. The contribution from adsorption effects can be expressed with its variance as:

$$\sigma^2_{ads} = \frac{C \cdot r_c^{\,2} \cdot v_i^2}{D_i} \qquad (11)$$

where C is a constant and a function of the capacity factor ($k=(t_i-t_M)/t_M$ where t_i and t_M are the migration times of the analyte and a neutral marker, respectively) of the

sample ions, r_c is the radius of the capillary, v_i is the velocity of the solute i and D_i is the diffusion coefficient [Grossman and Colburn, 1992]. Adsorption of the analyte on the wall may result in tailing and non-Gaussian peaks and there are doubts whether this type of dispersion should be accounted for as a variance [].

If the sample zone has a conductivity (κ) which differs from that of the surrounding buffer, zone broadening will occur since the field strength inside and outside the zone will be different. The conductivity difference is caused by the difference in effective mobility between the sample ion and the electrolyte ion (if the sample ion is negative (positive) one should consider only the negative (positive) buffer ion). The peak becomes asymmetric with either fronting or tailing, i.e., a Gaussian concentration distribution is no longer observed. The zone broadening, expressed in its variance, is:

$$\sigma^2_{\Delta\kappa} = \frac{L^2}{16} \cdot \left(\frac{\Delta\kappa}{\kappa} \right)^2 \qquad (12)$$

where $\Delta\kappa$ is the conductivity difference between the electrolyte and the migrating zone and κ is the conductivity of the buffer. To minimize $\Delta\kappa$, the sample should have a low concentration and the electrolyte ion should have a mobility close to that of the sample ion.

Efficiency

In separation science, two related concepts for measuring the efficiency of a separation are widely used, plate height (H) and plate number (N). The width of the analyte zone may be expressed by the plate height (H).

$$H = \frac{\sigma^2}{L} \qquad (13)$$

where σ^2 is the variance of zone and L is the effective separation length to the detector. In an ideal separation the zone broadening of a solute should be as small as possible, which means that the variance of a zone should be kept at a

minimum. Low values of H are thus favorable and are in the μm range for high-efficiency separations.

In an optimized CE system the ions are separated in the buffer solution due to differences in effective mobilities and the diffusion is the only zone-broadening factor.

An alternative, commonly employed term in electrophoresis is the plate number:

$$N = \frac{L}{H} = \frac{L^2}{\sigma^2} \tag{14}$$

The plate number can be obtained at half the height or at the baseline of a peak by use of the following formula:

$$N = f \cdot \left(\frac{t_i}{w} \right)^2 \tag{15}$$

where w is the width of the peak at half the height or at the baseline, t_i is the migration time and the factor (f) is 5.54 at half the height and 16 when the width of the peak at the baseline is used. Theoretical predictable values are commonly in the range of $1 \cdot 10^5$-$5 \cdot 10^5$ m^{-1}.

Resolution

The resolution (R_s) is a quantitative measure of the degree of separation of two species and is defined as:

$$R_s = \frac{2 \cdot \Delta t_{mig}}{(w_1 + w_2)} \tag{16}$$

or:

$$R_s = \frac{1.18 \cdot \Delta t_{mig}}{(w_{1(1/2)} \pm w_{2(1/2)})} \tag{17}$$

where Δt_{mig} is the difference between the migration times of the analytes and w_1 and w_2 are the widths of the peaks at baseline; $w_{1(1/2)}$ and $w_{2(1/2)}$ are the peak widths at half the height. Accordingly, to achieve a high resolution Δt should be large, whereas the zone width should be kept at a minimum. R_s is dependent on the number of theoretical plates and the relative mobility difference of any two species [Giddings, 1969; Jorgenson and Lukacs, 1981; Guttman *et al.*, 1988]:

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{\Delta\mu_{app}}{\bar{\mu}} \qquad (18)$$

where $\Delta\mu_{app}$ is the difference in apparent mobilities of two solutes (for instance, enantiomers) and $\bar{\mu}$ is their average mobility. The R_s is made up of a selectivity term ($\Delta\mu_{app}/\bar{\mu}$) and an efficiency term (N). The selectivity term is determined by the properties of the analytes and the buffer, whereas the efficiency term is determined by the properties of the buffer and the instrument and other factors.

PRESENT STATE OF HIGH-PERFORMANCE CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE) is one of the most promising separation techniques in the field of separation science. The number of publications has grown rapidly and international meetings are held annually. Virtually all kind of molecules can be analyzed by CE, charged, as well as non-charged (following complexation with charged molecules in CE and CEC). The usefulness is reflected in the diverse fields in which the technique is employed, for instance, environmental sciences, biochemistry, molecular biology, clinical diagnosis, biotechnology and forensic medicine. By the introduction of commercial CE apparatus in the mid 1980's the technique has been established not only as a complement to high-performance liquid chromatography (HPLC) but sometimes also as a substitute when speed and separation power are enhanced by the using of CE.

Like HPLC, CE is amenable to automation, on-line detection and quantification of the separated species. Further, CE, as well as HPLC, is readily adapted to a preparative mode. However, the mass load is lower in CE that might hamper subsequent analyses, but can be overcome by special techniques.

In comparison to slab gel electrophoresis, CE has been considered to have a low sample throughput since only one sample can be analyzed at a time, but by use of arrays of capillaries and monitoring modes such as imaging detector or fast laser scanning detection several samples can be analyzed simultaneously. Interest in these detection modes has grown rapidly since the start of the Human Genome Project (HUGO) where CE with its inherent potential for high resolution and fast analyses has contributed substantially to decrease the planned time for the project.

The usefulness of CE is not restricted to separations, but is useful also for studies of interactions between molecules; enzyme kinetic analyses and topology studies of DNA molecules, i.e., molecules of the same size migrate with different rates in a gel (or polymer solution) due to different molecular shapes.

The three capillary electrophoresis modes, which were used in this work, are discussed briefly below.

LIGAND-EXCHANGE CAPILLARY ELECTROPHORESIS (LECE)

In recent years the general interest in enantiomer separations of drugs and clinically important chiral compounds is growing since the discovery that only one of the optical isomers has physiological effect while the counterpart isomer has none or side effects. Generally, separation of optical isomers is achieved by two different approaches called *direct* and *indirect* enantioseparation. Direct separation involves the formation of diastereomeric complexes of the enantiomers with a chiral molecule. Indirect enantioseparation involves the formation of diastereomeric derivatives through a covalent reaction of the enantiomers with a chiral reagent. The separation can be then carried out with non-chiral chromatographic or electrophoretic systems.

To accomplish enantiomeric separation the enantiomers must interact with a chiral selector to form diastereomeric complexes with different association constants and, thus, ΔG values. Chiral recognition generally depends on interactions acting simultaneously at three or more points between the enantiomer and chiral selector (the 'three-point-interaction' rule of *C.E. Dalgliesh* [1952]). At least one of these interactions must be stereo-selective in order to form diastereomeric complexes and thereby separate the enantiomers.

Enantioseparation by ligand exchange mechanism is based on multicomponent chelate complexes, consisting of a central ion, e.g., Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, and at least two chiral bifunctional ligands. A chelate complex (semi-complex) for enantioseparation is formed by mixing, e.g., an amino acid, such as proline, hydroxyproline with a salt of copper or other heavy metals. The concentration of the chelator and central ion must be in right proportion, i.e., often the concentration of the chelator is twice that of the central ion [Gozel *et al.*, 1987].

Enantioseparation depends on the different stability of the enantiomers in the semi-complex. The analyte and selector form a ternary complex as follows [Horimai *et al.*, 1997]:

$$[CL_n\text{-}M] + CA \rightleftharpoons [CL_{n-1}\text{-}M\text{-}CA] + CL \quad (19)$$

where *CL* is the chiral ligand, *M* is the central ion and *CA* is the chiral analyte. Analyte and chiral selector interact with each other through coordinate-covalent bonds in possible combinations with hydrophobic interaction, hydrogen bonds, electrostatic or van der Waals interactions.

