Journal of Chromatography, 623 (1992) 221–228
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 480

Development of a flavoprotein column for chiral separation by high-performance liquid chromatography

Nariyasu Mano, Yoshiya Oda, Naoki Asakawa, Yutaka Yoshida and Tadashi Sato

Department of Physical and Analytical Chemistry, Tsukuba Research Laboratories, Eisai Co., Ltd., 5-1-3 Tokodai, Tsukuba, Ibaraki 300-26 (Japan)

Toshinobu Miwa

Department of Pharmaceutical Research Laboratory, Eisai Co., Ltd., 2-1 Takehaya-machi, Kawashima-cho, Hashima-gun, Gifu 483 (Japan)

(First received March 26th, 1992; revised manuscript received June 25th, 1992)

ABSTRACT

A new chiral stationary phase using flavoprotein, a glycoprotein present in chicken egg-white, was developed for high-performance liquid chromatography. This column could achieve baseline separations of acidic (ketoprofen), weakly acidic (warfarin) and neutral (benzoin) compounds. In the flavoprotein-conjugated silica gel column, the capacity factor and enantioselectivity for several model drugs were greatly influenced by the pH and the concentration of organic solvents and salts in the mobile phase, and the optimum conditions for chiral separation varied from compound to compound. However, this column shows good stability to pH variation and to organic solvents, and it should be applicable for the chiral separation of many compounds in the reversed-phase mode. This column can directly separate optical isomers with an aqueous mobile phase, so it should be very useful in the fields of pharmacokinetics and clinical chemistry.

INTRODUCTION

Many pharmaceutical drugs have asymmetric centres, but most of them are used clinically in racemic form. However, drug enantiomers can have qualitatively or even quantitatively different physiological actions, so chiral resolution has become an important subject in the development and use of pharmaceutical drugs. Some direct chiral separations have been achieved by high-performance liquid chromatography (HPLC) in the normal-phase mode, but laborious pretreatments were required in the application of these methods to biological samples for pharmacokinetic and pharmacological research. Further, many of the direct chiral separation columns have very high selectivity and cannot be used with a wide range of compounds. Therefore, there is an urgent requirement for direct chiral separation columns with a wide selectivity in the reversed-phase mode, for rapid and simple determination of enantiomers of clinically relevant drugs. Such columns would be very useful for research in the fields of pharmacokinetics and pharmacology.

Recently, various chiral stationary phases that can be used under reversed-phase conditions have been developed, such as protein-conjugated columns, cyclodextrin-conjugated columns [1,2], and a triacetylcellulose column [3]. Protein-conjugated columns have been proved useful by Allenmark *et al.*, who developed a bovine serum albumin (BSA)-conjugated column [4], and by Hermansson, who developed an α_1-acid glycoprotein (α_1-AGP)-con-

Correspondence to: Dr. N. Mano, Department of Physical and Analytical Chemistry, Eisai Co., Ltd., 5-1-3 Tokoda, Tsukuba, Ibaraki 300-26, Japan.

0021-9673/92/$05.00

jugated column [5]. A BSA-conjugated column has been used to resolve enantiomers of acidic compounds [4], and an α_1-AGP-conjugated column to resolve racemic amines [5,6]. On the other hand, Miwa *et al.* have developed a highly effective column for chiral recognition by using ovomucoid, an acidic glycoprotein found in chicken egg-white [7]. An ovomucoid column, which is now commercially available, can resolve acidic and basic enantiomers [8,9], and is relatively stable to variation of pH, to heat, and to organic solvents [7,10]. Kirkland *et al.* have reported that an ovomucoid column has a higher stability and better chiral resolution than an α_1-AGP-conjugated column [11]. Oda *et al.* have recently performed on-line simultaneous determination and resolution of enantiomers of verapamil and its metabolites in plasma with an ovomucoid column and a column-switching technique [12].

As mentioned above, protein-conjugated columns, such as the ovomucoid column, can be useful for pharmacokinetic studies because of their applicability to a reasonably wide range of drug enantiomers in the reversed-phase mode. However, there are still many enantiomers that cannot be resolved by commercially available protein-conjugated columns. Therefore, we still require new materials with a high chiral recognition ability and a wider range of selectivity when used in the reversed-phase mode.

