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Capillary Electrochromatography with Gradient Elution

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A capillary electrochromatograph incorporating a gradient-forming system generally employed in HPLC is described, and the use of gradient elution in reversed phase electrochromatography is demonstrated by the separation of PTH-amino acids and steroid hormones. The gradient former employs two reciprocating displacement pumps to control the composition of the eluent in the reservoir at the column inlet with time in a controlled manner. Thus, the composition of the mobile phase flowing through the column and driven by electrosmotic forces can be changed with time in a controlled fashion as customary in HPLC with gradient elution. The design of the system allows also for isocratic elution by pumping the eluent of constant composition through the cavity at the column inlet and thus continuously supplying fresh buffer. The eluent gradient is generated by the two pumps and a 10 μL mixer. From there the liquid passes at a flow rate of 0.1−0.2 mL/min through the 17 μL cavity housing the column inlet and an electrode. The flow of the mobile phase was electrosmotic at an effective overall electric field strength of 500−1500 V/cm through a 50 μm × 20/12 cm capillary column packed with 3.5 μm octadecylated silica particles. Gradient profiles generated in this manner were highly reproducible. The same-day and day-to-day reproducibilities of the electrosmotic flow were found to be better than 3%. The use of the capillary electrochromatographic system was demonstrated with isocratic and gradient elution for the separation of complex mixtures of biologically interesting substances. The influence of the column temperature on the electrosmotic flow velocity and retention of PTH-amino acids was also investigated.

Capillary electrochromatography (CEC) is an electrokinetic separation technique that is expected to combine the versatility offered by HPLC with the high plate efficiency of CZE.[1−3] The technique employs fused-silica capillaries packed with a stationary phase that has fixed charges at the surface. Thus, the flow of the eluent across the column is generated and maintained by electrosmotic means upon application of high electric field. The unique flow field allows the use of long capillary columns packed with small particles, resulting in significant gains in peak capacity. As the mobile phase flow originates at the surface of the stationary phase, band spreading is significantly less than in conventional HPLC.[3,4] The sample components are separated due to differences in their partitioning between the mobile and the stationary phases and, if they are charged, also due to differences in their electrophoretic mobilities.[5]

No equiresolutive chromatogram can be obtained by isocratic elution in the chromatographic separation of a mixture containing several components of widely differing retention behavior, and this has been refered to as the "general elution problem".[6] In order to separate all components without compromising analysis time, resolution, and peak sensitivity, gradient elution is employed to increase gradually and in a controlled manner the eluent strength during the chromatographic run. To realize the full potential of CEC, it is therefore necessary to employ instrumentation having gradient elution capability for the separation of complex mixtures. Indeed, the application of a high electric field additionally to a pressure-driven gradient[7] has been found to result in enhanced separation efficiency. Electrosmotically driven solvent gradients for CEC have also been obtained by merging two electrosmotic flows that are generated in open fused-silica tubes and regulated by computer controlled voltages of 0−50 kV.[8]

We have modified a commercially available capillary electrophoresis unit by incorporating a gradient former with HPLC pumps so that the eluent flow is driven solely electrosmotically through the column during the gradient run. Use of instrument is demonstrated by isocratic and gradient separation of PTH-amino acids and steroid hormones.

EXPERIMENTAL SECTION

Chemicals and Materials. HPLC-grade acetonitrile, methanol, and chlorosulfonic acid were from Fisher Scientific (Springfield, NJ). Reagent-grade acetone and sodium dihydrogen phosphate monohydrate were from J. T. Baker Inc. (Phillipsburg, NJ), formamide was from Eastman Kodak Co. (Rochester, NY), and sodium hydroxide was from Mallinckrodt (Paris, KY). Water was purified by a NanoPure unit (Barnstead, Boston, MA). Neat aqueous phosphate buffer was prepared as a 50 mM stock solution, the pH was adjusted to the desired value ±0.05 pH unit with 1 M sodium hydroxide, and the buffer solution was filtered

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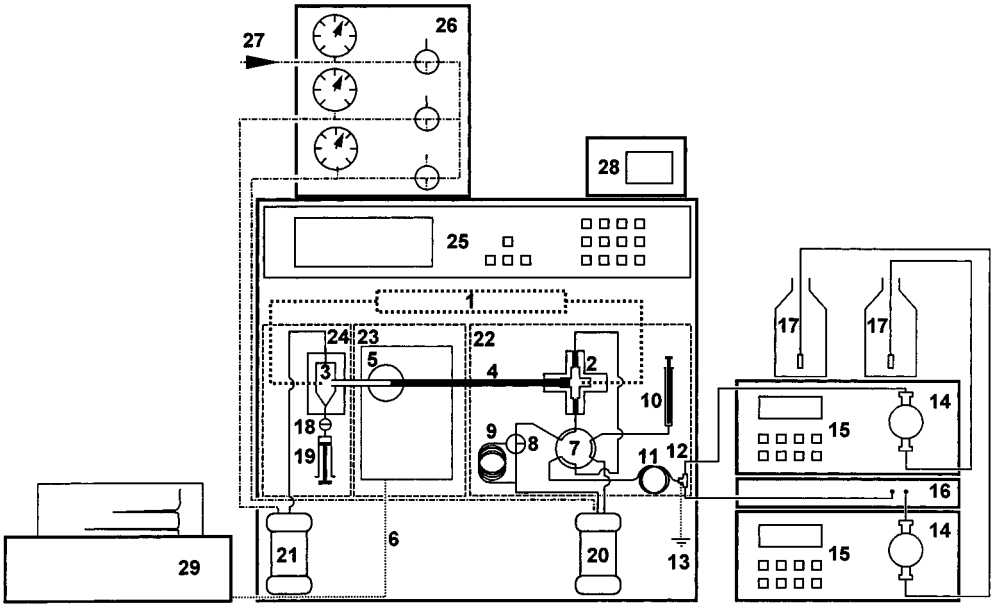

