Study on Open Tubular Capillary Affinity Liquid Chromatography

Mingliang Ye, Hanfa Zou*, Zhen Liu, Zhengdeng Lei, and **Jianyi Ni**
National Chromatographic R. & A. Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116011, China

Abstract

A novel mode of affinity chromatography (AC) based on an open tubular capillary column (OTAC) is demonstrated. The OTAC column is prepared by immobilizing Cibacron blue F3GA onto the inner surface of a 50-µm-i.d. capillary column. The AC experiment is performed on a capillary electrophoresis instrument by using its pressure system as the driving force. Bovine serum albumin and lysozyme (Lys) are successfully separated with stepwise gradient elution. The relative standard deviation (RSD) for the elution time of the retained Lys is 0.08%, and good repeatability of its peak area and peak height with an RSD value lower than 2.12% for 10 consecutive runs is observed. The loading capacity and detection limit for the retained Lys are approximately 36 ng and 8.6 ng, respectively. It is also found that the amount of protein adsorbed is unaffected by the flow rate of the loading buffer, and OTAC can be used for the fast determination of biopolymers. Some of the advantages of OTAC over conventional modes of open tubular capillary liquid chromatography are that the detection sensitivity and loading capacity of a sample can be greatly improved, because the relatively large inner diameter of the capillary can be adopted and the whole capillary column can be used to adsorb the solute in OTAC.

Introduction

Open tubular columns (OTCs) were first proposed for gas chromatography (GC) by Golay (1) in 1959. Since then, OTCs have quickly replaced packed columns (PCs) in GC for most analytical applications because of the high efficiency that can be obtained with OTCs for similar analysis times and inlet pressures. The advantages of OTCs over PCs in high-performance liquid chromatography (HPLC) have also been demonstrated theoretically and experimentally (2–7). However, the development of open tubular capillary liquid chromatography (OTLC) was not as rapid compared with that of GC because of the difficulties in the construction, detection, and injection associated with OTLC columns. From chromatographic theory, the inside diameter of an OTLC column must be the same as the diameter of conventional PCs having an optimal inner diameter of approximately 2 µm for column efficiency (8,9). Therefore, it is very difficult to prepare capillary columns of such a small diameter with the appropriate coating materials that have the sufficient retentive and mass loadability characteristics needed. Furthermore, more sensitive detectors such as on-column laser-based optical detectors are needed because of the small diameter. Although OTLC experiments were first presented as early as 1979 by Knox et al. (2), the insufficient detection sensitivity and low mass loadability of OTCs still inhibit the development of OTLC.

The separation mechanism of affinity chromatography (AC) is based on the biospecific interactions between the immobilized ligands and the solutes, which is different from that of other liquid chromatographic (LC) modes. Stepwise gradient elution is typically used in AC. After the injection of a sample, the solutes that have no affinity interaction with the ligand on the affinity column are first flushed out with an eluent having low elution strength, then the solutes that have affinity interaction are able to be adsorbed on the ligands. Afterwards, the eluent is changed to a buffer having strong elution strength, and the adsorbed solutes are washed off. Plate theory is not valid in AC because of the use of stepwise gradient elution. We have recently found that an OTC with an inner diameter of 50 µm can be used in AC (10). An open tubular capillary affinity chromatographic (OTAC) column is prepared by coating a layer of triazine dye on the inner surface of the capillary wall. Bovine serum albumin (BSA) and lysozyme (Lys) have been successfully separated in this system. The latest progress of this novel mode of AC is reported in this study.

Experimental

Material

A fused-silica capillary (50-µm i.d., 365-µm o.d.) was obtained from Yongnian Optic Fiber Plant (Hebei, China). BSA, Lys, and Cibacron blue F3GA were purchased from Sino-American Biotechnology Co. (Beijing, China). γ-Glycidoxypropyltrimethoxysilane was purchased from Sigma Chemical Co. (St. Louis, MO).

*Author to whom correspondence should be addressed: e-mail zouhfa@mail.dlptt.ln.cn.

Instruments

The OTAC experiments were all performed on the capillary electrophoresis (CE) instrument P/ACE system MDQ (Beckman, Fullerton, CA). This instrument can apply gas pressure as high as 100 psi to one or both ends of the capillary. The mobile phase was driven with the pressure system at a pressure of 0.5 to 30 psi. Data collection and instrument controlling were performed with P/ACE System MDQ version 1.5 software. The equipped detector was a diode array detector.

