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Journal of Chromatography A, 687 (1994) 223–232

JOURNAL OF CHROMATOGRAPHY A

Studies of ovomucoid-, avidin-, conalbumin- and flavoprotein-conjugated chiral stationary phases for separation of enantiomers by high-performance liquid chromatography

Nariyasu Mano[a,*], Yoshiya Oda[a], Naoki Asakawa[a], Yutaka Yoshida[a], Tadashi Sato[a], Toshinobu Miwa[b]

[a]*Department of Physical and Analytical Chemistry, Tsukuba Research Laboratories, Eisai Co., Ltd., 5-1-3 Tokodai, Tsukuba, Ibaraki 300-26, Japan*

[b]*Department of Pharmaceutical Research Laboratory, Eisai Co., Ltd., 2-1 Takehaya-machi, Kawashima-cho, Hashima-gun, Gifu 483, Japan*

First received 16 May 1994; revised manuscript received 15 August 1994

Abstract

The effects of organic modifiers, buffer salts and pH on the retention and chiral separation of four protein-immobilized chiral stationary phases (CSPs), an ovomucoid–CSP, an avidin–CSP, a conalbumin–CSP and a flavoprotein–CSP, were investigated. Both retention and enantioselectivity were affected by alteration of the mobile phase conditions, and it was elucidated that the hydrophobic and ionic interactions between enantiomers and chiral recognition moieties in immobilized protein molecules were important to the chiral separation of each CSP. The protein bindings of enantiomers for four native proteins were also examined with chiral separation chromatography using a commercial ovomucoid–CSP (Ultron ES-OVM). The racemate which showed significant differences in protein binding abilities among its enantiomers was excellently resolved by chromatography.

1. Introduction

Chiral separations of drug enantiomers by high-performance liquid chromatography (HPLC) have shown great progress in recent years [1–5], and the mechanisms of some of the chiral separation modes have been clarified step by step [6,7]. It is known that protein columns can be used for the separation of drug enantiomers within a broad range [8] and can be used under reversed-phase conditions [9]. However, the mechanism of chiral separation with protein-conjugated columns has not yet been clarified, because proteins are complex biopolymers consisting of a number of *l*-amino acids residues, and they are capable of many interactions with small molecules, such as hydrophobic and electrostatic interactions. It is known that many enzymes can recognize each drug enantiomer [10], which may be due to differences in affinities for the active sites in the enzymes [11]. Wainer and co-workers have described that a human serum albumin (HSA)-conjugated chiral stationary phase (CSP) undergoes an allosteric action in the retention of drug enantiomers [12] and reported that good correlations were obtained

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(94)00782-9

between retention expressed as $k'/(k'+1)$ and the extent of albumin binding for benzodiazepines and coumarins [13]. We therefore considered that protein binding was essential for retention and chiral separation on protein–CSPs.

We have developed four protein-conjugated CSPs, namely an ovomucoid-conjugated CSP (OVM–CSP) [14], an avidin-conjugated CSP (AVI–CSP) [15], a conalbumin-conjugated CSP (CON–CSP) [16] and a flavoprotein-conjugated CSP (FLA–CSP) [17], and have described their usefulness for the analysis of drug enantiomers in biological samples [18–20].

In this study, we used four model compounds, (±)-ketoprofen (KP), (±)-propranolol (PP), (±)-chlormezanone (CM) and (±)-benzoin (BZ) (Fig. 1). We compared the retentions and chiral separation properties of four CSPs with alteration of the mobile phase properties, such as organic solvents, buffer concentration, buffer anion and pH. The hydrophobic interactions and the ionic interaction between enantiomers and chiral recognition moieties were important for chiral separation with each CSP. In addition, protein bindings of enantiomers for native proteins were examined, and the enantiomers which displayed a great protein binding ability showed strong retention in chromatography. Also, each CSP had a similar property in that the enantiomers which showed significant differences in protein binding abilities were excellently resolved by chromatography.

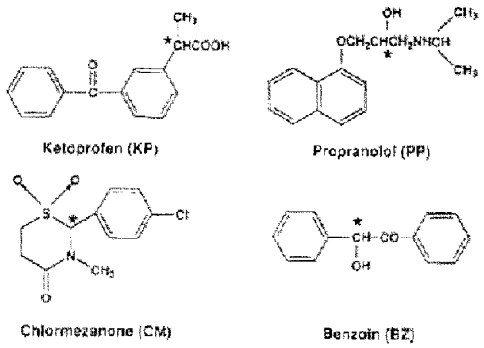

Fig. 1. Structures of model compounds. The chiral centres are indicated by asterisks.

