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Evaluation of an ODS Column Modified with Zwitterionic/Nonionic Mixed Surfactants and Its Application to Direct Injection Determination of Inorganic Anions

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An octadecylsilica (ODS) column modified with zwitterionic/nonionic mixed surfactants was evaluated for the direct injection determination of inorganic anions in biological fluids by ion chromatography. A zwitterionic surfactant (sulfobetaine-type) and a nonionic surfactant (polyoxyethylene-type) were used for a stationary-phase modification. When aqueous electrolyte solutions with concentrations of sub-mM to several mM were used as a mobile phase, the zwitterionic surfactant coated on the ODS surface exhibited unique separation selectivity for ionic species, while the nonionic surfactant coated on the ODS might have formed a hydrophilic network over the ODS surface and restricted matrix proteins from adsorbing on the stationary phase. Consequently, the mixed surfactant-modified column system allowed an efficient ion chromatographic separation of inorganic anions as well as a size-exclusive removal of column-fouling proteins. This separation system was applied to the direct injection determination of UV-absorbing anions in human saliva. The detection limits for nitrite, nitrate, iodide and thiocyanate were 3.1, 2.7, 4.5 and 25 μM, respectively, with UV detection at 210 nm (injection volume; 20 μl), and their relative standard deviations for 5 replicate measurements of saliva samples spiked with 100 μM each of those anions were 1.4, 0.9, 2.2 and 5.5%, respectively.

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Introduction

The identification and quantification of inorganic and organic species in biological fluids are subjects of interest as chemical speciation analysis in metallomics.[1] So far, in fact, their concentration levels have been widely used as indicators for diagnosing our health or disease. That is, ionic species, such as amino acids, polyamines, organic acids, catecholamines, and their metabolites in serum, saliva and urine can be correlated with diseases as metabolic error.[2-6] High-performance liquid chromatography (HPLC) including ion chromatography (IC) is generally used for the determination of such ionic species. Under general mobile-phase conditions, however, proteins in biological fluids are often irreversibly adsorbed on a conventional separation column, resulting in a drastic deterioration of the column efficiency, which requires a tedious, pretreatment step, such as analyte extraction and matrix elimination.[7]

Although such pretreatments are of importance to overcome experimental difficulties, they involve risks, such as contamination from analytical reagents and the alteration of unstable species. Thus, considerable efforts have also been dedicated to the development of direct sample injection methods without any sample pretreatment. To date, various types of restricted-access stationary phases with a protein-repelling hydrophilic exterior have been exploited, mainly for therapeutic drug analysis and monitoring,[8-17] although the use of micellar mobile phases that solubilize proteins has also been investigated.[18,19] Such efforts also involve a column switching technique that has the advantage of preconcentrating trace analytes.[20]

Furthermore, it has been reported that aggregates of biomolecules, such as proteins and bile acids, irreversibly adsorbed on a conventional reversed-phase column, often works as a restricted-access type stationary phase, while providing some additional separation functions, such as ion-exchange separation and/or enantiomeric separation.[21-24] The present authors' group has demonstrated that an octadecylsilica (ODS) column modified with a zwitterionic bile acid derivative (CHAPS; 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) allowed a size-exclusive removal of column-fouling proteins as well as an efficient separation of small analytes, such as inorganic anions and drugs.[25,26] Such separation characteristics of the CHAPS-modified ODS column may be related to the special structure of sophisticated biomolecules that possess various functional groups and a unique carbon skeleton. In the present work, another attempt was made to prepare a multi-functional stationary phase with separation capability similar to the CHAPS-coating only by

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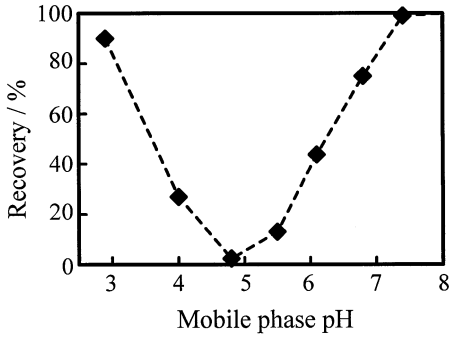

Fig. 1 Effect of the mobile-phase pH on the recovery of β-amylase from the C18SB/Tween 80-modified ODS column. Test sample, 1000 mg l^{-1} of β-amylase; mobile phase, 5.0 mM of sodium hydrogenphosphate with different pH values; detection, UV absorption at 230 nm.

