Journal of Chromatography A, 660 (1994) 275–281
Elsevier Science B.V., Amsterdam

CHROMSYMP. 2879

Retention and enantioselective properties of ovomucoid-bonded silica columns

Influence of physical properties of base materials and spacer length

Jun Haginaka*, Chikako Seyama and Tokiko Murashima

Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Koshien Kyuban-cho, Nishinomiya 663 (Japan)

Hiroya Fujima and Hiroo Wada

Shinwa Chemical Industries Ltd., 50 Kagakatsu-cho, Fushimi-ku, Kyoto 612 (Japan)

ABSTRACT

The influence of the physical properties of base silica materials and spacer length on chiral separation of enantiomers on ovomucoid (OVM)-bonded materials was investigated. With regard to the pore size of the base silica materials, the 300-Å materials gave higher enantioselectivity than the 120-Å materials, despite the smaller amounts of bonded OVM proteins. However, higher resolution was obtained with the latter materials. With regard to the spacer length, aminopropyl (AP)-, aminobutyl-, N-(4-aminobutyl)-3-aminopropyl- and N-(6-aminohexyl)-3-aminopropyl-silica gels were activated by N,N′-disuccinimidyl carbonate (DSC) and the proteins were bound. The first two materials showed excellent chiral resolution properties for the solutes tested, and the AP materials gave higher enantioselectivity and resolution. On the other hand, only oxprenolol enantiomers were slightly resolved on the last two materials. Also, AP-silica gels activated by DSC were compared with glycerylpropyl (GP)-silica gels activated by 1,1′-carbonyldiimidazole. The former materials gave higher resolution for acidic and basic solutes despite the lower enantioselectivity, whereas for the uncharged hexobarbital the latter materials gave higher enantioselectivity and almost equal resolution. The above results reveal that the 120-Å base silica gels are more suitable than the 300-Å base silica gels for obtaining larger amounts of bonded OVM proteins and that a less hydrophobic spacer such as an AP group and a hydrophilic spacer such as a GP group are suitable.

INTRODUCTION

Protein-bonded stationary phases including albumins such as bovine serum albumin (BSA) [1] and human serum albumin [2] and mucoids such as α_1-acid glycoprotein [3] and ovomucoid [4] from egg white have been developed for the separation of enantiomeric forms. Among these, an ovomucoid-bonded silica column is of special interest because of its long-term stability and because it is suitable for separating a wide range of enantiomeric mixtures [5–7]. It is well known that many factors such as the physical properties of the base silica materials, spacer length and bonding method affect the resolution of enantiomeric pairs on protein-bonded stationary phases [8–10]. In this study, we investigated the in-

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(93)E0770-U

fluences of these factors on the retention, enantioselectivity and resolution of various solutes on ovomucoid-bonded silica columns.

EXPERIMENTAL

Reagents and materials

Ibuprofen, ketoprofen, chlorpheniramine maleate and hexobarbital were kindly donated by Kaken Pharmaceutical (Tokyo, Japan), Chugai Pharmaceutical (Tokyo, Japan), Essex Nippon (Osaka, Japan) and Teikoku Chemicals (Osaka, Japan). Tolperisone hydrochloride was purchased from Sigma (St. Louis, MO, USA). The structures of the racemic compounds used in this study are shown in Fig. 1. OVM proteins from egg white were purchased from Eisai (Tokyo, Japan). N,N′-Disuccinimidyl carbonate (DSC) and 1,1′-carbonyldiimidazole (CDI) were purchased from Sigma, 2-propanol, ethanol, methanol and acetonitrile of HPLC grade from Wako (Osaka, Japan) and 3-aminopropyl (AP)-triethoxysilane, 4-aminobutyl (AB)-triethoxysilane and 3-glycidoxypropyltrimethoxysilane from Chisso (Tokyo, Japan). N-(4-Aminobutyl)-3-aminopropyl (ABAP)-trimethoxysilane and N-(6-aminohexyl)-3-aminopropyl (AHAP)-trimethoxysilane were kindly donated by Shin-etsu Chemical Industries (Tokyo, Japan). Silica gels were obtained from Shinwa Chemical Industries (Ultron-120, particle diameter 5 μm, pore size 120 Å, specific surface area 300 m^2/g, and Ultron-300, particle diameter 5 μm, pore size 300 Å, specific surface area 100 m^2/g). Other solvents and reagents were used as received.