2. Experimental

2.1. Materials

The solvents used were of HPLC grade. The four chicken egg white proteins were purified as described previously [21–24]. KP was purchased from Sigma (St. Louis, MO, USA), PP from Aldrich (Milwaukee, WI, USA), CM and BZ from Tokyo Kasei Kogyo (Tokyo, Japan), N,N-disuccinimidyl carbonate from Wako (Osaka, Japan) and N,N-disuccinimidyl suberate from Pierce (Rockford, IL, USA). The chiral column used in the protein binding study was an Ultron ES-OVM (5 μm, 150 mm × 4.6 mm I.D.), obtained from Shinwa Chemical Industries (Kyoto, Japan). The four protein CSP columns were prepared as described previously [14–17]. Nucleosil $5NH_2$ was purchased from Macherey–Nagel (Düren, Germany). Centrifree micropartition systems (MPS-3) for ultrafiltration were obtained from Amicon (Beverly, MA, USA).

2.2. Chromatographic analysis with protein columns

Chromatographic analysis was performed using a Tosoh (Tokyo, Japan) CCPM pump, a WISP 712 autosampler (Waters, Milford, MA, USA), a Uvidec 100-VI UV spectrophotometer (Japan Spectroscopic, Tokyo, Japan) and a C-R4AX integrating recorder (Shimadzu, Kyoto, Japan). Known amounts of enantiomers were dissolved in water–methanol solutions, each solution was diluted with water to a concentration of 20 μg/ml and 10 μl of these solutions were injected into the HPLC column. All chromatographic experiments were carried out at a flow-rate of 1.0 ml/min and at room temperature.

2.3. Assay of protein binding of enantiomers for native proteins

The protein bindings of enantiomers were measured as follows. All solutions for incubation were prepared with 50 m*M* phosphate buffer. The final concentration of racemates was 100 μg/ml and that of proteins was 10 mg/ml. A

1-ml volume of sample solution was incubated for 1 h at about 25°C, and the free concentration of enantiomers in the filtrate was determined by HPLC with chiral separation using an Ultron ES-OVM column after filtration using MPS-3. All operations were performed at ambient temperature with the mobile phase at a flow-rate of 1.0 ml/min.

3. Results and discussion

The retention and the chiral resolution of enantiomers with protein-immobilized CSPs were influenced by mobile phase conditions such as buffer concentration, buffer anion, organic modifier and pH. Variations in these conditions may change the interaction of solutes with the solid phase caused by the conformation of the protein and/or the electrostatic situation and the hydrophobicity of solutes.

The variations in the capacity factor (k') and the separation factor (α) with alteration of methanol content are shown in Table 1. The k' values of all compounds on every CSP increased with decreasing methanol content. Hence these four CSPs clearly undergo a reversed-phase separation. In addition, the α values also increased with decreasing methanol content in almost all instances, although PP vs. OVM–CSP

Table 1
Effect of methanol content on retention and chiral separation of enantiomers on four CSPs

CSP	MeOH (%)	KP		PP		CM		BZ	
		k'_1	α	k'_1	α	k'_1	α	k'_1	α
OVM	15	2.48	1.11	–	–	1.40	2.91	3.72	2.15
	20	1.64	1.00	67.67	1.07	0.93	2.14	2.33	1.96
	25	1.16	1.00	33.89	1.09	0.67	1.63	1.24	1.67
	30	0.87	1.00	19.27	1.11	0.60	1.18	0.81	1.47
	35	0.69	1.00	11.63	1.13	0.55	1.00	0.59	1.29
	40	0.58	1.00	8.23	1.14	0.45	1.00	0.49	1.20
AVI	10	–	–	4.87	1.02	4.60	1.20	4.25	1.02
	15	22.24	1.75	4.10	1.04	3.03	1.32	3.07	1.06
	20	12.92	1.59	3.46	1.03	1.94	1.44	2.17	1.06
	25	8.06	1.47	3.12	1.00	1.38	1.49	1.62	1.05
	30	5.05	1.37	2.61	1.00	0.98	1.53	1.21	1.00
	35	3.23	1.30	2.33	1.00	0.75	1.57	0.93	1.00
	40	2.39	1.25	2.11	1.00	0.63	1.56	0.76	1.00
CON	2	5.60	1.00	7.71	1.00	1.35	1.19	2.96	1.06
	4	4.48	1.00	6.45	1.00	1.19	1.16	2.54	1.06
	6	3.77	1.00	5.63	1.00	1.10	1.13	2.20	1.05
	8	3.30	1.00	4.87	1.00	1.10	1.00	1.98	1.05
	10	2.96	1.00	4.30	1.00	1.03	1.00	1.87	1.00
FLA	2	8.83	1.19	–	–	1.48	1.72	6.38	1.48
	4	6.59	1.18	31.85	1.00	1.28	1.66	5.36	1.45
	6	4.69	1.16	23.88	1.00	1.07	1.58	3.90	1.36
	8	4.31	1.14	21.34	1.00	1.04	1.54	3.54	1.33
	10	3.03	1.12	16.00	1.00	0.84	1.44	2.61	1.25
	15	1.79	1.07	12.05	1.00	0.72	1.31	1.84	1.19
	20	1.36	1.00	9.65	1.00	0.64	1.23	1.47	1.14

Chromatographic conditions: mobile phase, 20 m*M* phosphate buffer (pH 6.0)–methanol; flow-rate, 1.0 ml/min; detection, UV at 230 nm; sample amount, 200 ng in 10 μl. k'_1 = Capacity factor of first-eluted enantiomer; α = separation factor.

and CM vs. AVI–CSP showed opposite results. This trend of α values was clearly observed when neutral compounds were used on the OVM–, CON– and FLA–CSPs.