In this study, we used flavoprotein, which is present in chicken egg-white, as a ligand for chiral separation by means of reversed-phase HPLC. Flavoprotein is a glycoprotein which has 14% carbohydrate, consisting of mannose, galactose, and glucosamide. Its p*I* value is *ca.* 3.9–4.1 [13], which is similar to that of ovomucoid (3.9–4.3), and its molecular mass is 32 000–36 000. Interestingly, it also has the ability to bind to riboflavin [14]. This paper describes a new stationary phase for chiral resolution, flavoprotein-conjugated silica gel, which allows the separation of many drug enantiomers by means of reversed-phase HPLC.

EXPERIMENTAL

Apparatus

A Tosoh CCPM pump (Tosoh, Tokyo, Japan) equipped with a UVIDEC100-VI variable-wavelength UV spectrophotometer (Japan Spectroscopic, Tokyo, Japan) was used. A stainless-steel column (150 mm × 4.6 mm I.D.) was packed with flavoprotein-conjugated silica gel. The samples were injected with a WISP 712 Autosampler (Waters Assoc., Milford, MA, USA). The pH was measured with a TDA HM-60S pH meter (Toa Electronics, Tokyo).

Preparation of flavoprotein column

Flavoprotein-conjugated silica gel was prepared as follows: Nucleosil $5NH_2$ (2 g) and N,N-disuccinimidyl carbonate (3 g) reacted for 12 h in acetonitrile (50 ml) at room temperature in a rotary evaporator. The activated silica gel was collected by filtration and washed with acetonitrile and then with the coupling buffer (50 m*M* potassium phosphate buffer, pH 7.5). Flavoprotein (2 g) was dissolved in 50 ml of coupling buffer and then the activated silica gel was added. The mixture was allowed to react for 2 h at room temperature in a rotary evaporator, then the flavoprotein-conjugated silica gel was collected by filtration and washed with water and 2-propanol–water (1:2). This gel was packed into a stainless-steel column (150 mm × 4.6 mm I.D.) by a conventional high-pressure slurry-packing procedure.

Reagents and materials

Flavoprotein was purified from chicken egg-white [14]. Ketoprofen (KE), ibuprofen (IB), flurbiprofen (FL), and warfarin (WA) were purchased from Sigma (St. Louis, MO, USA). α,ε-Dibenzoyllysine (DB) and benzoin (BE) were from Tokyo Kasei Company (Tokyo). N,N-Disuccinimidyl carbonate was purchased from Wako (Osaka, Japan). Nucleosil $5NH_2$ was purchased from Macherey-Nagel (Düren, Germany). Organic solvents and water were of HPLC-grade, and other chemicals were of high purity.

Sample preparation

Known amounts of drug enantiomers were dissolved in methanol, and each solution was diluted with water or water–methanol (1:1) to a concentration of 20 ng/ml, and 10 μl were injected into the HPLC column.

RESULTS AND DISCUSSION

Protein-conjugated columns generally show higher chiral recognition ability than other HPLC col-

umns used under reversed-phase conditions. However, there are many drug enantiomers that cannot be resolved on the currently available protein-conjugated columns. Flavoprotein is a chicken egg-white glycoprotein with the capacity to bind riboflavin in a ratio of 1:1 [15] at pH 4.0 or above [14]. This protein plays an important role in the transfer of riboflavin from blood to egg-white [15]. It is very stable to heat, retaining its riboflavin-binding capacity after heating at 100°C and pH 7.0 for 15 min [14]. So we expected that flavoprotein would be a stable ligand for chiral recognition, and we chose this protein to develop a new chiral stationary phase for HPLC with an aqueous mobile phase.

Ketoprofen (KE), ibuprofen (IB), and flurbiprofen (FL) are antiinflammatory drugs used extensively in clinical medicine (Fig. 1). Fig. 2A–C shows the chromatographic resolution of these acidic profen enantiomers on the new column. KE was baseline-resolved with 50 m*M* KH_2PO_4 (pH 4.6) containing 10% ethanol for elution, as shown in Fig. 2A. But IB (Fig. 2B) and FL (Fig. 2C) were not appreciably resolved under the same conditions in spite of their similar structures.

α,ε-Dibenzoyllysine (DB), another carboxyl group-containing compound (Fig. 1), gave almost the same chromatogram as IB (Fig. 2D). Warfarin (WA), an anticoagulant, is a weakly acidic compound (Fig. 1). With WA, we obtained the dramatic result that the retention time of the second-eluted enantiomer was twice that of the first-eluted enantiomer (Fig. 2E). However, benzoin (BE), which is a neutral compound (Fig. 1), was not well resolved under the same conditions (Fig. 2F): its retention time was far shorter than those of the other compounds.

