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Characterization of a novel diol column for high-performance liquid chromatography

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Abstract

For the investigation of a diol phase (Inertsil Diol column) in hydrophilic interaction chromatography, urea, sucrose and glycine were used as test compounds. The chromatographic conditions were investigated for optimal column efficiency. The column temperature used in common reversed-phase liquid chromatography could also be used for the separation and the flow-rate should be adjusted to 0.3–0.5 ml/min to optimize column efficiency. It is suggested that the velocity of the hydrophilic interaction is slower than the hydrophobic interaction in RPLC. The addition of trifluoroacetic acid is effective for the retention of glycine, but ineffective for urea and sucrose. The diol phase exhibited sufficient chemical stability even if exposed to water in high percentage, and could be applied with isocratic elution for the separation/analysis of amino acids and glucose.

Keywords: Stationary phases, LC; Hydrophilic interaction chromatography; Amino acids; Glucose; Sugars

1. Introduction

Reversed-phase liquid chromatography (RPLC) is currently the most popular method in the field of high-performance liquid chromatography (HPLC). The commonly employed mobile phases for RPLC are different from those used for normal-phase liquid chromatography (NPLC). Typically, non-polar solvents such as hexane and chloroform are used for NPLC and polar solvents such as methanol and water are used for RPLC. Consequently, the elution orders present a striking contrast when a mixture consisting of compounds with various chemical properties is separated in the NPLC and RPLC modes.

Hydrophilic interaction chromatography (HILIC) was investigated as a new chromatographic concept by Alpert [1]. The retention mechanism in HILIC has not been definitely established due to the fact that complex interactions occur between the polar solute, solvent molecules of the mobile phase, the polar stationary phase and residual silanol on silica gel.

Many applications of HILIC have been reported, including the separation and analysis of polar pharmaceuticals [2], cosmetic samples [3], peptides [4–6], and carbohydrates with a diol phase [7–9].

Although most columns for carbohydrates are based on an amino acid-modified stationary phase since the first application to the analysis of a sugar mixture [10,11], some reports [7] relating to the separation of carbohydrates with a diol phase indicated high recovery and reproducibility. For the determination of sugar, refractive index detection has been widely used for non-UV-absorbing compounds. Lafosse et al. [8] introduced an evaporative light-

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scattering detector to the HPLC system where the separation and analysis of sugars were realized by gradient elution. Herbreteau et al. [9] used a mobile phase consisting of dichloromethane and methanol to separate raw sugars and polyols with both a polar stationary phase and a diol phase.

In this study, a new diol phase was investigated for optimizing HILIC separation/analysis and characterization. Several applications are also presented to demonstrate the utility of this column.

2. Experimental

2.1. Apparatus

The HPLC system consisted of a PU-610 pump, a CO-630 column oven, a UV-620 variable-wavelength UV detector (GL Sciences, Tokyo, Japan), a Shodex RI-74 refractive index (RI) detector (Showa Denko, Tokyo, Japan), and a Triathlon autoinjector (Spark Holland, Amsterdam, Netherlands). The newly developed diol column was Inertsil Diol (pore diameter, 100 Å; surface area, 430 m^2/g; pore volume, 1.01 ml/g; particle size, 5.1 μm; percent carbon, 20%) (GL Sciences). The column size was 150 mm×4.6 mm I.D.

2.2. Reagents

HPLC-grade acetonitrile was from Kishida (Osaka, Japan). Trifluoroacetic acid and triethylamine were used as additives to the mobile phase, and were obtained from Kishida. Urea, sucrose, glycine, glucose and all amino acids were from Sigma (St. Louis, MO, USA). Analytical-grade benzene was used as analyte for the chemical stability test of the diol phase.

2.3. Chromatographic conditions

The test sample, dissolved in acetonitrile–water (50:50, v/v), consisted of urea, sucrose and glycine at concentrations of 2.06, 2.21, and 2.07 mg/ml, respectively. To avoid any influence of baseline fluctuations of RI detection on the peak areas, the concentration used in the test was comparatively high. The injection volume was 5 μl.