Water purified with a Nanopure II unit (Barnstead, Boston, MA, USA) was used for the preparation of the eluent and sample solutions.

Preparation of silica gels having different spacer lengths

Ultron silica gel (5 g) was dried *in vacuo* over P_2O_5 at 150°C for 6 h and added to 120 ml of dry toluene. The mixture was heated at reflux until all the water had been removed as an azeotrope into a Dean–Stark-type trap. Next, the silylating agents described above, corresponding to 10 μmol/m^2 of the specific surface area, were added and reacted for 8 h, except for 3-glycidoxypropyltrimethoxysilane (48 h). The reaction mixture was cooled to room temperature, filtered and washed with toluene and methanol. The isolated silica gels were dried *in vacuo* over P_2O_5 at 60°C for 2 h. To the silica gels reacted with 3-glycidoxypropyltrimethoxysilane, 80 ml of perchloric acid (pH 3.0) were added and the mixture was refluxed for 4 h. The reaction mixture was cooled to room temperature, filtered and washed with water and methanol. The isolated 3-glycerylpropyl (GP)-silica gel was dried *in vacuo* over P_2O_5 at 60°C for 2 h. The obtained silica gels having different spacers are abbreviated as shown in Fig. 2.

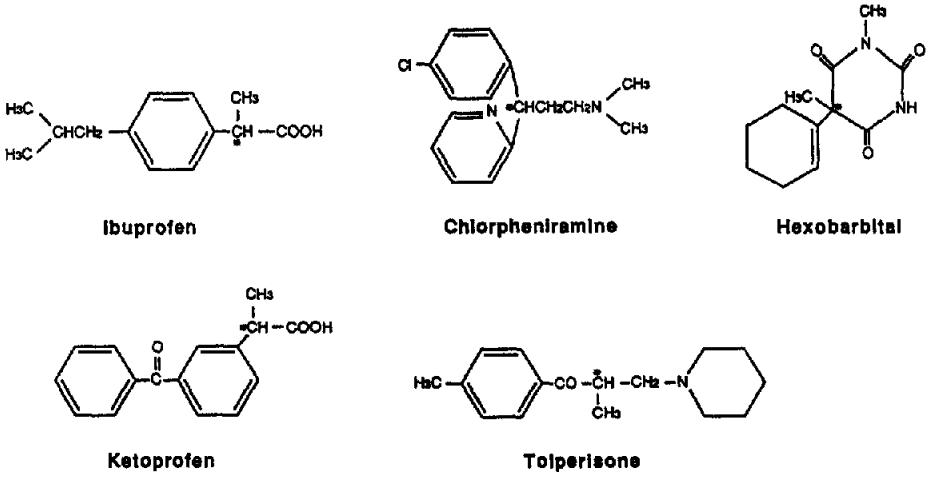

Fig. 1. Structures of the enantiomers studied.

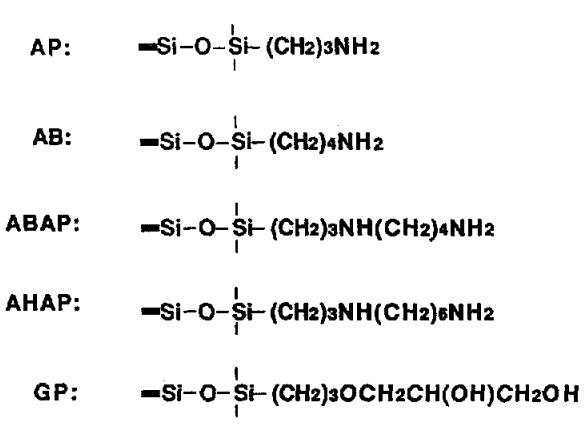

Fig. 2. Spacers used and their abbreviations.

