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# Separation of Peptides by Pressurized Capillary Electrochromatography

Kai ZHANG[1], Chao YAN[2], Zhi Chao ZHANG[1], Qin Sun WANG[1], Ru Yu GAO[1]*

[1] State key Laboratory of Elemento-Organic Chemistry, Nankai University, Tianjin 300071
[2]Unimicro Technologies, Inc., 4713 First Street, Pleasanton, CA 94566, USA

**Abstract:** A novel gradient pressurized capillary electrochromatography (pCEC) instrument was developed to separate peptides. Two gradient elution modes, hydrophobic and hydrophilic interaction mode in pCEC, were performed on this instrument. Baseline separation of six peptides was obtained on two gradient modes with C18 column and strong cationic exchange column respectively. The effects of mixer volume and total flow rate of pumps on resolution were also discussed.

**Keywords:** Pressurized capillary electrochromatography, peptide, separation, reversed phase, hydrophilic interaction.

Capillary electrochromatography (CEC), which combines the high selectivity of high performance liquid chromatography (HPLC) with high efficiency of capillary electrophoresis (CE), has received considerable interest and developed into a powerful separation technique. To further explore the potential of CEC, it is very important to develop the capability of gradient elution for successfully separating a wide variety of complex samples such as peptides and proteins. While it is very difficult that gradient elution is performed on commercially available CE equipment or "home-built" system[1-2]. In our laboratory, a novel special CEC instrument, in which a continuous gradient elution can be set up easily, has been developed. In this system, the mobile phases are driven by electroosmotic flow (EOF) and pressurized flow, since the retention factor, in theory, can be tuned well by adjusting pressure and electrical field. Some practical problems in CEC such as bubble formation and column dry-out can be overcome and CEC automatic operation is easier. Separation of peptides in CEC has received considerable attention in recent years [3-4]. In this work, six peptides were separated by gradient pCEC using C18 capillary column and strong cationic exchange (SCX) capillary column on this CEC instrument. The separation results were satisfactory.

## Experimental

All separations were carried out with a Trisep$^{TM}$ 2000GV CEC system which comprised

*E-mail: rygao@sina.com

a solvent gradient delivery module (two PU-1580 intelligent HPLC pumps purchased from JASCO, Japan), a high voltage power supply, a variable wavelength UV/Vis detector, a micro fluid manipulation module (including a 20 nL four ports injector) and a data acquisition module. A SCX capillary columns (250 mm × 100 μm i.d.) packed with 5 μm particles and ODS capillary columns (250 mm × 100 μm i.d.) packed with 3 μm particles were supplied from Unimicro Technologies. Inc. (Pleasanon, CA, USA). A negative voltage was added on the column outlet and the column inlet was grounded. Pressure was applied to the column inlet during the separation. Total flow rate of the two pumps was 30 μL / min. The wavelength of the UV/Vis detector was set at 214 nm. The injector has an internal loop of 20 nL.

Samples: peptides were obtained from Sigma (USA). Peptides were first dissolved in water to obtain a solution containing 1 mg / mL each of drugs, then were further diluted with mobile phase to give the drug an approximate concentration of 0.1 mg / mL. All these solution were filtered with 0.22 μm micro filter. All chemicals were of analytical-reagent grade.

Mobile phase solutions were first prepared adjusting the potassium dihydrogen phosphate buffer to the desired pH value or adding appropriate volume of trifluoroacetic acid (TFA) and then mixing with the appropriate amount acetonitrile. Mobile phase solution was degassed in an ultrasonic bath for 10 min before using.

**Results and Discussion**

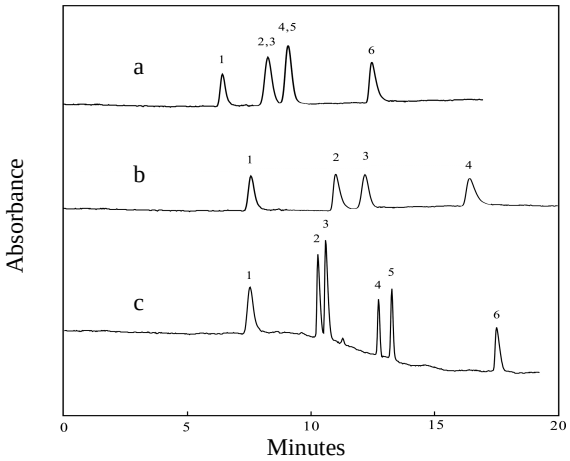
It is illustrated that the retention time of peptides decreased with increase of acetonitrile concentration in reversed phase pCEC (RP-pCEC) as shown in **Figure 1**. It was observed that peptide retention to the changes in acetonitrile concentration was so sensitive that the separation of similar peptides was poor on isocratic mode, while good resolution can be obtained for six peptides in gradient RP- pCEC.