Figure 1. Capillary electrochromatograph with gradient elution capability: 1, high-voltage power supply; 2, inlet reservoir with electrode; 3, outlet reservoir with electrode; 4, packed capillary column; 5, on-line sensing unit of UV detector; 6, detector output, 0−1 V; 7, sample injection valve; 8, purge valve; 9, restrictor; 10, syringe for introduction of sample or buffer; 11, capillary resistor; 12, static mixing tee; 13, grounding; 14, pumps; 15, pump control panels and readouts; 16, manometer; 17, eluent reservoirs; 18, switching valve; 19, syringe for buffer introduction; 20, waste reservoir at the inlet; 21, waste reservoir at the outlet; 22, thermostated inlet compartment; 23, detector compartment; 24, outlet compartment; 25, CEC instrument control panel; 26, gas pressure control; 27, gas inlet, 1.4 MPa nitrogen; 28, temperature control; 29, data acquisition. Line symbols: ⋯, electric wiring; —, liquid lines; — · —, gas lines; ---, separating lines between instrument compartments.

through a 0.45 μm pore size MicronSep nitrocellulose filter from Micron Separations Inc. (Westborough, MA). Eluents were prepared by appropriate dilution of the stock solution with water and acetonitrile without further adjustment of the pH. Steroid hormones and PTH-amino acids were obtained from Sigma (St. Louis, MO).

The Zorbax ODS stationary phase (3.5 μm, 80 Å or 5 μm, 300 Å) was a gift from Rockland Technologies Inc. (Newport, DE). Polyimide-coated fused-silica capillaries of 375 μm o.d. and 50 or 75 μm i.d. were obtained from Quadrex Corp. (New Haven, CT).

Instrumentation. Electrochromatography was carried out by using a Model ABI 270A-HT capillary electrophoresis unit (Perkin Elmer/Applied Biosystems, Foster City, CA) that was modified to allow its use also for CEC and μ-HPLC with isocratic and gradient elution as illustrated by the flow sheet in Figure 1.

The original ABI instrument comprises three compartments: at the right, the inlet compartment (22) with autosampler and the oven; in the middle, the detector compartment (23) with the cell for on-column UV detection (5); and at the left, the outlet compartment (24) housing the outlet reservoir, which is connected to the vacuum system. The instrument is equipped with a 1−30 kV high-voltage power supply (1) and 190−700 nm variable-wavelength UV detector (6). The autosampler assembly, comprising the sample carousel for sample/buffer vials and the electrode/capillary holder, could not be pressurized during CEC operation. The electrode/capillary holder was removed from the capillary oven and placed inside the instrument without disconnecting it from its electronic control board to avoid software error during operation. The outlet reservoir was also removed from the left compartment and the vacuum line disconnected.

A PEEK cross of 17.2 μL internal volume, part no. P730 from Upchurch Scientific (Oak Harbor, WA) was used as the inlet reservoir (2). The two horizontal ports of the cross were used for the capillary column (4) and the platinum electrode connected to the high-voltage power supply (1). The vertical openings of the cross were connected to ports 1 and 4, respectively, of a Model 8125 six-port valve (7) (Rheodyne, Cotati, CA) by means of two 200 mm × 75 μm fused-silica capillaries. Port 2 of the six-port valve (7) was connected to the inlet of a three-way purge valve (8), part no. 02-0124 (Alltech, Deerfield, IL). One outlet of this purge valve (8) was connected to a 150 cm × 50 μm i.d. fused-silica capillary (9), whereas the other outlet was connected to a PEEK tube, 60 mm long, 1.59 mm o.d., 0.508 mm i.d. The ends of the fused-silica capillary (9) and the PEEK tube were connected to a Tefzel T-piece, part no. P632 (Upchurch Scientific). The third port of the Tefzel T-piece was connected with the help of a PEEK tubing, 300 mm long, 1.59 mm o.d., 0.508 mm i.d., from Upchurch Scientific to the inlet waste reservoir (20) made of a 100 mm long × 17 mm i.d. glass-bore column from Omnifit (Toms River, NJ).

The outlet reservoir (3), of 3 mL internal volume having appropriate ports for the capillary column, platinum electrode, and inlet and outlet lines for the mobile phase, was made from 48 × 30 × 30 mm PEEK block (McMaster-Carr Supply Co., New Brunswick, NJ) and mounted on a plexiglass plate with slits to adjust its position. The outlet reservoir was filled with the mobile phase by means of a plastic syringe, which was connected to the reservoir by a 100 mm long, 1.59 mm o.d., 0.508 mm i.d. PEEK tubing and a switching valve, part no.V101S, from Upchurch Scientific. The outlet waste reservoir (21) was made of a 50 mm × 17 mm i.d. Omni glass tube (Omnifit). Both reservoirs for waste mobile phase (20, 21) could be pressurized up to 200 psi with helium jointly or individually by means of three three-way ball valves, part no. 4566K85 (Parker Hannifin Corp., Jacksonville, AL), and the pressures were monitored with three gauges of 0−400 psi range, Part No 3851K33 (McMaster-Carr Supply Co.).

The gradient former consisted of two Model 305 piston pumps (14) from Gilson Medical Electronics Inc. (Middletown, WI), a Model 805 manometric module (16), and a 10 μL stainless steel

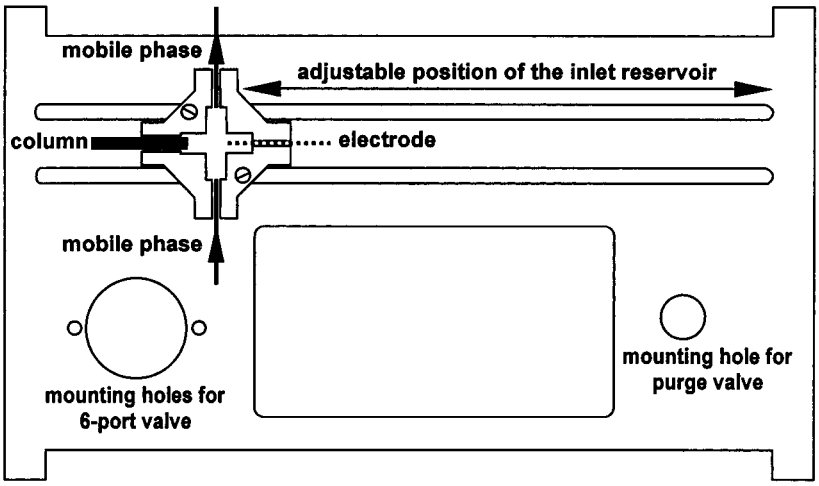

Figure 2. Illustration of the inlet mobile phase reservoir with mounting plate to allow for the adjustment of axial position.