Activation of silica gels having amino or hydroxyl groups

AP-, AB-, ABAP- and AHAP-silica gels were activated by DSC. Amounts of 5 g of the gels were slurried in 70 ml of acetonitrile and reacted with 5 g of DSC for 24 h at 30°C. The reaction mixture was filtered and washed with acetonitrile, water, methanol and dichloromethane. GP-silica gels were activated by CDI. Amounts of 5 g of the gels were slurried in 30 ml of dioxane. To the mixture, 4 g of CDI dissolved in 20 ml of dioxane were added and reacted for 4 h at 30°C. The reaction mixture was filtered and washed with dioxane and methanol. Both silica gels were dried *in vacuo* over P_2O_5 at 60°C for 2 h.

Preparation of OVM-bonded materials

OVM was bound to DSC-activated AP-, AB-, ABAP- and AHAP-silica gels as follows: 2 g of the DSC-activated silica gels were slurried in 20 m*M* phosphate buffer (pH 6.8). To the mixture, 2 g of OVM proteins dissolved in 20 ml of the same buffer were added slowly and stirred for 15 h at 30°C. Similarly, OVM was bonded to CDI-activated silica gels as follows: 2 g of the CDI-activated GP silica gels were slurried in 20 m*M* phosphate buffer (pH 7.8). To the mixture, 2 g of OVM proteins dissolved in 20 ml of the same buffer were added slowly and stirred for 15 h at 30°C. Both reaction mixtures were then filtered and washed with water and methanol. The isolated AP, AB, ABAP and AHAP materials were dried *in vacuo* over P_2O_5 at 40°C for 6 h.

These OVM-bonded materials were packed into a 150 or 100 × 4.6 mm I.D. stainless-steel column by the slurry packing method.

Chromatography

The HPLC system used was composed of an LC-9A pump, an SPD-6A spectrophotometer, a SIL-6B autoinjector, a C-R4A integrator and an SCL-6B system controller (all from Shimadzu, Kyoto, Japan). The flow-rate was maintained at 0.8 or 1.0 ml/min. Detection was performed at 220 or 254 nm.

Capacity factors were calculated from the equation $k' = (t_R - t_0)/t_0$, where t_R and t_0 are the elution times of retained and unretained solutes, respectively, and k'_1 and k'_2 correspond to the capacity factors of the first- and second-eluted peaks, respectively. The retention time of unretained solute, t_0, was measured by injecting a solution whose organic modifier content was slightly different from that of the eluent used. The enantioseparation factor was calculated from the equation $\alpha = k'_2/k'_1$. Resolution was calculated from the equation $R_s = 2(t_2 - t_1)/(t_{w1} + t_{w2})$, where t_{w1} and t_{w2} are the widths of the first- and second-eluted peaks, respectively. All separations were carried out at 25°C using a CO-1093C column oven (Uniflows, Tokyo, Japan).

The eluents, which are prepared by using phosphoric acid–sodium dihydrogenphosphate or sodium dihydrogenphosphate–disodium hydrogenphosphate and organic modifier, are specified in the tables.

Sample preparation

A known amount of racemic solute was dissolved in methanol or water and the solution was diluted with the eluent to the desired concentration. A 20-μl aliquot of the sample solution was loaded on to the column. The amount loaded was less than 0.5 μg.