Separation of peptides on SCX column was also investigated. It was observed that the retention of peptides on this SCX column increased with increase of acetonitrile concentration as shown in **Figure 2**. It is demonstrated the separations of peptides are based on mixed mode of hydrophilic interaction, ionic exchange[5] and electromigration. In opposition to gradient RP-pCEC, The gradient elution on SCX-pCEC was performed by decreasing acetonitrile concentration in mobile phase. **Figure 2** demonstrates the electrochromatograms of separation of peptides by isocratic and gradient elution on SCX column. It is obvious that gradient SCX-pCEC is more effective and useful.

From **Figure 1** and **Figure 2**, the plate number in isocratic pCEC is not very high, for example, the plate number of **2** (Gly-Arg-Gly-Asp) is 12700 N/m in RP-pCEC; and that of **4** (Gly-Cys) is 8500 N/m in SCX-pCEC. But, gradient elution capability improves the separation efficiency and increases the separation range tremendously.

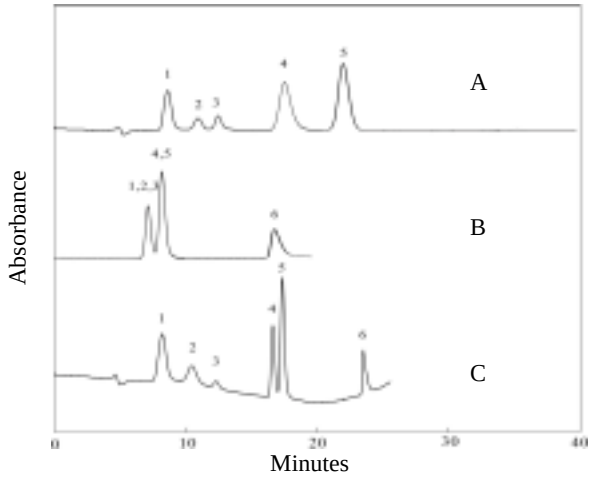
In this separation system, a gradient elution is generated with two micro-HPLC pumps. The mobile phase is mixed in a micro-mixer. After split, a fraction of gradient mobile phase enters the capillary column under controlling of pressure. In the experiment, it was observed that the effects of total flow rate of pumps and mixer volume on resolution of peptides were obvious, as shown in **Table 1**.

**Figure 1** Separation of six peptides on isocratic and gradient pCEC on C18 column




Experiment conditions: column: C18, 3 μm, 250 mm × 100μm I. D.; mobile phase: a: 0.1% TFA in 12 % $CH_3CN$; b: 0.1 % TFA in 3 % acetonitrile; c: (A) 0.1% TFA, (B) 0.1 % TFA in 25% acetonitrile; liner gradient: 10-20 % B in 3 min, 20-60 % B in 4 min; pressure added on-column: 1500 psi; voltage: 2 kV; room temperature. Peaks: 1 Gly-Gly-Gly; 2 Gly-Arg-Gly-Asp; 3 Arg-Gly-Asp; 4 Gly-Arg-Gly-Asp -Ser-Pro-Lys; 5 Gly-Arg-Gly-Asp -Ser-Pro; 6 Met-Met.

**Figure 2** Separation of six peptides on isocratic and gradient pCEC on SCX column




Experiment conditions: Column: SCX, 5 μm, 250 mm × 100 μm I. D; mobile phase: A: 75% (v/v) acetonitrile in 15 mmol/L $KH_2PO_4$ buffer (pH 2.5); B 50% (v/v) acetonitrile in 15 mmol/L $KH_2PO_4$ ( pH 2.5); C: gradient: 80% (v/v) acetonitrile in 4 min, 80% (v/v) acetonitrile to 40% (v/v) acetonitrile in 6 min, buffer: 15 mmol/L $KH_2PO_4$ ( pH 2.5); flow rate: 0.03 mL / min; voltage: 10 kV; pressure: 1000 psi. Peaks: 1 Met-Met; 2 Gly-Leu; 3 Leu-Gly-Gly; 4 Gly-Cys; 5 Gly-Gly-Gly. 6 Gly-Arg-Gly-Asp-Ser-Pro-Lys.

**Table 1** Effect of volume of mixer and total flow rate of pumps on resolution in gradient pCEC

| Volume of mixer ( μL ) | Total flow rate of pumps (μL / min) | Resolution $_{4,5}$ |
|---|---|---|
| 2.2 | 30 | 1.40 |
| 2.2 | 100 | 1.12 |
| 20 | 30 | 2.78 |
| 20 | 100 | 2.42 |
| 50 | 30 | 3.03 |
| 50 | 50 | 2.84 |
| 50 | 100 | 2.75 |

The experiment conditions were the same as **Figure 1**C

## Conclusion

This study demonstrates that six peptides can be separated successfully by gradient RP-pCEC and gradient SCX-pCEC on our special CEC instrument respectively. Gradient pCEC is powerful and useful for complicated sample separation, which is difficult in isocratic elution mode. Optimizations of instrument's fittings and operation condition are also very important for micro-instrument.

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