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Narrow open tubular column for high efficiency liquid chromatographic separation

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We report a great feature of open tubular liquid chromatography when it runs using an extremely narrow (e.g., 2-μm inner diameter) open tubular column: more than 10 million plates per meter can be achieved in less than 10 min and under an elution pressure of ca. 20 bar. The column is coated with octadecylsilane and both isocratic and gradient separations are performed. We reveal a focusing effect that may be used to interpret the efficiency enhancement. We also demonstrate the feasibility of using this technique for separating complex peptide samples. This high-resolution and fast separation technique is promising and can lead to a powerful tool for trace sample analysis.

Advanced analytical tools can lead to new ways to conduct biotech research. Open tubular liquid chromatography (OTLC) could be such an analytical tool since theoretical studies[1-4] have predicted that open tubular (OT) columns offer the best means of achieving high separation efficiencies for liquid chromatography (LC). It has been predicted that the optimal inner diameter (i.d.) of the OT column is in the range of 1 to 2 μm. However, OTLC has rarely been exploited using columns in this i.d. regime due to the challenges (such as picoliter-volume detection, nano-capillary column preparation, low sample loading capacity, etc.) of utilizing such narrow capillaries. Surface-roughened[5] and/or porous layer OT columns[1, 3, 6, 7] have been used to improve the loading capacity, and high peak capacity results have been obtained.[8, 9] Here we show that we can achieve extraordinarily high efficiencies simply by reducing OT column diameter; plate heights of less than 0.1 μm have been obtained in less than 10 min and under an elution pressure of ~300 pounds per square inch (psi) or ca. 20 bar.

We exhibit here the extraordinarily high efficiencies we can obtain when we perform OTLC using a 2-μm-i.d. OT column. Figure 1 presents the experimental apparatus we used to carry out this experiment. A 6-port valve with an external loop volume of 2 μL was used for sample injection. A laser-induced fluorescence (LIF) detector, as previously reported[10], was employed to monitor the separation process. An Agilent 1200 HPLC pump coupled with a flow splitter served as a gradient pump. The column was coated with trimethoxy(octadecyl)silane.

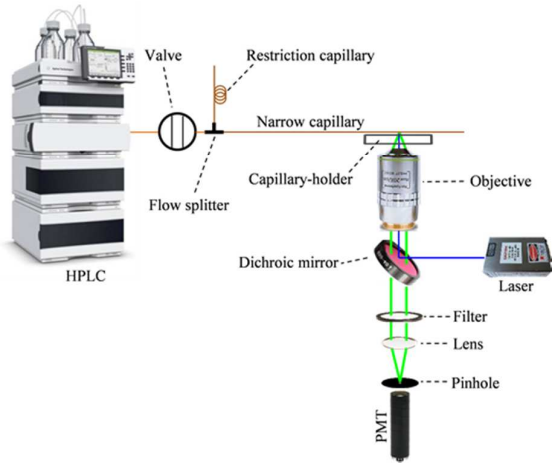

Fig. 1 Schematic diagram of the experimental apparatus. The narrow capillary was a 2 μm i.d. and 150 μm o.d. column. A 10-cm-long 20-μm-i.d capillary was used as the restriction capillary. The capillary connecting the tee and injection valve was a 180 μm i.d. and 360 μm o.d. capillary. Through the tee, a small portion of the 2 μm i.d. and 150 μm o.d. was inserted into the 180 μm i.d. and 360 μm o.d. capillary to avoid dead volume between these two capillaries.

Figure 2A presents four isocratic separation chromatograms for eleven fluorescently labelled amino acids under different elution pressures. All eleven peaks (except for peaks 4 and 5 in the top chromatogram) were nicely resolved with resolutions ($=\Delta t/(w_{½}+w'_{½}$, where $w_{½}$ and $w'_{½}$ are the widths of two peaks and Δt is the distance between the two peaks) greater than

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0.6. The separation was completed in less than 10 min under an elution pressure of 300 psi, while the separation was

