Enantiometric Separation with Use of Stationary Phase Coated with Micellar Bile Salt for Microcolumn Liquid Chromatography

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A novel method for enantiometric separation using an ODS stationary phase coated with bile salt micelles in high-performance liquid chromatography has been developed, where bile salt surfactants such as sodium cholate and sodium deoxycholate were used for micelle formation. The present method was applied to the separation of the enantiomers of 1,1′-binaphthyl-2,2′-diyl hydrogenphosphate (BNDHP) and *N*-dansylphenylalanine by using acetonitrile–water solution as the mobile phase. The dependence of the capacity factors and separation factors on the acetonitrile concentration in the mobile phase was examined for BNDHP enantiomers. These factors decreased with increasing acetonitrile concentration, which resulted in the change of separation characteristics of BNDHP enantiomers. Although BNDHP and *N*-dansylphenylalanine enantiomers were separated, similar enantiomers such as 2,2′-dihydroxy-1,1′-binaphthyl and other *N*-dansylamino acids were not separated in the present separation system.

In the previous papers,[1—5] micellar bile salts and micellar bile acid derivatives have been used as the mobile phase additives for enantiometric separation by liquid chromatography (LC). From these experimental results and some consideration on the kinetic behaviors of the enantiomers in the separation column, it was suggested that the existence of four phases in the LC column system, viz., bulk solvent, ODS (octadecylsilica) stationary phase, micelles in the mobile phase, and micelles adsorbed on the ODS stationary phase, should be taken into account in the enantiometric separation. Furthermore, it was elucidated that the partition between the micelle in the mobile phase and the solvated ODS stationary phase was predominant under some separation conditions, while the partition between the bulk solvent in the mobile phase and the micelles adsorbed on the ODS stationary phase might be predominant in some other cases. Since bile surfactants generally form reversed micelles in some polar solvents,[6—9] the outsides of the micelles are hydrophobic, which results in strong adsorption onto the ODS surface. Consequently it is considered that the micellar bile surfactants were adsorbed on the ODS stationary phase surface by hydrophobic interaction and the adsorbed micelles somewhat contributed to the enantiometric separation.

Hence, in the present work, we have tried to coat the bile micelles on the ODS and to use the ODS coated with a micellar bile salt as the stationary phase for enantiometric separation, where any bile salt is not contained in the mobile phase. This new separation system has been applied to the separation of the enantiomers of 1,1′-binaphthyl-2,2′-diyl hydrogenphosphate and *N*-dansylphenylalanine.

Experimental

Apparatus. A microcolumn LC system was assembled with a microfeeder (MF-2; Azumadenki Kogyo, Tokyo) equipped with a 0.5 ml gas-tight syringe (MS-GAN 050; Ito, Fuji) as a pump, a microvalve injector with an injection volume of 0.02 μl (ML-552; JASCO, Tokyo), a 15 cm×0.35 mm i.d. separation column packed with Develosil ODS-5 (5 μm; Nomura Chemical, Seto), an absorbance detector (UVIDEC-100V; JASCO) with a modified flow cell, and a data processor (Chromatopac C-R4AX; Shimadzu, Kyoto). The separation microcolumn was prepared from fused-silica tubing by a slurry packing method as reported previously.[10] The flow rate of the mobile phase was 2.8 μl min^{-1}. The separation was carried out at room temperature, viz., 20—25 °C.

Reagents. All reagents used in the present work were of analytical reagent grade and were obtained from Wako Pure Chemical Industries (Osaka), unless otherwise stated. These reagents were used without further purification. Purified water was prepared by using a Milli-Q deionization system (Nihon Millipore Kogyo, Tokyo). Bile salt surfactants, sodium cholate (NaC) and sodium deoxycholate (NaDC), were purchased from Wako Pure Chemical Industries. Optical isomers, (*R*)-(−)-1,1′-binaphthyl-2,2′-diyl hydrogenphosphate ((*R*)-(−)-BNDHP), (*S*)-(+)-BNDHP, (*R*)-(+)-2,2′-dihydroxy-1,1′-binaphthyl ((*R*)-(+)-DHBN), and (*S*)-(−)-DHBN were obtained from Tokyo Chemical Industry (Tokyo). Dansyl-DL-phenylalanine (DL-Phe) and *N*-dansyl-L-phenylalanine (L-Phe) were obtained from Sigma (St. Louis, Mo, USA). These isomers were dissolved in acetonitrile–water solution (50:50) or in acetonitrile–0.13 M (1 M=1 mol dm^{-3}) ammonium acetate mixture (20:80).

