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Creation and characteristics of phosphatidylcholine stationary phases for the chromatographic separation of inorganic anions

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Abstract

New stationary phases for chromatographic separation of anions, obtained by loading liposomes made from dimyristolyphosphatidylcholine (DMPC) onto reversed-phase packed columns (C_{18} and C_{30}) are reported. Mono- and divalent anions were used as model analyte ions and retention data for these species were obtained using the DMPC stationary phases and used to elucidate the separation mechanisms involved in this chromatographic system. The DMPC stationary phases can separate anions by either a solvation-dependent mechanism or an electrostatic ion-exchange mechanism, depending upon the relative magnitudes of the negative electrostatic potential ($\Psi_{(-)}$) of the phosphate moiety (P^-) and the positive electrostatic potential ($\Psi_{(+)}$) of the quaternary ammonium groups (N^+) on the headgroup of DMPC. If $\Psi_{(+)} > \Psi_{(-)}$, such as in case where $\Psi_{(-)}$ has been reduced either by binding of eluent cations (e.g., H^+ or divalent cations) onto the P^- group of DMPC or by steric screening when a C_{30} reversed-phase material was used to support the DMPC, then the overall electrostatic surface potential (and hence also the effective anion-exchange capacity) was generally large and the anions were separated on the basis of an electrostatic mechanism. However, if $\Psi_{(+)}$ was similar to $\Psi_{(-)}$, such as in the case of using a C_{18} reversed-phase support and monovalent cations as eluent cations, then the overall electrostatic surface potential and the effective anion-exchange capacity were very small and the analyte anions were separated on the basis of a solvation-dependent mechanism. The DMPC stationary phases were found to be suitable for the direct determination of iodide and thiocyanate in highly saline water samples, such as seawater samples.

Keywords: Stationary phases, LC; Retention mechanisms; Phosphatidylcholine stationary phases; Inorganic anions; Dimyristolyphosphatidylcholine

1. Introduction

Phosphatidylcholines (PCs), or lecithins, are naturally occurring zwitterions, found widely in animal

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tissues. It has long been known that PCs are interactive with ions (especially inorganic anions) and these interactions can modify the functioning of various membrane-related physiological processes [1]. As observed experimentally, in both whole muscle [2–4] and single muscle [1] fibres, the anion–PC interactions can cause an increase in the muscle twitch tension. The strength of the anion–PCs interactions has been shown to increase in the order $ClO_4^- > SCN^- > I^- > NO_3^- > Br^- > Cl^- > F^- > SO_4^{2-}$ [5]. This suggests that a chromatographic stationary phase based on phosphatidylcholines should show the same sequence of separation selectivities for inorganic anions. Such a stationary phase can be obtained by sterically entrapping liposomes made from PCs in gel beads [6], by covalently coupling PCs onto a specific support materials [7–9], or by using the so-called avidin–biotin interactions [10]. These phosphatidylcholine stationary phases have been used for evaluating interactions occurring between lipid membranes and particular organic solutes (such as drugs, peptides, or proteins) [6–11]. However, the possibility of using phosphatidylcholine stationary phases for the separation of inorganic anions has not yet been evaluated.

In this study, we have created a new type of phosphatidylcholine biomimetic stationary phase by loading liposomes made from dimyristolyphosphatidylcholine (DMPC) onto a reversed-phase chromatographic column and this has been used for the separation of inorganic anions. Mono- and divalent anions were chosen as model ions and these have been separated with unique selectivity, showing that the DMPC stationary phase recognises the analyte ions in a manner that is quite distinct from that for conventional anion-exchange columns.

2. Experimental

2.1. HPLC system

Chromatographic separations were performed using a high-performance liquid chromatographic (HPLC) system obtained from Shimadzu (Kyoto, Japan). This instrument consisted of a LC-10AT system comprising a Shimadzu LC-10AT pump, a Shimadzu SIL-10A auto-injector, a Shimadzu CTO-10A column oven, and a Shimadzu CR-6A Chromatopac data system. A Shimadzu SPD-6A UV–visible detector operated at 210 nm was used for the detection of the analyte ions.

2.2. Reagents

DMPC of purity >99% was used to establish the stationary phase and was obtained from Funakoshi (Tokyo, Japan). Other reagents used to prepare samples and eluent were of analytical grade and were obtained from Wako (Osaka, Japan). Deionized water used to prepare the samples and eluents was obtained using a WG261 Autostill water purification system (Yamato, Tokyo, Japan).

