Korean J. Chem. Eng., **16**(1), 22-27 (1999)

CORRELATION OF HETP AND EXPERIMENTAL VARIABLES IN PREPARATIVE LIQUID CHROMATOGRAPHY

Kyung Ho Row† and Chong Ho Lee

Department of Chemical Engineering, Inha University, 253 Yonghyun-Dong, Nam-Ku, Inchon 402-751, Korea
(Received 15 January 1998 • accepted 15 June 1998)

Abstract – HETP (Height Equivalent to a Theoretical Plate) is widely designated as the column efficiency in chromatographic separations. The effects of experimental variables such as concentration, injection volume, flow rate and composition of the mobile phase, column diameter, and column length on HETP were investigated by preparative liquid chromatography. Water and methanol as an organic modifier were used as the mobile phase. A sample of thymidine was injected into C_{18} columns with different dimensions. From fifty experimental runs, it was shown that the larger amounts of sample and higher flow rates increased HETP and an optimum HETP existed at a column diameter of 7.8 mm for preparative packings used in this experiment. The experimental values of HETP were correlated into a quadratic equation with the interaction terms based on the logarithmic experimental values of the experimental variables; its regression coefficient was 0.952. The experimental variables were simulated from the correlation equation, and their effects on HETP were discussed.

Key words: HETP, Correlations, Experimental Variables, Preparative Liquid Chromatography

INTRODUCTION

High-performance liquid chromatography (HPLC) is widely used for the analysis of various kinds of materials, specially pharmaceuticals. The main advantages of HPLC include its simplicity, accuracy, versatility, reproducibility, and selectivity. Using this powerful technique, numerous attempts have been made to purify many different mixtures. In preparative chromatography, sufficient samples are treated for substantial use. It is easily expected that resolution and column efficiency could be degraded by increasing the amounts of sample. Usually, large amounts of purified materials require stringent operating conditions different from those for analytical-scale instruments. Resolution, separation speed, and sample loading are, in general, three major interrelated factors of chromatographic separation. For any one of these to be emphasized, the others must be compromised [Synder and Kirkland, 1979]. Normally, sample loading on an analytical scale requires mg order of sample weight. To keep such good resolution in preparative scale as in analytical separation, sample isolations can be carried out in a larger column with the same particle sizes. This requires a very large pressure drop to pump the mobile phase through a chromatographic column.

The number of theoretical plates (N) in a chromatographic column can be determined from the experimental peak. N is proportional to column length (L). The HETP (Height Equivalent to a Theoretical Plate) is defined by the ratio of L to N, and it is related to column efficiency. Small HETP values mean more efficient columns with large N values. Under specific column packings and samples to be separated, HETP is greatly influenced by operating variables and column dimension, which need to be optimized in terms of resolution and sample loading. Although a number of operating variables influence HETP, their effects on HETP were reported in the limited number of variables [Gau, 1995; Guillaume, 1995a, b, 1996; Valiente, 1994]. Since preparative systems usually use a large column, the effect of its dimension is very critical, but it is hardly revealed to clarify its effect on HETP. The experimental variables considered in this work were sample concentration, injection volume of sample, flow rate of mobile phase, quantity of organic modifier in mobile phase, column diameter, and column length. These factors affecting HETP were interrelated into correlation equations. This work also shows the effects of the experimental variables on HETP calculated from the best-fit equation in three-dimensional plots.

†To whom correspondence should be addressed.
E-mail: rowkho@dragon.inha.ac.kr

METHODOLOGY

To investigate the effects of the experimental variables on HETP, three correlation equations were tested as shown in Table 1. They were a linear equation, a quadratic equation, and a quadratic equation with interaction terms based on the logarithmic values of each variable, respectively. The experimental variables were sample concentration (x_1), injection volume of sample (x_2), flow rate of mobile phase (x_3), quantity of organic modifier in mobile phase (x_4), column diameter (x_5), and column length (x_6). The resulting equations were obtained by Mathematica (Ver.2.2) and compared to each other in terms of the regression coefficient, r^2, defined as follows:

$$r^2 = \frac{[\Sigma(x_i - \bar{x})(y_i - \bar{y})]^2}{[\Sigma(x_i - \bar{x})^2][\Sigma(y_i - \bar{y})^2]} \quad (1)$$