A honey sample dissolved in acetonitrile–water (50:50, v/v) was filtered through a 0.25 μm filter, and a 10 μl sample was injected.

The void time was determined from the time of the first deviation from the baseline.

3. Results and discussion

3.1. Chromatographic properties of the diol phase in HILIC

In order to optimize the chromatographic performance of the diol phase, urea, sucrose and glycine were used as test compounds. The compounds have a high water solubility, and never remain on the stationary phase in RPLC. Although the anomeric separation of monosaccharides on the diol phase results in a complicated chromatogram, sucrose exhibited a symmetrical peak and sufficient retention.

3.1.1. Effect of acetonitrile content on the retention factor k

Fig. 1 shows that the retention factor *k* of the test compounds increases with increasing MeCN concentration in the mobile phase. This is behavior opposite to that in RPLC. Perhaps the behavior is due to the solubility of the test compounds in the

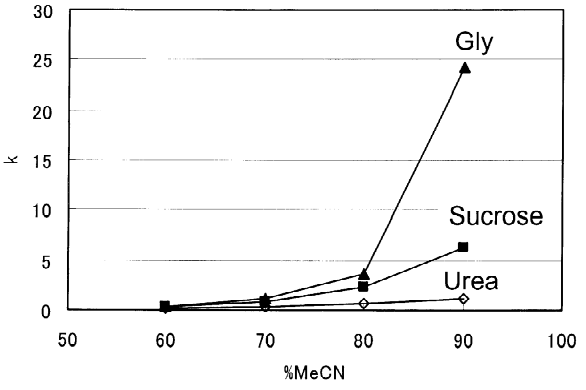

Fig. 1. Relationship between retention factor and acetonitrile concentration. Column, Inertsil Diol 150×4.6 mm I.D., 5 μm; mobile phase, acetonitrile–water (w/w); flow-rate, 1.0 ml/min; column temperature, 40 °C; detection, RI detector.

stationary phase increasing with decreasing water concentration in the mobile phase.

3.1.2. Effect of column temperature

In chromatography, the k value is strongly dependent on the column temperature. Generally, an increase in column temperature can lead to a decrease in the value of k because of the increase in the solubility of the analyte in the mobile phase. When using the diol column, the typical relationship between the k value and the column temperature is shown in Fig. 2a where a higher separation temperature results in reduced sample retention, especially for sucrose.

Increasing the column temperature is desirable for increased column efficiency because mass transfer of the solute increases with decreasing viscosity of the mobile phase. Plates number were measured at various temperatures (Fig. 2b). The maximum column efficiency is achieved at the minimum point of the plate height curve where the optimal temperature can be obtained. The optimal temperatures for urea, sucrose and glycine were 40, 50 and 60 °C, respectively. Urea exhibited the lowest temperature of 40 °C, and at temperatures higher than 40 °C, the plate height increased with increasing column temperature. The reason for this is that a higher temperature results in a lower retention and relatively increased extra column dispersion. Furthermore, the plate height for glycine decreased with increasing temperature due to decreased resistance to mass transfer in the stationary phase.

3.1.3. Optimizing the flow-rate for maximum column efficiency

According to the van Deemter equation, the flow-rate of the mobile phase directly effects the column efficiency. In the case of an ODS column with 4.6 mm inner diameter, a flow-rate of 1.0 ml/min or less is usually used to obtain the optimum plate number. The new diol column, however, exhibited different features, as shown in Fig. 3. Over a range of 30 to 60 °C separation temperature, the optimum flow-rate appeared at 0.3 ml/min. This suggests that the velocity of the distribution between stationary and mobile phases is dominated by stationary phase mass transfer in the separation process using the diol column. Another important result was that the optimal flow-rate was independent of the column temperature. The experimental results show that the

