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Note

Evaluation of reversed-phase column performance using *p*-hydroxybenzoate esters and tartrazine in water-enriched solvent

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In the past few years chemically bonded reversed-phase columns have become the most popular columns in liquid chromatography. They have several advantages. The bonded stationary phases are quite stable and insoluble in most solvents. The equilibration of the column with solvent is rapid. The hydrophobic interaction provides good separation for organic compounds. The difficulties with chemically bonded reversed-phase columns as well as other types of column are differences in capacity factor (k') and efficiencies from one column to another and the changing of column performance with the passage of time. Therefore any column should be tested not only when the column is new to see how it is performing with regard to other columns, but also as it is used to ensure its stability so that the data generated at different times can be compared with each other. In other words, the column performance must be checked with standards.

There is quite a large variety of standards which can be chosen to evaluate a column[1–3]. In principle, the selected compounds should be able to reveal the physical and chemical characteristics of the chromatographic system; the selected compounds should be well behaved in the tested column so that reproducible results can be easily obtained; the k' values of these compounds should be spread in the practical k' range (about 2 to 10); the mobile phase required by the standards should be made from commonly used solvents and in typical compositions.

Tartrazine, methyl-*p*-hydroxybenzoate, ethyl-*p*-hydroxybenzoate, propyl-*p*-hydroxybenzoate, and *n*-butyl-*p*-hydroxybenzoate which are dissolved in water-enriched solvent are used as standards in our laboratory. These compounds are well behaved in a reversed-phase column with methanol–water eluents. The mobile phase used to run the test solution is methanol–water (55:45). The k' values of the compounds are well spread in the practical k' range. The physical and chemical characteristics of the chromatographic system divulged from the data generated from these compounds are discussed below.

EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) M-45 solvent delivery system was used with a Valco sample valve and a 10-μl sample loop. The chromatograms were

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monitored by a Gilson (Middleton, WI, U.S.A.) Model HM variable-wavelength UV detector with an 8-μl dead volume detector cell. The Spherisorb 10 μm ODS column (25 cm × 5.0 mm) was obtained from HPLC Technology (Lomita, CA, U.S.A.).

Reagents

The methanol was supplied by MCS Manufacturing Chemists Inc. (Cincinnati, OH, U.S.A.), tartrazine, methyl-*p*-hydroxybenzoate, ethyl-*p*-hydroxybenzoate and propyl-*p*-hydroxybenzoate were from Aldrich (Milwaukee, WI, U.S.A.) *n*-butyl-*p*-hydroxybenzoate was from Sigma (St. Louis, MO, U.S.A.). Potassium nitrate was from J. T. Baker (Pittsburgh, PA, U.S.A.). Polystyrene sulfonate was from Scientific Polymer Products (Ontario, NY, U.S.A.).

Data acquisition system

The chromatographic data were converted into digital form and stored in an LSI-11 microcomputer and then transferred to a DEC-10. The data corresponding to each peak were fit by the Cram–Chesler eight-parameter equation[10,16]. Then this equation was numerically integrated by using Gauss–Legendre polynomials to obtain the retention time and variance of the peaks.

Chromatographic conditions

The solvent was methanol–water (55:45, v/v). The flow-rate was calibrated by buret and stop watch. The temperature was 22°C. The solutes were detected by absorbance at 254 nm.

RESULTS AND DISCUSSION

Before one can properly explore the information in the chromatogram, it is necessary to be clear about the fundamentals of retention. When the solutes traverse the column, they spend time in the flowing mobile phase, in the interstitial space, in the stagnant mobile phase and the stationary phase. The total retention time t_R is the sum of those times:

$$t_R = t_{fm} + t_{sm} + t_s$$

where t_{fm} is the time the solute stays in the flowing mobile phase, t_{sm} is the time the