Next we examined the character of the flavoprotein column in more detail by using KE, DB, WA, and BE as model compounds. First we examined the effect of the concentration of organic

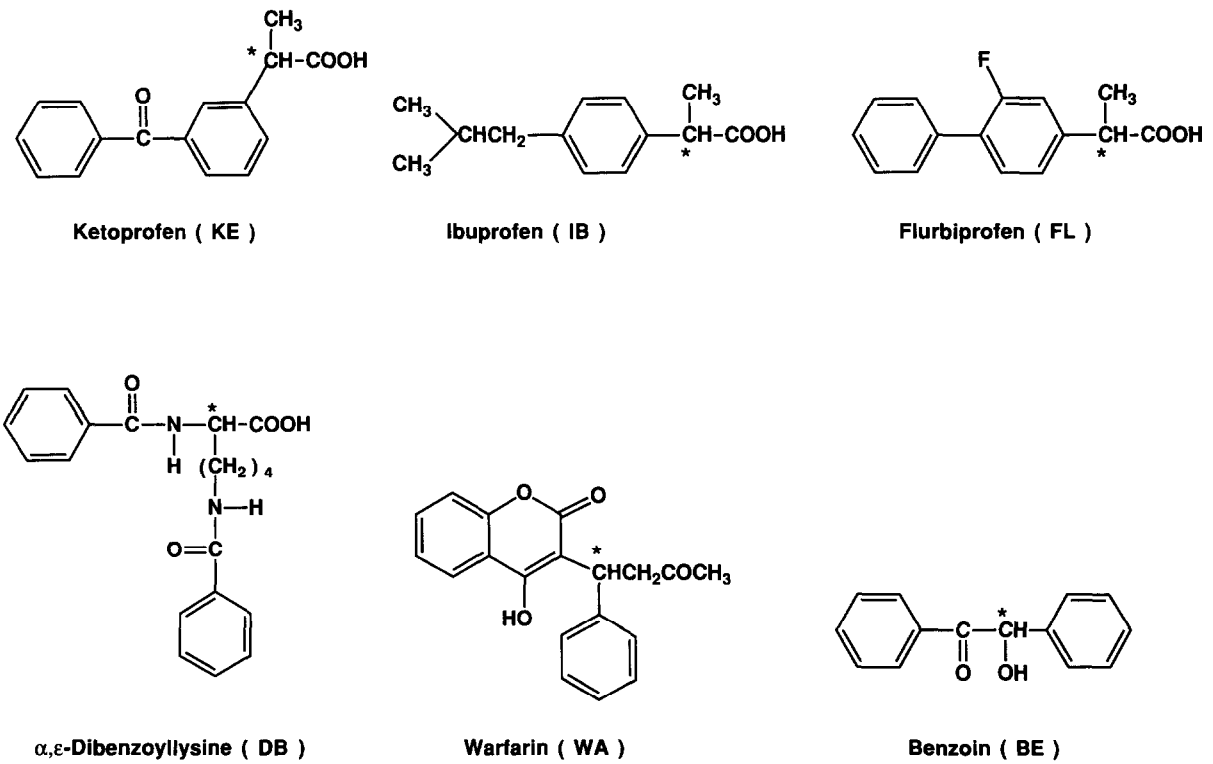

Fig. 1. Molecular structures of compounds resolved by HPLC on a flavoprotein-conjugated column. The chiral centres are indicated by asterisks.