2.3. Column preparation

DMPC (600 mg) was dissolved in 15 ml chloroform in a round-bottomed flask. The DMPC was dried by rotary evaporation of the chloroform and the residue redissolved in 10 ml diethyl ether. The ether was evaporated slowly to produce a DMPC film on the surface of the flask (a finer lipid film is believed to be produced by the two-step evaporation procedure). The film was flushed with nitrogen and kept under vacuum for at least 4 h, and was then hydrated by dispersal in 10 ml of a water–methanol (95:5, v/v) solution containing 10 m*M* NaCl to form multilamellar liposomes. The complete hydration was ensured using a vortex mixer (Yamato, Tokyo, Japan). The multilamellar liposomes were passed 10 times through two stacked polycarbonate filters of 100-nm pore size (Nuclepore, Costar, MA, USA) at room temperature in a high-pressure vesicle extruder (Lipex Biomembranes, Vancouver, Canada) to obtain the unilamellar liposomes. The unilamellar liposomes were added to 2 l of the 10.0 m*M* NaCl water–methanol (95:5, v/v) solution and were then used to prepare the DMPC columns by passing this solution through a reversed-phase C_{18} HPLC column (250×4.6-mm I.D., 5-μm particle size, L-column, CERI, Tokyo, Japan) and also a reversed-phase C_{30} HPLC column (250×4.6-mm I.D., 5-μm particle size, Nomura, Seto, Japan) at a constant flow-rate of 1.0 ml/min for about 30 h. Using the breakthrough method [12], the C_{18} column was found to contain 0.71 mmol DMPC/column and the C_{30} column was

0.73 mmol DMPC/column. After completion of the HPLC separations in this study, 70 ml of chloroform were introduced into each column to remove DMPC from the columns. The effluents were collected and the solvent removed by rotary evaporation and the residue dried and weighed. The amount of the residue was found to be 0.68 and 0.71 mmol for the C_{18} column and the C_{30} column, respectively. Phosphorus elemental analysis of the residues confirmed them to be DMPC. These results indicated that the DMPC was immobilized on the reversed-phase stationary phase.

3. Results and discussion

3.1. Effects of the hydrophobic chain length of the supporting reversed-phase material

Sodium salts of seven UV-absorbing anions ($S_2O_3^{2-}$, SCN^-, I^-, NO_3^-, NO_2^-, BrO_3^- and IO_3^-) were dissolved in deionized water and were analysed in duplicate using the C_{18}/DMPC and C_{30}/DMPC stationary phases with 10.0 m*M* aqueous NaCl solution as eluent in each case. Both stationary phases showed an ability to separate the model anions, but differed in the selectivity of the separation. The C_{30}/DMPC stationary phase gave the retention order $IO_3^- < BrO_3^-/NO_2^- < NO_3^- < I^- < S_2O_3^{2-} < SCN^-$, while the C_{18}/DMPC stationary phase gave the retention order $IO_3^- = S_2O_3^{2-} < BrO_3^- = NO_2^- = NO_3^- < I^- = SCN^-$ for the model anions. Plots of logarithm of retention factor (k') versus logarithm of concentration of NaCl ([NaCl]) are shown in Fig. 1. Plots for the C_{18}/DMPC column (Fig. 1a) show that $\log k'$ for the analyte ions increased slightly as log [NaCl] increased, whilst for the C_{30}/DMPC column (Fig. 1b), $\log k'$ decreased linearly as log [NaCl] increased. Slope values for the monovalent anions (BrO_3^-, NO_2^-, NO_3^-, I^- and SCN^-) were all -0.56, and for the divalent anion ($S_2O_3^{2-}$) was -1.53.

These experimental data suggested that the length of the hydrophobic chain of the supporting reversed-phase material could influence the separation mechanism of the DMPC stationary phases for the separation of inorganic anions. Fig. 2 shows a schematic

