Theoretical Analysis of Chromatographic Peak Asymmetry and Sharpness by the Moment Method Using Two Peptides

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Abstract The analyses of peak shapes in chromatography are useful in operating chromatographic system. The asymmetry and sharpness of a chromatographic peak are estimated by the reversed-phase adsorption of two standard peptides (angiotensin II bradykinin) on C_{18}. In this work, the average particle diameters of C_{18} were 5 and 15 μm, while the pore sizes were 100 and 300 Å. The composition of the mobile phase was 50/50 vol. % of a binary mixture of acetonitrile and water with 0.1% TFA, and the particles were packed in a stainless column (4.6 × 150 mm). The third and the fourth central movement were calculated from the chromatographic elution curves by moment analysis. The peak asymmetry was determined by two theoretical calculations: the asymmetry factor by elution peak analysis and skewness with moment analysis. The sharpness was estimated by the fourth central moment. In this work, the most acceptable skewness was calculated when the pore size was 300 Å. The large excess was observed on small pore size.

Keywords: packings, moment analysis, peptides, asymmetric factor, skewness, excess

INTRODUCTION

Chromatogram evaluation is a possible method to analyze a peak shape. The analysis of peak shapes helps not only to gain qualitative and quantitative data; it can also explain the operation of the chromatographic system. Furthermore, it can be useful when investigating the mathematical parameters and mass-transfer characteristic of the chromatographic system. A peak shape is characterized by the respective statistical moments, third and fourth central moments, the peak skewness and excess calculated from them, the asymmetry factor calculated from peak widths at various peak heights and the standard deviation of the peak [1].

Most of the actual chromatographic peaks are not symmetrical. Therefore, it is necessary to introduce an asymmetric factor for the skewness, which characterizes the peak shape. Before the examination of the asymmetric factor it is important to analyze the reasons behind the actual asymmetry. The most important reasons for the presence of the asymmetry are the column overload, the heterogeneity of the stationary phase surface, the heterogeneity of the column packing, and the effects of the extra-column. Significant tailing, due to extremely slow mass transfer kinetics, can only be observed when the apparent plate number is uncommonly small. The slow mass transfer may explain the peak asymmetry in chro-

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matography, but generally the tailing or fronting presence has to be attributed to the other factors [2].

Heterogeneity will not always result in strongly tailing profiles when the isotherms are linear on the elution profile. A chromatographic peak approaches a Gaussian peak when the elution profile was linear. The effect of slow mass transfer, adsorption–desorption kinetics, and dispersion on the band profile were characterized by Felinger by combining the stochastic model of chromatography with a mobile phase dispersion [2]. Peak asymmetry can be evaluated by means of the empirical peak shape models. Often, quantity analyses can be obtained with other types of calculations or with simple graphical measurements, which quickly describe the peak asymmetry [1,2].

The pharmaceutical functions of the two peptides that were used in this study are as follows. The main function of bradykinin is to increase the sensation of pain. Bradykinin also sensitizes free nerve endings, making them hypersensitive to heat and light touch while creating an overall sensation of soreness. It is presence in blood launches a chain of reactions that result in the production of angiotensin II and III, both being molecules that raise blood pressure. In addition to its role in raising blood pressure, angiotensin II promotes the overgrowth of cells, called hypertrophy, in the heart and blood vessel walls as well as in the kidneys. The hypertrophic response to angiotensin II is a major problem leading to heart and kidney failure, and atherosclerosis [3]. The reversed-phase high-performance liquid chromatography (RP-HPLC) is the most widely used analytical technique for the separa-

tion and isolation of such peptides [4].

The purpose of this work was to survey the effect of the third and fourth central moment from the chromatographic elution curves of two peptides according to different particle sizes (5, 15 μm), pore sizes (100, 300 Å) and flow rates (0.5, 0.75, 1.0, 1.25, 1.5 mL/min). Also, the skewness and excess, as well as the asymmetry factor were determined according to particle and pore sizes.

THEORY

The chromatographic peaks were analyzed by the method of moments [5-8]. In general, in order to completely characterize an arbitrary probability distribution function, all moments are required. The first moment about the origin, μ_1, is the center of gravity of the distribution curve with respect to the origin or the mean by the differentiation procedure of the Laplace-transformed residence-time distribution function [7,8]. The second moment about the mean, μ' or σ^2, indicates the spread of the distribution curve or relative, normalized variance by the differentiation procedure of the Laplace-transformed residence-time distribution function [9]. The third central moment, μ'_3, draws a conclusion about the asymmetric level of peaks and direction. The skewness (S) is determined from the analysis of the third moment, whereas the analysis of the fourth moment provides information on the shape (sharpness) and the excess or kurtosis of the elution profile. The peak is expressed as a function of time and height, $f(x)$. The general moment of a chromatographic peak is as follows:

$$\mu_n = \frac{\int_0^\infty (x - \mu_1)^n f(x)dx}{\mu_0} \tag{1}$$

where μ_0 is the area covered by the peak.