The effects of organic modifiers of the mobile phase on the retention and separation of enantiomers on the four CSPs are shown in Table 2. This examination also showed that these four CSPs were involved in a reversed-phase separation mode. In the case of using straight-chain alcohols as organic modifiers such as methanol, ethanol and 1-propanol, an increase in the hydrophobicity of their alcohols induced a decrease in the α values. These results were the same as those obtained in the experiment with variation in methanol content mentioned above. On the other hand, using 2-propanol resulted in α values the same as or lower than those obtained using ethanol. Different results in chiral separation were sometimes induced by adding acetonitrile to the mobile phase. The α valve of PP using acetonitrile was larger than that using methanol, in spite of the significantly weaker retention on OVM–CSP, and FLA–CSP using acetonitrile also promoted better enantioselectivity for BZ than with 1-propanol with almost the same retention capacity.

From the results obtained in these studies, the increase in α values with decreasing hydrophobicity in the mobile phase suggests that the hydrophobic interaction in chiral recognition cavities significantly affected the difference in affinity for protein–CSPs. Addition of methanol as an organic modifier resulted in better resolution than that of other straight-chain alcoholic solvents on every CSP. Methanol is the most polarized and the smallest molecule among the organic solvents generally used in HPLC, hence it may be able to elute with minimum interference from hydrophobic interactions between the

Table 2
Effect of organic modifiers on retention and chiral separation of enantiomers on four CSPs

CSP	Solvent	KP		PP		CM		BZ	
		k'_1	α	k'_1	α	k'_1	α	k'_1	α
OVM	Methanol	1.64	1.00	67.67	1.07	0.93	2.14	2.33	1.96
	Ethanol	1.14	1.00	13.82	1.00	0.56	1.18	1.00	1.49
	1-Propanol	0.74	1.00	1.19	1.00	0.35	1.00	0.55	1.00
	2-Propanol	0.91	1.00	3.98	1.00	0.48	1.00	0.78	1.42
	Acetonitrile	0.71	1.00	4.01	1.15	0.37	1.00	0.58	1.00
AVI	Methanol	12.92	1.59	3.46	1.03	1.94	1.44	2.17	1.06
	Ethanol	4.52	1.31	2.44	1.00	1.14	1.11	1.50	1.00
	1-Propanol	1.97	1.00	1.66	1.00	0.66	1.00	1.05	1.00
	2-Propanol	2.92	1.11	2.31	1.00	0.89	1.00	1.38	1.00
	Acetonitrile	1.89	1.00	1.44	1.00	0.53	1.00	0.87	1.00
CON	Methanol	5.60	1.00	7.71	1.00	1.35	1.19	2.96	1.06
	Ethanol	4.15	1.00	5.49	1.00	1.18	1.12	2.43	1.06
	1-Propanol	3.51	1.00	4.31	1.00	1.12	1.00	2.18	1.00
	2-Propanol	4.60	1.00	4.03	1.00	1.22	1.00	2.46	1.00
	Acetonitrile	4.73	1.00	6.03	1.00	1.28	1.00	2.43	1.00
FLA	Methanol	3.03	1.12	16.00	1.00	0.84	1.44	2.61	1.25
	Ethanol	1.93	1.10	8.07	1.00	0.73	1.15	1.77	1.14
	1-Propanol	1.37	1.00	3.91	1.00	0.62	1.00	1.22	1.00
	2-Propanol	2.02	1.10	6.54	1.00	0.71	1.00	1.75	1.13
	Acetonitrile	1.26	1.00	4.50	1.00	0.56	1.00	1.21	1.09

Chromatographic conditions: mobile phase, 20 m*M* phosphate buffer (pH 6.0)–organic solvent (OVM, 80:20; AVI, 80:20; CON, 98:2; FLA, 90:10); flow-rate, 1.0 ml/min; detection, UV at 230 nm; sample amount, 200 ng in 10 μl. k'_1 = Capacity factor of first-eluted enantiomer; α = separation factor.

solutes and the chiral recognition cavities on proteins immobilized on a silica support. On the other hand, the results for PP vs. OVM–CSP and CM vs. AVI–CSP in Table 1 were exceptional cases and suggest that methanol as an organic modifier did not interfere much with the specific interactions in the chiral recognition cavities in those cases. Acetonitrile was sometimes a good organic modifier in the mobile phase. The reason for this seems to be that the interference mechanism with acetonitrile on the hydrophobic interaction between solutes and chiral recognition moieties in the solid phase differs from that with methanol, and the hydrophobic interaction in cavities having a specific affinity was influenced by the hydroxyl group in the alcohol.