Preparation of Separation Column Coated with Micellar Bile Salts. NaC or NaDC (100 mM) dissolved in pure water was passed through the Develosil ODS-5 microcolumn for 30 min at a flow rate of 2.8 μl min^{-1}. Then the column was washed with pure water at least for 60 min at the same flow rate. Subsequently, the eluent was just changed to the mobile phase solution employed for enantiometric separation. In order to test the stability of the micellar bile salt coated stationary phase, 10% acetonitrile aqueous solution was passed through the column for 20 h, and the elution was monitored at 210 nm by a UV absorption detector. If the micelles coated on the ODS surface were desorbed, the signals of the bile salts might be detected as some baseline change. However, no baseline change was observed during the test period. This result indicates that the

micelle-coated stationary phase was stable without remarkable degradation. Furthermore, the micelle-coated column could be used for at least 6 months with almost the same separation characteristics.

Results and Discussion

Separation of BNDHP Enantiomers. A characteristic property of bile salt surfactant is their ability to form reversed micelles by means of ion-ion and ion-dipole interaction together with hydrogen bonding.[6—9] It is also known that bile salt micelles have helical structures.[9] The critical micelle concentrations (CMC) of these bile salts in pure water are 2-13 mM.[11,12] According to the results obtained in our previous works,[3—5] as mentioned earlier, micellar bile salts are adsorbed on the ODS by hydrophobic interaction, and form some heterogeneous stationary phase. Therefore, if the bile salt micelles on the ODS stationary phase still keep their helical structures, the enantiomers would be recognized by the micelles coated on the ODS even without bile salt in the mobile phase.

Figure 1 demonstrates the enentiometric separation of BNDHP by the microcolumn LC using the micellar bile salt-coated stationary phase and 10% acetonitrile aqueous solution as the mobile phase. The results are shown for the stationary phases coated with NaC (A) and NaDC (B). As can be seen in Fig. 1, BNDHP enantiomers could be separated by the micellar bile salts-coated stationary phase, even though bile salts were not contained in the mobile phase of acetonitrile–water solution. These results indicate that the micelles coated on ODS surface still keep their helical structures.

The retention times for BNDHP are summarized in Table 1 in terms of the mobile phases of acetonitrile–water mixture (10 : 90) and pure water. When pure water was utilized as the mobile phase for the separation of BNDHP enantiomers, the enantiometric separation was still possible, but the retention times of the analytes were longer compared to those in the acetonitrile-water mixture mobile phase. That is, (*R*)-(−)-BNDHP and (*S*)-(+)-BNDHP were eluted at 47 and 87 min in pure water, respectively, when the NaDC coated stationary phase was used. From the data in Table 1, it is estimated that the separation factors for (*R*)-(−)-BNDHP were 1.12 and 1.26, when NaC and NaDC were used, respectively.

The effect of the acetonitrile concentration in the mobile phase on the retention times are shown in Fig. 2. It is noted that the retention times of the BNDHP enantiomers were varied with acetonitrile concentration, viz., they became shorter at the higher concentration of acetonitrile.

It is further noted here that the mobile phase of the higher acetonitrile concentration could not be used in the present system, because the micelles adsorbed on the stationary phase were desorbed slowly with time. The present system allowed to use the mobile phase containing acetonitrile up to 27% and at the higher concentration the enantiometric separation of BNDHP was unsuccessful because of the desorption of the adsorbed micelles.

Dependence of Capacity Factors and Separation Factors on Acetonitrile Concentrations. The dependence of the capacity factors k'_R and k'_S for (*R*)-(−)-BNDHP and (*S*)-(+)-BNDHP, respectively, on the concentration of acetonitrile in the acetonitrile–water mixture mobile phase are summarized in Fig. 3, where the micellar bile salt coated stationary phase was used. As is seen in Fig. 3, the capacity factors decrease with increasing the acetonitrile concentration in the mobile phase.

The separation factor ($\alpha = k'_S/k'_R$, $k'_S > k'_R$) at different acetonitrile concentration can be calculated by using the capacity factors of k'_S and k'_R for (*S*)-(+)-BNDHP

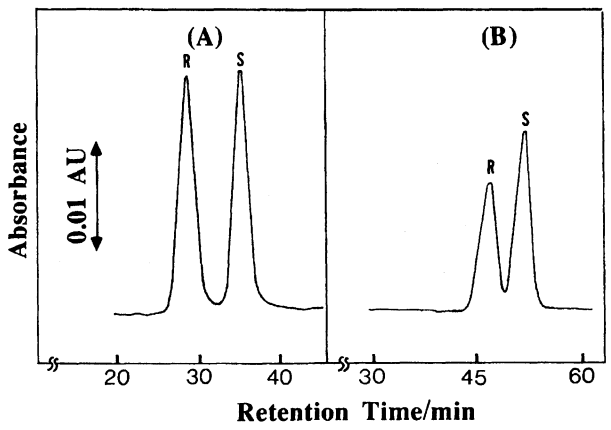

Fig. 1. Separation of BNDHP enantiomers by using 10% acetonitrile aqueous solution as the mobile phase. Stationary phase: Develosil ODS-5 (150×0.35 mm i.d.) coated with NaDC (A) and NaC (B), Flow rate: 2.8 μl min^{-1}, Analytes: (*R*)-(−)-BNDHP and (*S*)-(+)-BNDHP.