The use of optically active copper (II) complexes was first described by *V.A. Davankov and Rogozhin* [1971] for the resolution of enantiomeric amino acids by liquid chromatography. This chiral separation principle has been widely used in HPLC by either adding a chelate complex to the mobile phase or by applying ligand-exchange chiral stationary phases (CSPs) to resolve enantiomeric compounds [Hare and Gil-Av, 1979; LePage *et al.*, 1979; Lindner *et al.*, 1979]. *R.N. Zare and co-workers* reported optical resolution by ligand-exchange capillary electrophoresis (LECE) first using copper (II) complexes [Gassman *et al.*, 1985; Gozel *et al.*, 1987]. The authors used an L-histidine-Cu(II) complex as an additive to the BGE for the chiral separation of dansyl-amino acids. The chiral separation is attributed only to the formation of diastereomeric ternary mixed copper(II) complexes possessing different stability constants. The equilibria existing in the solution can be written as:

$$[Cu(L\text{-}A_x)_2] + L\text{-}A_y \rightleftharpoons [Cu(L\text{-}A_x)(L\text{-}A_y)] + L\text{-}A_x$$

$$[Cu(L\text{-}A_x)_2] + D\text{-}A_y \rightleftharpoons [Cu(L\text{-}A_x)(D\text{-}A_y)] + L\text{-}A_x \qquad (20)$$

where L-A_x is the selector amino acid, L-A_y and D-A_y are the enantiomers of the amino acid separated in the forms of chelate complexes.

The first direct separation of underivatized amino acids was reported by *M.G. Schmid and G. Gübitz* [1996], using L-proline- and L-hydroxyproline-Cu(II) complexes as chiral selectors. Eleven amino acids containing aromatic groups were successfully resolved into their enantiomers. Using Cu(II) complexes of L-proline, L-hydroxyproline or aspartame, *C. Desiderio and co-workers* [1994] separated 2-hydroxy acid enantiomers. An interesting approach has recently been presented by *S. Krasensky and co-workers* [1995] to improve the detection sensitivity. A capillary containing Cu(II)acetate and L-proline or aspartame was coupled to a capillary

without a chiral selector at the detector side by a bifurcated block. This circumvented interferences in detection by the chiral selector. In the chiral separation of hydroxy acids, this system achieved a detection limit of 10^{-18} mole of mandelic acid, *m*-methoxymandelic acid, 3-phenyllactic acid and 3-indolelactic acid. Recently, the combination of ligand-exchange and host-guest interactions has been investigated as a new tool for chiral separations [Horimai *et al.*, 1997].

The limitation of the very effective technique of LECE is the prerequisite of chelate complex-forming groups in the analyte.

CAPILLARY ELECTROCHROMATOGRAPHY (CEC)

Some general viewpoints. In many respects, capillary electrochromatography (CEC) is a hybrid separation technique with advantages from both high-performance capillary chromatography and capillary electrophoresis [Tsuda *et al.*, 1982; Knox and Grant, 1987; Cohen *et al.*, 1987b]. Electrochromatography was first suggested by *Synge and Tiselius* [1950] and *Pretorius and co-workers* [1974]. It uses an electroendosmotic-driven flow, instead of a pressure driven flow, to propel the mobile phase through the column. Electroendosmotic flow is generated in the electrical (Helmholtz) double layer at charged solid-liquid interfaces. The separation mechanism in CEC is primarily based on differential interaction (e.g., partition) between two phases. If the solutes are charged, they can also be influenced by the electric field, resulting in differential migration of the solutes due to electrophoresis. CEC offers the same stationary phases with different chromatographic properties and broad application range of retention mechanisms and selectivities typical of chromatography without using an expensive HPLC pump. As in free zone capillary electrophoresis small inner diameter (10-100 μm) columns can be employed to minimize thermal gradients originated from Joule heating, thereby reducing zone broadening significantly. Low-conductivity buffers can also be employed to suppress this broadening [Hjertén *et al.*, 1995a].

CEC provides several important advantages over microcolumn high performance liquid chromatography (μHPLC). The electroendosmotic flow is

generated uniformly along the capillary; therefore, there is no pressure drop in the column. Whether the flow velocity profile is strictly plug-like is an open question. The electroendosmotic flow velocity is virtually independent of the capillary diameter or, for packed capillaries, the particle diameter of the stationary phase. In contrast, the pressure-driven flow velocity through a packed bed depends directly on the square of the particle diameter and inversely on the column length.

In open tubular-CEC the stationary phase is attached in a thin layer to the inner wall of the capillary column. The method has a low capacity but gives a high resolution when the inner diameter is small, which, however, affords low sensitivity upon on-tube UV-detection due to the very short light path [Everaerts *et al.*, 1989; Mayer and Schurig, 1992; 1993; 1994]. Alternatively, the capillary column can be packed with particulate material containing the stationary phase (packed-CEC), which generally consists of inorganic particles (e.g., silica beads) [Knox and Grant, 1987; 1991]. Packed beds have a high loading capacity, but the classical chromatographic zone broadening effects are relatively large. Additionally, none of the standard methods to pack columns with small beads give sufficiently uniform beds in narrow bore tubes. Besides, frits are needed to support the bed and pressurized electrode chambers are required to avoid bubble formation. Many of these drawbacks are eliminated when the stationary phase is made up of a polymeric network (continuous beds [Hjertén *et al.*, 1989b; Mohammad and Hjertén, 1994; Ericson *et al.*, 1997], also called continuous polymer rods, 'monoliths' [Svec and Frechet, 1992; Rathone and Horváth, 1997] and continuous column support [Fields, 1996; Minakushi *et al.*, 1997] or gels [Fujimoto, 1995; Fujimoto *et al.*, 1995; 1996]). One of several reasons for the good chromatographic properties is that the particles are very tiny (0.2-0.5 μm) which means a small but not negligible eddy diffusion in the van Deemter equation:

$$H=A+B/v+C\cdot v \qquad (21)$$

A corresponds to the eddy diffusion; B/v is the longitudinal diffusion while $C\cdot v$ is the resistance to mass transfer [van Deemter *et al.*, 1956]. Eddy diffusion often gives a large contribution to the total plate height, but it is smaller, the smaller the beads, i.e.,

from this point of view the continuous beds should be preferable to conventional packed beds which usually consist of beads with diameters in the range 3-5 μm.

Particulate media, including continuous beds (=continuous polymer rods, silica rods, monoliths). Only a few papers have been published about preparation of chromatographic beds *in situ* in a column tube (avoiding an expensive synthesis of beads and the cumbersome packing) because of the great difficulty to combine low flow resistance with high efficiency. The first successful method was published as late as 1989 [Hjertén *et al.*, 1989b]. The "continuous polymer beds" had the property that the resolution increased upon an *increase* in flow rate. They are built up of small particles as are conventional packed beds, but with the difference that the particles are covalently linked and often attached to the column tube wall to avoid supporting frits.

Definition of homogeneous separation media and some examples. It is difficult to make a stringent definition of homogeneous media. For our purposes a less rigorous, but practical definition is acceptable: a homogeneous separation medium is one which is visually transparent, i.e., no elements large enough to be visible in a light microscope or/and cause sufficient light scattering to make the medium opalescent (on the molecular level all media are heterogeneous). According to this definition, solutions of non-charged or charged polymers, such as polyacrylamide, dextran, polyvinylpyrrolidone, methylcellulose, polyvinylalcohol, starch and non-charged or charged gels, for instance, those obtained by crosslinking of these and other polymers, are homogeneous. Agarose gels exhibit slight opalescence at low concentrations, which becomes more pronounced at higher concentrations. The obvious difficulty to decide whether they should be classified as homogeneous gels at high concentrations shows the weakness of the definition. Experience tells us, however, that gels of agarose at concentrations as high as 12 % still give narrow zones upon electrophoresis of proteins and can, therefore, be regarded as homogeneous media.

According to theoretical considerations [Hjertén, 1990; Gaš *et al.*, 1995], polymer solutions [Hjertén *et al.*, 1989b; Palmer, 1997; Potocek *et al.*, 1998] and gels [Fujimoto, 1995; Fujimoto *et al.*, 1995; 1996], such as agarose and polyacrylamide, should behave as ideal chromatographic media because they are not made up of particles, being thus physically and macroscopically homogeneous. Therefore, the

eddy diffusion should be zero in gels. The effective pore size in homogeneous gels is much smaller than the average diameter of the channels between the (non)porous beads in packed beds and, thereby, the residence time of the analytes in the mobile phase is shorter, i.e., the resolution is higher. Observe also that the mobile phase cannot be transported through a homogeneous gel by a hydrodynamic flow (because of the high back pressure), only by an electroendosmotically-generated flow in the gel.