The effects of buffer concentration in the mobile phase on k' and α values on every CSP are shown in Table 3. The k' and α values of CM and BZ were not substantially changed. The k' values of KP on the OVM–, AVI– and CON–CSPs decreased with increasing buffer concentration, whereas those on the FLA–CSP were not changed. Although the k' values of PP on FLA–CSP decreased with increasing buffer concentration, those on the other three CSPs did not vary, in contrast to the results with KP. On the other hand, with AVI–CSP, the α values of PP increased with increase in buffer concentration, whereas those of all the others were not substantially altered. These results suggest that the ionic interaction between protein–CSPs and ionic solutes contributed to the retention capacity.

Changes in the buffer anion in the mobile phase also affected the retention of ionic solutes on the four CSPs (Table 4). The k' of KP increased when borate buffer was employed in comparison with the use of phosphate buffer on

Table 3
Effect of buffer concentration on retention and chiral separation of enantiomers on four CSPs

CSP	Buffer concentration (m*M*)	KP		PP		CM		BZ	
		k'_1	α	k'_1	α	k'_1	α	k'_1	α
OVM	5	1.68	1.00	32.75	1.08	0.69	1.64	1.31	1.68
	10	1.52	1.00	30.04	1.07	0.71	1.63	1.33	1.68
	20	1.16	1.00	33.89	1.09	0.67	1.63	1.24	1.67
	50	1.05	1.00	30.00	1.08	0.69	1.67	1.36	1.68
	100	1.01	1.00	23.15	1.07	0.69	1.64	1.31	1.69
AVI	5	22.73	1.60	2.98	1.00	2.00	1.43	2.19	1.06
	10	17.78	1.59	3.06	1.00	2.01	1.45	2.20	1.06
	20	12.92	1.59	3.46	1.03	1.94	1.44	2.17	1.06
	50	10.05	1.57	3.13	1.06	2.00	1.49	2.17	1.06
	100	8.29	1.52	2.83	1.07	1.96	1.53	2.10	1.06
CON	5	8.12	1.00	8.43	1.00	1.50	1.30	3.18	1.11
	10	6.98	1.00	10.49	1.00	1.56	1.51	3.31	1.12
	20	5.60	1.00	7.71	1.00	1.35	1.19	2.96	1.06
	50	5.11	1.00	8.48	1.00	1.54	1.12	3.28	1.06
	100	4.45	1.00	7.50	1.00	1.43	1.15	3.15	1.05
FLA	5	2.85	1.15	22.96	1.00	0.91	1.41	2.70	1.26
	10	3.13	1.16	18.40	1.00	0.90	1.42	2.73	1.27
	20	3.03	1.12	16.00	1.00	0.84	1.44	2.61	1.25
	50	2.80	1.13	13.47	1.00	0.89	1.43	2.64	1.28
	100	3.22	1.13	10.94	1.00	0.91	1.44	2.77	1.30

Chromatographic conditions: mobile phase, phosphate buffer (pH 6.0)–methanol (OVM 75:25; AVI, 80:20; CON, 98:2; FLA, 90:10); flow-rate, 1.0 ml/min; detection, UV at 230 nm; sample amount, 200 ng in 10 μl. k'_1 = Capacity factor of first-eluted enantiomer; α = separation factor.

Table 4
Effect of buffer anions on retention and chiral separation of enantiomers on four CSPs

CSP	Buffer	KP		PP		CM		BZ	
		k'_1	α	k'_1	α	k'_1	α	k'_1	α
OVM	Phosphate	1.16	1.00	33.89	1.09	0.67	1.63	1.24	1.67
	Acetate	3.15	1.00	19.74	1.09	0.71	1.63	1.31	1.66
	Borate	9.46	1.00	15.91	1.11	0.66	1.68	1.22	1.66
	Tartrate	1.00	1.00	24.04	1.05	0.75	1.63	1.35	1.62
	Citrate	1.01	1.00	26.23	1.09	0.71	1.00	1.37	1.69
AVI	Phosphate	15.38	1.58	2.87	1.05	1.99	1.45	2.20	1.07
	Acetate	7.22	2.27	1.68	1.00	1.45	1.30	1.92	1.00
	Borate	–	–	1.15	1.00	1.93	1.30	2.17	1.00
	Tartrate	10.07	1.53	2.67	1.06	1.96	1.56	2.19	1.00
	Citrate	6.30	1.60	3.38	1.07	1.92	1.57	2.16	1.04
CON	Phosphate	7.24	1.00	5.80	1.00	1.37	1.12	3.24	1.00
	Acetate	10.26	1.00	5.18	1.00	1.40	1.19	3.13	1.00
	Borate	20.14	1.00	5.58	1.00	1.39	1.19	3.20	1.00
	Tartrate	6.49	1.00	7.05	1.00	1.52	1.14	3.51	1.00
	Citrate	4.42	1.00	8.78	1.00	1.36	1.00	3.33	1.00
FLA	Phosphate	3.03	1.12	16.00	1.00	0.84	1.44	2.61	1.25
	Acetate	5.14	1.11	13.84	1.00	0.94	1.41	2.96	1.22
	Borate	6.72	1.13	18.35	1.00	0.90	1.34	2.70	1.21
	Tartrate	3.43	1.13	11.69	1.00	0.90	1.40	2.70	1.27
	Citrate	2.61	1.15	14.38	1.00	0.78	1.72	2.89	1.30