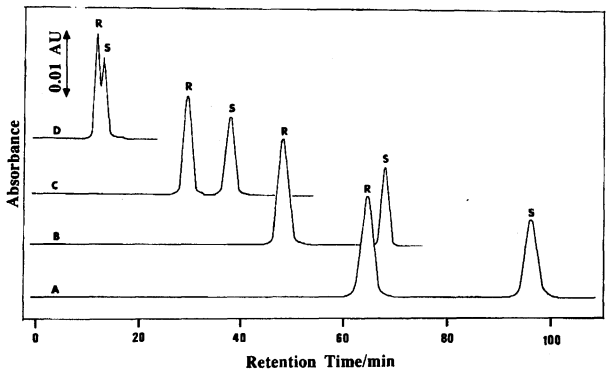

Fig. 2. Enantiometric separation of BNDHP at different acetonitrile concentration in the mobile phase. Separation conditions are the same as in Fig. 1(A) except the mobile phase. Mobile phase: (A) pure water only, (B) acetonitrile–water (4 : 96), (c) acetonitrile–water (12 : 88), (D) acetonitrile–water (25 : 75).

Table 1. Retention Time for BNDHP Enantiomers Obtained by the Microcolumn LC System Using the ODS Stationary Phase Coated with NaC and NaDC and Mobile Phase of Acetonitrile–Water Mixture (10:90) and Pure Water

Enantiomers	Mobile phase	Retention time/min	
		NaC-coated	NaDC-coated
(*R*)-(−)-BNDHP	Acetonitrile–water	47.16	29.19
(*S*)-(+)-BNDHP	Acetonitrile–water	52.76	35.93
(*R*)-(−)-BNDHP	Purewater	86.87	47.37
(*S*)-(+)-BNDHP	Purewater	100.95	85.72

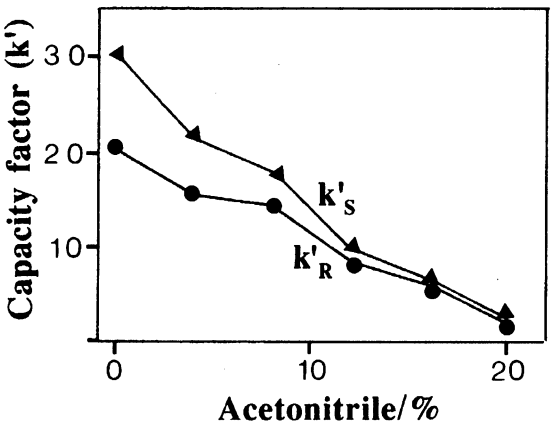

Fig. 3. Relationship between the capacity factors (k') and the acetonitrile concentration in the mobile phase. The experimental conditions are the same as in Fig. 2.

and (*R*)-(−)-BNDHP, respectively, in Fig. 3. The results are shown in Fig. 4. As is seen in Fig. 4, the separation factor decreases at the higher acetonitrile concentration in the mobile phase.

In order to explain the results obtained in Figs. 3 and 4, the retention mechanisms in liquid chromatography using reversed micellar mobile phase should be taken into account. In our previous works,[3—5] the existence of four phases in the separation column was considered,

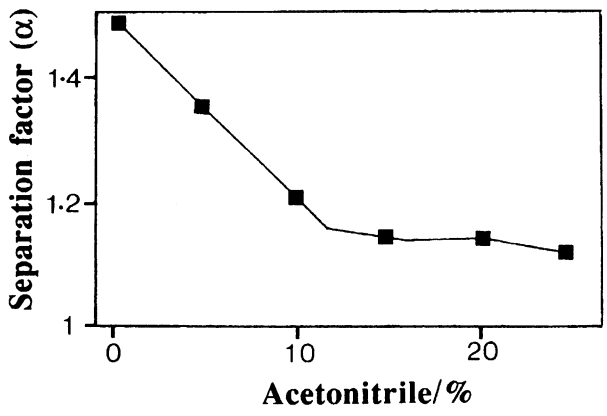

Fig. 4. Separation factor (α) as a function of the acetonitrile concentration in the mobile phase. The experimental conditions as are the same in Fig. 2.