Table 1 shows the regression coefficients, and the correlation

Table 1. Correlation equations used in this work

Type of equations	Eq. no.	Equations	r^2
Linear equation in natural logarithm	(4)	$HETP=1.897+0.076\ln x_1+0.088\ln x_2+0.381\ln x_3-0.082\ln x_4-2.7971\ln x_5+0.849\ln x_6$	0.826
Quadratic equation in natural logarithm	(5)	$HETP=24.746-0.066\ln x_1-0.011(\ln x_1)^2+0.136\ln x_2-0.027(\ln x_2)^2-1.006\ln x_3+0.588(\ln x_3)^2+0.328\ln x_4-0.078(\ln x_4)^2-7.638\ln x_5+1.326(\ln x_5)^2-5.820\ln x_6+0.597(\ln x_6)^2$	0.907
Quadratic equation with interaction terms in natural logarithm	(6)	$HETP=5.725-7.353\ln x_1-0.018(\ln x_1)^2-1.894\ln x_2-0.073\ln x_1\ln x_2-0.049(\ln x_2)^2-0.469\ln x_3-0.057\ln x_1\ln x_3+0.022\ln x_2\ln x_3+0.279(\ln x_3)^2+16.039\ln x_4+0.574\ln x_1\ln x_4-0.008\ln x_2\ln x_4+0.412\ln x_3\ln x_4-0.169(\ln x_4)_2-2.725\ln x_5+0.854\ln x_1\ln x_5+0.302\ln x_2\ln x_5-1.443\ln x_3\ln x_5+1.143\ln x_4\ln x_5+5.556(\ln x_5)^2-9.270\ln x_6+0.707\ln x_1\ln x_6+0.227\ln x_2\ln x_6+0.440\ln x_3\ln x_6-3.016\ln x_4\ln x_6-3.431\ln x_5\ln x_6+2.061(\ln x_6)^2$	0.952

equations [Eq. (4)-(6)]. The last equation with the highest regression coefficient was used to investigate the effects of the experimental variables on HETP in three-dimensional plots.

EXPERIMENTAL

Thymidine purchased from Sigma (St.Louis, MO, U.S.A.) was chromatographically pure. The solute was dissolved in HPLC-grade water and the concentration was 500-10,000 μg/ml (ppm). HPLC-grade water and methanol were obtained from Baker (Phillipsburg NJ, U.S.A.).

The preparative HPLC system consisted of an HPLC metering pump (Model 2396-26, TSP), a U6K injector (2 ml sample loop), a back pressure regulator (Tescom Co., Model 26-1722), which can be controlled up to 6,000 psi, a rotameter, and a UV detector (Young-In Sci. Co., Korea). Absorbance was monitored at 254 nm with a sensitivity of 2 and 0.001 a.u.f.c. The data aquisition system was CHROMATE (Ver.2.1, Interface Eng., Korea) installed in a Pc. All separations were done at ambient temperature. Stainless steel columns packed with octadecylsilica of 40-63 μm particle size (YMC Co.) were used.

The modifier concentrations ranged from 1 to 20%. Sample volumes of 50-1,000 μl were injected directly into HPLC. The elutions were performed by using an isocratic mode at flow rates of 1.5-5.5 ml/min. Table 2 summarizes the ranges of experimental conditions. Fifty experiments were carried out to obtain the experimental values of HETP.

RESULTS AND DISCUSSION

HETP is defined by the ratio of column length to the number of theoretical plates, N, and calculated from the following

Table 2. Experimental variables and ranges

Variables	Ranges
Injection volume	0.05-1.0 ml
Concentration	0.5-10 mg/ml
Sample amount	0.1-10 mg
Flow rate of mobile phase	1.5-5.5 ml/min
Methanol percentage	1-20 (v/v,%)
Column diameter	4.6-9.8 mm
Column length	100-500 mm