Chromatographic conditions: mobile phase, 20 m*M* buffer (pH 6.0)–methanol (OVM, 75:25; AVI, 90:10; CON, 98:2; FLA, 90:10); flow-rate, 1.0 ml/min; detection, UV at 230 nm; sample amount, 200 ng in 10 μl. k'_1 = Capacity factor of first-eluted enantiomer; α = separation factor.

every CSP, and the order of the increase in k' was OVM > AVI > CON > FLA. The strengths of retention using the borate buffer were about nine times those using phosphate buffer on OVM–CSP, about four times on AVI–CSP, about three times on CON–CSP and about double on FLA–CSP. This order agrees with the amounts of mannose in the carbohydrate [25]. The carbohydrate moiety of glycoproteins is known to form a carbohydrate–borate complex with the borate anion, and this protein surface change may be related to the above phenomenon. On the other hand, in contrast to the result with KP, the k' value of PP on the OVM– and AVI–CSPs was the lowest when the borate buffer was employed, although on the CON– and FLA–CSPs it was not substantially changed compared with the result with the phosphate buffer. Regarding the α values, the OVM–, CON– and FLA–CSPs did not display much variation, whereas AVI–CSP showed different results attributable to the change in buffer anion.

Table 5 shows the results for k' and α values obtained on changing the pH of the mobile phase. The p*I* values of OVM, AVI, CON and FLA are 3.9–4.3, 9.5–10.0, 6.05–6.6 and 3.9–4.1, respectively [25]. However, KP was most strongly retained at pH 4 on every CSP. This may indicate that the same amino acid residue in the four proteins is related to the retention of KP. KP has a carboxylic acid group in its molecule, so that its p*K* value is about 4–5. Consequently, the retention of KP increased with pH change from 7 to 4 in the mobile phase because its hydrophobicity increased. However, at pH 3, the retention of KP became weak; we consider

Table 5
Effect of pH on retention and chiral separation of enantiomers on four CSPs

CSP	pH	KP		PP		CM		BZ	
		k'_1	α	k'_1	α	k'_1	α	k'_1	α
OVM	3.0	2.96	1.07	0.01	1.00	0.50	1.40	0.66	1.27
	4.0	4.04	1.00	1.61	1.00	0.55	1.35	0.88	1.53
	5.0	2.85	1.00	2.92	1.00	0.53	1.36	0.87	1.59
	6.0	0.91	1.00	16.25	1.10	0.53	1.36	0.82	1.49
	7.0	0.39	1.00	60.72	1.20	0.54	1.43	0.80	1.41
AVI	3.0	14.26	1.23	0.00	1.00	1.84	1.71	2.05	1.00
	4.0	34.69	1.38	0.70	1.00	1.81	1.59	2.04	1.05
	5.0	30.08	1.46	0.86	1.00	1.78	1.57	2.05	1.07
	6.0	15.38	1.58	2.87	1.05	1.99	1.45	2.20	1.07
	7.0	7.29	1.61	7.24	1.00	1.90	1.45	2.11	1.06
CON	3.0	22.65	1.00	0.15	1.00	1.71	1.00	3.26	1.00
	4.0	24.74	1.00	0.58	1.00	1.45	1.12	3.17	1.00
	5.0	19.16	1.00	2.06	1.00	1.45	1.23	3.11	1.08
	6.0	7.24	1.00	5.80	1.00	1.37	1.12	3.24	1.00
	7.0	2.66	1.00	2.66	1.00	1.42	1.18	3.27	1.00
FLA	3.0	11.58	1.12	0.00	1.00	0.95	1.05	2.10	1.13
	4.0	23.20	1.34	0.96	1.09	0.89	1.21	2.87	1.00
	5.0	12.39	1.27	4.83	1.07	0.87	1.36	2.86	1.19
	6.0	3.48	1.15	15.63	1.00	0.85	1.47	2.96	1.28
	7.0	1.42	1.00	–	–	0.94	1.53	3.01	1.29

Chromatographic conditions: mobile phase, 20 m*M* phosphate buffer–methanol (OVM, 70:30; AVI, 80:20; CON, 98:2; FLA, 90:10); flow-rate, 1.0 ml/min; detection, UV at 230 nm; sample amount, 200 ng in 10 μl. k'_1 = Capacity factor of first-eluted enantiomer; α = separation factor.

that alteration of the conformation of the protein itself based on the dissociation of an amino acid residue, which was attributed to the non-specific interaction, contributed to this phenomena. For PP, the k' values increased with increasing pH, although CON–CSP showed a different tendency. The pK of PP is 9.5; therefore, the hydrophobicity of the enantiomers themselves increases on approaching alkaline conditions. However, CON–CSP has a weaker hydrophobic interaction for retention in comparison with the other three CSPs; thus the change in the hydrophobicity of the protein itself seems to affect retention more than those of PP. On the other hand, the α values of KP on AVI–CSP changed with alteration of the buffer anion, although the other CSPs were not substantially affected except for CM vs. OVM- and FLA–CSPs. KP has a carboxylic acid group in the molecule, and the enantioselectivity of KP may decrease owing to inhibition due to the access of buffer anions containing a carboxylic acid to the chiral recognition cavity in AVI–CSP.