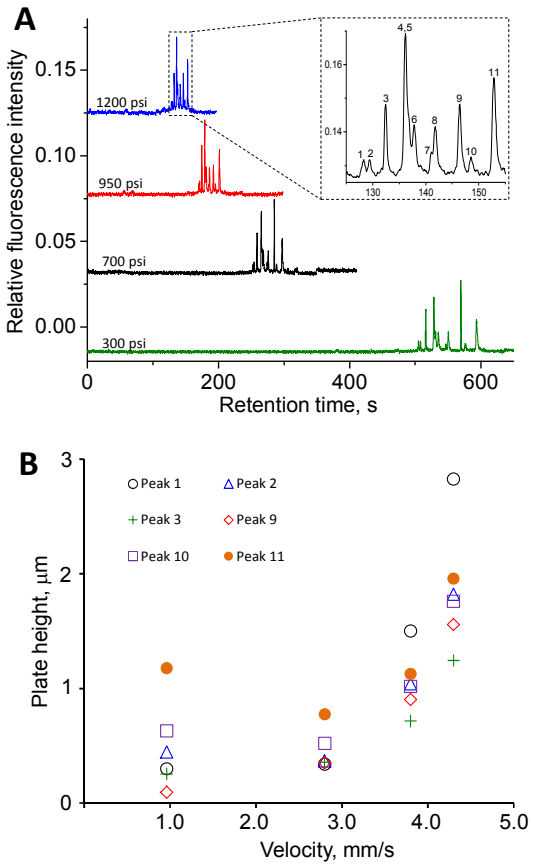

Fig. 2 Effect of pressure on separation. (A) chromatograms under different elution pressures. The separation column had a total length of 48 cm (44 cm effective), an o.d. of 150 µm and an i.d. of 2 µm. 10 mM NH_4HCO_3 in DDI water was used as mobile phase A, and acetonitrile was used as mobile phase B. Isocratic elution was performed using 20% mobile phase B and 80% mobile phase A. The external loop of the injection valve had a volume of 2 µL. The splitter had an estimated splitting ratio of $1{:}2.8\times10^4$, corresponding to an actual injection volume of 7.1 pL. The sample was a mixture of histidine (1), asparagine (2), glycine (3), tyrosine (4), arginine (5), alanine (6), tryptophan (7), valine (8), isoleucine (9), phenylalanine (10) and leucine (11) dissolved in 10 mM NH_4HCO_3; each at 6.5 µM. The inset expanded the peak portion of the top chromatogram to show clearly how the peaks were separated. (B) plate height plotted against mobile phase velocity. The velocity measurement data are presented in Figure S1.

completed in less than 3 min under an elution pressure of 1200 psi. Many peaks had widths (peak-width at half-maximum) of ~0.5 s or less. Figure 2B presents plate height as a function of mobile phase velocity. The data points were obtained from the column length (45 cm) divided by the number of theoretical plates of each peak. The number of theoretical plates (N) of a peak equals $5.54\ (t_R/W_{1/2})^2$, where t_R and $w_{½}$ are the retention time and width of the peak, and plate number for all peaks are presented in Table S1. Many data points had plate heights of less than 1 µm. Peak 9 at u = 0.93 mm/s had a plate height of 0.094 µm - corresponding to 1.1×10^7 plates/m and 2.0×10^4 plates/s. It is worth pointing out that all these chromatograms were obtained under isocratic elution; this is required for plate height calculations[11]. Narrower peaks and increased resolutions can be obtained if gradient elution was utilized (e.g., many peaks in Figure 4A had FWHM of ~0.3 s.) It is also important to notice that the entire separation can be complete in less than 10 min.

Ultrahigh efficiencies[12] have been obtained in ultrahigh performance liquid chromatography (UPLC) and a slip flow model[13] has been utilized to interpret these high efficiencies. Slip flow may have contributed to the efficiency enhancement in our experiment, but it alone cannot account for the entire enhancement because some peaks in Figure 2 have widths narrower than diffusion-caused bandwidths (in which a plug flow was assumed).

We propose a "focusing effect" that may facilitate us to interpret our high efficiency results. To unveil this effect, we deliver an analyte band to the OT column at two different distances to the detector. We allow the analyte band to diffuse for a pre-set period of time so that we can obtain a band with a desired width. We then elute the broadened band across the detector for chromatogram measurement.