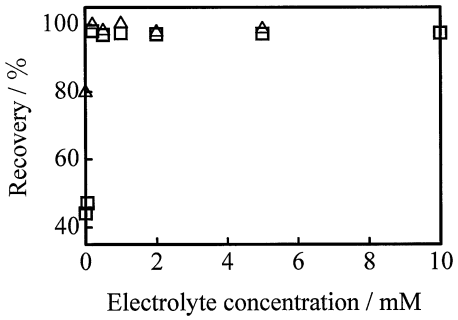

Fig. 2 Effect of the concentration of the electrolyte in the mobile phase on the recovery of proteins from the C18SB/Tween 80-modified ODS column. Test samples: □, 1000 mg l^{-1} of β-amylase; △, 1000 mg l^{-1} of albumin. Mobile phase, sodium dihydrogen phosphate solutions at pH 7.4; detection, UV absorption at 230 nm.

utilizing simple artificial surfactants as stationary-phase modifiers, where two artificial surfactants with different interactive properties (a sulfobetaine-type zwitterionic surfactant and a polyoxyethylene-type nonionic surfactant) were employed.[27] The developed stationary phase was applied to a direct injection determination of anions in human saliva.

Experimental

Apparatus

The IC system used was similar to that in previous work.[28] The system consisted of a pump (Model LC-10AD, Shimadzu, Kyoto, Japan), a sample injector (Model 7725, Rheodyne, Cotati, CA, USA) with a 20 µl loop, and a UV absorption detector (Model 870-UV, Jasco, Tokyo, Japan). In the present experiments, an ODS column (L-column; 4.6 mm i.d. × 250 mm long, Chemicals Evaluation and Research Institute, Tokyo) modified with zwitterionic/nonionic surfactants was employed as the separation column. Several electrolyte solutions were tested as the mobile phase.

Reagents

In the present experiment, two surfactants, such as octadecyldimethylammonium propanesulfonate (C18SB, sulfobetaine-type) from Aldrich (Tokyo) and polyoxyethylene(20)sorbitol monooleate (Tween 80, polyoxyethylene-type) from Wako Pure Chemicals (Osaka, Japan), were used as ODS column modifiers. Proteins used as matrices for the artificial biological fluid were β-amylase, albumin, carbonic anhydrase and Blue Dextran from Sigma (St. Louis, MO, USA). Inorganic salts were of analytical-reagent grade from Wako Pure Chemicals. These reagents were used without further purification.

Column preparation by two-step dynamic-coating procedure

As mentioned in our previous paper,[29] sulfobetaine-type zwitterionic surfactants that possess both ammonium and sulfonate groups exhibit anion selectivity similar to those observed for cationic surfactants, while nonionic surfactants are known to be useful as a protein-repellent.[30] In this experiment, the following two-step dynamic-coating procedure was adopted to bring out respective separation characteristics. In the first step, a water/acetonitrile (80/20) mixture solution containing 2 mM of C18SB was passed through an ODS column for 4 h at a flow rate of 0.7 ml min^{-1}. In the second step, an aqueous solution containing 1% of Tween 80 and 2 mM of C18SB was passed through the column for 2 h at the same flow rate. Subsequently, the modified ODS column was rinsed with water for at least 10 h to remove any excess of surfactant. This procedure proved to be most effective for preparing columns with high separation efficiency and physical stability.