RESULTS AND DISCUSSION

Amounts of bonded OVM proteins

Table I shows the amounts of bonded OVM proteins on the various materials. With regard to comparison of the base silica gels having pore sizes of 120 and 300 Å (AP-120 and AP-300,

TABLE I

AMOUNTS OF BONDED OVM PROTEINS ON VARIOUS MATERIALS

Spacer[a]	Pore size (Å)	Bonded protein (μmol/g)
AP-120[b]	120	4.1
AP-300[b]	300	1.3
AB	120	4.4
ABAP	120	3.9
AHAP	120	0.9
GP	120	2.0

[a] AP = 3-aminopropyl; AB = 4-aminobutyl; ABAP = N-(4-aminobutyl)-3-aminopropyl; AHAP = N-(6-aminohexyl)-3-aminopropyl; GP = glycerylpropyl.
[b] 120 and 300 refer to the average pore sizes of the base silica materials.

respectively), the former materials showed about three times larger amounts of the OVM proteins than the latter materials. As the coverages of AP phases of the AP-120 and AP-300 silica materials were about 1000 and 350 μmol/g, respectively, there are good correlations between the coverages of the AP phases and the amounts of bonded OVM proteins. Note that only 0.4% of the AP phases should be used for binding of the OVM proteins. For the preparation of BSA-bonded materials, Thompson *et al.* [8] compared 100- and 300-Å base silica materials, which resulted in amounts of the bonded proteins of 3.2 and 1.1 μmol/g, respectively. Taking into account the molecular masses of OVM (M_r 28 000) and BSA (M_r 68 000), our results agreed well with those of Thompson *et al.* [8]. The AP, AB and ABAP materials showed almost identical amounts of bonded OVM proteins, whereas the AHAP material showed smaller amounts, owing to the lower coverages of the AHAP than the AP, AB and ABAP phases. The GP materials showed about half the amounts of bonded proteins compared with the AP and AB materials. Taking into account the coverages of GP phases (*ca.* 500 μmol/g), the activation method does not affect the protein coverages. OVM proteins were bound to the AP, AB, ABAP and AHAP materials by a urea bond. On the other hand, the OVM proteins were bound to the GP materials by a carbamate bond. The results obtained above reveal that the amounts of bonded OVM proteins are mainly dependent on the coverages of the spacer phases.

Comparison of base silica materials

Table II shows the influence of pore size of base silica materials on the retention, enantioselectivity and resolution of ketoprofen, chlorpheniramine and hexobarbital. The eluent used was 20 m*M* phosphate buffer containing 10% and 5% of ethanol for the AP-120 and AP-300 materials, respectively. The retention properties of acidic, basic and uncharged solutes could be

TABLE II

COMPARISON OF BASE SILICA MATERIALS FOR RETENTION, ENANTIOSELECTIVITY AND RESOLUTION OF VARIOUS SOLUTES ON OVM-BONDED MATERIAL

HPLC conditions: column, OVM-bonded materials packed into a 100 mm × 4.6 mm I.D. stainless-steel column; eluent, 20 m*M* phosphate buffer–ethanol [90:10 (v/v) for the AP-120 materials and 95:5 (v/v) for the AP-300 materials]; flow-rate, 0.8 ml/min.

Column	Compound	pH 3.2[a]			pH 4.0[a]			pH 5.1[a]			pH 6.0[a]			pH 6.9[a]		
		k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s
AP-120	Ketoprofen	23.6	1.34	3.00	32.3	1.24	2.58	13.0	1.11	1.15	3.54	1.00		1.16	1.00	
	Chlorpheniramine				0.19	1.77	0.59	2.05	1.88	2.51	11.2	2.09	5.80	41.3	2.12	7.28
	Hexobarbital	0.46	1.23	0.53	0.61	1.22	0.76	0.68	1.19	0.69	0.80	1.26	1.10	0.82	1.40	1.91
AP-300	Ketoprofen	8.26	1.49	1.92	15.3	1.45	2.24	6.28	1.32	1.88	1.44	1.17	0.80	0.47	1.00	
	Chlorpheniramine				0.30	1.00		1.72	1.95	2.46	6.67	2.33	3.92	25.3	2.49	4.05
	Hexobarbital	0.28	1.00		0.34	1.29	0.53	0.46	1.34	0.68	0.44	1.41	0.82	0.50	1.56	1.72

[a] Buffer pH.