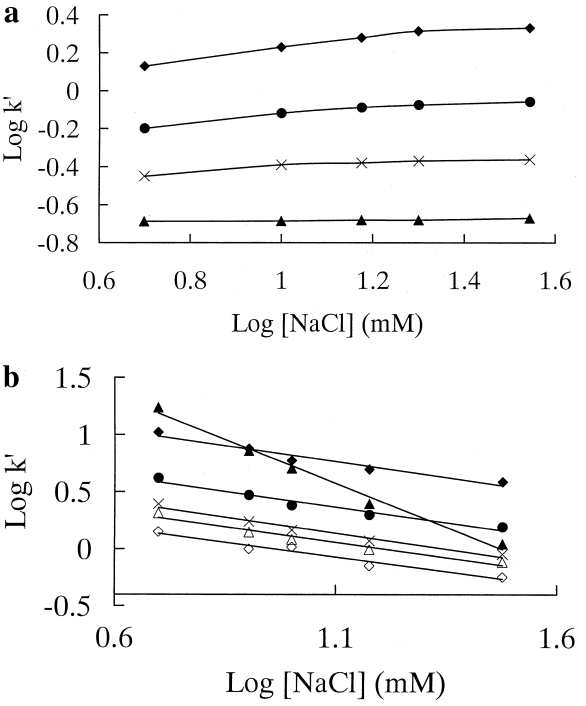

Fig. 1. Plots of $\log k'$ versus log [NaCl] for separations of anions using the C_{18}/DMPC (a) and C_{30}/DMPC (b) stationary phases. C_{18}/DMPC stationary phase: C_{18}-packed column (250×4.6-mm I.D.) coated with DMPC; C_{30}/DMPC stationary phase: C_{30}-packed column (250×4.6-mm I.D.) coated with DMPC. Flow-rate: 1.0 ml/min. Sample: 0.1 m*M* each of $NaIO_3$, NaI, $NaBrO_3$, $NaNO_2$, $NaNO_3$, NaSCN, and $Na_2S_2O_3$; injection volume: 20 μl. Detection: UV absorbance at 210 nm. Plots for (a): ♦, SCN^-; ●, I^-; ×, $BrO_3^-/NO_2^-/NO_3^-$; and ▲, $S_2O_3^{2-}/IO_3^-$; for (b): ♦, SCN^-; ●, I^-; ×, NO_3^-; △, NO_2^-; ◇, IO_3^-; and ▲, $S_2O_3^{2-}$.

illustration of the different ways in which the DMPC could be adsorbed on the C_{18} and C_{30} materials. With the C_{18} column the length of the hydrophobic tail on the DMPC is such that the positively charged groups (i.e., the quaternary ammonium groups, N^+) and the negatively charged groups (i.e., the phosphate moieties, P^-) of DMPC are both exposed to the mobile phase and can interact fully with the analyte ions. Under these conditions, the close proximity of the two functional groups causes the positive electrostatic potential produced by N^+ (designated as $\Psi_{(+)}$) to be offset strongly by the negative electrostatic potential produced by P^- (designated as $\Psi_{(-)}$). As a result, the overall surface potential (designated by $\Psi_{(\text{total})}$), which also determined the

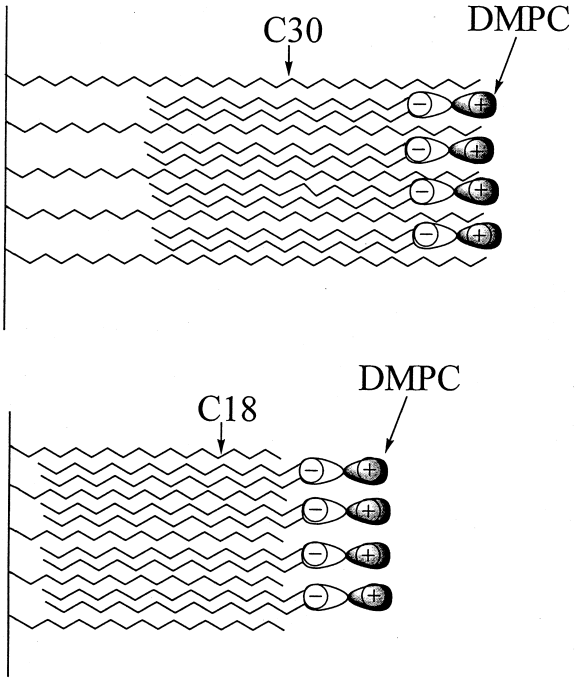

Fig. 2. Schematic illustration of possible positions of the positively and the negatively charged groups on the headgroups of DMPC in the C_{18} and the C_{30} stationary phases.

effective anion-exchange capacity of the stationary phase, is small. Analyte anions were therefore separated on the basis of a solvation-dependent mechanism. Anions having small free energies of hydration, such as iodide and thiocyanate, are retained readily by DMPC whereas analyte anions having large free energies of hydration, such as thiosulfate, showed weak retention. However, if the DMPC stationary phase was established using a C_{30} reversed-phase support the P^- groups of DMPC can be expected to be sterically hindered by the longer hydrophobic chain. This results in a diminished value $\Psi_{(-)}$, leading to a more positive value of $\Psi_{(\text{total})}$, so that analyte anions are retained by a conventional anion-exchange mechanism.

