Introduction

In HPLC, analytes injected into a separation column broaden naturally during the separation procedure, and this band spreading is inevitable theoretically. Reducing the band broadening of analytes is one of the most critical tasks in HPLC to enhance both the separation efficiency and sensitivity.

Advanced HPLC column technologies that have been developed to suppress the band spreading include columns packed with small particles, core-shell type packing, and monolithic columns.

Meanwhile, in capillary electrophoresis (CE), various methods have been developed that leads to compression of a long sample plug into the narrow band together with a concentration of analytes. In these on-capillary concentration methods, the reducing sample zone width is achieved by the electrophoretic migration of the analytes under a heterogeneous field. That is, the variation in the migration velocity in the heterogeneous field during the analysis is a key factor for reducing the sample zone width, or on-capillary concentration.

Capillary electrochromatography (CEC) is a separation method that combines HPLC and CE methods. In pressurized CEC (pCEC) using an HPLC pump system, the heterogeneous field is easily introduced into a column through gradients of the mobile phase composition. Therefore, on-capillary concentration methods for CE should be applicable to pCEC and may be potentially useful for reducing the sample zone width spread in the column.

Keywords Analyte zone sharpening, angiotensin, dynamic pH junction, electrochromatography, step gradient

Experimental

Reagents and chemicals

Three analyte peptides, namely, angiotensin II (pI = 6.74), [Asn1, Val5]-angiotensin II (pI = 8.14), and angiotensin III (pI = 8.80), were purchased from the Peptide Institute (Osaka, Japan). Acetonitrile, phosphoric acid, potassium dihydrogen phosphate, and disodium hydrogen phosphate (Wako, Osaka, Japan) were used for the mobile phases. The pH values of phosphate buffer in this manuscript indicate those of the aqueous solution before mixing with acetonitrile.

Apparatus and procedure

The apparatus used in this paper was almost the same arrangement described in our previous work. Briefly, the...
apparatus was composed of two pumps (LC-20AD, Shimadzu, Kyoto, Japan), an injector (Model 7520, Rheodyne, Cotati, CA), a UV detector (CE-2075, Jasco, Tokyo, Japan), a high voltage power supply (HCZE-30PN0.25, Matsusada Precision, Shiga, Japan), two laboratory-made splitters, and a laboratory-made capillary column. The capillary column (i.d. 0.15 mm, packed length 150 mm) was packed with ODS particles (d.p. 5 μm, Capcell pak C18 SG120, Shiseido, Tokyo, Japan). High voltage was applied to the reservoir at the column outlet and the splitter at the column inlet was grounded for safety.

Procedure of zone sharpening

The procedure and mechanism of the zone sharpening is shown in Fig. 1. At first, a basic mobile phase was supplied to the column and analytes (peptides) were injected (Fig. 1(A)). The step gradient program was started at the time of the sample injection and the delay time for the change in the mobile phase composition provided the separation in the basic condition. Under basic conditions, the peptides have negative charges and the application of negative voltage at the outlet induced their electrophoretic migration to the inlet (Fig. 1(B)). Then, the acidic mobile phase was supplied to the column in a step gradient (Fig. 1(C)). Since velocity of the acidic/basic interface is faster than that of analytes interacting with the stationary phase, the acidic zone overtakes the analyte zone. In the acidic region, the charges of peptides alter to positive and the analytes migrated electrophoretically to the outlet (Fig. 1(D)). Therefore, around the acidic/basic interface, the direction of electrophoretic migration was in the opposite direction and the peptide zone was focused (Fig. 1(E)). After focusing of the analyte zone at the relatively upstream side, the next analyte zone was then focused (Fig. 1(F)).

Results and Discussion

Effect of pH on zone sharpening

Typical zone sharpening of [Asn1, Val5]-angiotensin II is shown in Fig. 2. In Fig. 2, the mobile phase supplied to the column was switched from basic (pH 8.9) to acidic (pH 2.9) at \( t = 0 \) s (a delay time of approximately 30 s was required for the change of the mobile phase composition at the column inlet). Figure 2(A) shows the chromatogram of [Asn1, Val5]-angiotensin II without application of voltage, i.e., the peptide was analyzed in HPLC with a step gradient of the mobile phase. Under this setup, the analyte was eluted after the mobile phase alternation. Figure 2(B) shows the result with the application of \(-6\) kV. As clearly shown in Fig. 2(B), the band zone of [Asn1, Val5]-angiotensin II was reduced and the peak height increased. Here, we defined the enhancement factor of peak height, EFH, as follows:

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EFH = \frac{\text{peak height with applied voltage}}{\text{peak height without applied voltage}}
\]

The EFH value for Fig. 2(B) was approximately 3.0. That is, zone sharpening was successfully achieved.

As shown in Fig. 1, the change in electrophoretic migration direction is a key factor for the zone sharpening studied in this manuscript. Therefore, the effect of the mobile phase pH on the sharpening was evaluated. Figure 3 shows the chromatograms of [Asn1, Val5]-angiotensin II with basic mobile phases of various pHs (the pH of the acidic mobile phase was kept at 3.2). As shown in Fig. 3, zone sharpening was observed with the mobile phases of pH 9.0 and 8.9. However, the effect was not obtained when the mobile phases of pH 7.7 and 6.9 were used. Since the pI of [Asn1, Val5]-angiotensin II is 8.14, the analyte had an almost neutral or slightly positive charge in the latter mobile phases. Therefore, the step gradient of the mobile phase
did not produce the change in the direction of electrophoretic migration (or change in the sign of the charge), and no zone sharpening was achieved.