namely, bulk solvent, solvated ODS stationary phase, micelles in the mobile phase, and micelles adsorbed on the ODS stationary phase. In the present system, however, there exist no micelles in the mobile phase. Thus only three phases exist in the present separation system, i.e., bulk solvent, solvated ODS stationary phase, and micelles adsorbed on the ODS. Therefore, the capacity factor (k') can be expressed as follows,

$$k' = \frac{V_{LS}[L_S]}{V_m} K_{B-LS} + \frac{V_{MS}[M_S]}{V_m} K_{B-MS}, \quad (1)$$

where V_{LS}, V_{MS}, and V_m are the volumes of the solvated ODS stationary phase, the micelles adsorbed on the ODS, and the bulk solvent in the mobile phase, respectively, $[L_S]$ and $[M_S]$ the concentrations of the solvated ODS and the micelles adsorbed on ODS, respectively, K_{B-LS} the partition coefficient of an analyte between the bulk solvent and the solvated ODS stationary phase, and K_{B-MS} the partition coefficient of an analyte between the bulk solvent and the micelles adsorbed on the ODS stationary phase.

Under a fixed condition, $V_{LS}[L_S]$, and V_m are constant, and thus the capacity factor (k') (and so the separation factor) depends on the partition coefficients (K_{B-LS} and K_{B-MS}) and $V_{MS}[M_S]$ (the amount of the micelles adsorbed on the ODS stationary phase). As is seen in Figs. 3 and 4, the capacity factors and separation factors became smaller with increasing the acetonitrile concentration in the mobile phase. These results may be interpreted in two ways. One is that the partition coefficient becomes smaller by increasing acetonitrile concentration in the mobile phase, and the other is that the amount of the micelles adsorbed on the ODS stationary phase is slightly reduced in the stationary phase by the long time elution of the acetonitrile–water solution. At present, it is not clear that either of these two mechanisms is predominant, although the former may be possible.

The present system was also applied to enentiometric separation of related compounds, e.g., 2,2′-dihydroxy-1,1′-binaphtyl (DHBN), but they could not be eluted from the column. This may be because the partitions of DHBN isomers into the solvated ODS stationary phase is much larger than those of BNDHP isomers.

In the previous papers,[3—5] we reported the enan-

tiometric separation of BNDHP using the micellar bile salt mobile phase, where acetonitrile–water mixture was used as the mobile phase and various bile salts were added as the additives. Comparing the present results with those reported previously,[3—5] the retention times of BNDHP were longer and their separation characteristics were poorer when the micellar bile salt coated stationary phase developed in the present work was used. These results indicate that the helical structures of the bile salt micelles on the ODS are deteriorated in the heterogeneous stationary phase produced by the dynamic coating method, although such structures are still to some extent maintained for chiral recognition ability. According to the previous results,[3] DHBN provided the larger separation factor or capacity factor compared to BNDHP when the micellar bile salt mobile phase was used. This suggests that the partition of DHBN to the stationary phase is significantly larger than that of BNDHP. The reason why DHBN was not eluted from the column of the micellar bile salt coated stationary phase may be explained by the larger partition due to the hydrophobic interaction between DHBN and the heterogeneous stationary phase.

Separation of Phenylalanine Enantiomers. Figure 5 demonstrates the enantiometric separation of *N*-dansyl-DL-phenylalanine by using 20% acetonitrile solution as the mobile phase, where the NaDC coated stationary phase was used. The D-isomer was eluted before the L-isomer, and the separation factor was 1.09. This system was also applied to separation of other *N*-dansyl DL-amino acid enantiomers, but successful resolution could not be achieved.

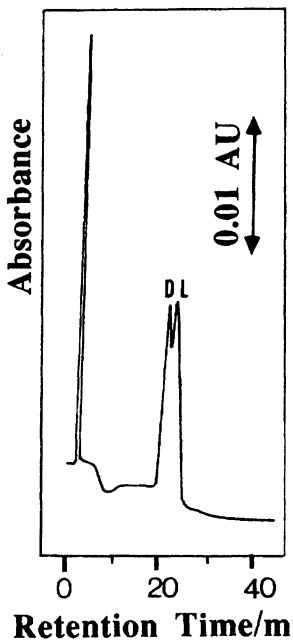

Fig. 5. Enantiometric separation of *N*-dansyl-DL-phenylalanine by using 20% acetonitrile aqueous solution as the mobile phase. The stationary phase was coated with NaDC, and other experimental conditions are the same as in Fig. 1.

Conclusion

The ODS stationary phase coated with micellar bile salts was successfully applied to separation of the enantiomers of 1,1′-binaphthyl-2,2′-diyl hydrogenphosphate and *N*-dansylphenylalanine in microcolumn liquid chromatography. Separation characteristics significantly depended on the acetonitrile concentration in the mobile phase. The present system, however, failed to separate 2,2′-dihydroxy-1,1′-binaphtyl isomers. Thus, the separation mechanisms in the present system using the bile salts-coated stationary phase should be elucidated to explore wider applicability. It is noted here that the micellar adsorbed stationary phase developed in the present work is also applicable to ion chromatography and such studies are now in progress.[13,14]

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