$$S = \frac{\mu'_3}{\mu'^{3/2}_2} \tag{2}$$

The skewness (S) is a measure of the asymmetry of a distribution. It can be calculated as a function of the third and second central moments. If it is equal to zero, the distribution is symmetrical. If it has a (+) value, the function is skewed toward smaller values of x and in that case is a tailing function. If S has a (-) value, the function is skewed towards higher values of x.

$$E = \frac{\mu'_4}{\mu'^2_2} - 3 \tag{3}$$

The excess (E) or kurtosis (K) is a measure of the peakness of a distribution compared to the normal distribution. If E is positive, the distribution is more peaked than the corresponding normal distribution, but if E is negative the distribution is less peaked than the corresponding normal distribution. The standard de-

viation $\sigma = \sqrt{\mu_2}$. S and E are also used to express any distribution function in terms of the corresponding normal distribution according to the Gram-Charlier series [10-11].

Eluted peaks often deviate from this ideal shape and the peak skewness is described qualitatively by the so-called asymmetry factor, A_s, which is "fronting" when $A_s < 1$ or "tailing" if $A_s > 1$. Depending on the case, the commonly applied acceptance criterion is for the asymmetry factor to be in the following range:

0.8 < A_s < 1.5-1.8.

$$A_s = b/a \tag{4}$$

This equation calculates 10% of an eluted peak height. The value of 'a' is the distance from the front of the peak to the peak center and 'b' is the distance from peak center to the rear of the peak.

MATERIALS AND METHODS

The two standard peptides, angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) and bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), were purchased from Sigma (St. Louis, MO, USA). The molecular weights of the two peptides, anigotensin II ($C_{50}H_{71}N_{13}O_{12}$ xH_2O) and bradykinin ($C_{50}H_{73}N_{15}O_{11}$) were 1,046 and 1,240, respectively. The HPLC grade solvent, acetonitrile, was from Ducksan Pure Chemical (Kyungki-Do, Korea). Trifluoroacetic acid (TFA) was purchased from Sigma (St. Louis, MO, USA). Water filtered by a Milipore ultra pure water system (Milipore, Bedford, MA, USA) was used. Four standard peptides, 5 mg respectively, were dissolved in 1 mL of water, then the concentration of the solutions was adjusted to 5,000 μg/mL, respectively. A constant injection volume (3 μL) of the mixture solutions were used throughout the study.

In this experiment, the HPLC was performed using a Waters 600S solvent delivery system (Waters, Milford, MA, USA). A 2487 UV dual channel detector (Waters, Milford, MA, USA) was used. The data acquisition system was a Millenium[32] (Waters) system installed in a HP Vectra 500 PC. The water was filtered by an ultra-pure water system (Milipore, Bedford, MA, USA). The mobile phases were degassed with helium. The flow rate of the mobile phase was 1 mL/min, and was monitored at the fixed wavelength of 215 nm. The column was purchased from Alltech Co. The column size was 4.6 × 150 mm and was packed with C_{18}-pore sizes 100, 300 Å, and particle diameters 5, 15 μm. All the experimental runs were carried out in ambient temperature. The dead volume (V_0) was the retention volume of 20 μL of acetonitrile.

RESULTS AND DISCUSSION

Parameter estimations were made for the reversed-phase adsorption of two peptides, on a C_{18} RP-HPLC

Table 1. The third central moment (μ'_3)

ν	Angiotensin II			Bradykinin		
	100 Å, 5 μm	300 Å, 5 μm	300 Å, 15 μm	100 Å, 5 μm	300 Å, 5 μm	300 Å, 15 μm
0.50	57.02	38.59	582.32	152.85	33.29	956.96
0.75	15.38	0.39	308.99	43.55	16.94	375.94
1.00	6.92	2.15	15.12	36.40	0.67	85.68
1.25	6.23	-0.07	127.26	9.78	-0.95	135.69
1.50	2.54	0.80	63.49	1.58	0.49	60.16

Table 2. The fourth central moment (μ'_4)

ν	Angiotensin II			Bradykinin		
	100 Å, 5 μm	300 Å, 5 μm	300 Å, 15 μm	100 Å, 5 μm	300 Å, 5 μm	300 Å, 15 μm
0.50	1188.27	915.63	34879.96	3097.93	1028.67	56317.65
0.75	222.07	202.99	13502.64	672.07	340.74	19364.63
1.00	77.47	102.71	2436.05	523.11	100.69	4785.49
1.25	60.03	57.58	3681.23	111.61	64.56	4504.27
1.50	29.84	10.59	2240.82	26.51	19.96	2115.77

column. The third and fourth central moments were determined from chromatographic elution curves by moment analysis. The experimental flow rates of the mobile phase were 0.5, 0.75, 1.0, 1.25, and 1.50 mL/min. The mobile phase composition was fixed at 50/50 (vol.). It was made with water in 0.1% TFA/acetonitrile in 0.1% TFA.