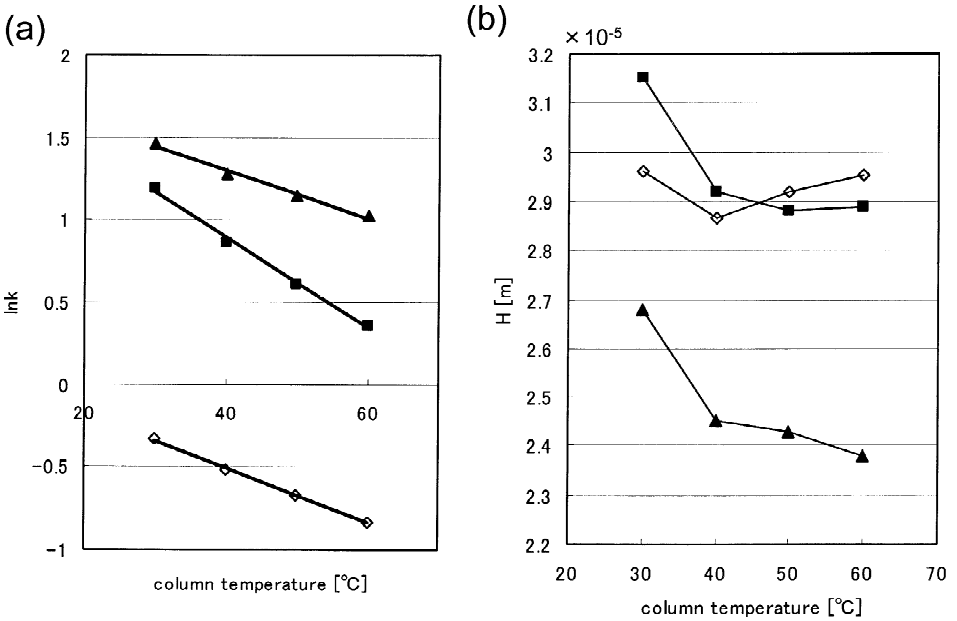

Fig. 2. Effect of column temperature (a) on ln k and (b) on plate height (H). Mobile phase, acetonitrile–water (80:20, w/w); flow rate, 1.0 ml/min; detection, RI detector. (◊) Urea, (■) sucrose, (▲) glycine.

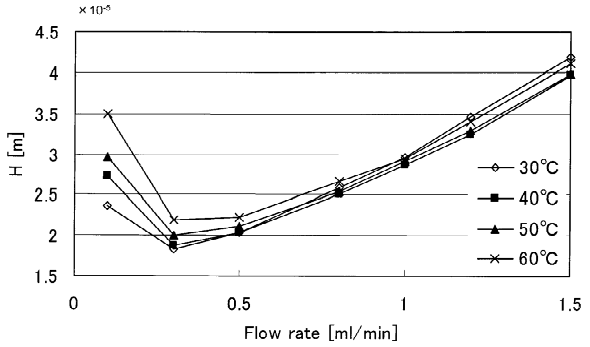

Fig. 3. Plate height (H) dependence on the flow-rate. Column, Inertsil Diol 150×4.6 mm I.D., 5 μm; mobile phase, acetonitrile–water (80:20, w/w); detection, RI detector; sample, sucrose.

plate height is smaller for lower separation temperatures.

3.1.4. Addition of trifluoroacetic acid to the mobile phase

Additives to the mobile phase are effective with respect to the retention time in HILIC. There is, however, a problem with precipitation due to the reduced solubility in a high concentration of MeCN when a buffered mobile phase is used. Therefore, trifluoroacetic acid (TFA), which is soluble in MeCN, was used as an additive, and the effect on retention time was investigated.

