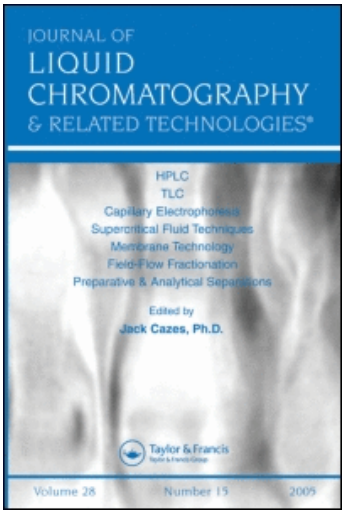

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Liquid Chromatographic Separation of Polar Organic Compounds Using Strong Anion-Exchanger as the Stationary Phase and Pure Water as the Mobile Phase

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LIQUID CHROMATOGRAPHIC SEPARATION OF POLAR ORGANIC COMPOUNDS USING STRONG ANION-EXCHANGER AS THE STATIONARY PHASE AND PURE WATER AS THE MOBILE PHASE

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ABSTRACT

A unique high performance liquid chromatographic system for the separation of polar organic compounds is described. Strong anion-exchanger was used as the stationary phase and pure water was used as the mobile phase. When the strong anion-exchanger was conditioned with pure water as the mobile phase, the fixed ionic site with its counterion functioned as a zwitterionic stationary phase.

This facilitated the separation of the polar organic compounds without need for ionization of the analytes. A strong commercial anion-exchanger (polyacrylate-based type) and a laboratory-made one (ODS-based type; obtained by dynamically coating hexadecyltrimethylammonium salts on the reversed-phase ODS surfaces) were chosen to represent the stationary phases. Five purine and pyrimidine bases and eight nucleosides were chosen to represent the polar organic analytes. All of these polar organic analytes were successfully separated and rapidly eluted with pure water as the mobile phase. Separation mechanisms involved were investigated and the experimental results suggested that electrostatic interaction was the main mechanism for separation in polyacrylate-based stationary phase; while, electrostatic and hydrophobic interactions were dual mechanisms for separation in ODS-based stationary phase.

INTRODUCTION

Ion-exchangers are widely used as stationary phases for high performance liquid chromatographic (HPLC) separations. Ions are the main targets (analytes) for separation where the ion-exchange model or the ion-exclusion model is used. An ion-exchanger normally consists of three elements; the solid particle, the fixed ionic site, and the counterion.[1] In the ion-exchange model, the fixed ionic site is used for attaching the analytes to the stationary phase and the counterion is used to release the analytes from the stationary phase. In this case, the mobile phase used must contain the same ion species as the counterion. In the ion-exclusion model, both the fixed ionic site and the counterion are used to selectively attract the analytes coming to the stationary phase. In this case, pure water can be used as the mobile phase. However, studies by Turkelson and Richard on ion-exclusion HPLC of organic acids[2] and Dumont and Fritz on ion-exclusion HPLC of polar organic compounds[3] have shown that the targeted analytes were strongly retained when only water was used as the mobile phase. To obtain a feasible retention for the targeted analytes, Dumont and Fritz[3] suggested using very short columns. However, this will inhibit the separation efficiency of ion-exclusion HPLC. Only for ion-exclusion HPLC of carbonate ions[4,5] is pure water mobile phase used effectively.

Only strong cation-exchangers in hydrogen form are used as the stationary phase for ion-exclusion HPLC.[5] Possibilities of using strong anion-exchangers as the stationary phase with pure water as the mobile phase for HPLC

separation have not previously been investigated. In previous studies, we used strong-positively/strong-negatively charged zwitterionic surfactants as the stationary phase for HPLC. The experimental results obtained showed that both the inorganic ions[6,7] and the polar organic compounds[8] could be successfully separated with pure water as the mobile phase. For this type of stationary phase, both the strong positively charged site (quaternary ammonium) and the strong negatively charged site (sulfonate) were fixed to the solid particles.