Tables 1 and 2 present the results of the calculations of the moments according to the elution curves using Eq. (1). Table 1 shows the result of the third central moment and Table 2 illustrates the data concerning the fourth moment of the two peptides. The results showed that the calculated moments with a smaller pore size were greater than those computed with a larger pore. Furthermore, the moments of large particles were greater than those of smaller particles.

Figs. 1 and 2 illustrate the asymmetry factors calculated according to Eq. (4). The asymmetry factor of the elution peak decreased as the flow rate of the mobile phase increased. Consequently, the tailing phenomenon was more important as the flow rate got smaller. In other words, the Gaussian-shaped symmetry peak was eluted out as the small flow rate increased. The asymmetry factor showed the same tendency for the 100 Å, 5 μm and 300 Å pore size, 15 μm particle size. This study observed that the peaks had superior symmetry with smaller particles sizes and larger pore sizes. The best asymmetry factor was calculated when the pore size was 300 Å and the particle diameter was 5 μm for this work.

In this work, most elution peaks looked like Gaussian-shaped curves, but the values calculated with Eq. (2) were slightly distorted shape. Even if some calculated
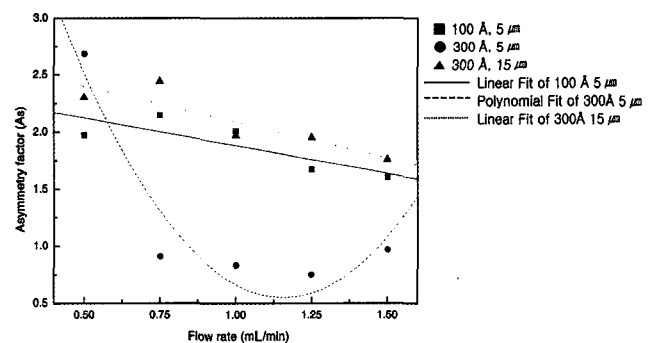
Fig. 1. Plot of the asymmetry factor (A_s) at various particle and pore sizes. (Angiotensin II)
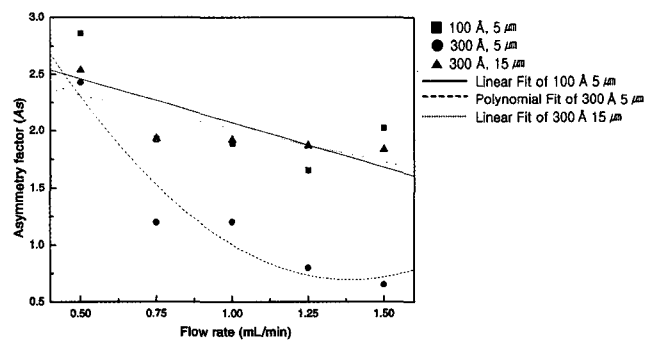
Fig. 2. Plot of the asymmetry factor (A_s) at various particle and pore sizes. (Bradykinin)
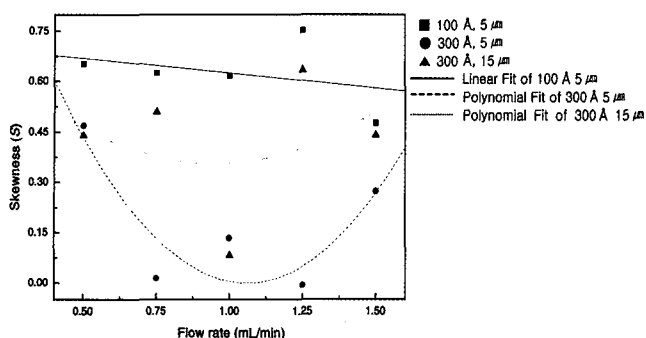
Fig. 3. Plot of the skewness (S) at various particle and pore sizes. (Angiotensin II)
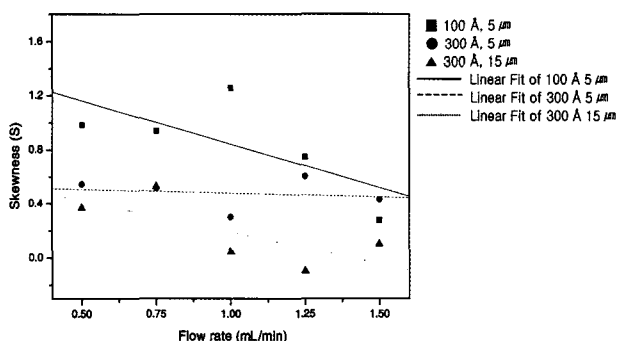