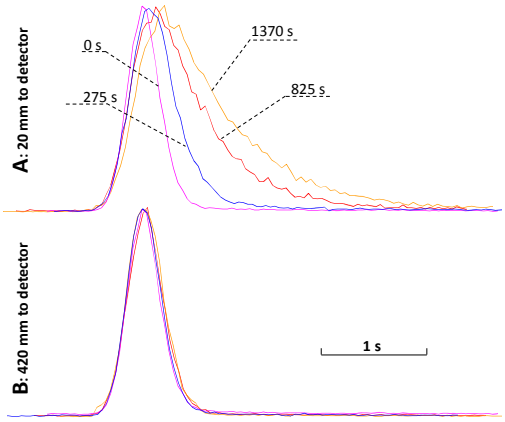

Fig. 3 Evidence of focusing effect. (A) A fluorescein band was injected as in Fig. 2 and eluted to a position at ca. 20 mm away from the LIF detector. After the band was parked there for 0, 275, 825 or 1370 s, it was eluted out to detector for chromatogram measurement. After all the chromatograms were normalized and aligned, they were displayed in (A). (B) A fluorescein band was injected as in Fig. 2 and eluted to a position at ca. 420 mm away from the LIF detector. After the band was parked there for 0, 275, 825 or 1370 s, it was eluted out to detector for chromatogram measurement. All the chromatograms were aligned in (B). The scale bar applies to all chromatograms.

Figure 3 presents the results. We see clearly in Figure 3A that the band got broadened by diffusion; a longer parking time induced a broader peak. All peaks in Figure 3B exhibited the same width; this indicates that analyte bands that have been diffused to various widths are focused to a fixed/equilibrated width. It should be pointed out that the peaks shown in Figure 3A represent only partially focused bands, because these bands were parked at ca. 20 mm away from the detector and as they were eluted to the detector they did not have adequate time to be focused to the equilibrated bands. We suspect that the focusing was effected by a gradient formed between sample matrix and eluent;

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Figure S2 presents some results supporting the above hypothesis.

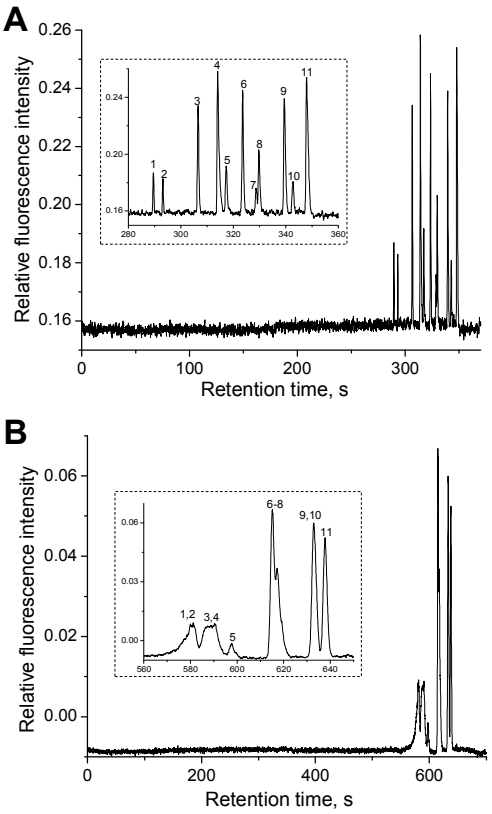

Fig. 4 Effect of inner diameter on resolution. Both the 5-um-i.d. and 2-um-i.d. columns had a total length of 48 cm (44 cm effective), and 150-μm o.d. Gradient profile was mobile phase B increased from 0% to 50% from 0 to 1.5 min, stayed at 50% B from 1.5 min to 2 min, and then decreased from 50% to 0% from 2 min to 2.5 min. For separation using the 2-um-i.d. column, the elution pressure was 600 psi and concentration of each amino acid was 6.5 μM. For separation using the 5-um-i.d. column, the elution pressure was 100 psi and concentration of each amino acid was 0.3 μM. All other conditions were the same as in Figure 2.

Reducing the column diameter is essential to increasing the separation efficiencies. Figure 4 presents a performance comparison between a 2-μm-i.d. and a 5-μm-i.d. column. Using a 2-μm-i.d. column, all peaks were baseline resolved, except that peaks 7 and 8 were partially resolved. Using a 5-μm-i.d. column, most peaks (peaks 1 and 2, peaks 3 and 4, peaks 6, 7 and 8, and peaks 9 and 10) were unresolved. Figure S3 presents a performance comparison between a 2-μm-i.d. and a 5-μm-i.d. column with isocratic elution. We have also tested a 10-μm-i.d. column, and as expected the separations were poorer than using a 5-μm-i.d. column.