Visco-Jet Mixer (12) (The Lee Co., Westbrook, CT). The outlet of the mixing TEE (12) was connected to the port 2 of the six-port valve by a 50 μm i.d., 600 mm long fused-silica capillary (11). The delay volume of the system arising from the volume of the mixer and the connecting tube was estimated as 12.06 μL. In order to prevent damage to the HPLC pumps, the mixer was kept at ground potential (13) and the 600 mm long segment of fused-silica capillary served as an electrical resistor to avoid electric shock or significant current losses. The applied voltage, the detector, and the oven temperature were all controlled by using the original control panel of the ABI instrument. The gradient program was controlled by the HPLC pumps (14). The detector output was recorded with a Model C-R3A integrator (29) (Shimadzu, Columbia, MD).

Figure 2 provides a schematic illustration of the inlet reservoir which along with the six-port valve (7) and the purge valve (8) was mounted on a 155 × 90 × 4 mm plexiglass plate secured in the oven by means of four set screws while allowing for the alignment of the capillary column with the sensing unit of the detector. Two 140 mm long and 3 mm wide slits permitted the alignment of the inlet reservoir to accommodate straight columns of 80−180 mm length horizontally in the oven whereas coiled columns could be used without length limitations.

Figure 3 illustrates the heating unit of the electrochromatograph. The oven of the unmodified ABI instrument (30−33) is capable of thermostating the sample electrode compartment at temperatures in the range of 25−60 °C whereas the detector compartment is not thermostated at all. In order to diminish the axial temperature gradient, an additional heating system was installed. Compressed air at a pressure of ~5 psi (34) was heated with a 50 W tubular electric heater (35) and brought to the detector compartment through a 3.2 mm i.d. Teflon tube (Upchurch Scientific). The electrical heater was controlled by a Model CN 9000A digital programmable temperature controller (28) (Omega, Stamford, CT), and the temperature was monitored with a iron−constantan low-noise thermocouple (37) (Omega).

Procedures. *(1) Packing Capillary Columns.* Polyimide-coated fused-silica capillary tubing of 375 μm o.d. and 50 μm i.d. was obtained from Quadrex Corp. (New Haven, CT). A temporary retaining frit was made at the end of a 25−30 cm long fused-silica capillary by sintering a thin slug of 2 μm nonporous silica particles (Glycotech, Hamden, CT) in a propane and oxygen flame by using a Type 3A blowpipe (Veriflo Corp., Richmond, VA). A slurry

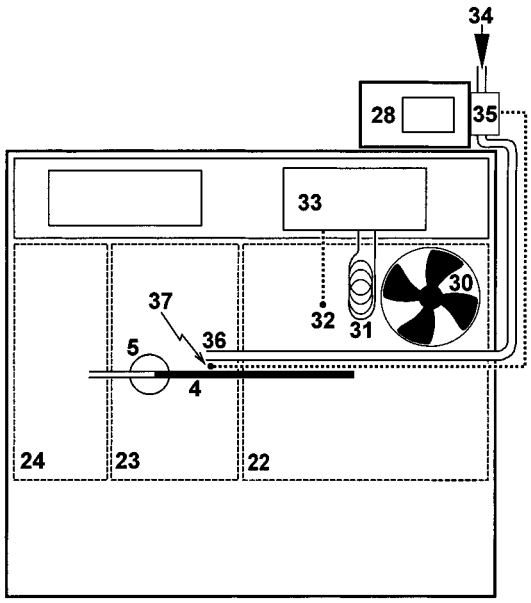

Figure 3. Temperature control of the CEC instrument. Parts represented by numbers up to 29 are described in Figure 1. Here explanation is given only for higher numbers. The original temperature control of the ABI instrument: 30, air fan; 31, heating coil; 32, thermocouple; and 33, temperature control electronics. Additional temperature control for the detector compartment: 34, 0.34 MPa inlet for compressed air; 35, heat exchanger; 36, heated air outlet; 37, thermocouple.

reservoir of 43 mm length, 6.35 mm o.d., and 2 mm i.d. and having an internal volume of 135 μL was fabricated from a stainless steel tubing (Alltech, Deerfield, IL) and a pair of column end fittings (Valco, Houston, TX), with the holes of the fittings drilled to a diameter of 0.5 mm. The open end of the capillary was connected to the slurry reservoir with ~2 mm of the capillary protruding into the reservoir. A 10 mg sample of the Zorbax ODS stationary phase was suspended in a mixture of 110 μL of acetone and 110 μL of toluene, sonicated for 5 min, and transferred to the slurry reservoir. The column was packed with acetone at a constant pressure of 65 MPa using an Altex 110A pump (Beckman, Fullerton, CA) for 30 min. Then, the flow of the packing solvent was stopped, and after slowly releasing the pressure, the column was disconnected from the slurry reservoir. Subsequently, it was connected directly to the pump and washed first with acetone for 30 min and then water at 50 MPa. An outlet retaining frit was

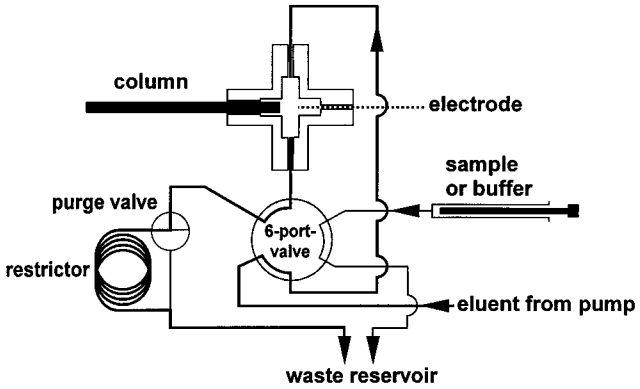

Figure 4. Sample introduction and flow control system for operating the instrument with pressure-driven flow. In this arrangement, the six-port valve is in position I, while the purge valve is closed.

sintered at a distance of 9 cm from the end of the column that was connected to the pump by heating with a Nichrom filament ring (Omega) while the column was still being flushed with water. Then, an inlet frit was sintered at an additional distance of 12.5 cm. After, the flow of water was stopped and the pressure was slowly released, the column was connected to the pump again but with the flow direction reversed to remove the excess stationary phase. A 2 mm length of the polyimide outer coating immediately after the outlet frit was removed by dissolving with 1 drop of chlorosulfonic acid to make a window for on-column detection. Finally, the capillary between the temporary retaining frit and the column inlet frit was cut off, and the outlet frit was trimmed to leave a length of exactly 8 cm between the detection window and the column outlet.