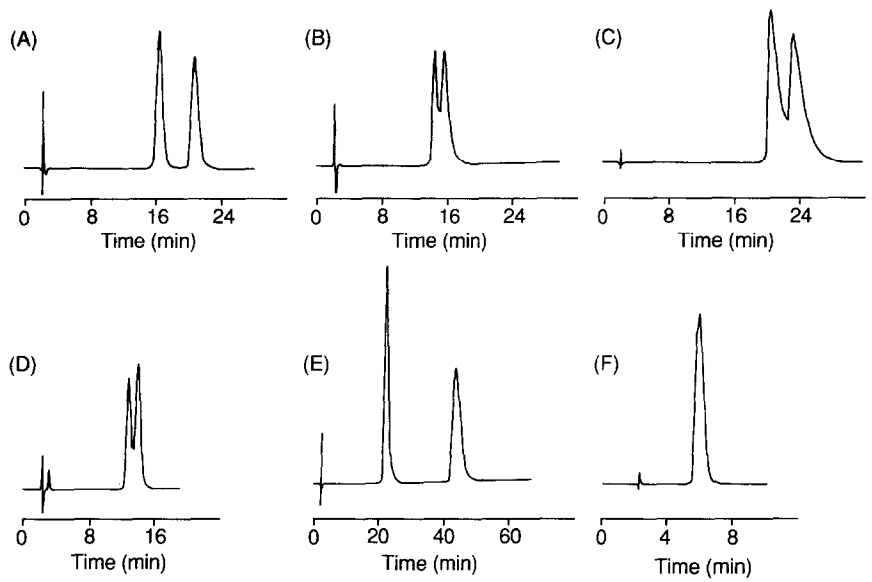

Fig. 2. Chromatograms of each compound on a flavoprotein-conjugated column: (A) KE; (B) IB; (C) FL; (D) DB; (E) WA; (F) BE. Chromatographic conditions: mobile phase, 50 m*M* KH_2PO_4 containing 10% ethanol; UV detection wavelength 230 nm; flow-rate, 1.0 ml/min; column temperature, room temperature.

solvent in the mobile phase on the capacity factor (k') and enantioselectivity. The column operation was apparently reversed-phase, as shown in Table I, because all the drugs showed longer retention times with a reduction in the concentration of organic solvent in the mobile phase. DB and BE, which could not be well resolved by elution with 50 m*M* KH_2PO_4 (pH 4.6) containing 10% ethanol, as mentioned above, showed increased separation factors (α value) and resolution (R_s value) with decreasing concentration of ethanol; this stronger retention of DB and BE led to better chiral separation, as described later. On the other hand, KE and WA did not show a regular variation of α values and R_s values, and the best resolution was obtained at 6% ethanol. However, these compounds were retained too strongly at 6% ethanol, and the peaks were considerably broadened. So the optimum concentration for chiral separation of KE and WA appears to be *ca.* 10% ethanol.

The performance of this column was greatly affected by the pH of the mobile phase. We examined the effect of the pH of the eluent containing 10% ethanol on the chiral separation. Fig. 3A shows the effect of pH on the retention of KE and DB. The *pK* value of a carboxylic acid is generally *ca.* 4 or 5, so KE and DB carry a negative charge above pH 4.0. The k' values of KE and DB decreased with increasing pH from pH 4.0. As noted in the Introduction, the p*I* value of flavoprotein is 3.9–4.1, so its charge is negative over pH 4.0 and positive below pH 4.0. Therefore, these results may reflect electrostatic repulsion between the ionized carboxylic acid (KE or DB) and the negative charge of the flavoprotein. On the other hand, the flavoprotein column most strongly retained KE and DB at pH 4.0. This is reasonable because this solid phase shows its maximum hydrophobicity at pH *ca.* 4.0.

The best pH for the chiral separation of KE was 4.6, and its R_s value was 3.04 (Table II), though the retention of KE was the strongest at pH 4.0 in this pH range. That is to say, the pH that gave the best chiral separation was different from the pH that gave rise to the strongest retention. This seems to show that the separating ability of the chiral recognition site is independent of the total charge of the protein molecule. On the other hand, DB gave the best resolution (R_s 1.53) at pH 4.0, at which value the retention was also strongest, and DB could not be resolved at pH values above 5.0. It was clear that the

TABLE I

EFFECT OF CONCENTRATION OF ORGANIC SOLVENT IN THE MOBILE PHASE ON CAPACITY FACTOR AND ENANTIOSELECTIVITY

k'_1, first-eluted enantiomer; k'_2, second-eluted enantiomer. Chromatographic conditions: mobile phase, 50 m*M* KH_2PO_4 containing ethanol at the concentration shown; other conditions were the same as in Fig. 2.

Ethanol conc. (%)	k'_1	k'_2	α	R_s
Ketoprofen (KE)				
4	23.94	30.95	1.29	2.09
6	10.94	15.00	1.37	5.23
8	8.60	11.08	1.29	4.09
10	6.84	8.38	1.23	2.97
12	5.82	6.81	1.17	2.08
Dibenzoyllysine (DB)				
4	13.13	15.00	1.14	1.94
6	9.13	10.22	1.12	1.25
8	6.90	7.56	1.10	1.05
10	5.46	5.83	1.07	0.76
12	4.45	4.65	1.04	0.55
Warfarin (WA)				
4	44.64	101.27	2.27	4.99
6	16.41	35.43	2.16	8.13
8	12.00	26.27	2.19	7.07
10	9.01	17.38	1.93	5.68
12	7.01	13.02	1.86	6.77
Benzoin (BE)				
4	5.69	6.50	1.14	1.02
6	3.21	3.49	1.09	0.71
8	2.84	3.03	1.07	0.64
10	2.51	2.60	1.04	0.40
12	2.32	2.32	1.00	0

optimum conditions for chiral separation differ from compound to compound.