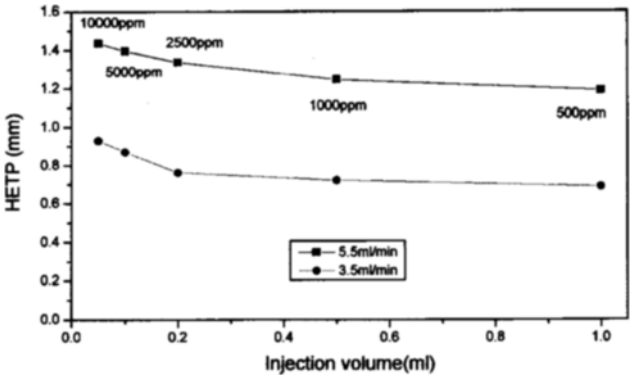

Fig. 1. Effect of injection volume on HETP.
(0.5 mg sample amounts, 20% MeOH, 500×7.8 mm I.D.)

relationship,

$$HETP = L/N = L/\{5.54(t_R/w_{1/2})^2\} \quad (2)$$

where L denotes the column length, t_R the retention time, and $w_{1/2}$ the peak width at half height.

The sample amount in units of mg is the multiplication of injection volume (ml) and its concentration (ppm). In Fig. 1, at a constant sample loading of 0.5 mg, the injection volume and the corresponding concentration were varied. When injection volume decreases, that is, sample concentration increases, HETP increases, which implies that samples should be injected as relatively large volumes of lower concentration, rather than as smaller volumes of more concentrated solutions. This approach reduces the effect of overloading at the column-bed inlet, thus increasing column loadability and performance. For a constant injection volume, the concentration effect is clearly shown in Fig. 2. The main difference between analytical and preparative chromatographies is the amount of sample injected into the system. Normally, more than mg order of sample should be separated in the preparative system. Concentration overload is sometimes not desirable because the nonlinearity of an equilibrium isotherm causes degradation of the resolution. Retention time is almost constant in the range of analytical loading (μg). Beyond this amount, peak area is larger, so peak width is longer, which deteriorates the column efficiency.

The column efficiency is dependent on the flow rate of the mobile phase. Throughput, which is related to the flow rate, is an important factor. Fig. 3 shows a van Deemter plot, or

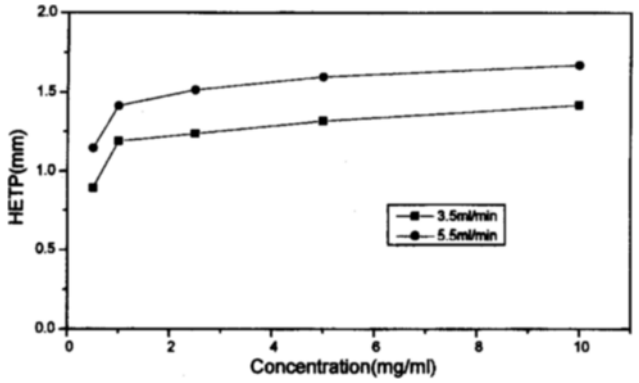

Fig. 2. Effect of concentration on HETP.
(1.0 ml, 5 % MeOH, 500×9.8 mm I.D.)

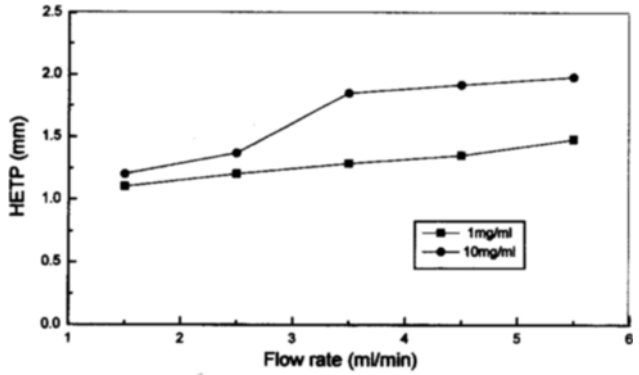

Fig. 3. Effect of flow rate on HETP.
(1.0 ml, 5 % MeOH, 500×9.8 mm I.D)

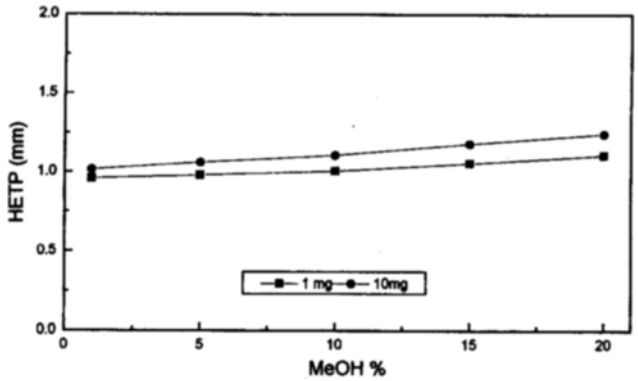

Fig. 4. Effect of mobile phase composition on HETP.
(1.0 ml, 4.5 ml/min, 500×9.8 mm I.D.)

