

**NEW APPROACHES IN ANALYSIS OF BIOMOLECULES USING
CAPILLARY ELECTROPHORESIS –METHODODOLOGICAL
STUDIES**

Ph.D. Thesis

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INTRODUCTION

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). 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 for similar analyses where the speed and separation power are enhanced by the use 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 the 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 where CE with its inherent potential for high resolution and fast analyses has contributed substantially to decrease the time-schedule of the project.

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

AIMS OF THE STUDY

The aims of the present study were as follows:

- To investigate the applicability of capillary electrophoresis for separations of biomolecules.
- To emphasize the usefulness of capillary electrophoresis for the separation of biomolecules which differ in size and chemical character.
- To study the ligand-exchange mechanism for separation of free α -amino acids by capillary electrophoresis and electrochromatography employing a newly synthesized and a chemically activated chiral selector and to investigate the separation parameters and optimize the enantiomer separation of α -amino acids using ligand-exchange mechanism.
- To prepare an electrochromatographic column *in situ* in the capillary filled with an entirely homogeneous gel having appropriate pore sizes and number of charged groups (including borate concentration for separation of neutral ribonucleosides) to generate electroosmotic flow.
- To investigate the isoelectric focusing method and to develop a new injection protocol 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 and to employ the new capillary isoelectric focusing method in high-resolution separation of hemoglobin variants of diagnostic importance.

APPLIED METHODS

LIGAND-EXCHANGE CAPILLARY ELECTROPHORESIS

Generally, separation of enantiomers 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.

Direct enantioseparation by a 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. Enantioseparation depends on the different stability of the enantiomers in the semi-complex. The analyte and the 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 first direct separation of underivatized amino acids involved L-proline- and L-hydroxyproline-Cu(II) complexes as chiral selectors. Eleven amino acids containing aromatic groups were successfully resolved into their enantiomers. Ligand-exchange mechanism has been studied in enantiomer separation of amino acids by both electrophoretic and electrochromatographic methods.

CAPILLARY ELECTROCHROMATOGRAPHY

In many ways, capillary electrochromatography (CEC) is a hybrid separation technique with advantages from both high performance capillary chromatography and capillary electrophoresis. It uses electroosmotic-driven flow, instead of a pressure driven flow, to propel the mobile phase through the column. Electroosmotic flow is generated in the Helmholtz double layer at the charged solid-liquid interface. The separation mechanism in this process 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 as does HPLC a broad 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 originating from Joule heating, thereby reducing zone broadening significantly.

The stationary phase can be attached in a thin layer to the inner wall of the capillary column, so-called open tubular-CEC. 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). Packed beds have a high loading capacity, but the classical chromatographic zone broadening effects are large. Additionally, none of the standard methods to pack columns with small beads give sufficiently uniform beds in narrow bore tubes and frits are needed to support the bed and pressurized electrode chambers to avoid bubble formation. Many of these drawbacks are eliminated when the stationary phase is made up of a polymeric network (continuous beds, also called 'monoliths', or gels). The *in situ* preparation of chromatographic beds is simple since it only requires that an aqueous monomer solution is sucked into the capillary and is polymerized. The support consists of very tiny particles (0.2-0.5 μm) covalently linked. The continuous beds have found application in several chromatographic modes and, more recently, also in electrochromatography.

Polymer solutions and gels, such as agarose and polyacrylamide gels, behave as ideal chromatographic media because they are not made up of particles, thus being macroscopically homogeneous, which results in less zone broadening.

A homogeneous separation medium is visually a transparent (like a polyacrylamide gel) or a slightly opalescent (like agarose gel) polymer network, i.e., no elements large enough to be visible in a light microscope or/and cause sufficient light scattering to make the medium opalescent.

CAPILLARY ISOELECTRIC FOCUSING

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 and microheterogeneity of protein therapeutics, and determination of isoelectric points. Capillary isoelectric focusing (CIEF) 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 might be generated. The primary CIEF experiments were performed in capillaries coated with neutral polymers, such as linear polyacrylamide, exhibiting no electroendosmotic flow (EOF).

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 conventional gel IEF.

After focusing is performed in a coated capillary as a first step, the whole length of the pH gradient formed is mobilized to pass the detection window for detection of the

focused zones (second step). In such a two-step IEF two techniques for mobilization have been described.

Using uncoated capillaries in single-step (“dynamic”) IEF, proteins are focused while being transported towards the detection point by EOF. The sample and ampholytes are 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. The addition of methylcellulose or hydroxymethylcellulose to the catholyte serves to coat the fused silica wall dynamically, thereby reducing protein adsorption and EOF.

RESULTS AND DISCUSSION

STUDY OF SMALL BIOMOLECULES

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

For the 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 an epoxide. Elemental analysis, NMR and IR spectra confirmed the structure of this new chiral selector, *N*-(2-hydroxyoctyl)-L-4-hydroxyproline (HO-L-Hypro). This selector compound was used in a complex of copper(II) ion in sodium phosphate as electrolyte.