elucidated by taking into account the isoelectric point of an ovomucoid protein (pI = 3.8–4.3) and the pK_a values of the solutes, as reported previously [11]. The capacity factors of all the solutes on the former materials are about double those on the latter materials, despite the higher organic modifier content. These results reveal that the retentions of solutes are dependent on the amounts of bonded OVM proteins. On the other hand, the AP-300 materials gave higher enantioselectivities than the AP-120 materials, despite the shorter retentions of the solutes on the former materials. This suggests that all the proteins bound to the AP-120 materials do not work effectively for chiral recognition of solutes. However, the resolution of basic and acidic solutes, except for ketoprofen at an eluent pH of 5.1, on the AP-300 materials was lower than that on the AP-120 materials. This might be because a solute might interact with the base silica materials, because of smaller amounts of the bonded proteins. We therefore prepared OVM-bonded materials by using base silica materials having an average pore size of 120 Å in the experiments described below.

Effect of spacer length for chiral recognition

Table III, IV and V show the influence of spacer length on the retention, enantioselectivity and resolution of acidic, basic and uncharged solutes, respectively, where ethanol is used as an organic modifier. It is well known [6,7,12] that organic modifiers affect the retention and enantioselectivity of solutes on an OVM-bonded column. We examined the influence of the type of organic modifier on the retentions and enantioselectivity of the solutes. Similar trends in retention and enantioselectivity were obtained with all five columns, and therefore only the data for ethanol are given in Tables III–V.

No enantioseparations of solutes on the ABAP and AHAP materials were observed except for a slight resolution of oxprenolol at an eluent pH of 6.9. Oda *et al.* [13] reported that a hydrophobic spacer with a C_6 carbon chain was suitable for obtaining higher enantioselectivity on avidin-bonded materials. We bound OVM to base silica materials by using the same spacer as Oda *et al.* [13]. However, the enantioselectivity obtained with the materials was poor, as with the ABAP and AHAP materials. Miwa *et al.* [14]

TABLE III

EFFECT OF SPACER LENGTH ON RETENTION, ENANTIOSELECTIVITY AND RESOLUTION OF ACIDIC SOLUTES ON OVM-BONDED MATERIAL

All materials were prepared by using the 120-Å base silica materials. HPLC conditions: column, OVM-bonded materials packed into a 150 mm × 4.6 mm I.D. stainless-steel column; eluent, 20 m*M* phosphate buffer–ethanol [90:10 (v/v) for the AP, AB, ABAP and AHAP materials and 95:5 (v/v) for the GP materials]; flow-rate, 1.0 ml/min.

Column	Compound	pH 3.2[a]			pH 4.0[a]			pH 5.1[a]			pH 6.0[a]			pH 6.9[a]		
		k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s
AP	Ibuprofen	7.17	1.37	2.69	11.7	1.34	2.88	7.75	1.17	1.34	2.58	1.08	0.54	0.84	1.00	
	Ketoprofen	23.0	1.34	3.05	34.2	1.21	2.32	14.0	1.09	1.00	4.04	1.00		1.27	1.00	
AB	Ibuprofen	9.54	1.27	1.86	14.2	1.27	2.07	8.74	1.14	1.10	3.19	1.00		0.87	1.00	
	Ketoprofen	27.8	1.27	1.99	37.7	1.18	1.33	16.1	1.07	0.54	5.13	1.00		1.47	1.00	
ABAP	Ibuprofen	13.7	1.00		21.9	1.00		16.9	1.00		5.07	1.00		1.57	1.00	
	Ketoprofen	29.9	1.00		46.6	1.00		28.2	1.00		8.24	1.00		2.71	1.00	
AHAP	Ibuprofen	13.2	1.00		19.8	1.00		14.1	1.00		4.86	1.00		1.57	1.00	
	Ketoprofen	29.9	1.00		44.5	1.00		24.5	1.00		8.19	1.00		3.21	1.00	
GP	Ibuprofen	4.24	1.29	1.14	7.08	1.34	1.01	4.52	1.23	0.69	1.37	1.00		0.32	1.00	
	Ketoprofen	15.7	1.54	2.57	27.2	1.47	2.63	11.5	1.28	1.52	2.49	1.11	0.32	0.84	1.00	

[a] Buffer pH.