Column preparation

The OTAC columns were prepared by the same procedures as the preparation of triazine dye substituted silica in HPLC (11,12) and triazine dye coated on the capillary in CE (l3), which are shown schematically in Figure 1. The 50-µm-i.d. capillary was first flushed with 1.0M KOH, H_2O, and 0.1M HCl for 3 h, 10 min, and 30 min, respectively. Then, the column was dried by flushing with N_2 and further rinsed with 5% (v/v) γ-glycidoxypropyltrimethoxysilane in a 0.1M sodium acetate buffer (pH 5.5) for 3 h at 90°C. Afterwards, the epoxide group was opened by flushing with an HCl solution (pH 3.0) for 1 h at 90°C. After rinsing with water for 5 min, the capillary column was flushed with 2mM Cibacron blue F3GA in a 0.1M Na_2CO_3 solution for 3 h at room temperature.

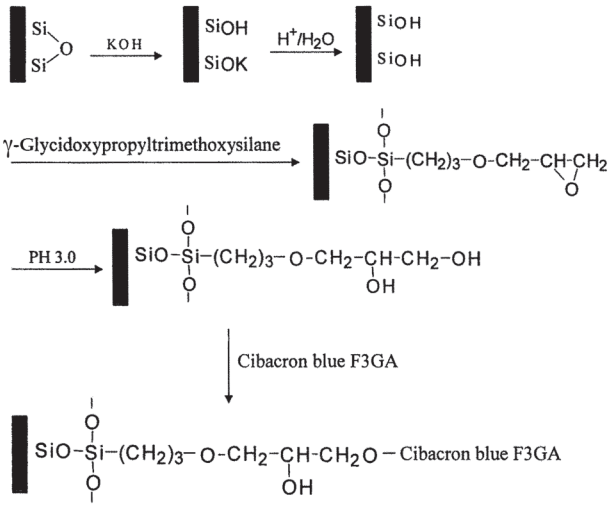

Figure 1. Scheme of the procedures for the preparation of the OTAC column.

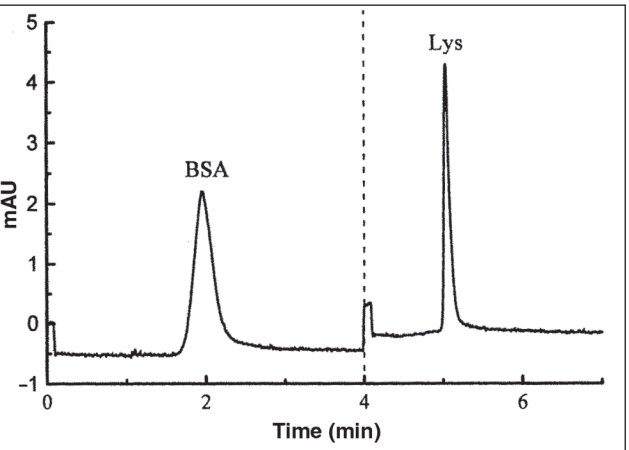

Figure 2. Chromatogram for the separation of BSA and Lys by OTAC.

Separation conditions

The loading buffer was a 10mM tris-HCl solution (pH 7.0), and the elution buffer was a 10mM tris-HCl solution (pH 7.0) containing 1.0M NaCl. The protein solutions were prepared with the loading buffer. Before injection, the column was first rinsed with the elution buffer for 5 min and then with the loading buffer for 2 min (both at a pressure of 10 psi). The samples were injected by applying a pressure of 0.5 or 1 psi. After injection, the column was first rinsed with the loading buffer and then with the elution buffer. The total length of the capillary was 60.5 cm, and the length from the injection side to the detection window was 50 cm. The detection wavelength was 208 nm. The temperature was set at 25°C.