3.2. Effects of the eluent cation

Fig. 3 shows two chromatograms for separation of the same sample obtained using 10.0 m*M* NaCl (left-trace) and 5.0 m*M* $CaCl_2$ (right-trace) as eluent; with C_{18}/DMPC as the stationary phase. Separations

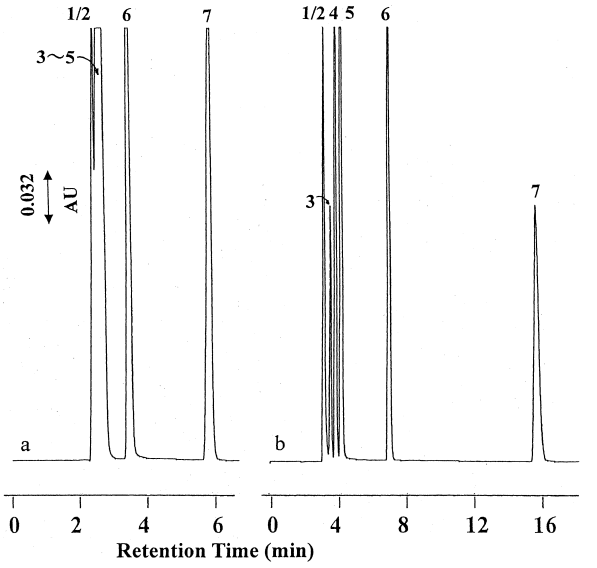

Fig. 3. Separation of anions using 10.0 m*M* NaCl (a) and 5.0 m*M* $CaCl_2$ (b) as eluent. C_{18}/DMPC stationary phase, other conditions as in Fig. 1. Peaks: 1=$S_2O_3^{2-}$, 2=IO_3^-, 3=BrO_3^-, 4=NO_2^-, 5=NO_3^-, 6=I^- and 7=SCN^-.

were also performed using the C_{30}/DMPC stationary phase and the chromatograms are shown in Fig. 4. As can be seen from these chromatograms, the $CaCl_2$

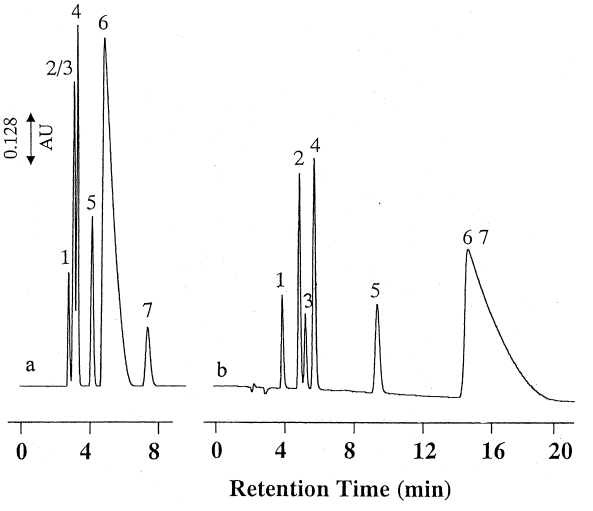

Fig. 4. Separation of anions using 10.0 m*M* NaCl (a) and 5.0 m*M* $CaCl_2$ (b) as eluent. C_{30}/DMPC stationary phase, other conditions as in Fig. 1. Peaks: 1=IO_3^-, 2=NO_2^-, 3=BrO_3^-, 4=NO_3^-, 5=I^-, 6=$S_2O_3^-$ and 7=SCN^-.

eluent gave longer retention times and thereby better separations of the analytes for both stationary phases. Plots of $\log k'$ versus log [NaCl] for the analyte anions using eluents containing varying concentrations of NaCl in the presence of a constant concentration (1.0 m*M*) of Ca^{2+} were constructed and straight lines were obtained in all cases. Negative slope values of -0.18 were obtained for the monovalent anions, whilst the divalent anion ($S_2O_3^{2-}$) showed a very small value of the slope and was also only weakly retained.

The concentration of hydrogen ions in the eluent exerted a strong effect on the retention of the analyte anions. For example, when a 10.0 m*M* NaCl eluent was used with the C_{18}/DMPC stationary phase, iodide and thiocyanate were eluted at 4.42 and 8.32 min, respectively. However, the retention times of these species increased to 10.82 and 33.78 min when a 10.0 m*M* HCl eluent was used. Note that the concentrated HCl eluent did not inflict damage to the liposome loading ODS column. This was tested by analysing the same sample with the NaCl eluent before and after the column had been used with the HCl eluent. No changes in retention times or separation efficiency were observed. Plots of $\log k'$ versus log [NaCl] obtained by maintaining the concentration of H^+ at a constant volume of 0.1 m*M* were constructed and straight lines with negative slope values of -0.41 for monovalent anions were obtained with the exception of nitrite, for which the slope was -0.19. Again, the divalent anion thiosulfate showed a very small slope and weak retention.