TABLE IV

EFFECT OF SPACER LENGTH ON RETENTION, ENANTIOSELECTIVITY AND RESOLUTION OF BASIC SOLUTES ON OVM-BONDED MATERIAL

All materials were prepared by using the 120-Å base silica materials. HPLC conditions: column, OVM-bonded materials packed into a 150 mm × 4.6 mm I.D. stainless-steel column; eluent, 20 m*M* phosphate buffer–ethanol [90:10 (v/v) for the AP, AB, ABAP and AHAP materials and 95:5 (v/v) for the GP materials]; flow-rate, 1.0 ml/min.

Column	Compound	pH 4.0[a]			pH 5.1[a]			pH 6.0[a]			pH 6.9[a]		
		k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s
AP	Chlorpheniramine	0.33	1.36	0.72	2.56	1.75	3.33	8.73	1.92	5.82	40.4	2.03	7.64
	Tolperisone				0.76	1.90	3.04	2.53	1.51	2.57	12.0	1.33	2.58
AB	Chlorpheniramine	0.44	1.41	1.04	3.40	1.74	3.77	13.2	1.89	4.84	56.5	1.97	4.60
	Tolperisone				0.96	1.75	2.60	3.74	1.41	1.74	16.4	1.27	1.54
ABAP	Chlorpheniramine	0.63	1.00		3.83	1.00		14.4	1.00		43.2	1.00	
	Tolperisone	0.13	1.00		1.32	1.00		4.88	1.00		12.1	1.00	
AHAP	Chlorpheniramine	0.62	1.00		5.55	1.00		23.7	1.00		48.7	1.00	
	Tolperisone	0.16	1.00		2.09	1.00		9.92	1.00		14.5	1.00	
GP	Chlorpheniramine	0.66	1.00		2.96	1.76	2.37	12.6	2.01	3.07	45.7	2.18	5.00
	Tolperisone	0.41	1.61	0.26	1.07	1.77	1.39	4.35	1.60	1.64	13.6	1.42	2.10

[a] Buffer pH.

reported that OVM-bonded polymer gels, which contain a polyamine (pentaethylenehexamine), had excellent chiral recognition properties for the basic chlorprenaline and chlorpheniramine. On the other hand, enhanced retention of acid ketoprofen was obtained with the polymer gels but no chiral resolution was achieved because of the strong achiral interaction with the polymer matrices. These results suggest that the superfluous achiral interaction of a solute with a hydro-

TABLE V

EFFECT OF SPACER LENGTH ON RETENTION, ENANTIOSELECTIVITY AND RESOLUTION OF HEXOBARBITAL ON AN OVM-BONDED MATERIAL

All materials were prepared by using the 120-Å base silica materials. HPLC conditions: column, OVM-bonded materials packed into a 150 mm × 4.6 mm I.D. stainless-steel column; eluent, 20 m*M* phosphate buffer–ethanol [90:10 (v/v) for the AP, AB, ABAP and AHAP materials and 95:5 (v/v) for the GP materials]; flow-rate, 1.0 ml/min.

Column	pH 3.2[a]			pH 4.0[a]			pH 5.1[a]			pH 6.0[a]			pH 7.0[a]		
	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s	k_1'	α	R_s
AP	0.69	1.00		0.84	1.18	0.75	1.00	1.21	1.07	0.86	1.28	1.39	0.96	1.42	1.95
AB	0.68	1.15	0.34	0.86	1.18	0.54	1.06	1.20	0.75	1.03	1.25	0.70	1.09	1.40	1.18
ABAP	0.74	1.00		0.82	1.00		0.85	1.00		0.83	1.00		0.74	1.00	
AHAP	0.76	1.00		0.78	1.00		0.82	1.00		0.85	1.00		0.77	1.00	
GP	0.49	1.22	0.79	0.66	1.30	1.19	0.73	1.39	0.92	0.83	1.50	1.49	0.65	1.59	1.92

[a] Buffer pH.

phobic and/or positively charged spacer might diminish the chiral interaction of the solute with the OVM protein.