Fig. 5. Plot of the excess (E) at various particle and pore sizes. (Angiotensin II)

Fig. 4. Plot of the skewness (S) at various particle and pore sizes. (Bradykinin)

Fig. 6. Plot of the excess (E) at various particle and pore sizes. (Bradykinin)

data were scattered, the skewness of the elution peaks tended to be reduced according to various particle and pore size. When the S value approached zero, the peaks were more symmetric resembling Gaussian-shaped peaks. Figs. 3 and 4 illustrate that the small pore size of the packing material yielded a large S value, so more tailing. As a result, a larger pore size led to a smaller S value, which also generated more symmetrical elution peaks compared to others. The tailing was more pronounced with small particles and pore sizes. These results lead to interesting implications.

Generally, it was thought that a small pore size of packing material would elute a more symmetric peak, but such an experiment has not been previously conducted. Both the 'A_s' and 'S' were more symmetric on the peak using 300 Å/5 μm particles. For small particles with a large pore size, the narrow pores of a circumference particle came to be small and this was due to a low backpressure. If the backpressure becomes small, the solutes in a column will gush out so that there would be uniform and more symmetric peaks. Also, as illustrated in Figs. 3~4, it is interesting that the packing of small particles led to a larger S value. Hence, there is a need for further research on the exact influence of pores with various sizes.

The E value calculated by Eq. (3). As shown in Figs. 5 and 6, the pore sizes in the different packings were smaller, E was larger and the particle sizes had different
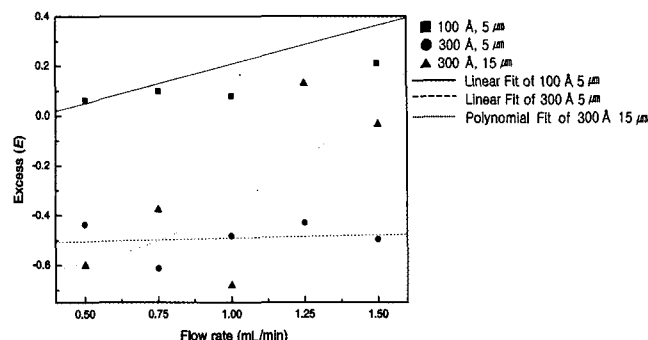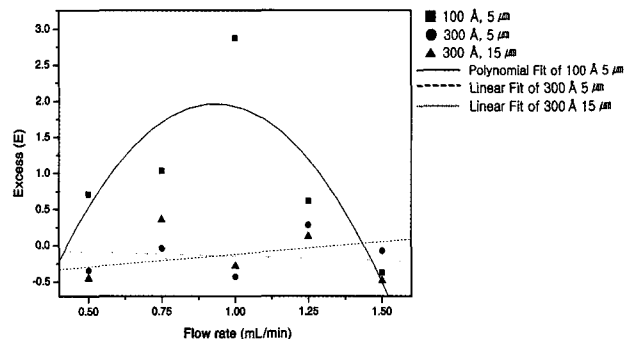
tendencies. The excess was larger when the pore sizes were much smaller. This means that the packing of a small pore size results in a steeper peak. Especially, the size of pore was a greater determinant than the particle size.

CONCLUSION

Parameter estimations were made for the reversed-phase adsorption of two standard peptides, angiotensin II and bradykinin, on C_{18}. We studied about chromatographic peak shape according to various packing materials. When the pore size in the packing material was smaller, S tended to be larger, and the peak shapes tend to be a tailing peak. The same behavior was observed with the study of particle size. An optimum size of packing materials can generate an adequate symmetric profile. In this work, the peak symmetry was best when the pore size was larger, e.g. 300 Å pore size particles. The excess explains the sharpness of a peak. If the excess is larger, the peak width is narrower and peak shape is steeper. The excess value was largest in a small pore size packing material.

499

Technology. This work was performed in the High-Purity Separation Laboratory of Inha University located in Incheon, Korea.

NOMENCLATURE

A_s : the asymmetric factor
E : the excess
S : the skewness
μ_1 : the first central moment
μ'_2 : the second central moment
μ'_3 : the third central moment
μ'_4 : the fourth central moment
v : flow rate [mL/min]

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