Results and Discussion

A stepwise gradient elution technique was used in this study. The sample solution was injected by applying a pressure of 0.5 psi for 10 s. The concentrations of BSA and Lys in the sample solution were both 500 µg/mL. The column was first flushed with the loading solution by a pressure of 5 psi. BSA eluted out at approximately 2 min because it did not have specific interaction with the triazine dye immobilized on the capillary column. Four minutes later, the mobile phase was changed to the elution buffer (the applied pressure was still set at 5 psi). The adsorbed Lys was eluted at 5 min. The chromatogram obtained is shown in Figure 2. Because the inner surface of the fused-silica capillary was smooth, the phase ratio of the silanized column was approximately 350 times smaller than that of commercial HPLC packings (6). In such conditions, the capacity factors of most solutes separated by conventional OTLC are so small that they are difficult to use for analytical purposes. Usually, the surface area of a capillary column has to be increased by various methods such as laying down a porous silica layer (5,6) in order to increase the retention and sample loadability. However, in OTAC, all of the inner surface of the capillary from the injection end to the detection window can be used to adsorb the solutes, and the retained solutes can be washed off in a sharp zone by a strong mobile phase. The concentration of the solutes in the zone may then be high enough to be detected. Although no effort was made to increase the inner surface area of the capillary in this study, the peak of Lys (which is adsorbed by the immobilized triazine dye) could be easily detected as shown in Figure 2. Therefore, the restriction of the phase ratio on OTAC was moderate. In order to obtain higher efficiency in conventional OTLC than in PC LC, the inner diameter of the capillary must be less than 5 µm according to chromatographic theory (2,5). However, plate theory is not valid for AC whenever step gradient elution is used. The inner diameter of the capillary with a 50-µm i.d. was adopted, and the peak of the retained Lys was relatively sharp (as shown in Figure 2). The peak width at the baseline was approximately 0.24 min. Therefore, the restriction of insufficient detection sensitivity on OTAC could also be improved, because a relatively large internal diameter of capillary can be used.

OTAC was performed with the CE instrument, and the mobile phase was driven by the gas pressure system. Therefore, the stability of the pressure was a key important factor. The repeatability of this system was investigated. The column was first rinsed with the elution buffer and loading buffer before injection as described in the Experimental section. The Lys sample solution with a concentration of 100 µg/mL was injected by applying a pressure of 1 psi for 3 min. Then, the column was flushed with the loading buffer with a pressure of 5 psi for 4 min and then flushed with the elution buffer at the same pressure. The relative standard deviation (RSD) for the elution time of Lys was 0.08% for 10 consecutive runs. This meant that the equipped pressure system was very stable. The RSDs of the peak area and the peak height were 2.09% and 2.12%, respectively, which were relatively poor compared with that for the elution time. The reason for this was that the UV absorption of the loading buffer and the elution buffer were different from each other, and when the frontal of the elution buffer reached the detection window, a disturbance of the baseline occurred. The Lys was flushed out by the elution buffer, and the peak appeared exactly at the disturbance zone of the baseline. Therefore, the measurement of the peak area and height were not very accurate. In order to minimize the influence of the baseline disturbance, the detection wavelength was set to 208 nm (at which the difference of the UV absorption of the two buffers was minimum).

The linear relationship of the peak area and peak height versus the amount of protein injected was investigated. It is instructed that a period of time is needed for the pressure to reach the applied value from zero. In order to decrease the influence of this period of time on the amount of protein injected, samples were injected at low pressure for a relatively long time. The Lys solution was prepared with the loading buffer with a concentration of 100 µg/mL. The sample was injected at 0.5 psi for 1.5 to 8 min. The amount of Lys injected can be calculated by multiplying the section area of the capillary, the concentration of the Lys, the injection time, and the velocity of the sample solution. It was assumed that the influence of the protein on the viscosity of the sample solution was neglected because the protein concentration was relatively low. Therefore, the velocity of the sample solution was equal to that of the loading buffer. The linear velocity of the loading buffer at the pressure of 0.5 psi was measured using BSA as a t_0 marker. The velocity measured was 2.64 cm/min. The experimental conditions were the same as that of the investigation of repeatability except for that of the sample solution injected. The chromatograms for the injection of the sample solution for 8 min and 7 min are shown in Figure 3. It is obvious that the column was overloaded when the sample solution was injected for 8 min (as can be seen from Figure 3A). Therefore, quantitative analysis can only be made to data collected by an injection time of less than 8 min. The good linear relationships of the amount of Lys injected (W) versus its peak area (A) and peak height (H) were observed with correlation coefficients of 0.9921 and 0.9997, respectively. Their calibration equation can be expressed as follows:

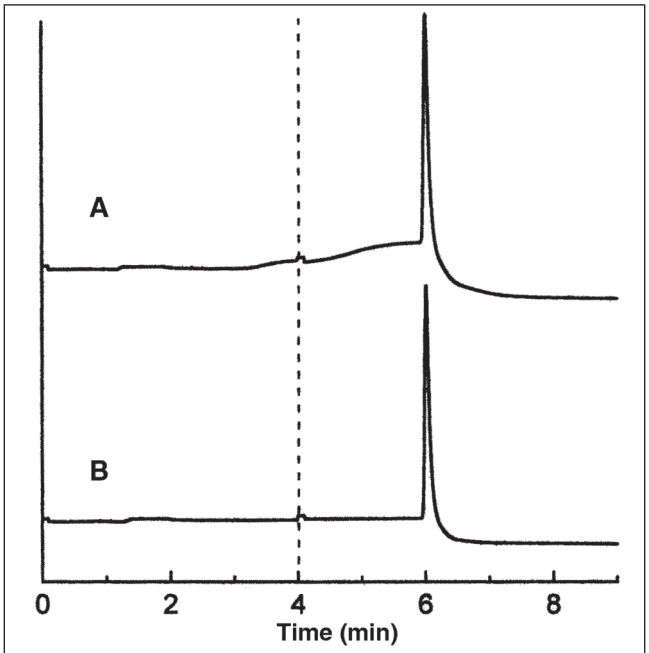

Figure 3. Chromatograms for the different amounts of Lys injected: 8 min (A) and 7 min (B).

$$A = 5.58W + 0.22,\ r = 0.9921 \qquad \text{Eq. 1}$$

$$H = 1.09W - 1.90,\ r = 0.9997 \qquad \text{Eq. 2}$$

Both of these equations do not go through the origin because of the baseline disturbance from switching the mobile phases. The linearity of relationship of W and H was better than that of W and A. Therefore, the calibration curve of W versus H was more preferable to use for quantitative analysis. The amount of Lys injected for 7 min was calculated to be 36.3 ng. In other words, the calibration curve for the injection of less than 36.3 ng Lys was linear. There are two ways to expand this linear range. Because the pressure drop was very small in OTAC, the length of the capillary column can be increased. The total inner surface area of the capillary will increase with an increase of the length of the capillary; therefore, the loadability of the adsorbed protein can be increased. The second way is to increase the specific surface area of the capillary wall. The phase ratio increases with an increase of the specific area, and the adsorption capacity also increases. The detection limit can be calculated from equation 2 in which the peak height equals three times the noise of the baseline in conventional chromatography. However, it is relatively difficult to accurately measure the detection limit because of the baseline disturbance from the switching of the eluents. In our experiment, 8.6 ng Lys was injected and well-detected. The separation was performed with an electrophoresis instrument, thus an electric field could be applied on the column and the peak of the retained solutes in AC could be further separated by the CE mechanism and shift away from the zone of the disturbance, thus making it possible to further improve the detection limit.

One of the advantages of using an OTC in LC is that the high flow rate can be applied because of the low pressure drop. Therefore, the analysis time can be very short. The analysis presented in this study can be separated into two stages after the samples are injected. The first stage is the stage of the loading sample when the column is flushed with the loading buffer, and the second stage is the elution stage when the column is flushed with an elution buffer. The flow rate (which is a result of the applied pressure) of these two stages can be different from each

other because the experiments were performed on an electrophoresis instrument. The influence of the applied pressure at the loading stage on the amount of Lys adsorbed was investigated. The influence of the pressure on the peak height is shown in Figure 4. It can be seen from Figure 4 that the influence was moderate with the range of pressure investigated. The column was flushed with the loading buffer for 4 min with a pressure from 5 to 30 psi, the RSD of the peak heights for Lys was 2.8%. This means that the influence of the loading buffer's flow rate on the amount of Lys adsorbed was not serious. When the pressure of the elution stage increased to 20 psi, the good linear relationship of the amount of Lys injected versus the peak height was also observed with a correlation coefficient of 0.9956. This means that the flow rate of the elution stage could also be increased. Fast separation of BSA and Lys was performed by applying a pressure of 20 psi at both the loading and elution stages (the obtained chromatogram is shown in Figure 5). The two proteins were successfully separated in less than 1.8 min. Therefore, OTAC can be used for the fast determination of biopolymer in biological samples.

Conclusion

A novel mode of AC was developed by coating a layer of ligand on the inner surface of a capillary column. The rewards of OTAC included high efficiency, low sample consumption, low eluent consumption, and high mass sensitivity because the diameter of the capillary column was very small. The flow rate can be very great because of the fast mass transfer in this system. The general ligand was immobilized in this study, and the specific ligand can also be immobilized in the future. A rapid separation of biopolymers by OTAC has been demonstrated. OTAC may also be a good choice for purification and the analysis of biopolymers in nanoliter and microliter biological samples.