(2) Equilibration of Column. The capillary column was mounted in the CEC unit and equilibrated with the mobile phase at a pump setting of 0.15 mL/min with the six-port valve in position I and purge valve closed as illustrated in Figure 4. The back pressure for a mobile phase containing 30% ACN was 20−25 MPa, and 10−12 MPa pressure drop over the restrictor forced a fraction of the mobile phase to flow through the column. Of course, the pressure built by the restrictor and the time required to equilibrate the packed-capillary column depends on the viscosity of the mobile phase employed. After ∼30 min, the flow was shut off and the purge valve opened cautiously to release the pressure slowly.

(3) Sample Injection. With the purge valve open and the six-port valve in position I, the flow rate of the pump was set to 0.1 mL/min, resulting in a back pressure of 10−12 MPa. Without pressurization of the waste reservoirs, the flow was allowed to stabilize for ∼15 min. The general procedure for injecting samples is illustrated schematically in Figure 5a. First, the six-port valve was turned to position II so that the flow of the mobile phase bypassed the inlet reservoir. Thereafter, the inlet reservoir was flushed with 100 μL of sample solution with the help of a 100 μL glass syringe (Hamilton, Bonaduz, Switzerland). A few picoliters of the sample were injected electrokinetically onto the capillary column upon application of 1 kV for 0.5−2 s. Then, the inlet reservoir was purged with ∼100 μL of the mobile phase and the six-port valve turned back to position I in order to flush the inlet reservoir continuously (Figure 5b) with the mobile phase.

(4) Isocratic Elution. The construction of the electrochromatographic system affords operation in either the isocratic or in the gradient elution mode. For isocratic elution, after the sample

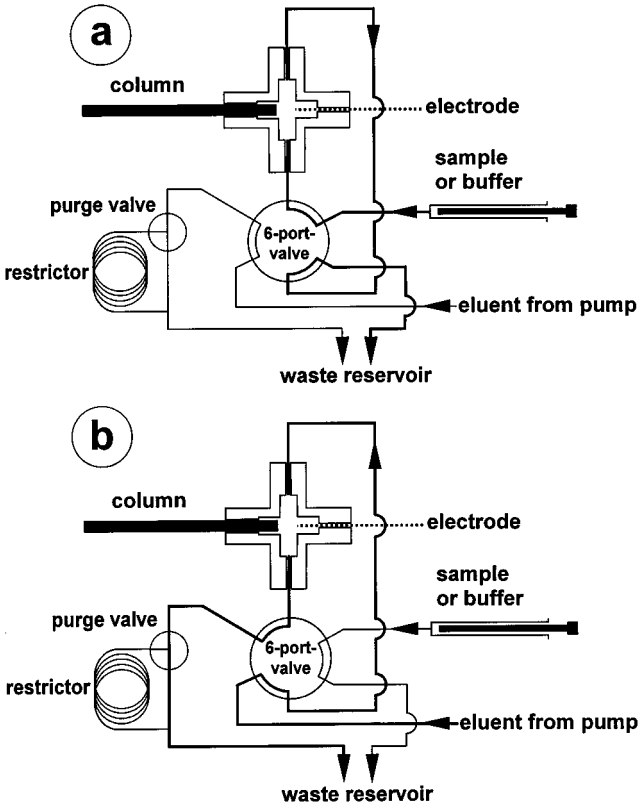

Figure 5. Schematic illustration of the sample introduction and eluent flow control system. (a) Arrangement for purging the inlet reservoir with the sample solution for subsequent electrokinetic injection and for flushing the reservoir with the mobile phase. The six-port valve is in position II and the purge valve is open; (b) arrangement for operation in the electrochromatographic mode with isocratic or gradient elution. The six-port valve is in position I, and the purge valve is open.

was injected and the six-port valve turned to position I, mobile phase flow at a constant composition was directed through the inlet reservoir. Upon pressurization of the inlet and outlet reservoirs with helium and application of voltage (10−30 kV), isocratic elution with electrosmotically driven flow proceeded while the inlet reservoir was continuously purged with the mobile phase at a flow rate of ∼0.1 mL/min.

(5) Gradient Elution. The gradient programmer is set to change the composition of the mobile phase in the inlet reservoir with time in order to obtain the desired eluent gradient. The gradient program and the application of the voltage commenced simultaneously and then the mobile phase from the inlet reservoir was drawn continuously into the column by electrosmotic means. Since, the composition of the mobile phase in the inlet reservoir changed with time according to the gradient program, the eluent composition at the column inlet also changed, producing reproducible gradient profiles.

Safety Considerations. All parts connected to the high voltage were housed in the Faraday cage of the ABI CE system, which ensured the automatic shutdown of the high voltage whenever it was opened. The 10 μL Visco-Jet mixer of the gradient system was connected to ground in order to avoid damage to the pumps by an electric shock. Tubing carrying liquid from the anodic and cathodic electrode reservoirs must not be interconnected in order to prevent electrical short circuit. Safety glasses have to be worn whenever fused-silica tubing is handled.