The principal difference between homogeneous gels and continuous beds (continuous polymer rods, silica rods, monoliths) is larger than that between packed beds and the continuous beds. We wish to emphasize this, since it might not be generally known that homogeneous gels and continuous beds (continuous polymer rods, silica rods, monoliths) represent two structurally completely different separation media.

CAPILLARY ISOELECTRIC FOCUSING (CIEF)

Isoelectric focusing (IEF) is a high-resolution technique for the separation of amphoteric compounds, such as complex protein and peptide mixtures. It is routinely used for characterization of biological extracts, monitoring protein purification, evaluating the stability or microheterogeneity of protein therapeutics, and determination of protein isoelectric points. Capillary isoelectric focusing (CIEF), as originally introduced by *S. Hjertén and M-D. Zhu* [1985], combines the high resolving power of conventional gel IEF with automation and quantitation. Since the internal wall of the fused silica capillaries is negatively charged electroendosmotic flow may be generated. The first CIEF experiments were performed in capillaries that had chemically treated inner walls. A coating with neutral polymers, such as linear polyacrylamide [Hjertén, 1985], exhibits no EOF.

As in gel IEF, proteins are separated according to their isoelectric points (p*I* differences as small as 0.01-0.001 can be detected) in a pH gradient formed by carrier ampholytes when an electric potential is applied [Hjertén *et al.*, 1986; 1987; Kilár and Hjertén, 1989a; 1989b; Kilár, 1991; 1994; Zhu *et al.*, 1992; 1993]. Ampholytes (amphoteric electrolytes) are molecules that contain both acidic and basic moieties.

The sample is normally introduced throughout the whole capillary along with the carrier ampholytes. To establish a pH gradient an acidic solution is in contact with the anodic end of the capillary and a basic solution with the cathodic end. When the electric field is applied the carrier ampholytes form a pH gradient and the amphoteric sample components migrate in the pH gradient to positions of where their net charge is zero (pH=their p*I* values). Axial diffusion of a protein out of the zone results in acquisition of charge and by electromigration the protein returns to the zone. Because of this focusing effect, CIEF zones are extremely narrow. The high resolving power is due to the focusing effect of the technique. Very high field strengths can be used to attain equilibrium within a few minutes, and the resolution is comparable to that of conventional gel IEF.

After focusing is attained in a coated capillary as a first step, in order to detect focused zones the whole length of the pH gradient formed should be mobilized to pass the detection window (second step). In such a two-step IEF two techniques for mobilization have been described. Chemical mobilization (also termed electrophoretic or ion addition) is carried out by changing the chemical composition of the anolyte or catholyte, causing a movement in the pH gradient that results in electrophoretic migration of focused zones [Hjertén *et al.*, 1987; Yao and Regnier, 1993]. Hydrodynamic mobilization transports focused zones by applying pressure at the capillary inlet or vacuum at the capillary outlet [Hjertén and Zhu, 1985].

Using uncoated capillaries in single-step ("dynamic") IEF, proteins are focused (ampholyte matrix formation) while being transported towards the detection point by EOF. *Thormann and co-workers* [1992] and *Mazzeo and Krull* [1992a; 1992b] developed this single-step IEF procedure in which the sample and ampholyte were introduced as a plug at the inlet of a capillary pre-filled with catholyte. Formation of the pH gradient and focusing of proteins into zones occur while the sample-ampholyte segment moves towards the detection point at the distal end of the capillary [Mazzeo and Krull, 1991; 1992a; 1992b; Mazzeo *et al.*, 1993; Thormann *et al.*, 1993; Molteni and Thormann, 1993; Molteni *et al.*, 1994; Wu and Pawliszyn, 1994; 1995]. The addition of methylcellulose (MC) or hydroxymethylcellulose to the catholyte serves to dynamically coat the fused silica wall, thereby reducing protein adsorption and EOF. It should be noted that this approach gives broader zones, due

to interaction of the proteins with the slightly charged capillary wall and other types of interactions.

It is possible to load significantly larger volumes in IEF than in most other CE modes since the solute can be mixed with the ampholyte solution and sucked into the entire length of the tube (in coated capillary two-step IEF).

Proteins have a tendency to aggregate at their p*I* because there is no electrostatic repulsion between them; therefore, precipitation is usually a disadvantage of IEF. CIEF has been used successfully to measure isoelectric points of proteins and for the separation of isoforms [Kilár and Hjertén, 1989a; 1989b, Kilár, 1991; Wehr *et al.*, 1997; Huang and Richards, 1997].

AIMS OF THE STUDY

In this thesis I will discuss some methods developed for separations of biomolecules. In life sciences there is a great interest in macromolecules, such as nucleic acids and proteins; their subunits (nucleotides, amino acids and peptides); small biomolecules that play important roles in the metabolism; drugs and the interactions between and the chemical reactions of biomolecules. Free α-amino acids (in enantiomer separation, **Paper I and II**), ribonucleosides (in electrochromatographic separation, **Paper III**), hemoglobin variants (in isoelectric focusing, **Paper IV**) were chosen as objectives of our study to provide a broad view of the usefulness of capillary electrophoresis.

The aims of the present study were as follows:

- To investigate the applicability of capillary electrophoresis for separations of biomolecules; for that purpose we have modified existing techniques and developed new separation methods; these studies have been combined with applications, such as investigations of interactions between proteins and peptides.
- To emphasize the usefulness of capillary electrophoresis for the separation of biomolecules which differ in size and chemical character.
- To point out that these methods have a broad application range because it is easy to adapt them to specific analytical problems.
- To study the ligand-exchange mechanism, successfully employed in HPLC, for separation of free α-amino acids by capillary electrophoresis and capillary electrochromatography.
- To employ a recently synthesized chiral selector for ligand-exchange capillary electrophoresis with improved selectivity.
- To investigate the separation parameters and optimize the enantiomer separation of α-amino acids using LECE

- To prepare an electrochromatographic column in situ in the capillary with a continuous bed having high capacity and low back pressure useful in electroosmotically- and pressure-driven modes
- To prepare an electrochromatographic column *in situ* in the capillary with an entirely homogeneous gel having appropriate pore sizes and number of charged groups to generate electroendosmotic flow and high borate concentrations for separation.
- To develop a capillary electrochromatographic method for separation of neutral ribonucleosides employing the well known property of borate compounds to form complexes with different stability constants between *cis* and *trans* hydroxyl and borate groups.
- To investigate the isoelectric focusing method and to develop a new injection protocol for CIEF in uncoated capillaries.
- To study the formation of pH gradients in uncoated capillaries using the invented injection protocol and to test ampholytes with different pH ranges from different sources.
- To employ the new capillary isoelectric focusing method in high-resolution separation of hemoglobin variants of diagnostic importance.

The results with small biomolecules will be presented followed by the studies of macromolecules. Both major parts of this work include four different CE methods: ligand-exchange capillary electrophoresis, capillary electrochromatography and capillary isoelectric focusing, affinity capillary electrophoresis, respectively.

Text, figures and tables of the published papers and the manuscript are referred to their Roman numbers (**Paper I**, **II, III** or **IV**) or to their pages in the Appendix (e.g., Table 1 on page A-4).

MATERIALS AND METHODS

CHEMICALS

All chemicals were of analytical grade used for buffer systems and reactions.

Aromatic α-amino acids, methylcellulose (MC) with a viscosity of 4,000 cP, vinylsulfonic acid, γ-methacryloxypropyltrimethoxysilane and allylglycidyl ether were purchased from Sigma Chemicals (St. Louis, MO, USA); 1,2-Epoxyoctane, dimethyl sulfoxide (DMSO) were from Aldrich (Deisenhofen, Germany); L-4-Hydroxyproline, copper(II) sulfate, diethylether, methanol were from Fluka (Buchs, Switzerland); ortho-phosphoric acid and sodium hydroxide were from Reanal (Budapest, Hungary); 3-Aminophenyl boronic acid (hemisulfate) and Servalyts were from Serva (Heidelberg, Germany); acrylic acid, sodium hydroxide, sodium dihydrogen phosphate and ammonium sulfate were from Merck (Darmstadt, Germany); agarose (D1, Special High-Electroendosmosis [SHE] and methoxylated agarose with the melting point 25.6 °C) was from Hispanagar (Burgos, Spain); Ampholines were from LKB (Bromma, Sweden); Pharmalytes were from Pharmacia (Uppsala, Sweden); AFSC hemoglobin electrophoresis control was from Isolab (Akron, OH, USA); and ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), piperazine diacrylamide and Bio-Lytes were from Bio-Rad Laboratories (Hercules, CA, USA). Ribonucleosides were kindly supplied by Dr. András Földesi (Dept. of Bioorganic Chemistry, Uppsala University).