If, for the strong anion-exchanger, a strong anionic species is used as the counterion with pure water as the mobile phase, the counterion will remain in the column and with the fixed ionic site will function as a strong-positively/strong-negatively charged zwitterionic stationary phase, even if a slight displacement of the counterion is produced by washing power and/or the polar nature of water.

A polyacrylate-based and an ODS-based strong anion-exchanger were chosen to represent typical stationary phases. Nucleosides and their bases were chosen to represent the polar organic analytes. These polar analytes were successfully separated using either of these strong anion-exchanger as the stationary phase with pure water as the mobile phase. The elution orders and the separation efficiencies for these analytes, when ODS-based strong anion-exchangers was used as the stationary phase, were very similar to those observed when using the bonafide strong-positively/strong-negatively charged zwitterionic surfactants as the stationary phase. However, they were different when the polyacrylate-based strong anion-exchanger was used as the stationary phase.

Experiments designed to investigate and explain separation mechanisms are described in this paper.

EXPERIMENTAL

Apparatus

The HPLC system used throughout this study was a Shimadzu (Kyoto, Japan) LC-10A system. It consisted of a LC-10AT pump, a sample injector (Cotati, California, USA) with a 100 μL injection loop, a CTO-10A column oven (the temperature was set as 25°C), a SPD-10A UV-vis detector, and a C-R6A Chromatopac.

Reagents

Adenosine, deoxyadenosine, guanosine, deoxyguanosine, cytidine, deoxycytidine, thymidine, and uridine (nucleosides) were obtained from Sigma (St. Louis, MO, USA). Adenine, guanine, cytosine, thymine, and uracil (bases) were obtained from Tokyo Chemical (Tokyo, Japan). These compounds dissolved in pure water were used as standard samples. Pure water used throughout this study was produced in the laboratory using a Milli-Q purification system (Millipore, Bedford, MA, USA). Pure water used as the mobile phase was stored in a clear polypropylene bottle (wall thickness, 0.7 mm, volume, 1000 mL).

The anions of sodium chloride, potassium phosphate (monobasic and dibasic), sodium sulfate, *p*-hydroxybenzoic acid, sodium taurodeoxycholate, and sodium dodecylsulfate, used as the counterions of the fixed ionic sites, were obtained from Wako (Osaka, Japan). Hexadecyltrimethylammonium chloride used for the laboratory-preparation of strong anion-exchangers was also obtained from Wako.

Column Preparations

In this study, two type of columns were used. One was a commercial strong anion-exchange column, Shim-pack IC-3A (150 × 4.6 mm i.d.; a polyacrylate-based resin with quaternary ammonium as the fixed ionic sites) obtained from Shimazu. The other was prepared in the laboratory by dynamic modification of an ODS-packed column (L-Column, 250 × 4.6 mm i.d.; Chemical Inspection & Testing Institute, Tokyo, Japan) with hexadecyltrimethylammonium chloride.

In this procedure, an aqueous solution containing 0.1 M hexadecyltrimethylammonium chloride was passed through the ODS-packed column for 60 min at a flow rate of 1.0 mL/min. This was then rinsed with pure water for one night (ca. 9 hours) at the same flow rate of 1.0 mL/min. Sulfate, chloride, phosphate, *p*-hydroxybenzoate, tauro-deoxycholate, and dodecylsulfate were used as the counterions.

In this procedure, an aqueous solution containing 10 mM of the anion (salt) was passed through the column for two hours as a flow rate of 1.0 mL/min. Pure water was then used as the mobile phase at the same flow rate for one night. These columns, with pure water as the mobile phase, were used for the analyte separations.