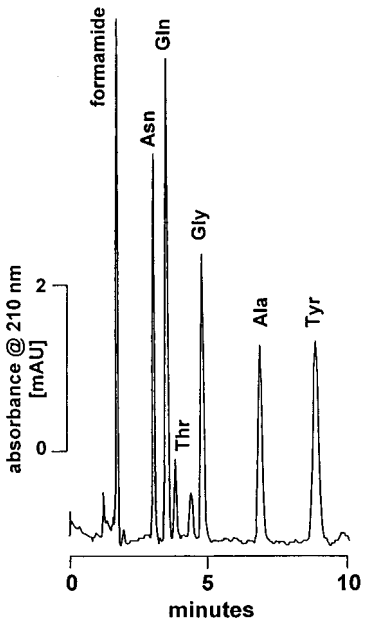

Figure 6. Electrochromatogram of six PTH-amino acids obtained by isocratic elution. Column, 50 μm × 20.7/12.7 cm packed with 3.5 μm Zorbax ODS having a pore size of 80 Å; mobile phase, 5 mM phosphate, pH 7.55, 30% acetonitrile; flow rate of the mobile phase through the inlet reservoir, 0.1 mL/min; voltage 10 kV; current, 1 μA; EOF velocity, 1.3 mm/s; temperature, 25 °C; UV detection at 210 nm; electrokinetic injection, 0.5 s, 1 kV. The peaks in the order of elution: formamide (1 μg/mL), PTH-asparagine, PTH-glutamine, PTH-threonine, PTH-glycine, PTH-alanine, and PTH-tyrosine. The concentration of the PTH-amino acids dissolved in the mobile phase was 30−60 μg/mL.

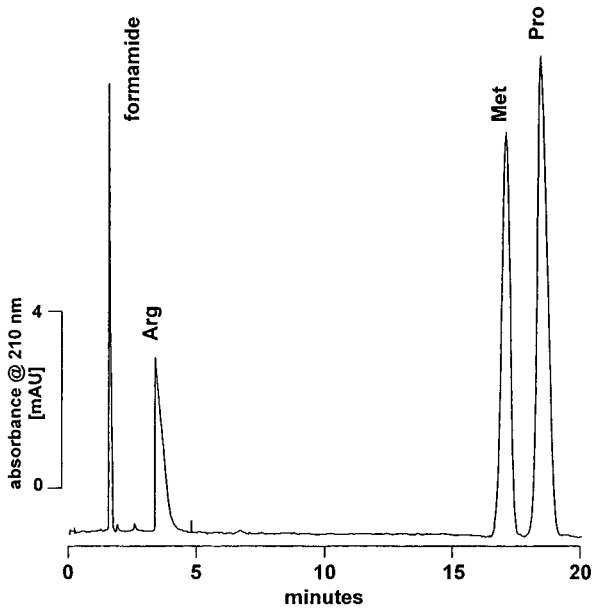

Figure 7. Isocratic separation of PTH-amino acids. Column, 50 μm × 20.7/12.7 cm packed with 3.5 μm Zorbax ODS particles having a mean pore size of 80 Å; mobile phase, 5 mM phosphate, pH 7.55, 30% acetonitrile; voltage, 10 kV; flow rate of the mobile phase through the inlet reservoir, 0.1 mL/min; current, 1 μA; temperature, 25 °C; detection, UV at 210 nm; electrokinetic injection, 0.5 s, 1 kV. The peaks in order of elution: formamide (1 μg/mL), PTH-arginine, PTH-methionine, and PTH-proline. The concentration of the PTH-amino acids dissolved in the mobile phase was 30−60 μg/mL.

Packed-capillary columns have to be fitted securely especially during packing and flushing them under high pressure.

RESULTS AND DISCUSSION

Isocratic Elution. *(1) Electrochromatographic Separation of PTH-Amino Acids.* The CEC instrument was used with isocratic elution to separate a mixture of six PTH-amino acids, and the results are illustrated by the electrochromatogram in Figure 6. The sample mixture was injected electrokinetically at 1 kV for 0.5 s and, the inlet reservoir having a capacity of only 17 μL was purged continuously with the mobile phase containing 5 mM phosphate, pH 7.55, in 30% ACN at a flow rate of 0.1 mL/min in order to maintain constant its composition at the column inlet during the chromatographic run. The reservoir at the outlet was rinsed with 5 mL of the mobile phase after every three runs. The detector wavelength was set at 210 nm, instead of the more commonly used 254 nm for PTH-amino acids in order to also detect formamide, which is used as the EOF marker. It is seen in Figure 6 that the PTH-amino acids are retained in order of increasing hydrophobicity, as expected, since they are not charged at pH 7.55.[9−11]

Figure 7 shows the electrochromatographic separation of PTH derivatives of arginine, methonine, and proline by isocratic elution under the same conditions as described above. The strong retention of PTH-methonine and PTH-proline suggests the need for gradient elution for the separation of a larger set of PTH-amino acids within an acceptable time window. It is seen in Figure 7 that PTH-arginine elutes at 3.4 min, and thus has the same elution time as PTH-glutamine under identical conditions; see Figure 6. At first sight this is surprising because in HPLC under comparable conditions (Ultrasphere ODS, 25 mM sodium acetate, pH 5.75[12]), the PTH-arginine peak elutes close to PTH-alanine peak. In our case, however, the positively charged guanidium group of PTH-arginine is subject to electrophoretic migration toward the cathode and this explains its relatively short retention time. On the other hand, PTH-arginine peak exhibits significant tailing due to silanophilic interactions between the positively charged side chain and the silanol groups at the surface of the stationary phase. The sample for the electrochromatogram in Figure 7 also contained PTH-aspartate and PTH-glutamate. However, due to their negatively charged side-chain carboxyls, these two PTH-amino acids were subject to counterdirectional electrophoretic migration and therefore most likely did not enter the column.

(2) Influence of Temperature on the Separation of PTH-Amino Acids. The significance of temperature as a major operational variable in liquid chromatography was recognized already in the 1960s.[13−16] Subsequent investigations were aimed mainly at elucidating the effect of column temperature on chromatographic retention and the selectivity of small molecules.[17,18] It has been established that both separation speed and efficiency could be

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enhanced by operating at elevated temperatures.[19,20] Since the effect of elevated temperature in electrochromatography elicited scant attention only, we tested our system with PTH-amino acids.