N-(2-hydroxyoctyl)-L-4-hydroxyproline (HO-L-Hypro) was synthesized in Prof. G. Gübitz laboratories (Graz, Austria) by a reaction between L-4-hydroxyproline and 1,2-epixyoctane in methanol at high pH value, i.e., an alkyl side chain was introduced to the L-4-hydroxyproline by reaction of the amino group with the epoxide (Figure 1). After purification of the crude product, the sodium salt was obtained as a colorless powder. The structure was confirmed by elemental analysis, as well as, by NMR and IR data.

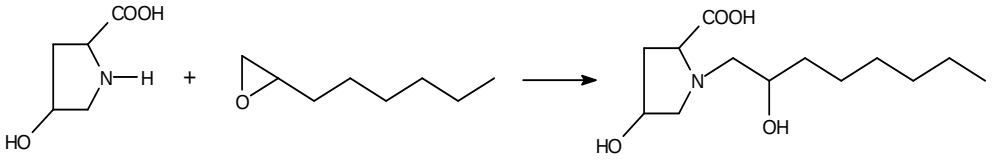

Figure 1. Synthesis scheme of N-*(2-hydroxyoctyl)-*L*-4-hydroxyproline*

N-(2-hydroxy-3-allyloxypropyl)-L-4-hydroxyproline (HAP-L-Hypro) was synthetized in a similar manner, L-4-hydroxyproline reacted with an equimolar amount of allylglycidyl ether (Figure 2).

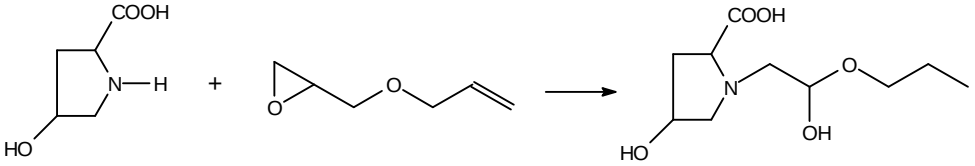

Figure 2. Synthesis scheme of N-*(2-hydroxy-3-allyloxypropyl)-*L*-4-hydroxyproline*

Aminomethylated nitrophenol pH markers having isoelectric points of 5.3, 6.4, 6.6, 7.2, 7.9, 8.6 and 10.4, were kindly supplied by K. Šlais (Brno, Czech Republic) (Figure 3).

p*I*	R_1	R_2	$N(R_3,R_4)$
5.3	4-CH_2-$N(R_3,R_4)$	2-Cl-6-NO_2	MOR
6.4	4-CH_2-$N(R_3,R_4)$	2-Cl-6-NO_2	MPIPE
6.6	NO_2	H	MOR
7.2	CH_3	NO_2	MOR
7.9	CH_3	NO_2	MPIPE
8.6	NO_2	CH_2-$N(R_3,R_4)$	MPIPE
10.4	NO_2	CH_2-$N(R_3,R_4)$	PIP

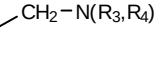

Figure 3. Structural formulas of the pI markers

Preparation of the hemoglobin variants from blood was made following the method of *Molteni and co-workers* [1994] (**Paper IV**). The mixture of normal hemoglobin variants in blood has isoelectric points between 6.7 and 7.4, the major component having a pI of 6.9. For more details see 'Experimental' in **Paper IV** on page A-28.

3-(acrylamido)phenylboronic acid (Figure 4) was synthesized according to the method published by *Igloi and Kössel* [1987] with some modification to increase the yield. 3-aminophenylboronic acid hemisulfate was reacted with acryloylchloride

at 0°C. Following purification, the solid material was used in preparation of a linear copolymer of APB and acrylic acid. NMR analyses gave spectral data characteristic of APB. More details are described in **Paper III** on page A-15.

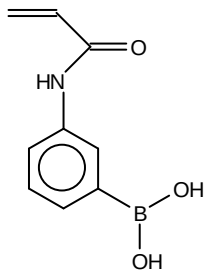

Figure 4. Structural formula of 3-(acrylamido)phenylboronic acid

CAPILLARY ELECTROPHORESIS INSTRUMENTATION

In this work three different types of CE instruments have been employed for the separations of biomolecules.

PrinCE (Prince Technologies, Emmen, The Netherlands) is a semi-automated CE apparatus with integrated software that operates the syringe pump (for washing and injection), the electrolyte carousel and the built-in power supply. For detection an external Bischoff Lambda 1000 UV/Vis (Analysentechnik, Leonberg, Germany) unit was coupled to it. In pressure-supported mode an external pressure device 812 bar) was also connected (**Paper II**). Data were collected with the Axxiom Chromatography 717 or 737 system (Moorpark, CA, USA). The control of temperature is not possible. Because the capillary must be put outside the equipment to reach the detector, the minimal length is as long as 50 cm. (**Papers I, II and IV**)

A fully automated ^{3D}CE system (Hewlett-Packerd, Palo Alto, CA, USA) equipped with a CEC pressure device (12 bar) and a diode array detector. An HPLC 6200 intelligent pump (Merck-Hitachi, Darmstadt, Germany) was used for capillary flushing at 70 bars. In pressure-supported mode 8-12 bars were applied at the capillary inlet. The applied voltage range was 7-30 kV. (**Paper II**)

A home-built CE apparatus based on a Linear Model 200 UV detector (Linear Instruments, Reno, NV, USA) employing a special interface with a ball lens focusing the light onto the capillaries can be manually operated only. A unique home-built tray for holding the electrolyte vessels was placed around the interface.

The capillaries with lengths between 8-20 cm in straight alignment are placed on the tray, which is covered with a lid during the run to avoid disturbing light to reach the photo diode. An external power supply (PALM 897 model for maximum 10 kV) was connected to the electrodes. The temperature was not controlled which often causes baseline drift. (**Paper III**)

CAPILLARIES

Capillaries with inner diameter 25, 50 or 75 μm were used with different effective (the length to the detection window) and total lengths (from 10 cm to 50 cm). The capillaries are coated externally with a polyimide layer to improve flexibility and controllability. The internal wall of the capillaries was untreated (uncoated capillary) (**Paper I, III**). Capillaries were ~~treared~~ **treated** with a bifunctional reagent (γ-methacryloxypropyltrimethoxysilane) in order to attach a continuous bed covalently (**Paper II**). A neutral polymer (methylcellulose) was used as buffer additive in 0.015 % to provide dynamic coating covering the inner wall by adsorption in the CIEF studies (**Paper IV**).

DETECTION

The UV absorbance detection based on measurement of the quantity of light transmitted through the capillary was used in all experiments. Detection was performed on-tube either at distinct wavelengths (208, 223, 254, 280, 415 nm) using Linear Model 200, Bischoff Lambda 1000 detectors and the dioade array detector in ^{3D}CE.

INJECTION MODES

The two most common, hydrodynamic and electrokinetic injection modes were used in the CE experiments. They usually afford good reproducibility. The samples were injected by pressure produced by a precision pump. In the commercial

CE instruments built-in algorithms correct the injection volume with high accuracy to improve reproducibility (pressure·time).

Electrokinetic injection was used when the capillary was filled with a continuous bed or a gel. EOF was utilized to inject neutral compounds.

PRACTICAL PERFORMANCE OF EXPERIMENTS

LECE in free solution

The selector, *N*-(2-Hydroxyoctyl)-L-4-hydroxyproline (HO-L-Hypro) was used as a complex with copper(II) ion in 10 m*M* concentration of sodium phosphate. A 10 m*M* $CuSO_4$ solution was mixed with an equal volume of 20 m*M* selector solution. The aqueous solution of amino acid racemate was injected prior to filling the capillary with the electrolyte solution containing the complexes. During the migration of free amino acid in the applied electric field semi-complexes form and the enantiomers are separated according to their different stability constants.