TABLE I

THE RETENTION TIMES OF SOLUTES WHICH ARE EXCLUDED FROM THE PORES OF THE PACKING

Flow-rate (ml/sec)	t_e *(sec)*	
	Polystyrene sulfonate	*Tartrazine*
0.0485	40.6	40.2
0.0333	58.0	57.0
0.0252	76.4	76.5
0.0168	114	111
0.00836	158	157

solute stays in the stagnant mobile phase and t_s is the time the solute stays in stationary phase. For an unretained solute the retention t_0 is the sum of t_{fm} and t_{sm}:

$$t_0 = t_{fm} + t_{sm}$$

When the solute is excluded from the pores, the retention time of the solute is t_{fm} which is commonly called t_e.

The first peak in the chromatogram of the test mixture is due to tartrazine. This compound is often used to measure the dead volume of the column. However, as indicated by Wells and Clark[4], injection of salts of low electrolyte concentrations in unbuffered eluent leads to the charged solute's being excluded from the pores of the packing, probably due to electrical charges on the stationary phase surface. Thus the retention time of tartrazine in unbuffered eluents is t_e. In Table I, the retention time of tartrazine is compared with the retention time of polystyrene sulfonate (molecular weight *ca.* $6 \cdot 10^6$) which is excluded from the pores due to its large size, at different flow-rates in an unbuffered methanol–water mixture. It is shown that the retention of tartrazine is essentially the same as the retention time of polystyrene sulfonate.

A second small negative peak occurs in the chromatogram of the test mixture, and is due to the injection of water-enriched samples which results in a peak from water. The water peak has been used to measure the dead volume of the column[5]. Because of the small molecular size of water, it undergoes total pore penetration. Therefore, the retention time of the water peak, t_0, and the retention time of tartrazine, t_e, can provide the information of interstitial porosity, ε_z, and the porosity ε_p:

$$\varepsilon_z = V_z/V_k = Ft_e/V_k$$

$$\varepsilon_p = V_{p_{max}}/V_k = F(t_0 - t_e)/V_k$$

where F is the flow-rate, V_z is the interstitial fluid volume, $V_{p_{max}}$ is the total intraparticle fluid volume and V_k is the empty column volume. By this method, the ε_z obtained from our column is 0.38 and ε_p is 0.23.

More precise procedures to measure the dead volume retention time, t_0, have been suggested by McCormick and Karger[6] by injection of a ^2H_2O-enriched sample and by Wells and Clark[4] by injection of sodium nitrate in buffered eluent. Nevertheless, the water peak also gives satisfactory quantitative information on t_0. Moreover, water is the most convenient resource one can obtain in the laboratory. For example, at 1.0 ml/min the value of t_0 obtained from a water peak is 177 sec while t_0 obtained from sodium nitrate in buffered eluent is 173 sec in our laboratory.

After obtaining t_0 and t_e, two important equilibrium distribution ratios can be calculated, the phase capacity factor k' and zone capacity factor k'' (ref. 7), k' is the ratio of the equilibrium quantity of solute in stationary phase to the equilibrium quantity of solute in mobile phase:

$$k' = \frac{t_R - t_0}{t_0}$$

k'' is the ratio of the equilibrium quantity of solute in the stationary zone to the equilibrium quantity of solute in the mobile zone, where the stationary zone consists of stationary phase, stagnant mobile phase and the support phase. The mobile zone is the flowing mobile phase:

$$k'' = \frac{t_R - t_e}{t_e}$$

While k' is the relevant parameter for retention, k'' is the more appropriate parameter for band dispersion. The k' and k'' of the parabens can be used as reference for column to column comparison or for comparison of column performance from time to time[8].