Since the built-in capillary oven of the ABI instrument allows the heating only of the right electrode compartment up to 60 °C as mentioned before, we installed an additional heating system to thermostat the detector compartment in order to guarantee a uniform column temperature over the whole length of the packed capillary. The temperature of the detector compartment was measured by a thermocouple mounted near the capillary column that was calibrated in the same way as the thermocouple of the built-in oven of the instrument. The four isocratic electrochromatograms in Figure 8 illustrate the effect of temperature on the separation of PTH-amino acids in the range of 25−53 °C where the EOF velocity increased almost linearly from 1.2 to 1.75 mm/s due to a decrease in the viscosity of the mobile phase with increasing temperature. With the exception of PTH-threonine, the separation of the amino acids was not adversely affected by the elevated temperature that facilitated an almost 3-fold increase in the speed of analysis upon increasing the temperature from 25 to 53 °C. This effect is similar to that observed in reversed phase chromatography due to enhancement of mass transport by raising the column temperature.[21] van't Hoff plots depicted in Figure 9 were straight lines and the retention enthalpies were evaluated (in kcal/mol) as −2.918 for PTH-asparagine, −4.12 for PTH-glutamine, −3.92 for PTH-threonine, −4.83 for PTH-glycine, −3.75 for PTH-alanine, and −3.42 for PTH-tyrosine. All enthalpy values are in the same range as that obtained with small molecules in reversed phase chromatography.[22]

(3) Stability of Electrosmotic Flow. In CEC, the mobile phase flows through a packed-capillary column by means of electrosmotic forces. Many operating parameters such as the electric field strength, the temperature, the pH and ionic strength of the mobile phase, and the concentration of organic modifiers affect the magnitude of the EOF.[23−26] We examined the stability and reproducibility of the EOF velocity in 44 electrochromatographic runs during five consequtive days of experimentation by using a 50 μm × 20.7/12.7 cm fused-silica capillary packed with 3.5 μm Zorbax ODS particles and 5 mM phosphate, pH 7.55, 30% ACN as the mobile phase. The results illustrated in Figure 10 show that the lowest and the highest EOF velocities were 1.14 and 1.35 mm/s, respectively, with a relative standard deviation (RSD) of 4.2%. In general, the EOF velocity stabilizes at a value ∼1.2 mm/s. One-day reproducibility ranged between 2.14 and 2.93 RSD and day-to-day reproducibility was 3.26% RSD.

Gradient Elution. *(1) Reproducibility of Gradient Profiles.* Gradient-forming systems are usually tested with a low-volume connection in place of the chromatographic column[8] and evaluating the rising detector signal obtained by a gradient run with a UV-absorbing tracer such as acetone in the gradient former. This procedure could not be employed in our case because the packed capillary column was also our "electrosmotic pump". The gradient elution system was therefore tested by using a capillary column packed with 6 μm Zorbax ODS particles having a mean pore diameter of 300 Å. During the gradient run, the composition of the mobile phase in the inlet reservoir changed from 10 mM phosphate buffer, pH 7.0, to 25% acetonitrile in the 10 mM aqueous phosphate buffer, pH 7.0, over a period of 10 min. The concentration change was monitored with 5% acetone in the gradient former by recording the UV absorbance of the column effluent at 254 nm, and the results are illustrated in Figure 11. Analysis of five chromatograms like that depicted in Figure 12 showed a RSD of 3% for the retention times of PTH-glycine and PTH-tyrosine peaks under gradient elution conditions. These results demonstrate that

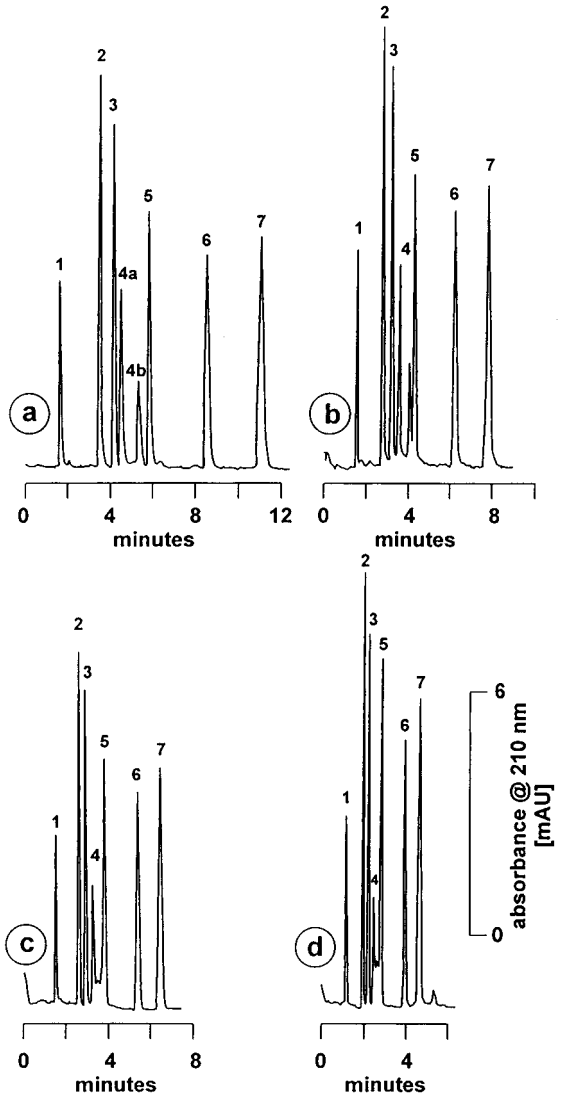

Figure 8. Effect of temperature on the separation of PTH-amino acids. Column, 50 μm × 20.7/12.7 cm packed with 3.5 μm Zorbax ODS particles having a mean pore size of 80 Å; mobile phase, 5 mM phosphate, pH 7.55, 30% acetonitrile; flow of the mobile phase through the inlet reservoir, 0.1 mL/min; voltage 10 kV; current, 1 μA; temperature (a) 25, (b) 35, (c) 45, and (d) 53 °C; UV detection at 210 nm; electrokinetic injection, 0.5 s, 1 kV. Sample: 1, formamide; 2, PTH-asparagine; 3, PTH-glutamine; 4, PTH-threonine; 5, PTH-glycine; 6, PTH-alanine; 7, PTH-tyrosine. The concentration of the PTH-amino acids dissolved in the mobile phase was 30−60 μg/mL.