LECE in continuous beds

In *in situ* preparation of a continuous bed, methacrylamide was used as a backbone monomer, piperazine diacrylamide as crosllinker, vinylsulfonic acid ~~**fro**~~ **for** the generation of the EOF and HAP-L-Hypro as ligand. The ~~**soltion**~~ **solution** of these compounds was sucked into the capillary up to the detection point. The polymerization was initiated with ammonium persulfate and TEMED. The shorter section between a capillary end and the detection window (8.5 cm) was filled with the continuous bed and, therefore, polarity and inlet/outlet positions were changed. (**Ide javaslom leírni, hogy mi lett így a pozitív és negatív pólus.)** A 50 m*M* sodium dihydrogenphosphate solution containing 0.1 m*M* Cu(II) at pH 4.6 was used as mobile phase. Aqueous solutions of amino acids were electrokinetically injected.

CEC

A solution of a polymer composed of acrylic acid (AA) and acryloyl-phenylboronic acid (APB) was mixed with 1 % hot aqueous agarose solution. This warm liquid was sucked into the capillary immediately and then cooled to room temperature to get a gel. The capillary was then prerun for a half an hour in order to obtain stable baseline. A sodium phosphate buffer was used in the electrolyte vessels

for the runs in which aqueous solution of ribonucleosides (adenosine, citidine, guanosine and uridine) were electrokinetically injected.

CIEF

A 50 cm long fused silica capillary was washed with 20 m*M* NaOH solution containing 0.015 % methylcellulose that dynamically coated the inner wall. The coating was consequently renewed between runs by washing the capillary with this MC solution. Separate aqueous solutions of ampholytes and the sample (aminomethylated nitrophenols or hemoglobin variants) were injected by pressure consecutively. NaOH (20 m*M*) and phosphoric acid (10 m*M*) were used as catholyte and anolyte, respectively.

RESULTS

STUDY OF SMALL BIOMOLECULES

*SEPARATION OF ENANTIOMERS OF FREE α-AMINO ACIDS USING LIGAND-EXCHANGE CAPILLARY ELECTROPHORESIS (**PAPER I**)*

Experimental precedents

The amino acids are the basic units of proteins and peptides but also of other substances. The production of optically pure enantiomers of amino acids is a highly important task of synthetical chemists. Most of the CE enantioseparation methods used for amino acids involve a chemical derivatization step in order to increase the UV absorbance of the analytes and the magnitude of the chiral recognition [Girelli and Sinibaldi, 1990; Guttman et al., 1988]. The detection of free α-amino acids can be accomplished by using UV spectroscopy in the range of 205-210 nm [Davankov and Zolotarev, 1978a; 1978b; 1978c; Lefebre *et al.*, 1978] or 254-260 nm [Davankov et al., 1979].

The direct way of separation (see **LIGAND-EXCHANGE CAPILLARY ELECTROPHORESIS**) was chosen in this thesis using the ligand-exchange mechanism with metal-chelate complexes as chiral selectors in CE. In previous studies L-hydroxyproline (L-Hypro) was taken as chiral amino acid forming chelate complex with Cu(II) ions for two reasons. Firstly, the *N*-substituted cyclic amino acids, e.g., proline and hydroxyproline, were found to display the largest enantioselective recognition ability in Cu(II) complexes from a large number of amino acid ligands studies [Davankov *et al.*, 1974; Kurganov *et al.*, 1978; Schmid and Gübitz, 1996]. Secondly, L-Hypro was preferred to L-proline on account of its more pronounced hydrophilic character. Our new approach is outlined below.

Results

Enantiomers of thirteen free α-amino-acids – DL-Phenylalanine, DL-*threo*-Dihydroxyphenylserine, DL-Phenylserine, DL-*o*-Methyl-tyrosine, DL-Hydroxy-tryptophan, DL-DOPA, DL-α-Methyl-DOPA, DL-*m*-Tyrosine, DL-*o*-Tyrosine, DL-*p*-Tyrosine, DL-α-Methyl-tyrosine, DL-Tryptophan, DL-Histidine – have been successfully resolved (for separation data see Table 1 on page A-4) by capillary zone electrophoresis with a new chiral selector, *N*-(2-Hydroxyoctyl)-L-4-hydroxyproline (HO-L-Hypro). The formation of complexes between the chelating amino acids and copper(II) ions was investigated at different concentration ratios and found to be constant in the ratio 2:1.

The required selector concentration of HO-L-Hypro was found to be about 10 mM for all amino acids since the copper complexes do not have disturbing UV-absorbance. The detection was possible at 208 nm.

The minimum detectable amount of the D-dihydroxyphenylalanine (DOPA) was 0.03 % in the sample of L-DOPA (see Figure 4 on page A-4). This enantiomer is inactive as anti-Parkinson drug and is, therefore, undesirable in medical production.

The influence of the HO-L-Hypro/Cu(II)-complex concentration on the resolution was investigated by increasing it gradually from 1 m*M* to 20 m*M*, using constant pH (4.3) and electrolyte concentration (5 m*M*) (see Figure 5 on page A-5). The resolution was higher with higher selector concentrations of the test amino acid (DL-α-Methyl-DOPA) but a plateau was reached above 8-10 m*M*. 10 m*M* selector concentration was chosen for all experiments.

The influence of electrolyte concentration on the result was investigated in the range from 1 m*M* to 20 m*M*. Higher electrolyte concentrations afforded higher resolution, but the migration times became much longer (at the same time high electrolyte concentration can cause Joule heating resulting in additional zone broadening and poorer resolution). Addition of organic modifiers did not show ~~an~~ **any** improvement in resolution.

There was a correlation between the isoelectric points (p*I*) of the amino acids and the optimal pHs for their separation. DL-histidine having a p*I* 7.64 gave a higher optimum pH value (6.0) than did the neutral amino acids. Their p*I* values are

in the range of 5.5-6.0, and the optimum pH values were found to be close to 4.3 (see Figure 6 on page A-5).

*SEPARATION OF ENANTIOMERS OF FREE α-AMINO ACIDS USING LIGAND-EXCHANGE CAPILLARY ELECTROCHROMATOGRAPHY (**PAPER II**)*

Experimental precedents

In the late 1980's a novel technology for *in situ* preparation of chromatographic beds, consisting of covalently linked polymer chains, was introduced [Hjertén *et al.*, 1989b]. These, so-called 'continuous beds' or 'monoliths', have found application in several chromatographic modes [Hjertén *et al.*, 1989b; Liao *et al.*, 1991; Hjertén *et al.*, 1993; Mohammad *et al.*, 1993; Li *et al.*, 1994, Mohammad *et al.*, 1995; Maruška *et al.*, 1999] and, more recently, also in electrochromatography [Hjertén *et al.*, 1995b; Ericson *et al.*, 1997]. There are, however, only a few applications of continuous beds for chiral separations by CEC. Chiral imprinted polymers have been developed, using various enantiomers as print molecules [Lin *et al.*, 1998; Schweitz *et al.*, 1998].

The ligand-exchange mechanism for the separation of enantiomers was first introduced into chromatography [Davankov and Rogozhin, 1971] then to capillary electrophoresis [Gassman et al., 1985; Gozel et al., 1987]. This elegant way to resolve optical isomers has been employed in CEC using a continuous bed.

Results

A chemically activated chiral selector, HAP-L-Hypro, was *in situ* co-polymerized with neutral (methacrylate), charged (vinylsulfonic acid) and cross-linking (piperazine diacrylamide) monomers by a free radical mechanism. During the polymerization a network of polymer chains formed building up small particles that are covelantly linked to each other and to the capillary wall. The interconnected ~~**nodules**~~ **modules** are large enough for high permeability ensuring low back pressure. Due to the rough surface of the microparticles their surface area is large which provides high binding capacity. Since the EOF originates from the sulfonic acid ligands the endosmotic velocity is independent of the pH of the mobile phase (cf. silica beds).