Fig. 3B shows the effect of the pH of the mobile phase on the k' values for WA and BE, which each have only one hydroxyl group as an ionic functional group. The k' value of the first-eluted enantiomer of WA did not change much between pH 4.0 and 4.6, but that of the second was considerably affected. WA (p*K* 5.03–5.06 [16]) was best resolved (R_s 6.99) at pH 4.6, a similar value to that for KE. As shown in Table II, KE and WA gave the best results in terms of the R_s and α values at pH 4.6. That is to say, the chiral recognition ability of this column for KE and WA is clearly highest at pH 4.6.

TABLE II

EFFECT OF pH OF THE MOBILE PHASE ON ENANTIOSELECTIVITY

Chromatographic conditions: mobile phase, 50 m*M* potassium phosphate buffer (pH as shown) containing 10% ethanol; other conditions were the same as in Fig. 2.

pH	α	R_s	pH	α	R_s
Ketoprofen (KE)			*Warfarin (WA)*		
3.8	1.17	1.46	3.8	1.55	2.94
4.0	1.23	2.09	4.0	1.73	4.48
4.2	1.26	2.33	4.2	1.84	4.97
4.6	1.27	3.04	4.6	1.98	6.99
4.8	1.23	2.08	4.8	1.97	5.43
5.0	1.19	1.72	5.0	1.91	5.03
5.6	1.09	0.84	5.6	1.66	4.10
Dibenzoyllysine (DB)			*Benzoin (BE)*		
3.8	1.11	1.16	3.8	1.00	0
4.0	1.13	1.53	4.0	1.00	0
4.2	1.13	1.31	4.2	1.00	0
4.6	1.09	1.01	4.6	1.00	0
4.8	1.06	0.64	4.8	1.05	0.48
5.0	1.00	0	5.0	1.07	0.57
5.6	1.00	0	5.6	1.10	0.68

Another compound which has a hydroxyl group, BE, could be resolved at pH values above 4.8, but its retention did not change much in the pH range 3.8–5.6. Interestingly, its R_s and k' values were increasingly enhanced with a rise of the pH of the mobile phase. So in the case of BE, the chiral recognition ability became higher with increasing pH, independent of retention.

The retention for each compound on this column was also greatly affected by the ionic strength of the mobile phase. Fig. 4 shows the effect of the salt concentration in the mobile phase for the retention of each enantiomer. The retention of acidic compounds, which carry a negative charge at the pH values examined, decreased with increasing salt concentration. This tendency was not observed with BE. On the other hand, the α values of these compounds were not greatly influenced in the salt concentration range 10–250 m*M*, except for DB at 250 m*M* (data not shown). Thus the ionic interactions between the drugs and the protein may be a minor factor in the chiral separation. The retention of these compounds is affected by electrostatic