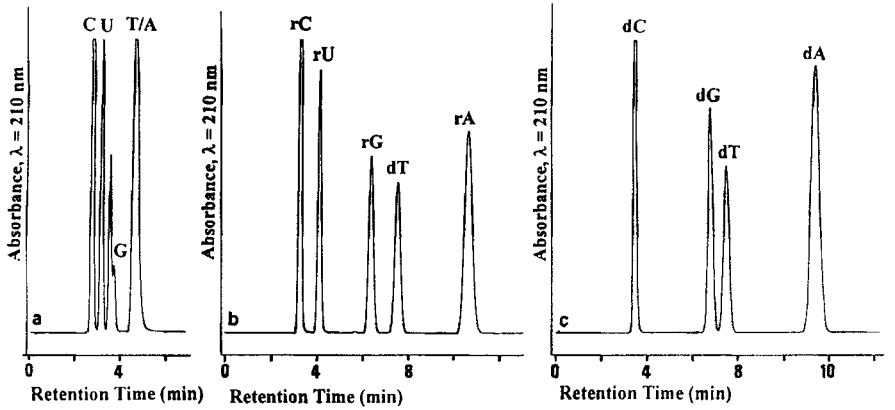

Figure 1. Chromatograms of bases (a), ribonucleosides and thymidine (b), and deoxyribonucleosides (c) obtained using ODS-based strong anion-exchanger as the stationary phase with pure water as the mobile phase. Column, ODS-packed column (250 × 4.6 mm i.d.) modified with hexadecyltrimethylammonium with sulfate as the counterion; mobile phase, pure water; flow rate, 1.0 mL/min; sample injection volume, 100 µl; analyte concentrations, 0.1 mM for each species (except for guanine which was prepared at its saturated concentration, because its solubility is very low); detection, UV-vis at 210 nm.

RESULTS AND DISCUSSION

Using Sulfate as the Counterion

Sulfate was chosen to represent the strong anions used as the counterion of the strong anion-exchangers (quaternary ammonium). Adenine (A), guanine (G), cytosine (C), thymine (T), and uracil (U); adenosine (rA), deoxyadenosine (dA), guanosine (rG), deoxyguanosine (dG), cytidine (rC), deoxycytidine (dC), thymidine (dT), and uridine (rU) were dissolved in pure water and analyzed. Typical chromatograms of the mixtures of the bases, ribonucleosides, and the deoxyribonucleosides are shown Figures 1 and 2.

Figure 1 shows the results obtained using the ODS-based strong anion-exchanger as the stationary phase; Figure 2 shows the results obtained using the polyacrylate-based strong anion-exchanger as the stationary phase.

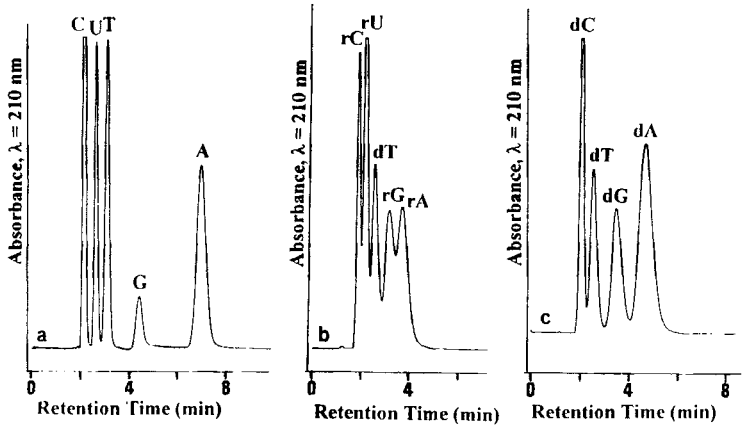

Figure 2. Chromatograms of bases (a), ribonucleosides and thymidine (b), and deoxyribonucleosides (c) obtained using polyacrylate-based strong anion-exchanger as the stationary phase with pure water as the mobile phase. Column, Shim-packed IC-3A (150 × 4.6 mm i.d.) with sulfate as the counterion. Other conditions were same as described in Figure 1.

When the ODS-based stationary phase was used, base-line separations for the bases (with the exception of A and T, which were coeluted), ribonucleosides and deoxyribonucleosides were always obtained. The elution orders were C > U > G> T = A for the bases; and rC > rU > rG > rA for the ribonucleosides; and dC > dG > dT > dA for the deoxyribonucleosides.

For each group (A, rA, dA; G, rG, dG; C, rC, dC; T, dT; and U, rU) of compounds, the base was the first eluted, followed by the ribonucleoside, and then the deoxyribonucleoside.