Enantiomers of nine free α-amino-acids – DL-phenylalanine, DL-asparagine, DL-serin, DL-threonine, DL-DOPA, DL-α-methyl-DOPA, DL-*p*-tyrosine, DL-α-

methyl-phenylalanine, DL-tryptophan – have been successfully resolved by capillary electrochromatography with a chiral selector, *N*-(2-Hydroxy-3-allylpropyl)-L-hydroxyproline (for separation data, see Table 1 on page A-10).

Endosmotic-, pressure-driven and pressure-facilitated modes were compared using DL-phenylalanine as a model compound. The resolution was highest in endosmotic-driven mode, whereas the efficiency was similar in endosmotic- and pressure-driven modes (Figure 2 on page A-9). Interestingly, in pressure-supported mode both the resolution and the efficieny were improved compered to those in the other modes.

Reduction of the length of the separation column in combination with high voltage and pressure provided the fastest separation (Figure 3 on page A-10).

*SEPARATION OF RIBONUCLEOSIDES IN AGAROSE-BASED GELS USING CAPILLARY ELECTROCHROMATOGRAPHY (**PAPER III**)*

Experimental precedents

Instead of using the conventional method to prepare a chromatographic bed for CEC, involving three very expensive and time-consuming steps (synthesis of beads, sieving the beads to uniform size, packing the column with these beds), gels can be formed *in situ* in capillaries.

In order to employ agarose as an electrochromatographic stationary phase charge moieties and ligand groups providing specific interactions with the sample components should be introduced. It is not a trivial problem to derivatize agarose because no gel will form if the classical methods attaching ligands to a preformed gel are applied to an agarose solution (the development of hydrogen bonds between the polymer chains is a prerequisite for gel formation but for sterical reasons derivatized polymer chains cannot come so close to each other that hydrogen bonds form, see structure of agarose in Figure 5). Therefore, the agarose was not derivatized and a preformed polymer, with the desired ligands: (i) carboxylic groups to generate electroendosmosis (to propel the mobile phase through the homogeneous gel); (ii) boronate groups to create electroendosmosis and form bonds with compounds containing two OH-groups in *cis*-position, was entrapped in an agarose gel.

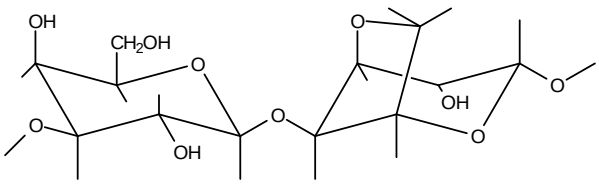

Figure 5. Structure of the repeating unit of agarose. Note the presence of the unusual sugar 3,6-anhydro-L-galactose.

Complex formation of boronic acids with *cis*-diol groups of sugars in solution is a property of these molecules, which has been studied extensively [Ferrier, 1978]. For the present purpose, one may summarize the chemistry of the process in terms of a pH-dependent esterification of a pair of stereochemically favorably

positioned hydroxyl groups with an organic boronic acid derivative [R-B(OH)$_2$] (Figure 6).

Figure 6. Reaction scheme of esterification of boronic acid derivatives

The reaction can occur even with single hydroxyl groups although to a smaller extent [Bergold and Scouten, 1983] which, however, can play some role in the entrapment of the polymers in agarose gels.

Results

In order to introduce borate ions as ligands for the separation of ribonucleosides 3-aminophenylboronic acid hemisulfate was reacted with acryloyl chloride (Figure 7) resulting in an activated monomer compound [Igloi and Kössel, 1987 with modifications] (see **Paper II**, Experimental on page A-15).

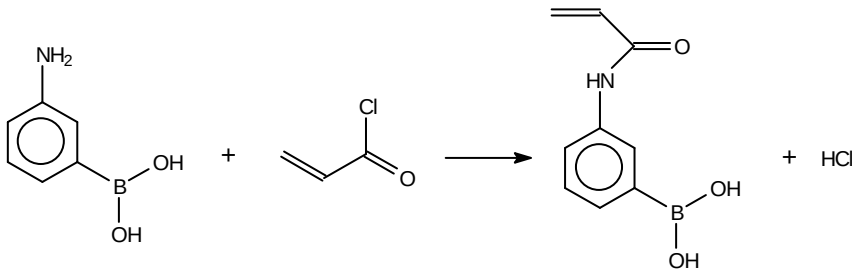

Figure 7. Synthesis scheme for chemical reaction of 3-aminophenylboronic acid and acryloyl chloride.

This compound can be co-polymerized with other acrylic monomers such as acrylic acid (AA). A co-polymer of acryloyl-aminophenyl boronic acid (APB) and acrylic acid (AA) was prepared with the addition of ammonium persulfate and N,N,N',N'-tetramethylethylenediamine via a free radical mechanism.

The capillaries were filled with a hot solution of agarose mixed carefully with the linear polymer solution of APB and AA and kept at low temperature to

initiate the gelation. The performance of the gel was constant during a period of 4-5 weeks.

To test the properties of the new homogeneous gel, CEC experiments in the frontal analysis mode were performed. Acetone was chosen as EOF marker due to its inert nature and, thus, did not interact with the separation matrix. Acetone was mixed with the buffer to obtain a 10 % (v/w) final concentration and used in the electrolyte vessel at the anodic end. Therefore, not a peak but a front of acetone solution was detected migrating with the velocity of electroendosmotic flow (see Figure 2 on page A-15). The experimental standard deviations in seconds were measured at $0.68 \cdot c_0/2$ (c_0 is the concentration of the analyte) and compared to those calculated for free diffusion using the Equation 9 (see Figures 3 on page A-17). The calculated diffusion broadening (σ_{diff}) was found to be higher than the measured experimental total broadening of the boundaries (σ_{exp}). The figure indicates that the originally plane boundary between the acetone phase and the gel phase at the start of the run was distorted during the run only by longitudinal diffusion but not by eddy diffusion.

A 1 % (w/v) aqueous solution of agarose was prepared by boiling in a water bath. A 100-µl aliquot of this solution was mixed at 80 °C with 190 µl of dialyzed aqueous solution of the ABP-AA polymer. The final concentration of agarose was about 0.3 % (w/v). The capillary was filled with this hot solution. To accomplish gelation, the capillary was kept at room temperature for 5 min and at 4 °C for an additional 5 min. The electrolyte vessels contained 5 m*M* sodium phosphate buffer (pH 7.8). Equilibration of the gel with this buffer and removal of UV-absorbing material were accomplished by a 25-min prerun at a field strength of 130 V/cm.

For testing the selectivity of this column, ribonucleosides (cytidine, adenosine, uridine and guanosine) were chosen because of their high UV-absorbance at 254 nm. They differ in their bases whereas the sugar moiety is the same interacting with boronate groups in the gel. The ribonucleosides are neutral at pH 7.8 and, therefore, migrate only under the influence of the electroendosmotic flow generated by the negative acrylic acid and boronate ligands. At pH 7.8 more tailing of the last two compounds (uridine and guanosine) was observed (Figure 6 on page A-22) whereas in runs at pH 7.6 better peak symmetry was recorded (Figure 8), although

other conditions were kept constant. It is known that the interactions are less pronounced at lower pH [Hjertén *et al.*, 19??]

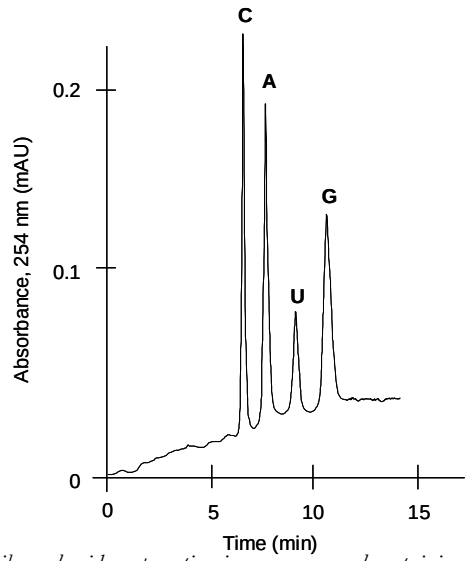

Figure 8. Electropherogram of ribonucleosides separation in an agarose gel containing boronate groups. 0.3% Agarose-APB-AA gel (entrapped polymer containing boronate and acrylic acid groups) Sample: C=cytidine, A=adenosine, U=uridine, G=guanosine. Buffer: 5 mM phosphate, pH 7.6. Capillary: 50 μm x 19(16) cm. Applied voltage: 2 kV; current: 4.2 μA.