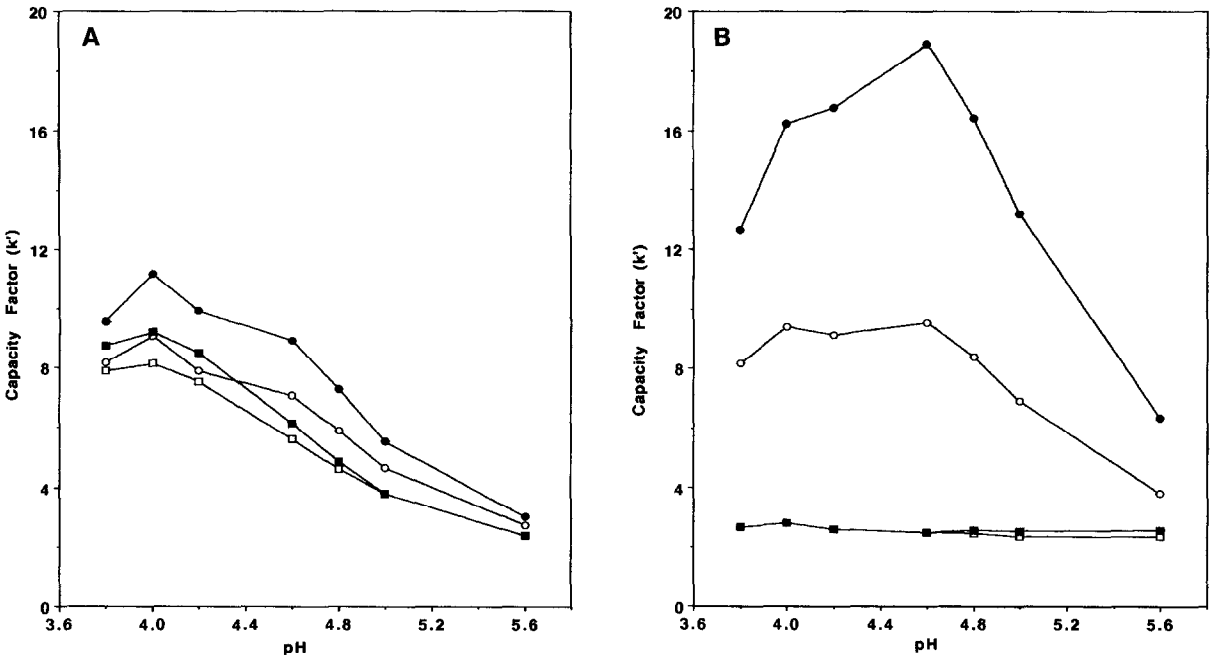

Fig. 3. Effect of pH of the mobile phase on capacity factors. (A) KE and DB: (○) first-eluted enantiomer of KE; (●) second-eluted enantiomer of KE; (□) first-eluted enantiomer of DB; (■) second-eluted enantiomer of DB. (B) WA and BE. (○) first-eluted enantiomer of WA; (●) second-eluted enantiomer of WA; (□) first-eluted enantiomer of BE; (■) second-eluted enantiomer of BE. Chromatographic conditions: pH of the mobile phase was as shown in the figure and other conditions were the same as in Fig. 2.

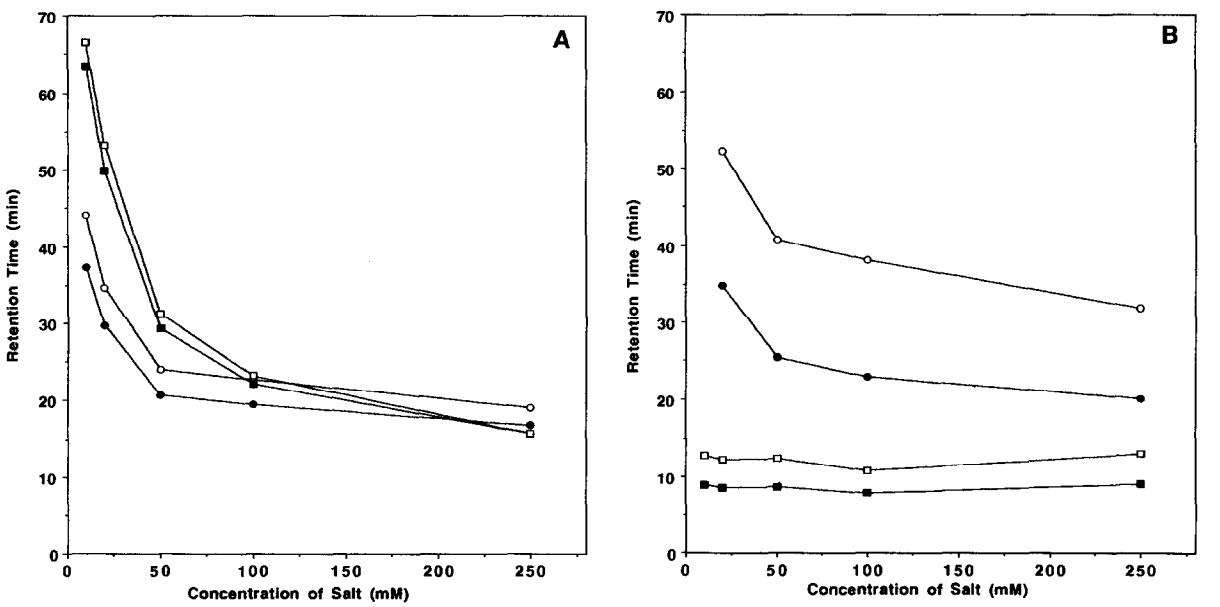

Fig. 4. Effect of salt concentration in the mobile phase on retention. (A) KE and DB: (●) first-eluted enantiomer of KE; (○) second-eluted enantiomer of KE; (■) first-eluted enantiomer of DB; (□) second-eluted enantiomer of DB. (B) WA and BE: (●) first-eluted enantiomer of WA; (○) second-eluted enantiomer of WA; (■) first-eluted enantiomer of BE; (□) second-eluted enantiomer of BE. Chromatographic conditions: salt concentration in the mobile phase was as shown in the figure, and ethanol concentrations were 10% (KE), WA), 6% (DB), and 4% (BE); other conditions were the same as in Fig. 2.