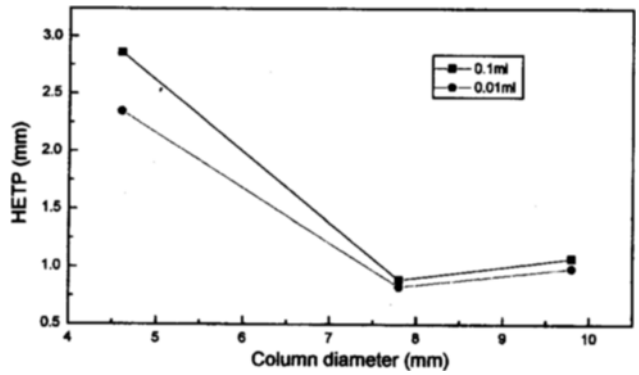

Fig. 5. Effect of column diameter on HETP.
(10 mg/ml, 5.5 ml/min, 5 % MeOH, 250 mm length)

H/u plot, Eq. (3), but the trend is almost linear. The retention time of the sample is longer at lower flow rates, but the peak width is almost not changed and the axial dispersion does not greatly contribute to peak broadening. So the larger the HETP, the higher the flow rate. At a flow rate lower than 1.5 ml/min, the retention time of thymidine was too long. The capacity of the pump could not deliver a mobile phase at a flow rate higher than 5.5 ml/min. Therefore, in this work, the range of flow rate was set between 1.5 and 5.5 ml/min.

Water was used as a mobile phase and methanol as an organic modifier in C_{18}-coated packings. A larger amount of the organic modifier decreases the retention time, because it is more adsorbed on the surface of the packings [Lee, 1995; Kim, 1995]. The content of the modifier can adjust the elution time of the sample, similar to flow rate. The effect of the volume content of methanol on HETP was slightly increased (Fig. 4). When the percentage of the organic modifier in the binary water-methanol system increases, or the viscosity of mobile phase decreases, the interfacial tension between the mobile and stationary phase decreases [Horvath, 1977]. Thus, in the two phases, the mass transfer rate of thymidine is higher by decreasing the polarity of the mobile phase and its viscosity. With an increase in the methanol percentage, the peak is negligibly broadened [Lee, 1996], but a decrease in the capacity factor results in a higher HETP.

The column in preparative chromatography should be large in diameter and length to treat more samples. Three different diameters of 4.5, 7.8, and 9.8 mm i.d. with the same column length of 250 mm were used to investigate the effect of column diameter as shown in Fig. 5. The column was packed in our laboratory. The dry packings were dumped into the column, and then the mobile phase was allowed to flow through the column. After they were unplugged, more packings were filled and mobile phase was pumped again. This operation was repeated until there was little empty space inside the column. A complex relationship between particle size, column length and diameter, sample injection techniques, and other factors influences the optimum diameter of small particles [Kirkland, 1976]. Even in the preparative packings of 40-63 μm, approximately 10 times larger than analytical packings, HETP was affected by several factors. A little increase in HETP of 9.8 mm i.d. might be attributed to the combined effect of the various factors mentioned above. It should be noted that the HETP with preparative packings is lower by two orders of magnitude than in analytical packings, although the effect of diameter is almost the same. The pressure drop was almost negligible in the preparative packings used in this work. Four columns (100×4.6, 200×4.6, 200×7.8, and 500×7.8 mm) were used to investigate the effect of column length on HETP, and the experimental result is shown in Fig. 6. As the column length is shorter, HETP decreases due to smaller eddy diffusion. As in Fig. 5, HETP is worse at a smaller diameter of the column.