The capacity factors of acidic and basic solutes on the AP, AB, ABAP and AHAP materials were increased with an increase in the hydrophobicity of the spacer, except for those of basic solutes on the AB materials. With regard to enantioselectivity and resolution, the AP materials gave higher enantioselectivity and resolution for acidic solutes than the AB materials. For basic solutes, the AP materials gave almost the same enantioselectivity as the AB materials and higher resolution. The retentions of hexobarbital were almost the same with the four materials, whereas the AP materials gave better enantioselectivity and resolution than the AB materials except for an eluent pH of 3.2.

The enantioselectivity and resolution with the GP material were compared with those for the AP materials. For acidic ibuprofen, the GP materials gave lower enantioselectivity and resolution than the AP materials, whereas for ketoprofen, the GP materials gave higher enantioselectivity and resolution except for resolution at an eluent pH of 3.2. For hexobarbital, the GP materials gave higher enantioselectivity and almost equal resolution. The lower column efficiency of the GP materials for acidic and basic solutes might be due to the smaller amounts of bonded OVM proteins, that is, the solute might interact with the base silica material. However, the GP materials are suitable for the chiral resolution of the uncharged hexobarbital. It is interesting that the ABAP materials, whose spacer length is very similar to that of the GP materials, has no chiral recognition properties. The results obtained above reveal that a less hydrophobic spacer such as an AP group and a hydrophilic spacer such as a GP group are suitable for binding of OVM proteins to silica gels.

In conclusion, the AP materials of pore size 120 Å gave the highest chiral recognition properties among the OVM-bonded materials tested. However, by diminishing the superfluous achiral interaction, including interaction with the base silica material, much more efficient OVM-bonded materials might be obtainable.

REFERENCES

1 S. Allenmark, *J. Liq. Chromatogr.*, 9 (1986) 425.
2 E. Domenici, C. Bertucci, P. Salvadori, G. Felix, I. Cahagne, S. Montellier and I.W. Wainer, *Chromatographia*, 29 (1990) 170.
3 J. Hermansson, *J. Chromatogr.*, 269 (1983) 71.
4 T. Miwa, M. Ichikawa, M. Tsuno, T. Hattori, T. Miyakawa, M. Kayano and Y. Miyake, *Chem. Pharm. Bull.*, 35 (1987) 682.
5 J. Haginaka, J. Wakai, H. Yasuda, K. Takahashi and T. Katagi, *Chromatographia*, 29 (1990) 587.
6 J. Iredale, A.-F. Aubry and I.W. Wainer, *Chromatographia*, 31 (1991) 329.
7 K.M. Kirkland, K.L. Nelson and D.A. McCombs, *J. Chromatogr.*, 545 (1991) 43.
8 R.A. Thompson, S. Anderson and S. Allenmark, *J. Chromatogr.*, 465 (1989) 263.
9 S. Andersson, S. Allenmark, P. Erlandsson and S. Nilsson, *J. Chromatogr.*, 498 (1990) 81.
10 S. Andersson, R.A. Thompson and S.G. Allenmark, *J. Chromatogr.*, 591 (1992) 65.
11 J. Haginaka, T. Murashima, Ch. Seyama, H. Fujima and H. Wada, *J. Chromatogr.*, 631 (1992) 183.
12 J. Haginaka, Ch. Seyama, H. Yasuda, H. Fujima and H. Wada, *J. Chromatogr.*, 592 (1992) 301.
13 Y. Oda, H. Ohe, N. Asakawa, Y. Yoshida, T. Sato and T. Nakagawa, *J. Liq. Chromatogr.*, 15 (1922) 2997.
14 T. Miwa, S. Sakashita, H. Ozawa, J. Haginaka, N. Asakawa and Y. Miyake, *J. Chromatogr.*, 566 (1991) 163.