Base-line separations for the bases were achieved, when the polyacrylate-based stationary phase was used, but base-line separations for the ribonucleosides and the deoxyribonucleosides were not achieved. The elution orders were C > U > T > G > A for the bases; and rC > rU > rG > rA for the ribonucleosides; and dC > dT > dG > dA for the deoxyribonucleosides. For each group of compounds, the ribonucleosie was the first eluted, followed by the deoxyribonucleoside, and then the base. A small peak which appeared at "void time" was always observed when the polyacrylate-based stationary phase was used. However, this peak was never observed when the ODS-based stationary phase was used.

Table 1

Effects of Counterions on the Retention Time (min) of the Analytes*

Analyte	Cl^-	SO_4^{2-}	*p*-HOB	TDC	DS	Cl^-	SO_4^{2-}
A	5.89	6.91	6.99	6.69	6.17	4.88	4.79
G	4.14	4.21	5.23	4.87	4.00	3.95	3.59
C	2.00	2.21	2.30	2.20	2.19	2.72	2.76
T	3.06	3.18	3.49	3.20	3.01	4.97	4.67
U	2.59	2.63	3.14	2.84	2.44	3.55	3.13
rA	4.01	3.75	3.71	3.72	3.99	9.53	10.58
rG	3.47	2.86	3.16	3.22	2.79	7.25	6.31
rC	2.17	1.88	1.85	1.88	1.86	3.15	3.25
rU	2.43	2.01	2.16	2.16	2.09	4.40	4.07
dA	4.85	4.60	4.58	4.59	4.34	10.83	11.36
dG	3.30	3.42	3.51	3.75	3.07	7.71	6.78
dC	2.30	2.02	2.08	2.02	2.03	3.43	3.48
dT	2.07	2.50	2.54	2.53	2.64	7.78	7.47

* Average of three measurements, deviations were found to be less than 1%. Chloride (Cl^-), sulfate (SO_4^{2-}), *p*-hydroxybenzoate (*p*-HOB), taurodeoxycholate (TDC), and dodecylsulfate (DS). The results shown in the last two columns (right-side) were obtained using the ODS-based stationary phase, the others were obtained using polyacrylate-based stationary phase. Other HPLC onditions were the same as described in Figure 1.

Mechanism Investigation

Hydrophobic and electrostatic properties of the stationary phase (counterion, fixed ionic site, and solid particle) and analytes determine the separation mechanism. The following experiments were designed to investigate these properties and their effects on the separation of the polar organic compounds.

To investigate the counterion further, anions with strong variation in size and hydrophobilities were also used. These were chloride, *p*-hydroxybenzoate (p-HOP), taurodeoxycholate (TDC), and dodecylsulfate (DS). The results of the separation of these polar organic compounds obtained using different species of anions as the counterion are summarized in Table 1.

For the ODS-based stationary phase, the fixed ionic site is physically bonded on the solid particle by hydrophobic interaction. When highly hydrophobic anions, such as TDC or DS are introduced to the column, a replacement of hexadecyltrimethylammonium by TDC or DS may occur. This will change the properties (the fixed ionic sites) of the stationary phase (ion-exchanger). To avoid such a problem, only chloride and sulfate were used as the counterions for the ODS-based stationary phase in this study. The retention time for the same species of the analyte differed with the species of counterion; this illustrating the counterion's contribution to analyte separations. However, no dramatic change in retention time for the same species of analyte was observed even when anion (TDC, DS) with higher hydrophobicity were used as the counterions. Thus, the effects of size and/or hydrophobicity of the counterions on the analyte separations are miniscule. The highest separation efficiency and the lowest relative standard deviation of the retention time (data not shown) are always obtained in both types of stationary phase with sulfate as the counterion. This indicates that sulfate is the most appropriate anion for this separation and that the electrostatic properties of the counterion is more important than its hydrophobicity.