The k' values of non-ionic compounds were not greatly affected by pH changes, but the best pH conditions for chiral separation of CM and BZ were different among the four CSPs. These results seem to show that amino acid residues related to non-specific interactions were different from amino acid residues related to chiral recognition in many instances.

The recoveries in the protein binding assay procedure are shown in Table 6, and indicate that this assay procedure could determine the concentration of the four racemates in the filtrate without adsorption on an ultrafiltration mem-

Table 6
Recoveries of four racemates with native protein binding assay

Racemate	Recovery (%)	
	1	2
KP	100.3 ± 2.2	100.4 ± 2.1
PP	95.8 ± 2.2	96.2 ± 2.2
CM	99.2 ± 1.1	101.2 ± 1.0
BZ	101.4 ± 2.6	101.0 ± 2.6

Chromatographic conditions: column, Ultron ES-OVM (150 mm × 4.6 mm I.D.); mobile phase, (KP) acetonitrile–20 m*M* phosphate buffer (pH 3.0) (10:100), (PP) acetonitrile–20 m*M* phosphate buffer (pH 6.8) (30:100) and (CM, BZ) ethanol–20 m*M* phosphate buffer (pH 4.6) (10:100); flow-rate, 1.0 ml/min; detection, UV at 230 nm; $n = 4$. 1 = First-eluted enantiomer; 2 = second-eluted enantiomer.

brane. These results were sufficient for investigating the difference in protein binding between enantiomers. The results of protein binding of enantiomers for four native proteins are shown in Table 7. A tendency for the bindings of ionic compounds for native proteins to be stronger than those of non-ionic compounds was observed except for the cases of PP vs. CON and FLA. OVM showed good capacities for binding to each compound, whereas the binding capacity of CON was very weak except for KP. AVI did not show a binding capacity for PP. In addition, the binding ratios of KP for AVI were opposite those of the others. On the other hand, compounds which had greater differences in protein binding between enantiomers were better resolved on protein columns in each instance, as

Table 7
Protein binding (%) of enantiomers for native proteins

Protein	pH	KP			PP			CM			BZ		
		1	2	2/1	1	2	2/1	1	2	2/1	1	2	2/1
OVM	3.0	19.6	19.5	0.99	3.1	2.8	0.90	2.0	6.8	3.40	4.8	10.8	2.25
	4.0	4.4	6.7	1.52	17.6	17.3	0.98	5.0	15.6	3.12	4.0	11.5	2.88
	5.0	3.7	6.0	1.62	18.6	18.5	0.99	6.0	17.1	2.85	5.2	12.8	2.46
	6.0	3.1	5.8	1.87	20.4	20.5	1.00	4.2	16.4	3.90	6.0	13.5	2.25
	7.0	0.0	0.1	–	23.0	23.6	1.03	4.7	23.7	5.04	4.9	10.6	2.16
AVI	3.0	60.8	45.5	0.75	0.0	0.0	–	9.4	30.4	3.23	11.9	12.4	1.04
	4.0	70.5	52.6	0.75	0.0	0.0	–	18.2	30.7	1.69	12.2	12.7	1.04
	5.0	71.2	54.1	0.76	0.0	0.0	–	26.9	32.9	1.22	14.7	15.7	1.07
	6.0	76.3	57.8	0.76	0.0	0.0	–	27.9	32.8	1.18	16.6	17.7	1.07
	7.0	74.2	53.0	0.71	0.0	0.0	–	29.7	31.9	1.07	16.2	17.5	1.08
CON	3.0	42.0	39.8	0.95	0.0	0.0	–	2.8	1.2	0.43	0.0	0.0	–
	4.0	17.5	17.6	1.01	0.0	0.0	–	1.0	0.0	–	0.0	0.0	–
	5.0	16.2	16.2	1.00	0.0	0.0		0.1	0.0	–	0.0	0.0	–
	6.0	9.4	10.0	1.06	0.0	0.0	–	0.9	0.0	–	0.0	0.0	–
	7.0	0.0	0.0	–	6.8	5.8	0.85	0.9	0.0	–	0.0	0.0	–
FLA	3.0	32.4	34.0	1.05	0.0	0.0	–	1.9	0.5	0.26	4.8	4.9	1.02
	4.0	33.6	41.2	1.23	15.3	13.7	0.90	4.0	4.8	1.20	13.0	14.0	1.08
	5.0	28.5	38.1	1.34	13.7	11.6	0.85	2.3	3.3	1.43	15.6	17.9	1.15
	6.0	25.7	38.5	1.50	34.0	31.9	0.94	2.8	6.2	2.21	17.7	22.1	1.25
	7.0	8.4	11.6	1.38	55.2	55.3	1.00	0.9	10.9	12.11	18.4	29.7	1.61

1 = Protein binding (%) of first-eluted enantiomer of each compound on Ultron ES-OVM; 2 = protein binding (%) of second-eluted enantiomer of each compound on Ultron ES-OVM; 2/1 = protein binding ratio.

shown in Fig. 2. The opposite binding ratios of KP for AVI (Table 7) suggest that the elution order of KP on AVI–CSP may be opposite to that on the other CSPs. OVM–CSP showed the best chiral recognition ability among the four CSPs using four enantiomers. However, AVI–CSP could achieve the chiral resolution of KP much better than OVM–CSP. This is due to the difference in the contributions to non-specific interactions. That is, AVI shows a smaller change in non-specific interactions than OVM on immobilization on the silica support.