A plot of log k' *vs.* the number of carbons in the alcohol part of the hydroxybenzoate esters yields a straight line. The slope is log $\alpha_{CH_2} \cdot \alpha_{CH_2}$ is called the hydrophobic selectivity. Hydrophobic selectivity has been investigated as a function of organic modifier type, modifier composition and temperature by Karger *et al.*[9]. It is found that hydrophobic selectivity is approximately independent of organic modifier and temperature when retention times are normalized. Therefore the hydrophobic selectivity measured under conditions when the organic modifier or temperature is different from the original test run can be compared with the original hydrophobic selectivity as long as the retention times are normalized. A change of the hydrophobic selectivity would indicate a chemical change of the hydrophobic stationary phase. A change in the intercept on the log k' axis without a corresponding change in slope indicates a change in the phase ratio. Moreover hydrophobic selectivity is the retention increment of one methylene group. Therefore it can give some hints to predict the retention of organic compounds. The α_{CH_2} value obtained from our column is 1.56.

The band dispersion is related to the efficiency of the column. The band dispersion can be expressed by the peak variance $\sigma^2_{t,tot}$ in time units. The precise value of $\sigma^2_{t,tot}$ can be obtained by a fit of certain functions to the data and calculating the second central moment with the aid of a computer. However, if the peaks are symmetrical, the $\sigma^2_{t,tot}$ is commonly expressed by the following approximation where t_w is the baseline width:

$$\sigma^2_{t,tot} = \frac{t_w^2}{16}$$

The data from the paraben peaks were fit by the Cram–Chesler equation[10,16] and the first moment and the second central moments were calculated to obtain t_R and $\sigma_{t,tot}$. These values are shown in Table II. The total dispersion consists of column dispersion and extra-column dispersion. Therefore, the total variance of the peak is the sum of the variance due to on column band broadening and the variance due to extra-column band broadening

$$\sigma^2_{t,tot} = \sigma^2_{t,col} + \sigma^2_{t,ext}$$

The variance of extra-column band broadening was evaluated by the method described by Kutner *et al.*[11]. The variance of the peaks $\sigma^2_{t,tot}$ and the square of the retention time t_R^2 are input into a linear least squares fit program. $\sigma^2_{t,ext}$ is obtained

TABLE II

RETENTION AND BAND BROADENING DATA

	Methyl-p-hydroxy-benzoate	*Ethyl-p-hydroxy-benzoate*	*Propyl-p-hydroxy-benzoate*	*n-Butyl-p-hydroxy-benzoate*
t_R(sec)	391	548	862	1479
$\sigma^2_{t,tot}$ (sec^2)	62.3	113	238	713
$\sigma^2_{t,col}$ (sec^2)	51.0	102	227	702
$\sigma^2_{t,ext}/\sigma^2_{t,col}$ (%)	22.2	11.1	5.0	1.6
k'	2.2	3.1	4.9	8.4
k''	3.5	4.9	7.8	13.3
A	3.20	3.69	3.89	3.62
C	0.077	0.081	0.080	0.080

from the intercept of $\sigma^2_{t,tot}$ at $t_R^2 = 0$. Due to the linear relationship between $\sigma^2_{t,tot}$ and t_R^2 of the parabens, one is able to determine $\sigma^2_{t,col}$ and $\sigma^2_{t,ext}$. The $\sigma^2_{t,ext}$ calculated from our paraben peaks is 11.3 $\pm$ 6.5 (one standard deviation) sec^2 with a linear correlation coefficient > 0.999. The precision of $\sigma^2_{t,ext}$ is increased by increasing the number of runs. The $\sigma^2_{t,ext}$ obtained from an average of 3 runs is 16.6 $\pm$ 1.4 sec^2. After subtracting $\sigma^2_{t,ext}$ from $\sigma^2_{t,tot}$, the variance of the column band broadening, $\sigma^2_{t,col}$ is obtained. The ratio of $\sigma^2_{t,ext}/\sigma^2_{t,col}$ indicates how significantly the extra-column broadening effect affects separation. As shown in Table II, extra-column band broadening has a significant effect on short retention compounds like methyl-*p*-hydroxybenzoate. Therefore, if one obtains a high value of $\sigma^2_{t,ext}/\sigma^2_{t,col}$ for the desired compounds which need to be separated, it is important to reduce extra column band broadening. The decrease of the extra-column band broadening effect not only improves the separation but also increases the sensitivity of detection.