Using the 2-μm-i.d. column, we separate three trypsin-digested peptide samples: 1 – trypsin-digested bovine serum albumin (BSA), 2 – trypsin-digested myoglobin, and 3 – mixture of 1 and 2. These chromatograms are presented in Figure 5. Close to 100 peptide peaks are identifiable in the top chromatogram; most of the peaks are located in a 6-min time window. All these separations are completed under an elution pressure of 300 psi and in less than 15 min.

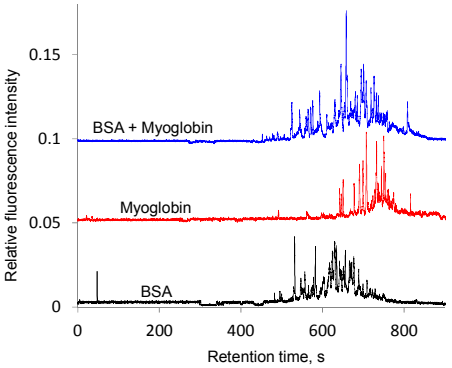

Fig. 5 Separation of protein digests. Gradient profile was mobile phase B increasing from 0 to 82.5% from 0 to 10 min. Sample was trypsin-digested BSA and myoglobin, each at 0.62 mg/mL. Elution pressure was 675 psi. All other conditions were the same as for the 2-μm-i.d. column in Figure 4.

In conclusion, we have discovered that the exceptionally high efficiencies (greater than 10^7 plates per meter) can be obtained when we use an extremely narrow (e.g., 2-μm-i.d.) capillary to perform OTLC. We have identified a focusing effect to interpret the efficiency enhancement. We have demonstrated the feasibility of using this method for separating complex peptide samples. The separations can usually be completed in less than 15 min on a simple experimental apparatus with an elution pressure of several hundred psi. It should be cautioned that care must be taken to prevent capillary from clogging when 2-μm-i.d. capillaries are used. Otherwise, the OTLC separations are very reproducible (see Figure S4). One can increase the column length to further improve the resolution or shorten the column length to enhance the separation speed (Figure S5). The resolution can also be improved by optimize the gradient profile (see Figure S6). Currently, a LIF detector is utilized to monitor the separations, which requires labeling of the analytes with a fluorescent dye. With the advancement of mass spectrometer (MS) and interfaces between nano-flow LC and MS, we expect that this narrow capillary OTLC will be able to couple with MS, providing a powerful tool for bioanalyses.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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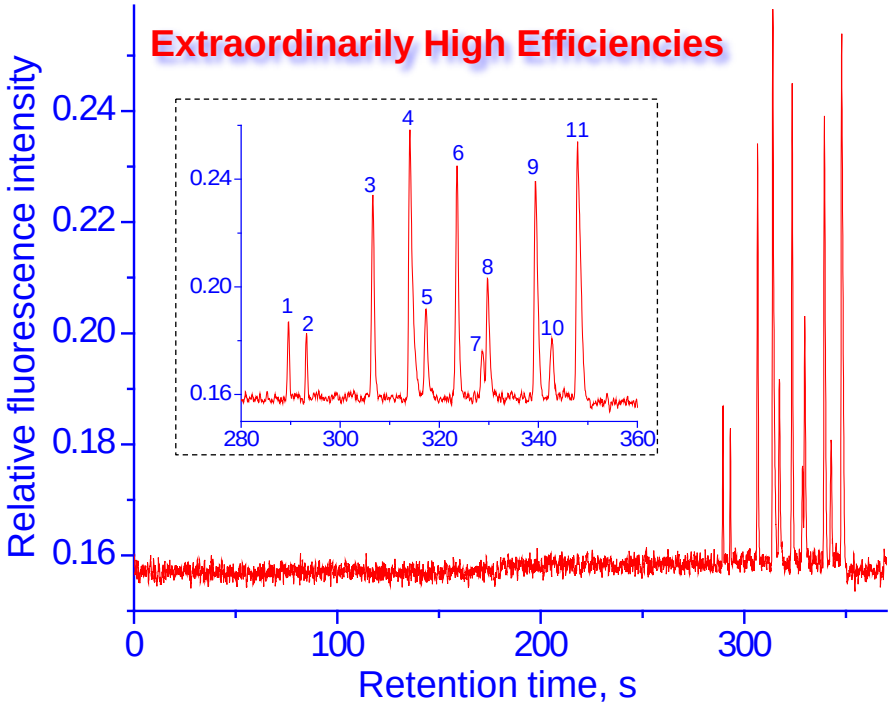
Extraordinarily High Efficiencies
Relative fluorescence intensity
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