To investigate the fixed ionic site and/or the solid particle, the mixtures of acetonitrile/water were used as the mobile phases. The concentration of the acetonitrile in the mobile phase was chosen as 1.0%, 3.0%, and 6.0% for the commercial column. In order to avoid desrobing the fixed ionic site (hexadecyltrimethylammonium) from the solid particle, only 1.0% acetonitrile aqueous solution was used for the laboratory-prepared column. Separation results are summarized in Table 2. For all of these analytes, retention times decreased with increasing concentration of acetonitrile in the mobile phase. However, the decrease in retention times for each analyte were different and depended on the type of stationary phase. When the ODS-based stationary phase was used, the retention time of both the nucleosides and their bases decreased largely. But when the polyacrylate-based stationary phase was used, the retention time of both the nucleosides and their bases decreased slightly. Previous studies on HPLC of nucleosides and their bases have shown that when the analytes separate, because of the hydrophobic interaction (the reversed-phase model), the retention time always decreased greatly when the amount of acetonitrile in the mobile phase is increased.[9-11]

According to the previous and the present experimental results, we may conclude that for the ODS-based stationary phase there is a relatively high hydrophobicity and for the polyacrylate-based stationary phase a very low hydrophobicity. The higher hydrophobicity of the ODS-based stationary phase resulted in a better (base-line) separation for the nucleosides.

Table 2

Effects of Acetonitrile Concentration on the Retention Time (Min) of the Analytes*

Analyte	0%	1.0%	3.0%	6.0%	0%	1.0%
A	6.91	6.42	5.63	4.68	4.79	4.16
G	4.21	4.14	3.86	3.60	3.59	3.40
C	2.21	2.18	2.17	2.16	2.76	2.72
T	3.18	3.03	2.84	2.66	4.67	4.18
U	2.63	2.59	2.51	2.42	3.13	3.08
rA	3.75	3.49	3.12	2.71	10.58	7.38
rG	2.86	2.78	2.59	2.37	6.31	4.97
rC	1.88	1.86	1.84	1.83	3.25	3.07
rU	2.01	2.01	1.94	1.88	4.07	3.61
dA	4.60	4.23	3.69	3.12	11.36	7.87
dG	3.42	3.25	2.96	2.63	6.78	5.23
dC	2.02	2.01	1.92	1.84	3.48	3.21
dT	2.50	2.42	2.31	2.22	7.47	5.76

* Average of three measurements, deviations were found to be less than 1.0%. Sulfate was used as the counterion for both of these columns. The results shown in the last two columns (right-side) were obtained using ODS-based stationary phase, the others were obtained using polyacrylate-based stationary phase. Other HPLC conditions were the same as described in Figure 1.

To investigate the analytes, buffers were used as the mobile phases. Whether the nucleosides and their bases are present in ionic or neutral form, depends on the mobile phase pH. When the mobile phase pH is less than 4 they are present as cation. When the pH is larger than 8 they are present as anions. When the pH is in the region 4 – 8 they are present as neutral species. Phosphate buffers were prepared at the following pH values 4.56, 6.01 and 8.33 and used as the mobile phases for the separation of these polar organic compounds. The elution order for these analytes observed using the phosphate buffers as the mobile phases were identical to the results observed when using pure water as the mobile phase. According to these experimental results (Table 3), we may conclude that the pH range of the these buffers covers the pH of the pure water used as the mobile phase. Therefore, we may indirectly conclude that the analytes in this HPLC were present as the neutral species.

Table 3

Effects of Mobile Phase pH on the Retention Time (Min)*

Analyte	pH4.56[a]	pH6.01[b]	pH8.33[c]	Water[d]
A	4.81	6.63	7.22	8.81
G	3.86	4.07	4.06	5.51
C	1.72	2.27	2.30	2.24
T	3.24	3.26	3.25	3.35
U	2.60	2.60	2.62	3.18
rA	3.65	3.79	3.90	3.72
rG	3.81	2.79	2.79	3.56
rC	1.96	2.11	1.84	1.88
rU	2.16	2.14	2.09	2.31
dA	4.82	5.38	5.49	4.59
dG	3.43	3.39	3.43	4.32
dC	1.89	2.17	2.16	2.03
dT	2.79	2.77	2.77	2.58

* Average of three measurements, deviations were found to be less than 1.0%. Mobile phase: (a) an aqueous solution containing 100 mM KH_2PO_4; (b) 90 mMKH_2PO_4 and 10 mM K_2HPO_4, (c) 100 mM K_2HPO_4, and (d) pure water (the counter ion of the fixed ionic site was phosphate). Polyacrylate stat'y phase. Other HPLC conditions were the same as described in Figure 1.