The efficiency of the column is commonly expressed in terms of the theoretical plate height (H) of the column:

$$H = \frac{L\sigma^2_{t,col}}{t_R^2}$$

(where L = column length). A large value of H indicates poor efficiency of the column. Poor efficiency of a column can be due to significant flow dispersion or slow mass transfer in the column.

The performance of the column can be evaluated in dimensionless terms introduced by Bristow and Knox[12]. It is called separation impedance, E,

$$E = h^2\varphi$$

where h is the reduced plate height, and φ is the column flow resistance factor:

$$h = \frac{H}{d_p}$$

$$\varphi = \frac{\Delta P\, t_0 d_p^2}{\eta\, L^2}$$

where ΔP is the pressure difference across the column, η is the viscosity of the eluent, and d_p is the average particle diameter. The separation impedance is a good parameter to use to compare the performance of columns. The lower the E value the better is the performance of the column. If the E value is high because φ is high, one suspects a blockage in the system or the packing contains an undue portion of fines. If E is high because h is high, it can be due to a slow mass transfer rate or significant flow dispersion. To distinguish between the latter two effects on h one has to obtain H_{col} at different flow-rates and fit the Van Deemter equation[13–15] to the data.

$$H_{col} = \frac{B'}{\mu_e} + A' + C'\mu_e$$

where μ_e is the interstitial linear velocity. The plate height equation is best given in the form of reduced plate height h and reduced velocity v. The Van Deemter equation can be rewritten as follows:

$$h_{col} = \frac{B}{v} + A + Cv$$

where

$$B = \frac{B'}{D_m}$$

$$A = \frac{A'}{d_p}$$

$$C = C'\frac{D_m}{d_p^2}$$

$$v = \frac{\mu_e d_p}{D_m}$$

D_m is the diffusion coefficient of solute in the solvent. The A term represents the flow dispersion. The Cv term represents the mass transfer terms. B/v is the axial diffusion term. The axial diffusion term is usually small and the B value is known experimentally[7] and theoretically[13] to be about 2 for porous material. By setting $B = 2$, the A and C values for parabens are given in Table II. For a reasonably well performing column, A is about 1 and C is about 0.1 (refs. 7 and 14). A larger value of A indicates that the column is poorly packed. A larger value of C indicates the presence of slow mass transfer between mobile zone and the stationary zone or slow adsorption–desorption kinetics. The A value obtained from the parabens (Table II) indicates that our column is not extremely well packed. The C value indicates fast mass transfer between mobile zone and stationary zone and fast kinetics for the paraben compounds. The C value of the parabens can be used as a reference to compare with other compounds. It the compound has about the same molecular size as the parabens and gives a large C value, it indicates very slow kinetics for that compound. Both the A

and C values can also be used as a reference to investigate the performance of the column from time to time. A dramatic change of the A value indicates the mechanical damage of the packed bed. A change of the C value indicates the change of the adsorption–desorption kinetics or pore structure.

CONCLUSION

The discussion above illustrates that by the injection of paraben standards at just one flow-rate (which takes less than 30 min) one can obtain quite a lot of information about the properties of the column. They are the interstitial porosity ε_{i}, the porosity ε_p, the phase capacity factor k', the zone capacity factor k'', the variance due to extra-column band broadening $\sigma_{t,ext}$, the theoretical plate height H, changes in phase ratio, the hydrophobic selectivity α_{CH_2}, the column flow resitance factor φ, and the separation impedance E. These properties are adequate to describe the column performance. Thus, the performance of a column can be compared from time to time or column to column. The data also can be used as a reference for optimization of the separation. Moreover, by injecting paraben standards at different flow-rates, one can obtain the flow-dispersion term, A, and mass transfer term, C, of the Van Deemter equation[13–15]. These two values can be used to probe the cause of band broadening.

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