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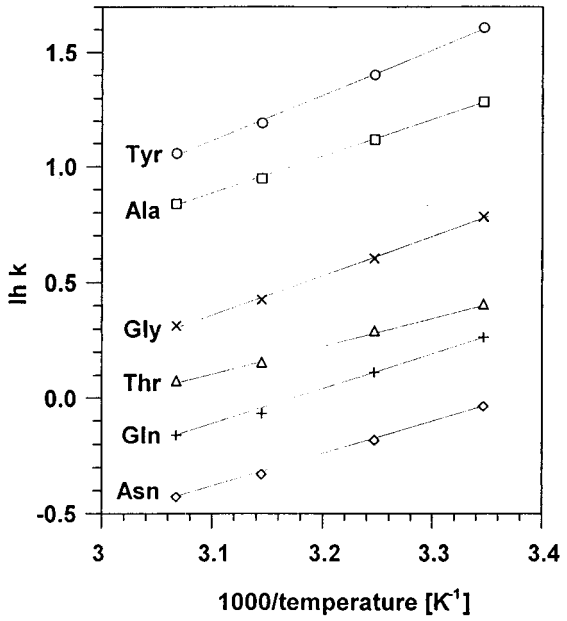

Figure 9. van't Hoff plots for PTH-amino acids in reversed phase CEC. Conditions as in Figure 8.

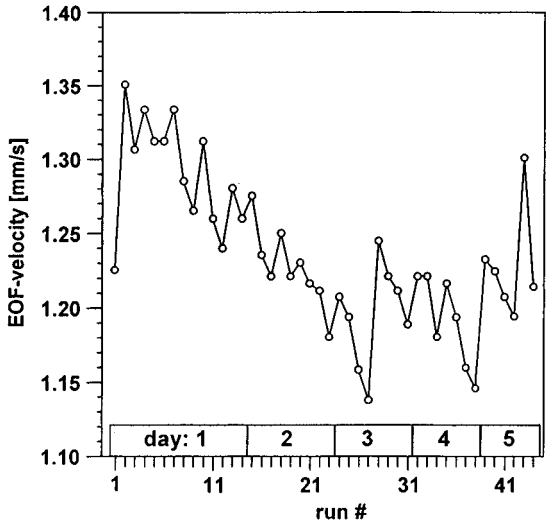

Figure 10. Reproducibility of electrosmotic flow through a packed capillary column. Column, 50 μm × 20.7/12.7 cm packed with 3.5 μm Zorbax ODS particles having a mean pore size of 80 Å; mobile phase, 5 mM phosphate, pH 7.5, 30% acetonitrile; flow rate of the mobile phase through the inlet reservoir, 0.1 mL/min; voltage 10 kV; current, 1 μA; EOF velocity, 1.236 mm/s (RSD = 4.2%, N = 44); temperature, 25 °C; UV detection at 210 nm; electrokinetic injection, 0.5 s, 1 kV. EOF tracer, formamide, 1 μg/mL.

gradient elution can be performed with a packed capillary column efficiently and reproducibly by means of electrosmotic pumping.

(2) Separation of PTH-Amino Acids and Steroid Hormones. Reversed phase HPLC is the method of choice for the separation and identification of PTH-amino acids from the Edman degradation of a peptide with phenylisothiocyanate.[27] Twelve PTH-amino acids covering the whole polarity spectrum from very polar (PTH-asparagine) to nonpolar (PTH-leucine) were separated in our system by gradient elution, and the electrochromatogram is depicted in Figure 12. As already mentioned above, most likely the velocity of the cathodic EOF was not high enough for the negatively charged PTH-aspartate and PTH-glutamate to enter the

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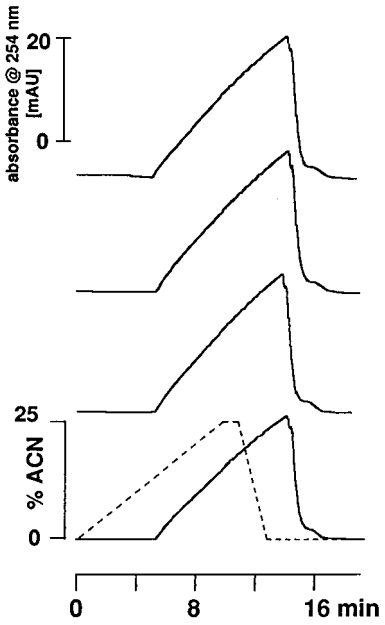

Figure 11. Reproducibility of gradient profile. Column, 50 μm × 14.4/11 cm packed with 5 μm Zorbax ODS particles having a mean pore size of 300 Å. Starting eluent (A), 10 mM phosphate, pH 7.0; gradient former (B), 10 mM phosphate, pH 7.0, 25% acetonitrile, 5% acetone; gradient, 0−100% B in 10 min, 100% B for 1 min, 100−0% B in 1 min at 0.1 mL/min; voltage 10 kV; current, 7 μA; temperature, 25 °C; UV detection, at 254 nm. The gradient profile at the column inlet is illustrated by the dotted line.

column with electrokinetic injection. In this study, the PTH-amino acids served only as model substances and no attempt was made to optimize the separation of all 20 PTH-amino acids of general interest. As seen in Figure 12, the 12 PTH-amino acids eluted in less than 15 min from a 50 μm i.d. capillary column packed with 3.5 μm Zorbax ODS particles having a mean pore size of 80 Å with a gradient from 30 to 60% acetonitrile in 5 mM phosphate buffer, pH 7.55. As an anisocratic elution technique, gradient elution facilitates faster separation than isocratic elution without compromising the resolution of the sample components. This can be seen by comparing the elution times of PTH-proline, isocratically in Figure 7 and by gradient elution in Figure 12. As seen in Figure 12, the EOF velocity changes during gradient run. This is expected in CEC due to the changes in the make-up of the eluent and also at the surface of the stationary phase. In our case, the EOF velocity shows only a modest fluctuation, which is believed to affect the separation to a much lesser degree than the rapidly changing organic strength of the mobile phase during the gradient run. A rigorous analysis of gradient elution with unsteady mobile phase flow velocity would require the solution of differential equations accounting for the temporal and spatial changes in the migration velocities of the sample components.