TABLE III

EFFECT OF ORGANIC MODIFIERS IN THE MOBILE PHASE ON CAPACITY FACTOR AND ENANTIOSELECTIVITY

k'_1, first-eluted enantiomer; k'_2, second-eluted enantiomer; n = plate number of first-eluted enantiomer. Chromatographic conditions: mobile phase buffer concentration 50 mM; pH 4.0 (DB), 4.6 (KE, WA), and 5.6 (BE), concentration of organic solvents, 4% (BE), 6% (DB), and 10% (KE, WA), other conditions were the same as in Fig. 2.

Compound	Solvent	k'_1	k'_2	α	R_s	n
KE	Methanol	12.35	15.42	1.25	2.01	1576
	Ethanol	9.25	10.69	1.16	1.39	1669
	1-Propanol	5.50	5.50	1.00	0	695
	1-Butanol[a]	–	–	–	–	–
	2-Propanol	7.00	7.82	1.12	0.87	1158
	tert.-Butanol	8.24	9.67	1.17	1.03	903
	Acetonitrile	5.82	6.35	1.09	0.79	1705
DB	Methanol	18.15	20.92	1.15	1.16	1176
	Ethanol	12.28	13.03	1.06	0.65	2383
	1-Propanol	6.95	6.95	1.00	0	1493
	1-Butanol	5.92	5.92	1.00	0	1921
	2-Propanol	9.17	9.60	1.05	0.55	2486
	tert.-Butanol	10.43	10.86	1.04	0.48	3574
	Acetonitrile	9.40	9.40	1.00	0	1105
WA	Methanol	16.39	32.60	1.99	5.61	851
	Ethanol	11.43	18.25	1.60	4.79	1409
	1-Propanol	6.23	6.49	1.04	0.40	2487
	1-Butanol[a]	–	–	–	–	–
	2-Propanol	8.33	10.78	1.29	1.92	795
	tert.-Butanol	10.04	13.53	1.35	2.21	661
	Acetonitrile	7.33	9.57	1.31	2.13	1053
BE	Methanol	4.11	6.00	1.46	3.01	1185
	Ethanol	3.71	5.36	1.44	2.81	869
	1-Propanol	2.53	3.06	1.21	1.76	1445
	1-Butanol	2.13	2.24	1.05	0.55	2208
	2-Propanol	3.06	4.24	1.39	2.54	1215
	tert.-Butanol	3.46	5.22	1.51	3.12	1258
	Acetonitrile	3.57	4.29	1.20	1.25	576

[a] 50 mM KH_2PO_4 (pH 4.6) containing 10% 1-butanol could not used because it was not mixed.

interactions, such as ion exchange and salting-out effects of the protein.

The effect of organic modifiers in the mobile phase on the retention and the enantioselectivity was examined (Table III). These results show that the k' values of all of the compounds examined decreased with increasing carbon number of the primary alcohol, and this agrees well with the data in Table I. In the case of primary alcohol solvents, the less hydrophobic solvents, methanol and ethanol, gave better R_s values than the more hydrophobic solvents, such as 1-propanol and 1-butanol. But in the case of branched-chain alcohol solvents, such as 2-propanol and *tert.*-butanol, this rule did not necessarily hold. This result was the same as that obtained with the ovomucoid column [10] and the conalbumin column [17]. These facts mean that the chiral separation of protein columns depends strongly on the kind of organic solvent used.

As described earlier, DB and BE did not give good resolution with 50 mM KH_2PO_4 (pH 4.6) containing 10% ethanol, as shown in Fig. 2D and F. Based on the above results, we considered that DB and BE might be effectively resolved by changing the pH and using a lower concentration of organic solvent. Indeed, we found that the enantiomers of DB were

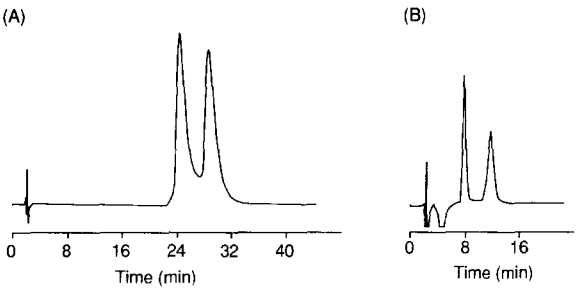

Fig. 5. Chromatograms of (A) DB and (B) BE on a flavoprotein-conjugated column. Chromatographic conditions: mobile phase, (A) 50 m*M* potassium phosphate buffer (pH 4.0) containing 6% ethanol, (B) 50 m*M* potassium phosphate buffer (pH 5.6) containing 4% *tert.*-butanol; other conditions were the same as in Fig. 2.