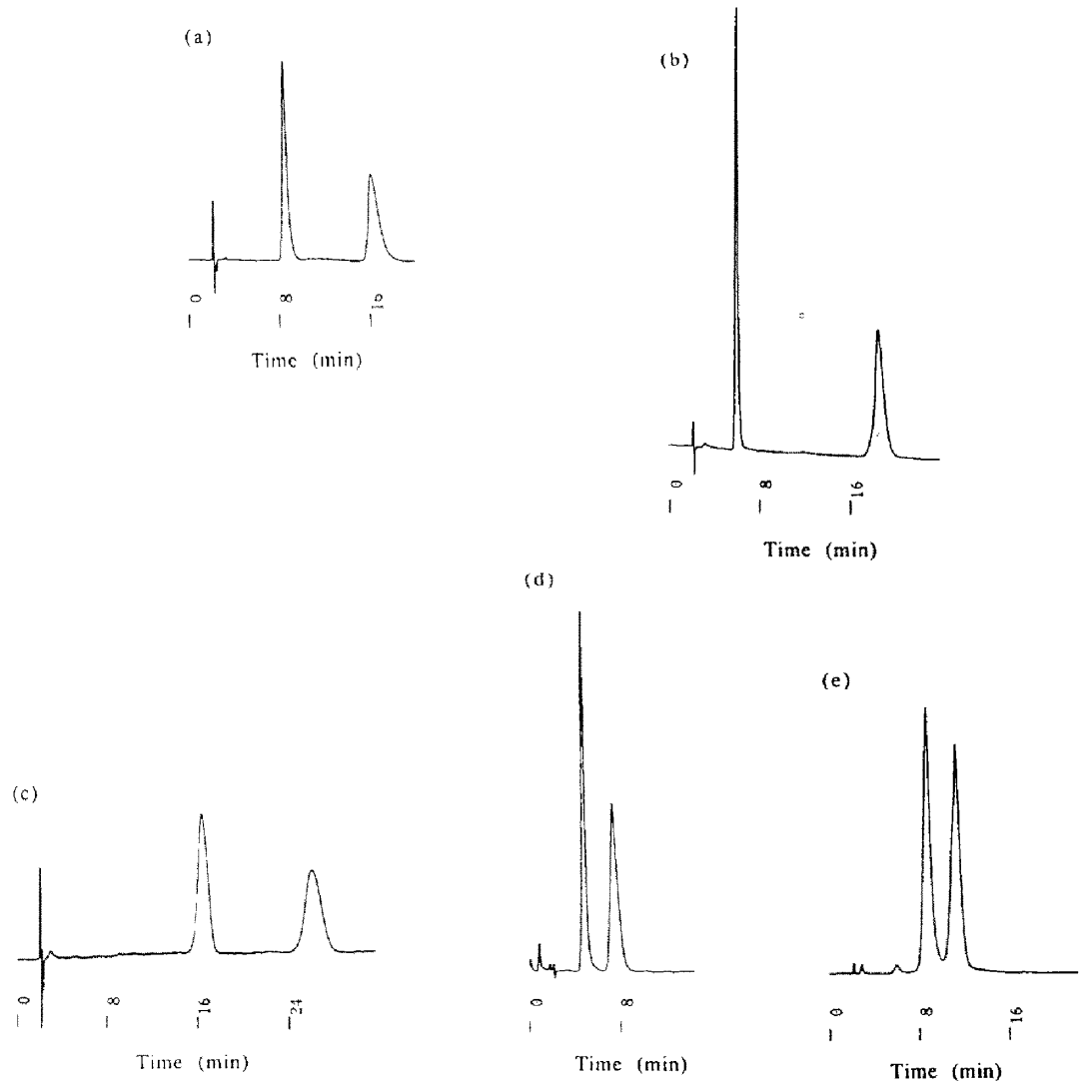

Fig. 2. Typical chromatograms obtained on protein-immobilized CSPs. Chromatographic conditions: column, (a, b) OVM–CSP (150 mm × 4.6 mm I.D.), (c) AVI–CSP (150 mm × 4.6 mm I.D.) and (d, e) FLA–CSP (150 mm × 4.6 mm I.D.); sample, (a, e) benzoin, (b, d) chlormezanone and (c) ketoprofen; mobile phase, (a) 20 m*M* phosphate buffer (pH 6.0)–methanol (85:15), (b) 20 m*M* phosphate buffer (pH 5.0)–methanol (90:10), (c) 20 m*M* phosphate buffer (pH 7.0)–methanol (80:20), (d) 20 m*M* phosphate buffer (pH 7.0)–methanol (98:2) and (e) 20 m*M* phosphate buffer (pH 6.0)–methanol (94:6); flow-rate, 1.0 ml/min; detection, UV at 230 nm; column temperature, room temperature; sample amount, 200 ng in 10 μl.