The hydrophobic properties of the nucleosides and their bases were previously investigated by Brown et al.[9,11] and they concluded that the nucleosides had higher hydrophobicities than their bases in HPLC, due to the "stacking" interactions. In this study, when using polyacrylate-based stationary phase for separations, the nucleoside was eluted faster than its base, this further illustrates the miniscule of hydrophobic effects of this stationary phase.

The analytes present in pure water are polar species, i.e., they have no charges. To achieve separation of these compounds when using electrostatic interaction, the analytes normally need to be converted into ionic species. However, in the single case where quaternary ammonium groups have been used as the stationary phase, the successful separation of the nucleosides were achieved using a neutral mobile phase.[12] We propose from this study that the polarities in the analytes are of critical importance in separation.

For the polyacrylate-based stationary phase, the analytes are separated by electrostatic interactions utilizing the polarities in the analytes with the fixed ionic site/counterion functioning as a zwitterionic stationary phase. This can be explained as follows:

> The fixed ionic site and the counterion functioned as a zwitterionic stationary phase producing a positive and a negative electrostatic field simultaneously in the column. The polar organic analytes, which have both positive and negative polarities are retained by the dual electrostatic fields (the stationary phase).
>
> The "net" (attraction – repulsion) electrostatic affinity of the analyte is extremely small, and as a result, the effective distribution of the analytes between the stationary phase and the mobile phase can be achieved with pure water as the mobile phase.
>
> Some of the analytes are strongly attracted by the stationary phase; these analytes cannot be effectively distributed to the mobile phase. They are replaced and eluted with the void time after the next injected sample enters the column. This phenomenon may be due mainly to the intrinsic properties of the polyacrylate-based stationary phase, because it is never observed when the ODS-based stationary phase was used.

For the ODS-based stationary phase, the hydrophobicity was relatively high. Hence, we may conclude that the hydrophobic interactions have also made a large contribution to the analyte separations. Nucleoside with higher hydrophobicity than its base was eluted slower than its base; this further shows the large scale hydrophobic effects of the ODS-based stationary phase. The polar organic analytes were separated with pure water as the mobile phase and the feasible retentions were achieved using a column of conventional size. This indicates the hydrophobic affinity of the analytes was much smaller than the hydrophobic affinity observed when using the conventional reversed-phase ODS columns. We propose the hydrophobic affinity for the polar organic analytes, in the present HPLC, is dramatically reduced by both the fixed ionic site and its counterion. The elution behaviours of the analytes, observed when using the ODS-based strong anion-exchanger as the stationary phase, were very similar to elution behaviours observed when using the bonafide strong-positively/strong-negatively charged zwitterionic surfactants immmobilized to ODS as the stationary phase. Hence, the dual mechanisms of electrostatic and hydrophobic interactions proposed in the previous paper[8] is suggested to explain the separation mechanisms for the ODS-based stationary phases.

CONCLUSION

A strong anion-exchanger can be used as the stationary phase in the unique condition where pure water is used as the mobile phase for HPLC. Polar organic compounds can be successfully separated and dual ionic sites (the fixed ionic site, and its counterion) are considered to be the main functionalizers for facilitating rapid separation and elution of the analytes.

A problem of this method that should be mentioned is that when samples containing both ions and polar compounds are injected into this HPLC system, the counterion is replaced by the analyte ion. This causes a small change in the retention times of the polar organic analytes. However, this problem is easily overcome by addition of large amounts of the counterion to the samples. The use of the bonafide strong-positively/strong-negatively charged zwitterionic surfactants as the stationary phase is an alternative solution to this problem.

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