Results and Discussion

Effect of the mobile-phase pH and the electrolyte concentrations on the recovery of proteins

Most proteins are susceptible to environmental changes in the separation media, and thus the mobile-phase conditions may provide significant influences on the elution of proteins from the separation column. Thus, several mobile phases were systematically examined in terms of their abilities to avoid the adsorption of proteins on the column. Figure 1 shows the effect of the mobile-phase pH on the recovery of β-amylase (1000 mg l^{-1}) from the column. In the recovery test, 5 mM of sodium hydrogen phosphate solutions with different pH values were employed as the mobile phases. The recovery values were calculated by comparing the peak area of β-amylase passed through the separation column with that passed through a Teflon tube with the same volume as the void volume of the separation column.[31,32] As can be seen in Fig. 1, the recovery was poor in the pH range 4 – 6, while β-amylase was well recovered above and below the pH region. Similar results were also obtained for albumin, carbonic anhydrase, and alcohol dehydrogenase. The poor recovery may be because proteins become hydrophobic in the pH region near the isoelectric point, which causes their adsorption on the column. Thus, further experiments were carried out at pH 7.4, while taking account of the physiological conditions.

The effects of the electrolyte concentrations in the mobile phase on the recovery of proteins were also examined by using phosphate buffers at pH 7.4, where β-amylase and albumin were used as test proteins. As can be seen in Fig. 2, the proteins were quantitatively recovered from the column, when the buffer concentration exceeded 0.2 mM. The poor recovery at concentrations lower than 0.2 mM might have been because the actual solution pH became below 7.0, due to the dissolution of carbon dioxide. Somehow, the present C18SB/Tween 80 mixed-modified column provided almost 100% recoveries over a concentration range of 0.2 to at least 10 mM. In the case of

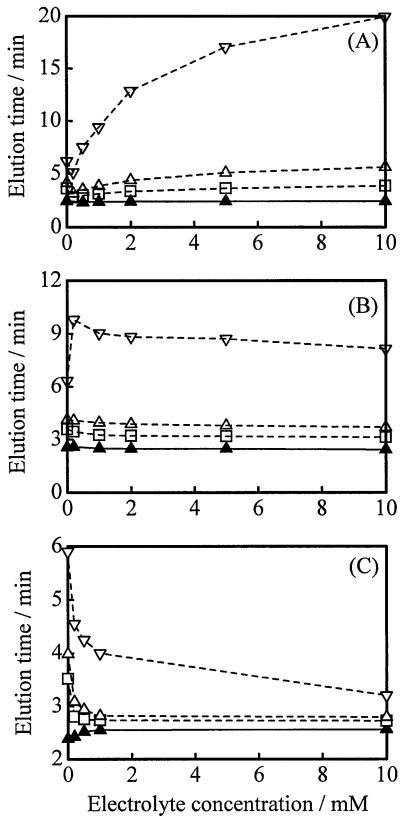

Fig. 3 Effect of the concentration of electrolytes in the mobile phase on the separation of inorganic anions. Test sample: an artificial saliva sample containing ▲, 1000 mg l^{-1} of β-amylase; □, 10 mM of NO_2^-; △, 10 mM of NO_3^-; ▽, 10 mM of I^-. Mobile phase: A, NaH_2PO_4; B, $NaClO_3$; C, $NaClO_4$; detection, UV absorption at 210 nm.

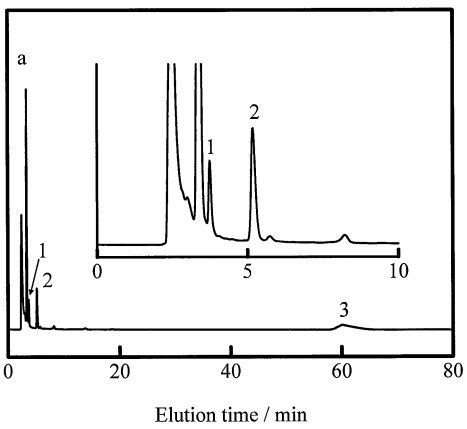

Fig. 4 Chromatogram of human saliva sample obtained by the C18SB/Tween 80-modified column system. Sample, human saliva sample collected from a healthy male; mobile phase, 5 mM sodium dihydrogen phosphate at pH 7.4; flow rate, 0.7 ml min^{-1}; injection volume, 20 µl; detection, UV absorption at 210 nm. Peaks: 1, NO_2^-; 2, NO_3^-; 3, SCN^-; a, matrix proteins.