Protein CSPs act in the reversed-phase mode, so the retention times of solutes were controlled by the concentration of the organic solvents in the mobile phase. However, these organic solvents interfere with the interaction between solutes and chiral recognition moieties in proteins. It is preferable to choose mobile phase components that produce an appropriate retention for analysis with less interference for affinity binding in chiral recognition cavities for the resolution of enantiomers on protein CSPs.

4. Conclusions

The retention properties and chiral separations of enantiomers on four protein CSPs were investigated, and these four CSPs were found to have different properties. The retentions of the enantiomers on each CSP were influenced by both hydrophobic interactions and ionic interactions on all non-specific parts in the solid phase, and chiral separations might also be influenced by the hydrophobic interactions and the ionic interactions and changes in conformation of the protein molecule itself, such as ionization or non-ionization of amino acid residues in chiral recognition moieties. The protein binding properties contribute substantially to the retention and chiral separation on each protein CSP.

Acknowledgement

The authors thank Mr. Hiroshi Inoue (Kawashima Factory, Eisai) for the purification and supply of the four chicken egg white proteins.

References

[1] J.I. Seeman, H.V. Secor, D.W. Armstrong, K.D. Timmons and T.J. Ward, *Anal. Chem.*, 60 (1988) 2120.
[2] S. Allenmark and B. Bomgren, *J. Chromatogr.*, 264 (1983) 63.
[3] J. Hermansson, *J. Chromatogr.*, 269 (1983) 71.
[4] Y. Okamoto and K. Hatada, *J. Liq. Chromatogr.*, 9 (1986) 369.
[5] N. Oi and H. Kitahara, *J. Liq. Chromatogr.*, 9 (1986) 511.
[6] A. Dobashi, Y. Dobashi and S. Hara, *J. Liq. Chromatogr.*, 9 (1986) 243.
[7] Th.R.E. Hampe, J. Schlüter, K.H. Brandt, J. Nagel, E. Lamparter and G. Blaschke, *J. Chromatogr.*, 634 (1993) 205.
[8] K.M. Kirkland, K.L. Neilson and D.A. McCombs, *J. Chromatogr.*, 545 (1991) 43.
[9] J. Iredale, A.-F. Aubry and I. Wainer, *Chromatographia*, 31 (1991) 329.
[10] C.-S. Chen, T. Chen and W.-Ru Shieh, *Biochim. Biophys. Acta*, 1033 (1990) 1.
[11] R.D. Knihinicki, K.M. Williams and R.O. Day, *Biochem. Pharmacol.* 38 (1989) 4389.
[12] E. Domenici, C. Bertucci, P. Salvadori and I.W. Wainer, *J. Pharm.. Sci.*, 80 (1991) 164.
[13] T.A.G. Noctor, M.J. Diaz-Perez and I.W. Wainer, *J. Pharm. Sci.*, 82 (1993) 675.
[14] T. Miwa, M. Ichikawa, M. Tsuno, T. Hattori, T. Miyakawa, M. Kayano and Y. Miyake, *Chem. Pharm. Bull.*, 35 (1987) 682.
[15] Y. Oda, N. Asakawa, S. Abe, Y. Yoshida and T. Sato, *J. Chromatogr.*, 572 (1991) 133.
[16] N. Mano, Y. Oda, T. Miwa, N. Asakawa, Y. Yoshida and T. Sato, *J. Chromatogr.*, 603 (1992) 105.
[17] N. Mano, Y. Oda, N. Asakawa, Y. Yoshida, T. Sato and T. Miwa, *J. Chromatogr.*, 623 (1992) 221.
[18] Y. Oda, N. Asakawa, Y. Yoshida and T. Sato, *Pharm. Res.*, 8 (1991) 997.
[19] Y. Oda, H. Ohe, N. Asakawa, Y. Yoshida, T. Sato and T. Nakagawa, *J. Liq. Chromatogr.*, 15 (1992) 2997.
[20] N. Mano, Y. Oda, H. Ohe, N. Asakawa, Y. Yoshida and T. Sato, *J. Pharm. Biomed. Anal.*, 12 (1994) 557.
[21] E. Frederiq and H.F. Deutsch, *J. Biol. Chem.*, 181 (1949) 499.
[22] T. Miwa, T. Miyakawa and Y. Miyake, *J. Chromatogr.*, 457 (1988) 227.
[23] M.B. Rhodes, P.R. Azari and R.E. Feeney, *J. Biol. Chem.*, 230 (1958) 399.
[24] M.B. Rhodes, N. Bennet and R.E. Feeney, *J. Biol. Chem.*, 234 (1959) 2054.
[25] Y. Sato and K. Watanabe, *Proteins Nucleic Acids Enzymes*, 23 (1978) 54.