The correlation equations with the six parameters, x_1-x_6, affect-

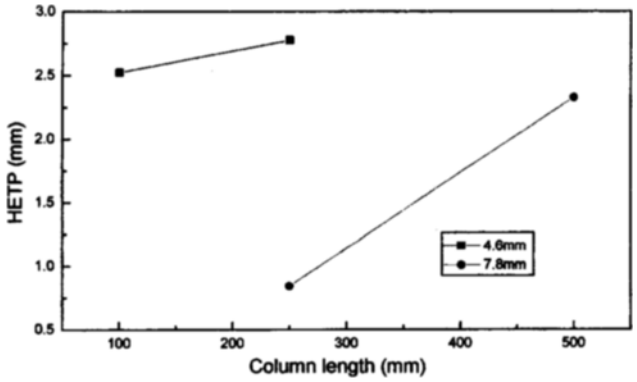

Fig. 6. Effect of column length on HETP.
(0.1 ml, 1 mg/ml, 5% MeOH, 5.5 ml/min)

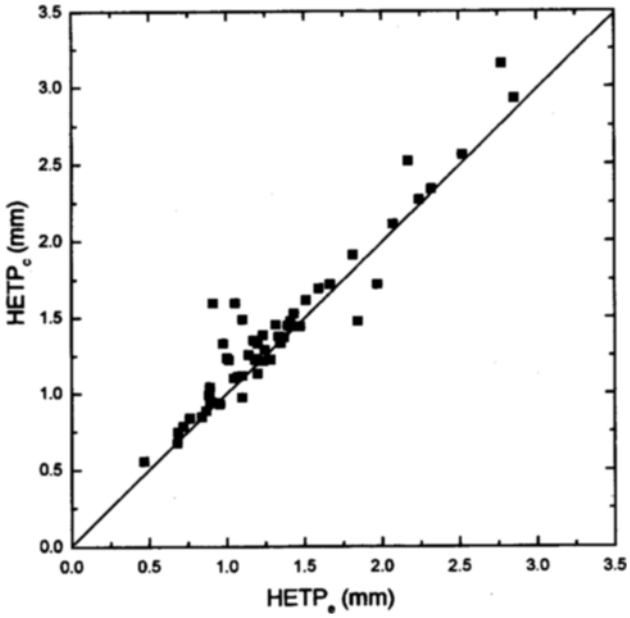

Fig. 7. Comparison of calculated values from Eq. (6) to experimental data.

ing HETP selected in this study are listed in Table 1. Small differences between the calculated HETP (HETP$_c$) from Eq. (6) and the fifty experimental data (HETP$_e$) were observed in Fig. 7. With this equation, the effects of the six experimental conditions were simulated. Fig. 8 shows the influence of sample concentration and injection volume. Column efficiency and retention time are independent of sample size in linear chromatography, while they decrease in the Langmuir isotherm [Guiochon, 1988]. The sample was thymidine, and the adsorption behavior of this sample was almost similar to its base, thymine. The experimental adsorption isotherm of thymine deviated slightly from linearity but might be expressed as the Langmuir isotherm [Row, 1995]. An increase in amount of the sample (sample concentration or injection volume) was accompanied by a larger plug. This resulted in an increase in the peak width, so lower column efficiency was achieved.

The column efficiency is expressed by the van Deemter equation,

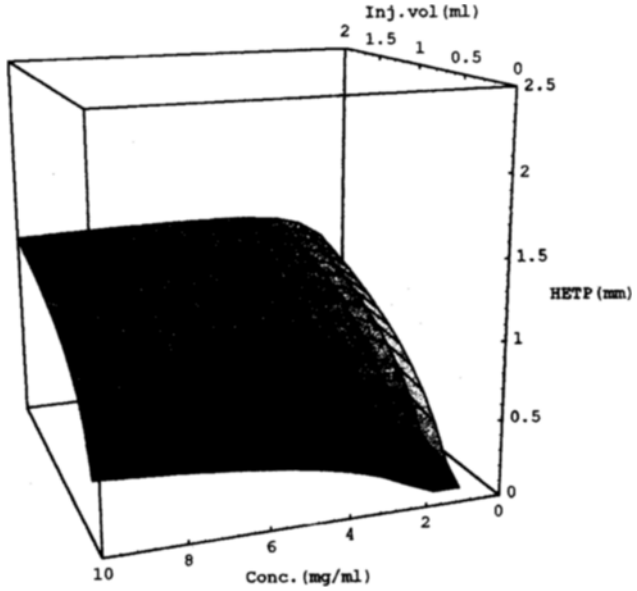

Fig. 8. Response surface of HETP to injection volume and concentration.
(3.5 ml/min, 5% MeOH, 4.6 mm diameter, 100 mm length)