These data indicated that some conventional anion-exchange effects could be introduced into the separation system by adding a sufficient amount of divalent cations or H^+ ions to the eluent. Sodium ions show only weak interactions with the phosphate groups of DMPC, whilst H^+ and the divalent cations are known to interact strongly with these groups [5]. When the C_{18} reversed-phase material was used as the support and an aqueous sodium salt was used as eluent, $\Psi_{(-)}$ largely offsets $\Psi_{(+)}$, giving a low value of $\Psi_{(\text{total})}$ and a predominantly solvation-dependent retention mechanism results. However, if the eluent contained a sufficient amount of strongly interacting cations such as H^+ or Ca^{2+}, then $\Psi_{(-)}$ decreases and $\Psi_{(\text{total})}$ becomes more positive, leading to a predominantly anion-exchange retention mechanism.

3.3. Effects of the eluent anion

Effects of the eluent anion were also examined. For the C_{18}/DMPC stationary phase, similar results to those obtained in our previous studies using a sulfobetaine type zwitterionic stationary phase were observed [13,14]. However, for the C_{30}/DMPC stationary phase, the plots of $\log k'$ versus log [eluent] were always observed as straight lines with negative slope values. For example, use of $S_2O_3^{2-}$, I^- and SCN^- as analytes and Na_2SO_4 as eluent gave slope values for I^- and SCN^- of -0.27, and -0.73 for $S_2O_3^{2-}$. These data again showed that a conventional anion-exchange mechanism contributes to analyte retention with the C_{30}/DMPC stationary phase.

3.4. Application to analysis of highly saline samples

The C_{18}/DMPC stationary phase showed little affinity for chloride and sulfate ions and thereby constituted an ideal chromatographic system for the direct determination of anions with higher retention, such as iodide and thiocyanate, in the presence of chloride and sulfate. Fig. 5 shows two chromato-

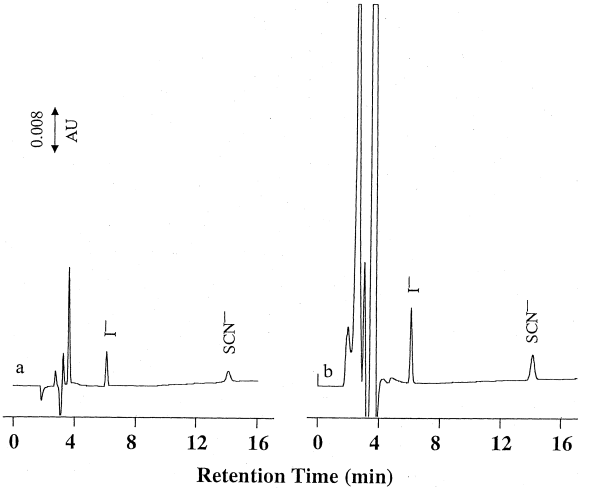

Fig. 5. Determination of 1.0 μ*M* each of iodide and thiocyanate in deionized water (a) and seawater spiked with 1.0 μ*M* each of iodide and thiocyanate (b). C_{18}/DMPC stationary phase, eluent: 10.0 m*M* $MgCl_2$. Other conditions as in Fig. 1. Peaks: (1) I^-; (2) SCN^-.

grams, one for the separation and detection of iodide and thiocyanate at the 1.0 μ*M* levels in deionized water sample (Fig. 5a) and the same concentration of these ions spiked into a seawater sample (Fig. 5b). The eluent was 10.0 m*M* $MgCl_2$, which is similar to the concentration of Mg^{2+} in seawater so as to stabilise retention times for the seawater samples. It can be noted that the presence of the saline matrix in Fig. 5b does not cause any broadening of the iodide and thiocyanate peaks.

4. Conclusions

Two biomimetic stationary phases obtained by immobilizing liposomes made from DMPC were created and their potential for separation of inorganic anions was evaluated. The separation mechanism was found to be dependent on the type of reversed-phase substrate used to support the DMPC, as well as on the type of eluent used. Both ion-exchange and solvation-dependent mechanisms were observed and these were rationalised in terms of the overall surface potential resulting from the adsorbed zwitterion. The developed stationary phase was shown to be useful for the direct determination of anions having low free energies of hydration in highly saline samples. Other possible applications of this new stationary phase, such as the separation and detection of peptides or proteins, are under evaluation by our group. Finally it is noteworthy that the biomimetic stationary phase was also applicable for the study of interactions occurring between ions and the phosphatidylcholine membrane [15].

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