CHAPS-modified columns, the applicable concentration range was narrower (0.2 – 1.0 mM), as mentioned in a previous paper.[26] Such an extension of the applicable concentration range would offer the advantages of a further improvement of the chromatographic separation.

Table 1 Analytical results for inorganic anions in human saliva determined by UV detection at 210 nm

	Concentration[a]/ mM	RSD[b], %	Detection limit/ mM
NO_2^-	0.088 ± 0.001	1.4	0.003
NO_3^-	0.350 ± 0.003	0.9	0.003
I^-	n.d.[c]	2.2	0.004
SCN^-	0.44 ± 0.02	5.5	0.02

a. Mean ± s.d. (n = 3).
b. The relative standard deviations for 5 replicate measurements of saliva samples spiked with each anion of 0.1 mM.
c. Not detected.

Effects of diverse electrolytes in the mobile phase on the separation of inorganic anions

The effects of diverse electrolytes in the mobile phase on the separation of inorganic anions were examined by using sodium dihydrogen phosphate, sodium chlorate, and sodium perchlorate.[33] An artificial saliva sample containing β-amylase (1000 mg l^{-1}), NO_2^- (10 mM), NO_3^- (10 mM), and I^- (10 mM) was prepared and analyzed by the IC system using each electrolyte mobile phase. The retention-time data are summarized in Fig. 3. Under each separation condition, β-amylase was rapidly eluted at an elution time of approximately 2.5 min, as can be seen in Fig. 3. On the other hand, it can be seen that the elution times of inorganic anions were significantly dependent on the types and concentrations of electrolytes added to the mobile phase.[33–35] In the NaH_2PO_4-eluent system, the elution times of anions became longer with an increase in the eluent concentration, while they were shortened at higher concentrations of the $NaClO_4$-eluent system. It can be stated here that the NaH_2PO_4-eluent may be useful for the mutual separation of analytes with a shorter retention time, whereas the $NaClO_4$-eluent can be used for rapid separation.

Direct injection determination of inorganic anions in human saliva

The present separation system was applied to the determination of UV-absorbing anions in human saliva. In this experiment, the NaH_2PO_4-eluent was chosen as the mobile phase to enhance the chromatographic resolution at a short retention time zone. Figure 4 shows the typical chromatogram for a human saliva sample obtained from a healthy male. As can be seen in Fig. 4, several peaks were detected with UV absorption at 210 nm, where NO_2^- with a short elution time could practically be separated from the sample matrices by using 5 mM of NaH_2PO_4, although the elution time of SCN^- became too long.

The analytical results for the concentrations of nitrite, nitrate, iodide, and thiocyanate in human saliva sample under the separation conditions listed in Fig. 4 are summarized in Table 1. The calibration graphs for nitrite, nitrate, iodide and thiocyanate with UV absorption detection at 210 nm were linear up to at least 2 mM, and their detection limits were 0.003, 0.003, 0.004 and 0.02 mM, respectively, estimated for a signal-to-noise ratio of 3. In addition, the relative standard deviations for 5 replicate measurements of saliva samples spiked with each anion of 0.1 mM were 1.4, 0.9, 2.2 and 5.5%, respectively. As a result, the concentrations of NO_2^-, NO_3^- and SCN^- detected in the saliva sample were 0.088 mM, 0.350 mM and 0.44 mM. Furthermore, in terms of the column durability, the C18SB/Tween 80-coated ODS column was found to be tolerable to a large number of

injections (> 100 times) without any deterioration in the column efficiency.

Conclusion

It was demonstrated that the C18SB/Tween 80 mixed surfactant-modified ODS column worked as a restricted-access type stationary phase with anion selectivity. The bulk of the proteins were eluted at the solvent front, while the resolution of inorganic anions was enhanced by using a proper electrolyte solution. In the present work, however, the mixed surfactant-modified ODS column was only applied to the separation of anions in human saliva.

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