As shown in Fig. 4, little change in retention time was observed for urea and sucrose; however, glycine

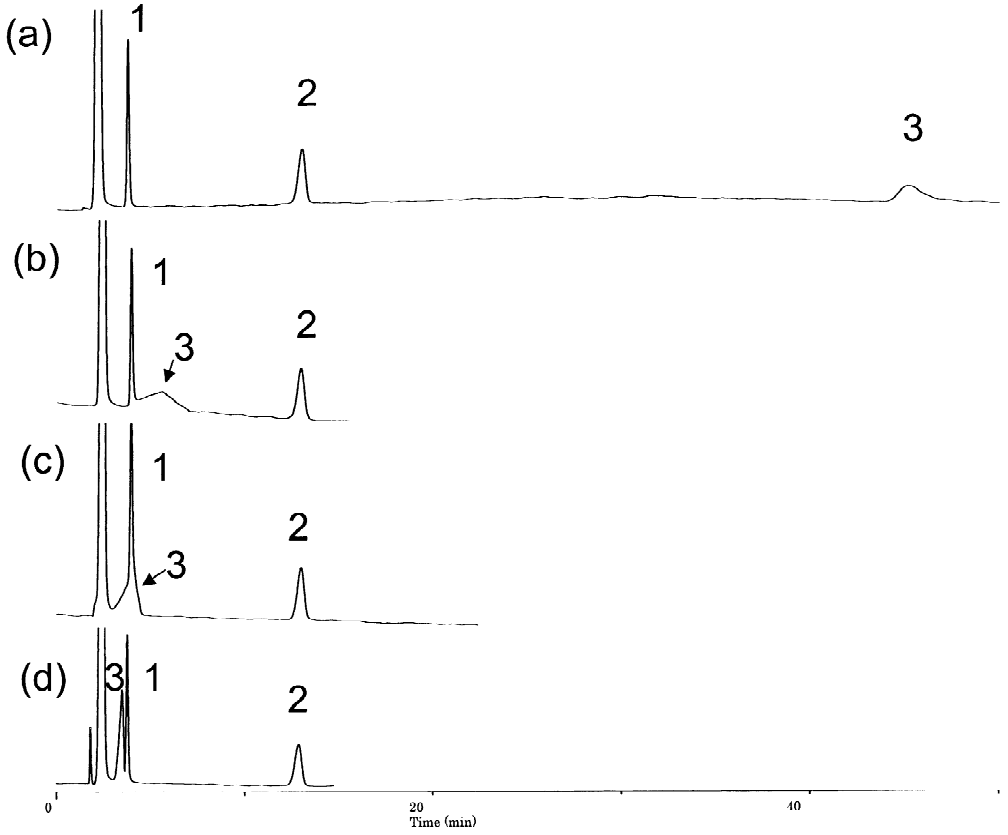

Fig. 4. Chromatograms obtained with different TFA concentrations: (a) no additive, (b) 0.001%, (c) 0.01% and (d) 0.1%. Column, Inertsil Diol 150×4.6 mm I.D., 5 μm; mobile phase, acetonitrile–water (90:10, w/w); detection, RI detector; flow-rate, 1.0 ml/min; column temperature, 40 °C. Peaks: 1=urea, 2=sucrose, 3=glycine.

was influenced significantly by TFA, and 0.001% TFA lead to a shorter retention time and a broader peak. When 0.1% TFA was used, glycine was eluted faster than urea. Once the mobile phase containing 0.1% TFA was delivered, the column required washing for at least 10 h to resume chromatographic performance.

3.1.5. Chemical stability of the diol phase

Loss of the bonded phase can be caused by hydration. There have been many attempts to enhance the stability of the stationary phase for RPLC. The purpose of these studies was to eliminate water from group bonding on the silica gel surface. Successful examples are a high carbon content [12] and a sterically protected bonded phase [13]. Hydrophilic phases such as a diol phase and an amino phase are readily degraded by water.

For HILIC, the diol phase is stable in mobile phases with a high concentration of MeCN, and degradation would occur after a few months. Inertsil diol, therefore, was tested in the reversed-phase mode with a mobile phase with a high concentration of water.