The theoretical plate numbers were often between 100-350,000 m^{-1}.

STUDY OF BIOPOLYMERS

*NEW INJECTION TECHNIQUE DEVELOPED TO ANALYZE HEMOGLOBIN VARIANTS BY CAPILLARY ISOELECTRIC FOCUSING (**PAPER IV**)*

Experimental precedents

Capillary isoelectric focusing has two different approaches; the two-step method requires coated capillaries, the focusing and the mobilization are separated in time; while the one-step method is performed in uncoated capillaries treated with neutral polymers as buffer additive ('dynamic coating') providing reduced EOF. In both approaches the ampholytes and the sample are mixed prior to injection (the capillary can be completely or partially filled with ampholytes) resulting in unwanted interactions that can change the isoelectric points of the proteins and destroy the resolution during the focusing. Additionally, during a development it is difficult to combine various concentrations of ampholytes having different pH ranges. All of these practical obstacles can be solved by using a new injection technique for CIEF in uncoated capillaries.

The course of the pH gradient is established by calibration with protein p*I* markers, the p*I*s of which had been measured by other methods. This technique is, however, not convenient because protein p*I* markers may change their p*I* values due to denaturation, for instance or conformational changes [Williamson *et al.*, 1973]. Recently, more stable p*I* markers were developed for p*I* estimation by using aminomethylated nitrophenols, which are highly soluble in water and have absorption at wavelengths where the proteins do not absorb [Šlais and Friedl, 1994; Caslavska *et al.*, 1994].

Besides normal hemoglobin variants characteristic to healthy adults, pathological and abnormal hemoglobins can be detected in some hereditary diseases (hemoglobin apathies, sickle cell anemia, thalassemias) caused by mutations in the globin chains. Employing CIEF these variants can be separated due to differences in their isoelectric points (HbA_{1c}=6.94, HbA=6.97, HbF=7.06, HbS=7.21, HbA_2=7.41, HbC=7.45 [Hempe and Craver, 1994]).

The method can be utilized in diagnosis of other diseases, such as diabetes mellitus. Glucose or glucose-6-phosphate molecules existing in blood can chemically react with an ε-amino group of lysine residues in globin chains via a non-enzymatic, irreversible formation of Schiff bases. The higher the concentration of glucose in blood the larger the number of glycosylated hemoglobin. The normal level of glycosylated hemoglobin (mainly as HbA_{1c}) is 4-6 % of the total hemoglobin while in diabetes mellitus this value can be increased up to 16-18 %. The quantitative analysis of HbA_{1c} can be easily accomplished by using CIEF [Finke *et al.*, 1998].

Results

Capillary isoelectric focusing experiments were performed in uncoated capillaries dynamically coated with methylcellulose. A novel injection protocol was developed in order to improve the resolution and the applicability of the method. The ampholytes and analytes were injected separately in three steps, i.e., ampholyte solutions (zone 2 and 4 in Figure 1 on page A-29) were injected before and after the sample (zone 3). The same type or different types of ampholyte solutions were applied consecutively as zones 4 and 2 with various lengths into the capillary. This injection set-up was called a 'sandwich' since two ampholyte zones straddle the sample zone. Approximately, 20-30 % of the whole capillary was filled with the 'sandwich' plug. Isoelectric focusing of the samples was then accomplished by applying the voltage. The pH gradient moves towards the cathode with the EOF.

Aminomethylated nitrophenols (Figure 2) were used in experiments to optimize the conditions for the injection protocol. The zones (2, 3 and 4) were injected by varying pressure and time in order to make different capillary geometries comparable. Using 75-μm capillaries ampholyte solutions (zones 2 and 4) were injected at 100 mbar for 7 and 15 sec, respectively, whereas the sample was injected at 50 mbar for 5 s. Using 50-μm capillaries ampholyte solutions (zones 2 and4) were injected at 50 mbar for 30 and 60 sec, respectively, while the sample was injected at 50 mbar for 5 s. In a series of experiments when same pH ranges were tested, sixteen ampholytes were applied as zones 2 and 4 (Table 1).

Table 1. Ampholytes having different pH ranges and from different sources.

Ampholyte	Source	pH range 2 — 11
Ampholine	LKB	3.5 — 10
Ampholine	LKB	3.5 — 5
Ampholine	LKB	4 — 6
Ampholine	LKB	5 — 7
Ampholine	LKB	7 — 9
Ampholine	LKB	9 — 11
Pharmalyte	Pharmacia	3 — 10
Pharmalyte	Pharmacia	4 — 6.5
Pharmalyte	Pharmacia	5 — 8
Pharmalyte	Pharmacia	6.5 — 9
Pharmalyte	Pharmacia	6.7 — 7.7
Pharmalyte	Pharmacia	8 — 10.5
Bio-Lyte	Bio-Rad	3 — 10
Bio-Lyte	Bio-Rad	3 — 5
Bio-Lyte	Bio-Rad	4 — 6
Bio-Lyte	Bio-Rad	5 — 7
Bio-Lyte	Bio-Rad	6 — 8
Bio-Lyte	Bio-Rad	7 — 9
Bio-Lyte	Bio-Rad	8 — 10
Servalyt	Serva	2 — 11
Servalyt	Serva	5 — 8

Ampholytes having narrow and broad pH ranges were tested for comparison by CIEF using the 'sandwich' injection (see Figures 2-5 on pages A-30-33). The lowest resolution was obtained with ampholyte solutions covering the lowest narrow pH range, pH 3-5 (see Figures 2a and 4a on pages A-30 and A-32). Somewhat better resolution of aminomethylated nitrophenols (dyes) was obtained by using of ampholyte solutions with higher, narrower or broader pH ranges. The resolution and the migration times of dye compounds were different when ampholyte solutions covering similar pH ranges from different sources were employed (for instance, see Figures 2b and 4b on pages A-30 and A-32). Although the pH ranges of the ampholytes did not cover the p*I* values of the seven dyes in several cases, separations of analytes were obtained to a certain extent in every experiment. In these cases the zones migrate in the gradient under zone sharpening [Hjertén, 19??].

The combination of ampholytes, using different pH ranges as zones 2 and 4, improved the separation of dyes. In the experiments with two different ampholytes a combination of a broad and a narrow pH range provided higher resolution using the 'sandwich' injection protocol than that obtained with simple injection of the mixture of ampholytes and dyes (see Figures 6b and 6a on page A-34). Significantly better resolution was obtained using lower concentration (2 %) of the ampholyte ampholytes (Figure 6c on page A-35). The combination of two narrow pH-range ampholytes can separate the seven dyes similarly to broad pH-range ampholytes, although the gradient did not cover every p*I* values (Figures 6d and 6e on pages A-35 and A-36).

All the four major compounds (HbA, HbF, HbS and HbC) of the hemoglobin standard were baseline separated. Among these variants minor constituents also formed probably composed by glycosylated proteins **(Nem pontosan értem.)**. Hemoglobin preparations from blood of controls and patients with diabetes mellitus were focused with high resolution employing the 'sandwich' injection protocol (see Figure 7 on page A-37). The highest resolution was obtained using a combination of a narrow (Pharmalyte 6.7-7.7) and a broad (Pharmalyte 3-10) pH range of ampholytes.

DISCUSSIONS

The objective to study biologically important molecules by capillary electrophoretic methods has been successful. Three CE modes were chosen to provide separations of small molecules, such as amino acids and nucleosides ~~are~~, and proteins. The final goal was not only to analyze some typical biomolecules but also to gain methodological knowledge in the field of separation science.

In continuation of the investigation of ligand-exchange capillary electrophoresis for separation of free amino acids an L-hydroxyproline derivative was synthesized as a chiral selector on the assumption that a long hydrophobic side chain bound to the nitrogen of L-hydroxyproline might improve the resolution. The alkyl side chain was introduced to the L-4-hydroxyproline by reaction of the amino group with the epoxide. The chiral selector, HO-L-Hypro has shown a significant improvement in chiral recognition compared to L-Hypro [Schmid and Gübitz, 1996], possibly due to additional binding forces in the side chain of the selector and hydrophobic interactions of the aromatic groups in amino acids. Improved resolution and shorter analysis time were obtained. The selector concentration was as low as 10 m*M*, which is one order of magnitude lower than that used in the experiments with L-Hypro.