almost baseline-resolved by elution with 50 m*M* potassium phosphate buffer (pH 4.0) containing 6% ethanol, and its R_s value was 1.78, as shown in Fig. 5A. On the other hand, the retention of BE was very much weaker than those of the other compounds. Further, the best resolution of BE was obtained at pH 5.6 in the range pH 3.8–5.6. Finally, elution with 50 m*M* potassium phosphate buffer (pH 5.6) containing 4% *tert.*-butanol was found to be the best for chiral separation; the R_s value was 3.12, as shown in Fig. 5B.

CONCLUSION

In this study, we prepared a new chiral stationary phase using a flavoprotein for reversed-phase HPLC, and examined the performance of this material. The results indicate that the retention and enantioselectivity are affected by various mobile phase conditions. The retention may be reflect the hydrophobic and coulombic interactions between the solutes and the solid phase. However, these interactions may not have much effect on the chiral separation. On the other hand, each compound requires characteristic conditions for chiral separation, so this may occur at distinctive chiral recognition sites that are different from the retention regions.

During this investigation, direct chiral separation of several acidic and neutral compounds was achieved by reversed-phase HPLC, though the resolution and optimum conditions for separation varied from compound to compound. Nevertheless, this material appears to have great potential for chiral separation, and further studies are planned to extend its range of applicability to other clinically used drugs.

ACKNOWLEDGEMENT

We are grateful to Mr. Hiroshi Inoue, Kawashima Factory, Eisai Co., Ltd., for the purification and supply of flavoprotein.

REFERENCES

1 W. L. Hinze, T. E. Riehl, D. W. Armstrong, W. DeMond, A. Alak and T. Ward, *Anal. Chem.*, 57 (1985) 237.
2 J. I. Seeman, H. V. Secor, D. W. Armstrong, K. D. Timmons and T. J. Ward, *Anal. Chem.*, 60 (1988) 2120.
3 R. Isaksson, P. Erlandsson, L. Hansson, A. Holmberg and S. Berner, *J. Chromatogr.*, 498 (1990) 257.
4 S. Allenmark, B. Bomgren and H. Boren, *J. Chromatogr.*, 264 (1983) 63.
5 J. Hermansson, *J. Chromatogr.*, 269 (1983) 71.
6 G. Schill, I. W. Wainer and S. A. Barkan, *J. Liq. Chromatogr.*, 9 (1986) 641.
7 T. Miwa, M. Ichikawa, M. Tsuno, T. Hattori, T. Miyakawa, M. Kayano and Y. Miyake, *Chem. Pharm. Bull.*, 35 (1987) 682.
8 T. Miwa, T. Miyakawa, M. Kayano and Y. Miyake, *J. Chromatogr.*, 408 (1987) 316.
9 T. Miwa, H. Kuroda, S. Sakashita, N. Asakawa and Y. Miyake, *J. Chromatogr.*, 511 (1990) 89.
10 J. Iredale, A. F. Aubry and I. Wainer, *Chromatographia*, 31 (1991) 329.
11 K. M. Kirkland, K. L. Neilson and D. A. McCombs, *J. Chromatogr.*, 545 (1991) 43.
12 Y. Oda, N. Asakawa, T. Kajima, Y. Yoshida and T. Sato, *Pharmaceutical Res.*, 8 (1991) 997.
13 Y. Sato and K. Watanabe, *Proteins, nucleic acids and enzymes*, 23 (1978) 54.
14 M. B. Rhodes, N. Bennett and R. E. Feeney, *J. Biol. Chem.*, 234 (1959) 2054.
15 W. P. Winter, E. G. Buss, C. O. Clagett and R. V. Boucher, *Comp. Biochem. Physiol.*, 22 (1967) 897.
16 V. J. Stella, K. G. Mooney and J. D. Pipkin, *J. Pharm. Sci.*, 73 (1984) 946.
17 N. Mano, Y. Oda, T. Miwa, N. Asakawa, Y. Yoshida and T. Sato, *J. Chromatogr.*, 603 (1992) 105.