$$HETP = A + B/u + Cu \quad (3)$$

where A is the eddy diffusion, B the axial diffusion, and C the resistance to mass transfer. In liquid chromatography, the axial diffusion is negligible and the mass transfer resistance is dominant. As shown in Fig. 9, the column efficiency decreased as the flow rate of mobile phase increased within the ranges of flow rate in this study. Specifically, HETP increased at larger content of methanol with higher flow rate. The effect of column dimension is plotted in Fig. 10. In preparative LC, sample loading usually can be increased using columns of larger internal diameter. The effect of internal di-

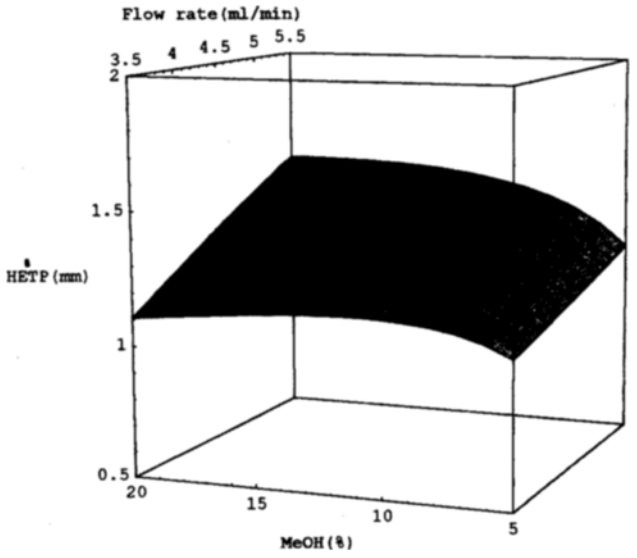

Fig. 9. Response surface of HETP to flow rate of mobile phase and MeOH(%).
(1 ml, 1 mg/ml, 7.8 mm diameter, 500 mm length)

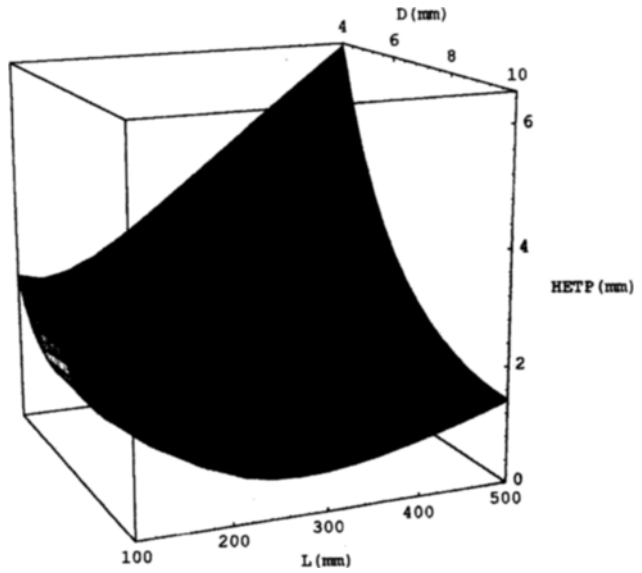

Fig. 10. Response surface of HETP to column diameter and length.
(0.1 ml, 1 mg/ml, 3.5 ml/min, 5 % MeOH)

ameter on column efficiency has not fully been understood yet. For the column packed with preparative packings (40-63 μm) in this experiment, the optimum column diameter and column length are dependent on each other. The sample in the mobile phase was not uniformly distributed in the column of lower diameter packed with larger packings, and in this experiment, an optimum value of HETP existed over the column dimension. Preparative operations are feasible with the larger column diameter. Also, separations in preparative scale can be performed if the same ratio of sample weight to packings contained in the column is maintained and remaining chromatographic parameters are held constant.

CONCLUSION

The experimental variables affecting HETP in this work were selected as sample concentration, injection volume of sample, flow rate of mobile phase, quantity of organic modifier in mobile phase, column diameter, and column length. The larger amounts of sample and higher flow rates of the mobile phase increased HETP, while the content of the organic modifier did not contribute a great change in HETP. The column diameter had an optimum HETP for the preparative packings used in this experiment. The experimental values of HETP might be correlated into the quadratic equation with the interaction terms based on the logarithmic experimental values. Specifically, the effects of diameter and length of column packed with preparative packings were evaluated from the correlation equation.