Fig. 5 shows the chemical stability test for the diol phase. Benzene was used as an analyte that could be retained on the propyl chain of the diol group. The mobile phase used was 25 m*M* phosphate buffer (pH 3.2)–MeCN (98:2, w/w). A low buffer pH is not desirable for the stationary phase because it readily causes hydrolysis.

The retention time of benzene would be shortened if a loss of the bonded phase occurred. No decrease in retention time or plate number was observed, even if 8000 ml of mobile phase flowed through the column.

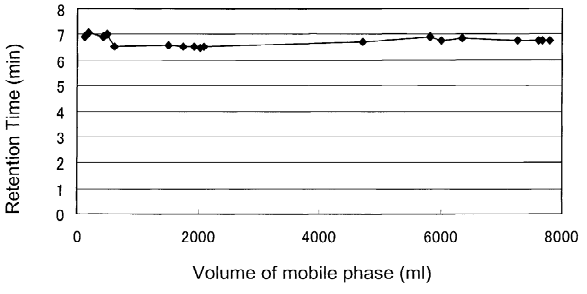

Fig. 5. Chemical stability of the Inertsil Diol phase. Mobile phase, acetonitrile–25 m*M* phosphate buffer (pH 3.2) (2:98, w/w); flow-rate, 1.0 ml/min; column temperature, 40 °C; sample, benzene; detection, UV at 254 nm.

3.2. Application

3.2.1. Analysis of amino acids

The diol column was applied to the separation/analysis of amino acids in the isocratic elution mode. Retention times of amino acids are shown in Table 1. All amino acids, except basic ones, could be separated under conditions of 80–90% MeCN. With more than 90% MeCN, peak shapes were greatly broadened. Basic amino acids could be separated under lower concentrations (40–60%) of acetonitrile. This means that the diol phase has hydrophilic features. In the chromatogram of an amino acid mixture, shown in Fig. 6, the elution order, on the whole, was reversed as compared with using an ODS column with the ion-paring method.

The retention times of acidic amino acids were short and peak shapes were generally broad. Peaks disappeared when applying more than 80% acetonitrile. We assume that the hydrogen bond between the hydroxyl group in the stationary phase and the carboxyl group greatly influences the behavior of acidic amino acids.

3.2.2. Analysis of glucose in a honey sample

The amino phase is, in general, popular for sugar analysis in HPLC. This phase has two shortcomings. One is a short column lifetime due to the fact that silica gel can dissolve in a high-pH solution, and the other is the formation of Schiff bases between the amino groups and the sugar or other carbonyl compounds. A diol phase, however, can overcome these problems, although retention is slightly weaker than on an amino phase. Glucose in a honey sample was quantified using the diol column.

The chromatogram of the honey sample obtained using the diol column is shown in Fig. 7. A calibration curve in the range 1.01–10.1 mg/ml was obtained with good linearity (correlation coefficient 0.999).

The use of a diol phase for sugar analysis results in a complicated chromatogram due to anomeric separation. To solve this problem, triethylamine was added to the mobile phase at a concentration of

Table 1
Retention times of amino acids obtained with the Inertsil Diol column

Amino acid	MeCN (%, in water)							
	30	40	50	60	70	80	85	90
Gly				2.8	3.7	6.4	11.1	26.4
Ala				2.7	3.5	5.7	9.2	20.1
Val				2.5	3.1	4.6	6.7	12.2
Leu				2.4	2.9	4.1	5.7	10.3
Ile				2.5	2.9	4.2	5.9	10.8
Ser				2.6	3.6	6.4	10.8	25.1
Thr				2.5	3.4	5.7	8.9	19.2
Phe				2.4	2.5	4.0	5.6	10.5
Tyr				2.5	3.1	4.8	7.3	15.8
Trp				2.5	3.0	4.4	6.4	13.2
Asp				1.5	1.8	–	–	–
Glu				1.8	2.2	–	–	–
Asn				2.6	3.5	6.4	10.8	26.6
Gln				2.6	3.5	6.5	11.1	26.5
Met				2.5	3.0	4.4	6.2	12.0
Lys	9.1	11.1	16.3	23.6				
Arg	10.1	12.2	17.7	32.6				

Column size, 150×4.6 mm I.D., 5 μm; column temperature, 40 °C; flow-rate, 1.0 ml/min.