(Mintha nem diszkutálnál sokat a paper II-ból? Vagy csak átsiklottam rajta?)

Although sodium phosphate used as background electrolyte has no or little buffer capacity in the range from pH 3 to 6 (pK_{a1}=2.12 and pK_{a2}=7.21) the pH dependence on resolution was obvious. By a careful choice of detection modes, probably all of the amino acids can be resolved into their enantiomers, employing the ligand-exchange mechanism.

High resolution and selectivity are not always the most important tasks in enantiomer separations. **(…. not always …. ?)** High sensitivity is also important. **(…. is ….?) Nem értem a kapcsolatot.** Most chiral drugs have only one active

enantiomer. The other enantiomer has often side effects. Ligand-exchange capillary electrophoresis is able to provide trace analyses of these unwanted enantiomers. **(Ezt a bekezdést az első bekezdés után javasolnám, hiszen ez egy általános bevezető gondolat.)**

Continuous beds for separation of enantiomers have several advantages over the conventional columns packed with silica-based beads. Although no methods for packing small inner diameter capillaries uniformly are known, the preparation of continuous beds is easy. It involves only a simple *in situ* polymerization providing smaller particles (0.002-0.005 mm) than that used in packed columns. Introduction of ligands for specific interactions and generation of electroendosmotic flow requires basic knowledge in organic chemistry. Silica-based packed columns for the HPLC mode provided similarly high enatioselectivity but extremely long retention times. That problem did not arise for the chiral CBs. Since the chiral CB has a high charge density able to generate high EOF in a broad pH range, the same column could be used in the HPLC as well as in the CEC mode, offering great flexibility.

In accordance with recent experiences [MacNair *et al.*, 1997; 1999] there was no significant difference in efficiency between endosmotic- and pressure-driven modes. The improved resolution upon a combination of EOF and pressure can hardly be explained at this stage of the development of continuous beds as electrochromatographic supports in CEC.

New homogeneous electrochromatographic media have been introduced. The electrochromatographic properties of agarose using acetone as inert EOF marker have been studied. The selectivity has been exemplified with ribonucleosides having hydroxyl groups in *cis* position known to react with boronate groups in the gel.

In the preparation of an agarose-based homogeneous gel for electrochromatography, the immobilization of the polymer ligand chains was probably accomplished by both physical entrapment and interaction with the agarose chains with the boronate groups [Bergold and Scouten, 1983] and phenyl groups (aromatic adsorption [Gelotte, 1960; Hjertén and Mosbach, 1962; Eaker and Porath, 1967]). No leakage of the polymer has been observed in a column used for several weeks, supporting the suggested interaction mechanism. For automated runs the agarose should be replaced by a low melting, methoxylated agarose [Chen *et al.*, 1996].

The pores in a gel are too small to permit a transport of the mobile phase by a hydrodynamic flow which, however, can be replaced by an electroendosmotic flow. Gels of agarose are among the homogeneous media that have the desired pore size, but they require derivatization with charged groups to afford satisfactory electroendosmotic velocity. They have also higher UV transmission in comparison with polyacrylamide gels, therefore, permitting convenient on-gel detection.

Because of the relatively small pore size of a gel the average time for an analyte to diffuse from one interaction site to another is short (much shorter than that in packed beds as mentioned above) which results in sharp and symmetrical zones (see the first two peaks in Figure 6 on page A-22). The zone width is affected also by the residence time on the stationary phase, i.e., the time the analyte is attached to an interaction site upon a collision. To fully utilize the potential advantages of the gels the nature of the interaction sites and the experimental conditions should be chosen to minimize the residence times in the stationary phase (high association and dissociation rate constants). Substances eluting late in electropherograms and chromatograms are often indications of strong interaction and can result in asymmetrical peaks, such as the last one in Figure 6 on page A-22. More symmetrical peaks, presented in Figure 8, were obtained at lower pH value (7.6) probably due to lower interactions suggesting that the separation mechanism is pH dependent.

Homogeneous agarose and polyacrylamide gels beds have the advantage that the eddy diffusion is zero. In addition, the distance between the walls of a gel pore is much smaller than the average distance between the beads in a packed bed or in a continuous bed. The time for an analyte molecule to diffuse from one interaction site to another is much shorter in this medium than in the other two beds; in other words, the resistance to mass transfer is smaller. The diffusional broadening (the second term in van Deemter equation) is smaller than that in free solution. Theoretically, agarose-based and other types of homogeneous gels thus approach the ideal chromatographic medium.

Capillary electrochromatography, based on partition or other mechanisms, may become a widely used method in the future for the separation of neutral sample components. Using mass spectrometry as detector of non UV-absorbing samples the

agarose based homogeneous electrochromatographic bed can be employed for the separation of mono- and oligosaccharides.

In both modes of capillary isoelectric focusing very impressive results have already been presented in previous studies. Some practical difficulties, such as unwanted interactions between ampholyte and sample molecules, prompted us to develop an injection protocol that can overcome these obstacles.

The advantages in the use of 'sandwich' injection are as follows: (i) a simple way to combine different ampholytes in a run; (ii) the proportions between leading and terminating ampholytes could be changed easily; (iii) the sample molecules interact with ampholyte molecules only a short time before injection; (iv) very high resolution can be achieved with thorough combination of ampholyte solutions.

Sixteen ampholyte solutions from different sources (Ampholine, BioLyte, Pharmalyte and Servalyt) having different pH ranges showed characteristic separation patterns with aminomethylated nitrophenols, i.e., the gradients were somewhat different (see inserts in Figures 2-5 on page A-30 to A-33). This was expected since the chemical structures of these ampholytes are known to be different. Using the 'sandwich' injection method it was easy to test numerous ampholyte solutions, to compare their pH gradients formed during the focusing and to combine ampholytes to improve the resolution.

The approach was proved to be useful for focusing of standard and other hemoglobin samples. The resolution of the hemoglobin variants was observed to be as high as that in other methods [Phillipou and Phillips, 1993; Hempe and Carver, 1994, Conti *et al.*, 1995; Castagnola *et al.*, 1995]. The method can, therefore, be utilized in quantitative measurements. The precision makes the method a possible alternative tool for clinical diagnosis of hemoglobin variants. The concentration of glycosylated hemoglobin was found to be significantly higher in blood from diabetes patients.

CONCLUSIONS

For investigation of biomolecules, capillary electrophoresis methods were developed and modified demonstrating the usefulness and that applicability of this separation technique.

Small molecules, such as amino acids and ribonucleosides have been studied by a simple free zone electrophoretic, an electrochromatographic methods and a new homogeneous electrochromatographic media, respectively. Both approaches are outstanding in their simplicity: (i) the sample compounds were injected as aqueous solutions; (ii) the detection was on-tube in the UV-range; (iii) the selectivity was high and (iv) the synthesis step is easy.

Proteins, as typical biopolymers, were studied by isoelectric focusing. A new injection protocol has been developed for uncoated capillaries providing good reproducibility and high resolution, as demonstrated for hemoglobin separations. This investigation included characterization of the pH gradient formed, using various ampholyte solutions for focusing of hemoglobin variants.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to:

- Professor József Belágyi, for accepting me as a research student and supporting my studies in his institute.
- Professor Ferenc Kilár, my supervisor, for introducing me in the field of scientific research and separation science, especially capillary electrophoresis and for his assistance and support in many different forms.
- Márta Szécsényi, for teaching me the everyday practice of chemistry in the labs; Erika Bak and Anikó Takátsy, for helping me when I was abroad; and all other members of the Institute, for their helpfulness and nice accompany.
- Dr. Ferenc Aradi, for his friendship and many discussions about science and life and for much advise.
- Professor Stellan Hjertén, for guarding me towards this thesis and being the most excellent tutor. Tusen tack!
- Professor Gerald Gübitz and Dr. Martin Schmid, for providing a friendly atmosphere in their labs and sharing their vast knowledge about chirality and chromatography. Special tanks to Martin for being my best friend in science and life during the years.
- Dr. Andor Mód, for his friendship and valuable assistance in the development of the isoelectric focusing method.
- My wife, Tünde, and my parents, for their continuous support and love.
- Fanni, my daughter, for most of all.

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APPENDIX