Effect of flow velocity

The separation of the mixture of angiotensin II, [Asn1, Val5]-angiotensin II, and angiotensin III was then investigated. Figure 4(A) shows the separation of the three peptides without application of voltage, and the peptides were eluted as broadened peaks. With the application of –9 kV (Fig. 4(B)), the elution of analytes was accelerated and the height of peak 1 ([Asn1, Val5]-angiotensin II) was enhanced (EFH for Fig. 4(B) was 3.5). However, the acceleration of peaks 2 and 3 brought about an overlapping phenomenon and relatively low enhancements of the peak heights were observed. Since zone sharpening is produced by heterogeneous electrophoretic migration in the column, as shown in Fig. 1, an increase in the electric field strength might be effective in enhancing the sharpening effect. When a higher voltage of –12 kV was applied to the column, the EFH of peak 1 increased to approximately 12 (data not shown). However, the peaks of 2 and 3 were overlapped completely.

As an alternative approach to enhancing the zone sharpening, decreases in pressurized flow velocity, \( v_p \), were investigated. That is, the relative electrophoretic velocity to the pressurized one was increased by the slowdown of the \( v_p \). In Fig. 4(B), the \( t_0 \)-based flow velocity was 1.3 mm/s. In Fig. 4(C), the flow rate was reduced to 0.8 mm/s. As clearly shown in Fig. 4(C), the application of –9 kV enhanced the heights of peaks 1 and 2, i.e., their EFH were approximately 10 and 6, respectively. However, the EFH of peak 3 was only 2.3. When the flow rate was further reduced to 0.6 mm/s, all the peaks were successfully enhanced without overlapping as shown in Fig. 4(D). The EFH values for peaks 1, 2, and 3 were approximately 10, 7, and 11, respectively. At the flow rate of 0.5 mm/s, EFH values of 12, 10, and 12 were achieved for peaks of 1, 2, and 3, respectively (Fig. 4(E)). The decrease in pressurised flow velocity was effective in enhancing the relative electrophoretic velocity of the peptides to the \( v_p \) when the same voltage was applied to the column. In other words, the decrease in the \( v_p \) was basically equivalent to the enhancement in the applied voltage. However, in Fig. 4(D, E), the peak 2 did not overlap the peak 3 in contrast to the case described in the last paragraph. Therefore, the effect of decrease in \( v_p \) was somewhat different from that of the increase in applied voltage. The decrease in pressurized flow velocity was effective in enhancing the zone sharpening effect, but further study is needed to gain a clear understanding of the detailed mechanism.

Selective zone sharpening

As shown in Fig. 2, the pH of the mobile phase is a key factor for the zone sharpening method. For successful zone sharpening, the \( pI \) value of the analyte peptide must be sandwiched between the \( pK_a \) values of the acidic and basic mobile phases. Here, selective zone sharpening was investigated. The \( pI \) values of
angiotensin II, [Asn₁, Val⁵]-angiotensin II, and angiotensin III are 6.74, 8.14, and 8.80, respectively. In Fig. 4, a pair of mobile phases with pH values of 3.1 and 9.2 were used. Therefore, all the analyte zones widths were reduced and the peak heights were enhanced. When the pH of the basic mobile phase is changed to below 8, it can be predicted that the zone of [Asn₁, Val⁵]-angiotensin II and angiotensin III will not be sharpened, or in other words, that only zone sharpening of angiotensin II will be observed.

Figure 5 shows the selective zone sharpening of angiotensin II with a step gradient of mobile phases of pH 3.1 and 7.1. When no voltage was applied to the column, peaks of angiotensin II and III were observed as an overlapped broadened peak as shown in Fig. 5(A). However, as clearly shown in Fig. 5(B), the application of voltage resulted in the enhancement of the peak height of angiotensin II only. Moreover, separation of angiotensin II and III was achieved. That is, selective zone sharpening was successfully demonstrated. It should be noted that the elution order in Fig. 5(B) was changed compared to that in Fig. 5(A). Since the charges of [Asn₁, Val⁵]-angiotensin II and angiotensin III stayed positive in both the acidic and basic mobile phases, they migrated toward the outlet end through the analysis. Thus, these peptides eluted faster than angiotensin II.

Conclusions

The dynamic pH junction method for CE was successfully utilized for reducing the peak zone width, or enhancing the peak height, in pCEC with a step gradient. The key of the proposed method is the pH values of the acidic and basic mobile phases, i.e., the pI value of the target analyte, or peptide, must fall within the pH range. When a suitable pair of pH values is used, selective enhancement of the peak height for the target analyte is possible. This technique will be useful for improving the signal-to-noise ratio of pCEC data.

In the field of CE, many on-capillary sample concentration methods have been developed. We have now studied the application of field amplified stacking and dynamic pH junctions in pCEC for reducing the peak width. The combination of CEC with other on-capillary concentration methods also has potential for high efficiency separations.

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