NOMENCLATURE

HETP, $HETP_c$, $HETP_e$: Height Equivalent to a Theoretical Plate, calculated form Eq. (6), and experimentally measured, respectively [mm]
L : column length [mm]
N : number of theoretical plates from Eq. (2) [−]
u : superficial velocity of mobile phase [cm/sec]
$w_{1/2}$: peak width at the half height [mm]
x_1 : sample concentration [ppm, μg/ml]
x_2 : injection volume of sample [ml]
x_3 : flow rate of mobile phase [ml/min]
x_4 : quantity of organic modifier in mobile phase [vol%]
x_5 : column diameter [mm]
x_6 : column length [mm]
t_R : retention time [mm]

ACKNOWLEDGEMENTS

This work was financially supported by KOSEF (Grant No. 971-1107-048-2) and performed in the High-Purity Separation Laboratory of Inha University (Inchon, Korea).

REFERENCES

Gau, Y. S., Sun, S. W. and Chen, R. R., "Optimization of High-Performance Liquid Chromatographic Separation for Progestogenic, Estrogenic, and Andogenic Steroids Using Factorial Design," *J. Liq. Chrom.*, **18**, 2373 (1995).

Guillaume, Y. and Guinchard, C., "A New Method to Study Column Efficiency and the Separation of Seven Compounds in Reserved-Phase Chromatography," *J. Chromatogr. Sci.*, **33**, 204 (1995a).

Guillaume, Y. and Guinchard, C., "Modelling Retention in Reversed Phase Liquid Chromatography in Relation to Temperature and Solvent Composition. Application to the Separation of Seven ρ-Hydroxybenzoic Esters," *J. Liq. Chrom.*, **18**, 3409 (1996).

Guillaume, Y. and Guinchard, C., "Study and Optimization of Column Efficiency in HPLC: Comparison of Two Methods for Separating Ten Benzoidiazepines," *J. Liq. Chrom.*, **17**, 1443 (1995b).

Guiochon, G., Golshan-Shirazi, S. and Jaulmes, A., "Computer Simulation of the Propagation of a Large-Concentration Band in Liquid Chromatography," *Anal. Chem.*, **60**, 1856 (1988).

Horvath, C. and Melander, W. R., "Liquid Chromatography with Hydrocarbonaceous Bonded Phases: Theory and Practice of Reversed Phase Chromatography," *J. Chrom. Sci.*, **15**, 393 (1977).

Kim, J. D., Row, K. H., So, M. S., Polunina, I. A. and Larin, A. V., "Chromatographic Behaviour of Deoxyribonucleosides with Respect to Organic Modifier Content in the Mobile Phase," *J. Liq. Chromatogr.*, **18**, 3091 (1995).

Kirkland, J. J., "Porous Silica Microspheres for High-Performance Size Exclusion Chromatography," *J. Chromatogr.*, **125**, 231 (1976).

Lee, Y. W., Row, K. H., So, M. S., Polunina, I. A. and Larin, A. V., "Reversed-Phase HPLC Retention of Deoxyribonucleosides as a Function of Mobile Phase Composition," *J. Liq. Chromatogr.*, **18**, 3077 (1995).

Lee, Y. W., So, M. S., Lee, J. W., Chung, S. T. and Row, K. H., "Retention Models of Capacity Factor with Different Com-

positions of Organic Modifier in RP-HPLC," *Korean J. Chem. Eng.*, **13**, 578 (1996).

Row, K. H. and Larin, A. V., "Experimental Study of Influence of Slight Deviation from Adsorption Isotherm Linearity on Elution Peak Profiles," *J. Chem. Eng. Japan*, **28**, 851 (1995).

Snyder, L. R. and Kirkland, J. J., "Introduction to Modern Liquid Chromatography," John Wiley & Sons, Inc., New York (1979).

Valiente, A. and Duprat, F., "Optimization of the Resolution of the Enantiomers of β-Dimethylaminobutyrophenone by HPLC on a β-Cyclodextrin Column," *J. Liq. Chrom.*, **17**, 1709 (1994).