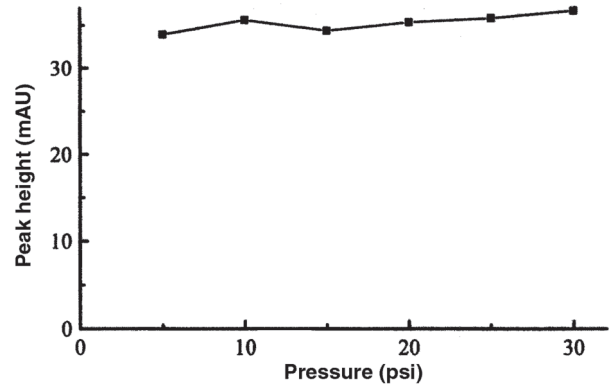

Figure 4. The influence of the pressure of the loading stage on the peak height of the retained Lys.

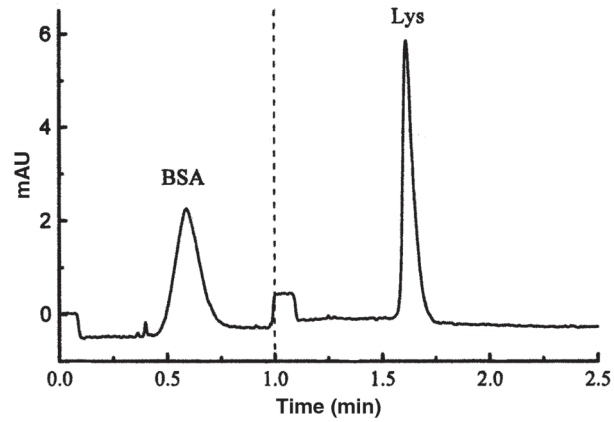

Figure 5. Chromatogram for the fast separation of BSA and Lys by OTAC.

Acknowledgments

The financial support from the Natural Science Foundation of China (No. 29635010) is gratefully thanked. Dr. Hanfa Zou is the recipient of the Excellent Young Scientist Award from the National Natural Science Foundation of China (No. 29725512).

References

1. M.J.E. Golay. *Gas Chromatography*. V.J. Coates, Ed. Academic Press, New York, 1958, pp 1–13.
2. J.H. Knox and M. Gilbert. Kinetic optimization of straight open-tubular liquid chromatography. *J. Chromatogr.* **186:** 405–418 (1979).
3. J.H. Knox. Theoretical aspects of LC with packed and open small-bore columns. *J. Chromatogr. Sci.* **18:** 453–61 (1980).
4. G. Guiochon. Conventional packed columns vs. packed or open tubular micro columns in liquid chromatography. *Anal. Chem.* **53:** 1318–25 (1981).
5. P.P.H. Tock, G. Stegeman, R. Peerboom, H. Poppe, J.C. Kraak, and K.K. Unger. The application of porous silica layers in open tubular columns for liquid chromatography. *Chromatographia* **24:** 617–24 (1987).
6. A.L.Crego, J.C. Díez-Masa, and M.V. Dabrio. Preparation of open tubular columns for reversed-phase high-performance liquid chromatography. *Anal. Chem.* **65:** 1615–21(1993).
7. Y. Guo and L.A. Colón. A stationary phase for open tubular liquid chromatography and electrochromatography using sol-gel technology. *Anal. Chem.* **67:** 2511–16 (1995).
8. J.W. Jorgenson and E.J. Guthrie. Liquid chromatography in open-tubular columns. Theory of column optimization with limited pressure and analysis time, and the fabrication of chemically boned reversed-phase columns on etched borosilicate glass capillary. *J. Chromatogr.* **255:** 335–48 (1983).
9. M. Novotony. Recent advances in micro column liquid chromatography. *Anal. Chem.* **60:** 500A–509A (1988).
10. M. Ye, H. Zou, Z. Liu, Z. Lei, and J. Ni. The preliminary study on the open tubular capillary affinity liquid chromatography. Submitted for publication in *Chinese J. Chromatogr.*
11. Y.D. Clonis, K. Jones, and C.R. Lowe. Process scale high-performance liquid affinity chromatography. *J. Chromatogr.* **363:** 31–36 (1986).
12. C.R. Lowe, M. Glad, P. Larsson, S. Ohlson, D.A.P. Small, T. Atkinson, and K. Mosbach. High-performance liquid affinity chromatography of proteins on Cibacron blue F3 GA bonded silica. *J. Chromatogr.* **215:** 303–316 (1981).
13. B. Lin, X. Zhe, and Y. Yang. Chinese Patent CN1085318A, 1992.

Manuscript accepted July 28, 2000.