Enantiomers of thirteen free α -amino-acids have been successfully resolved by capillary zone electrophoresis. The required selector concentration of HO-L-Hypro was found to be about 10 mM for all amino acids. The minimum detectable amount of the D-dihydroxyphenylalanine (DOPA) in the sample of L-DOPA was 0.03 %. The D-enantiomer is inactive as anti-Parkinson drug, therefore, is undesirable in medical production. 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 mM. 10 mM selector concentration was chosen for all experiments.

Higher electrolyte concentration resulted in higher resolution, at the expense of lower migration time. Addition of organic modifiers did not show improvement in resolution. A correlation between the isoelectric points (pI) of the amino acids and the optimal pHs for their separation was found.

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

A chemically activated chiral selector, *N*-(2-hydroxy-3-allyloxypropyl)-L-hydroxyproline (HAP-L-Hypro), was *in situ* co-polymerized with neutral, charged and cross-linking monomers by a free radical mechanism. During the polymerization a network of polymer chains formed building up small, non-porous particles covalently linked to each other and to the capillary wall. The interconnected nodules are large enough for high permeability ensuring low backpressure. 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.

Enantiomers of nine free α -amino-acids have been successfully resolved by capillary electrochromatography with the new chiral selector, HAP-L-Hypro.

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. Interestingly, in pressure-supported mode both the resolution and the efficiency were improved compared 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. Since the chiral continuous bed 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.

Comparing CE and CEC modes of ligand-exchange, the following conclusions can be made: Both approaches have provided high selectivity and short analysis time. CE mode offers higher resolution with reasonable migration times and the selector, HO-L-Hypro, was found to be more effective than that used in CEC mode. Run times were shorter in CEC

mode when the electrophoretic and endosmotic velocity was facilitated by additional hydrodynamic pressure applied.

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

In order to introduce borate ions as ligands for the separation of ribonucleosides, 3-aminophenylboronic acid hemisulfate was reacted with acryloyl chloride resulting in an activated monomer compound. A co-polymer of acryloyl-aminophenyl boronic acid (APB) and acrylic acid (AA) was prepared via free radical polymerization.

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. The theoretical plate numbers were often between 100-350,000 m⁻¹.

To test the properties of the new homogeneous gel, CEC experiments in the frontal analysis mode were performed using acetone as EOF marker. The experimental standard deviations were measured and compared to those calculated for free diffusion, using the Einstein equation. The calculated diffusion broadening was found to be higher than the measured experimental total broadening of the boundaries. The data indicate 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.

To test the selectivity of this column ribonucleosides which have high UV-absorbance at 254 nm, were chosen. They differ in their bases whereas the sugar moiety interacting with boronate groups in the gel is the same. 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. Four ribonucleosides were baseline separated.

Because of the relatively small pore size of this gel the average time for an analyte to diffuse from one interaction site to another is short (much shorter than that in packed beds) which results in a reduction of the third term in the van Deemter equation. 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.

STUDY OF BIOPOLYMERS

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

The ampholytes and analytes were injected separately in three steps, i.e., ampholyte solutions were injected before and after the sample. This injection set-up was called a 'sandwich'. The same type or different types of ampholyte solutions were applied consecutively to the capillary of various lengths. Approximately, 20-30 % of the whole capillary was filled with the 'sandwich' plug. Upon application of voltage isoelectric focusing of the samples started and the pH gradient began to move towards the cathode by the EOF.

Aminomethylated nitrophenols were used to optimize the conditions for the injection protocol. 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 pH gradients were somewhat different.

Ampholytes having narrow and broad pH ranges were tested for comparison with dye molecules. The lowest resolution was obtained with ampholyte solutions covering the lowest narrow pH range, pH 3-5. Somewhat better resolution was obtained with ampholyte solutions of higher narrow or broad pH ranges. The resolution and the migration times of the dye markers were different when ampholyte solutions covering similar pH ranges from different sources were employed. Although the pH ranges of the ampholyte did not cover the pI 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.

The combination of ampholytes, using different pH ranges, improved the separation of dyes. In the experiments with two different ampholytes a combination of a broad and a narrow pH range provided a higher resolution, using the 'sandwich' injection protocol, than that obtained with simple injection of the mixture consisting of ampholytes and dyes. Significantly better resolution was obtained using lower concentration (2 %) of the ampholytes. A combination of two narrow pH-range ampholytes separated the seven dyes similarly to broad pH-range ampholytes, although the gradient did not cover every pI values.

All the four major compounds of the hemoglobin standard were baseline separated. Among these variants minor peaks were also focused probably containing glycosylated proteins. Hemoglobin from blood of healthy individuals and patients with diabetes mellitus was focused with high resolution employing the 'sandwich' injection protocol. The highest resolution was obtained using a combination of narrow and broad pH ranges of the ampholytes.

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

CONCLUSIONS

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

The separations of small molecules, such as amino acids and ribonucleosides have been studied by free zone electrophoresis and electrochromatography, using continuous beds and new homogeneous gel media. Both approaches were 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 was 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 the characterization of the pH gradient formed, using various ampholyte solutions for focusing of hemoglobin variants.

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