0.1%. The addition of an amine can enhance the mutarotation rate and yield a non-anomeric separation [7]. Although a basic additive is undesirable for the silica gel, repeated injection did not lead to column degradation, as shown in Table 2.

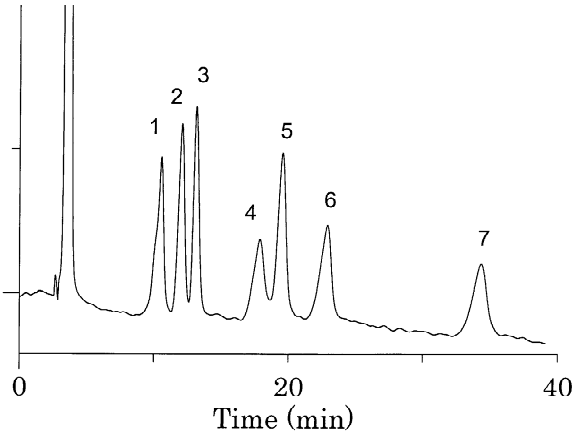

Fig. 6. Chromatogram of an amino acid mixture. Column, Inertsil Diol 150×4.6 mm I.D., 5 μm; mobile phase, acetonitrile–water (85:15, w/w); flow-rate, 0.6 ml/min; detection, RI detector; column temperature, 40 °C. Peaks: 1=Phe, 2=Met, 3=Val, 4=Thr, 5=Ala, 6=Ser, 7=citrulline.

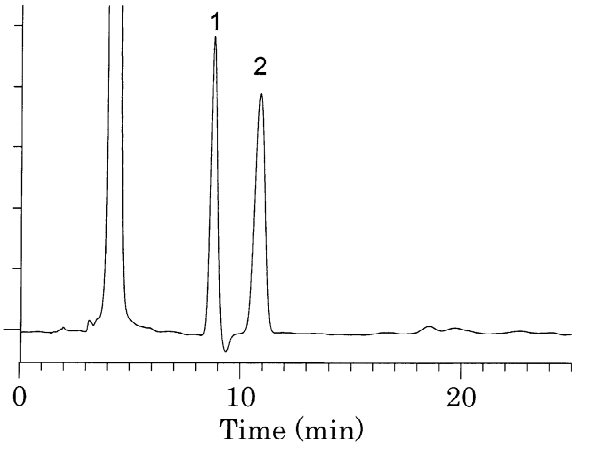

Fig. 7. Chromatogram of a honey sample. Column size, 150×4.6 mm I.D., 5 μm; mobile phase, acetonitrile–water (85:15, w/w) with 0.1% triethylamine; flow-rate, 0.5 ml/min; detection, RI detector; column temperature, 40 °C. Peaks: 1=fructose, 2=glucose.

Table 2
Reproducibility for the quantity of glucose in a honey sample

	RSD[a] (%)
Peak area	0.92
Retention time	0.65

[a] $n=80$.

4. Conclusions

Column temperature and flow-rate were investigated to optimize the column efficiency for the diol phase in HILIC. The column temperature used in common RPLC could also be used for separation based on the diol column. Flow-rates of 0.3–0.5 ml/min gave optimal column efficiency. This suggests that the velocity of the distribution between the stationary and mobile phases is dominated by stationary phase mass transfer in the separation process based on the diol column. Furthermore, the diol phase could be applied to the separation/analysis of amino acids and glucose using the isocratic elution method.

For most hydrophilic compounds, reversed-phase separation with an ODS column is used with a low concentration of organic solvent in the mobile phase. There are problems, however, with reproducibility and column degradation. HILIC with the diol phase is capable of solving these problems.

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