Another illustration for the use of capillary electrochromatography with gradient elution is seen in Figure 13. Five steroid hormones were separated by using a capillary column packed with 6 μm Zorbax ODS stationary phase having a pore size of 80 Å and with a gradient from 65 to 85% acetonitrile in 10 mM borate buffer, pH 8.0. The electrochromatogram in Figure 13 shows the changes in both the acetonitrile concentration and the EOF velocity in the course of the gradient run. As seen, the EOF velocity decreases almost 25% during the gradient run as a result of the increasing acetonitrile concentration in the eluent. This may be the reason for the relatively long retention and broadness

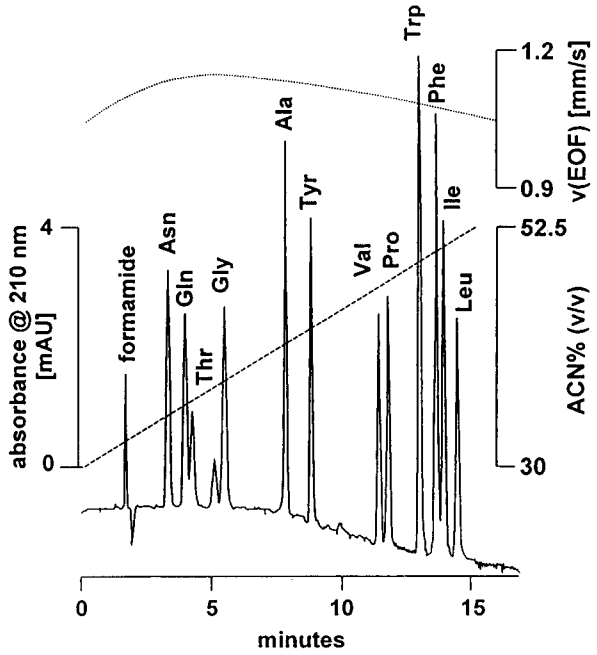

Figure 12. Capillary electrochromatography of PTH-amino acids with gradient elution. Column, 50 μm $\times$ 20.7/12.7 cm packed with 3.5 μm Zorbax ODS particles having a mean pore size of 80 Å. Starting eluent (A), 5 mM phosphate, pH 7.55, 30% acetonitrile; gradient former (B), 5 mM phosphate, pH 7.55, 60% acetonitrile; flow rate of mobile phase through inlet reservoir, 0.1 mL/min; gradient, 0−100% B in 20 min; voltage 10 kV; current, 1 μA; temperature, 25 °C; UV detection at 210 nm; electrokinetic injection, 0.5 s, 1 kV. Peaks in order of elution: formamide; PTH-asparagine; PTH-glutamine; PTH-threonine; PTH-glycine; PTH-alanine; PTH-tyrosine; PTH-valine; PTH-proline; PTH-tryptophan; PTH-phenylalanine; PTH-isoleucine; PTH-leucine. The concentration of the PTH-amino acids dissolved in the mobile phase was 30−60 μg/mL.

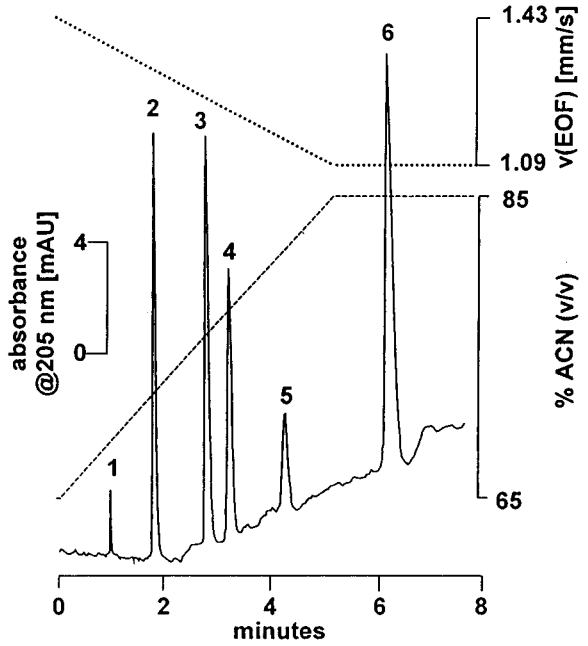

Figure 13. Capillary electrochromatography of steroid hormones with gradient elution. Column, 50 μm $\times$ 17.6/9.6 cm packed with 6 μm Zorbax ODS particles having a mean pore size of 80 Å. Starting eluent (A), 10 mM borate, pH 8.0, 65% acetonitrile; gradient former (B), 10 mM borate, pH 8.0, 85% acetonitrile; flow rate of the mobile phase through the inlet mobile phase reservoir, 0.1 mL/min; gradient, 0−100% B in 5 min, 100% B for 3 min; voltage 14 kV; current, 1 μA; EOF velocity, 1.43−1.09 mm/s; temperature, 25 °C; UV detection at 205 nm; electrokinetic injection, 0.5 s, 1 kV. Sample: 1, formamide; 2, corticosterone; 3, testosterone; 4, androsten-3,17-dione; 5, androstan-3,17-dione; 6, pregnan-3,20-dione. The concentration of the steroid hormones dissolved in the mobile phase was ~1 mg/mL.

of the last peak, pregnan-3,20-dione. The results suggest that due to the changing flow velocity the conditions in gradient CEC ought to be different from those that give optimal results in μ-HPLC.

CONCLUSIONS

The present work demostrates that by changing the mobile phase composition at the inlet reservoir of the CEC system, gradient elution can be performed efficiently and reproducibly with packed capillary columns by employing electrosmotic flow without the use of high-pressure pumps. The applicability of our system to the electrochromatography of complex mixtures of biomolecules is illustrated by the separation of mixtures of PTH-amino acid and human steroid hormone. The implications of this approach are likely to go further than gradient CEC since the instrument described here comes close to being able to function as a CEC and CZE unit operated at high voltage and also as a μ-HPLC unit with high pressure pumping capability. Since all three liquid phase analytical microseparation techniques employ columns of commensurate dimensions and liquids of similar properties, there is a high level of compatibility among them that facilitates the design of an analytical instrument that incorporates all three functions. The greek name *triskelion* would be appropriate for such an analytical device that can provide a reading on the sample composition by three different methods in a three-dimensional fashion. Besides the need for convenient, efficient, and fast multidimensional analysis, the compatibility of each method with mass spectrometry, which has become a *conditio sine qua non* in the design of instruments and methods for analytical separations, may accelerate further developments in this direction.

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