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Retention and enantioselective properties of racemic compounds on modified ovomucoid columns

II. Reaction with glyceraldehyde, formaldehyde and glutaric anhydride

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ABSTRACT

Modified ovomucoid (OVM) columns were prepared by reaction with glyceraldehyde, formaldehyde and glutaric anhydride. The retention and enantioselective properties of racemic compounds on these modified OVM columns were compared with those on an unmodified OVM column. The retentions of racemic compounds on the modified OVM columns were lower than or approximately equal to those on the unmodified OVM column (except for basic compounds on the OVM column reacted with glutaric anhydride). The modified OVM columns gave lower or approximately equal enantioselectivities than the unmodified OVM column for acidic and uncharged compounds, whereas the modified OVM columns gave higher enantioselectivity for basic compounds. These differences may be mainly attributed to changes in protein conformation, especially changes in chiral recognition site(s) as a result of modification. The results reveal that modification of OVM proteins may be effective for the chiral separation of basic compounds on an OVM column.

INTRODUCTION

An ovomucoid (OVM) column was prepared for chiral separations of racemic compounds by Miwa *et al.* [1]. Recently, OVM columns have been utilized for the chiral resolution of acidic, basic and uncharged compounds owing to the wider chiral recognition properties [2–8] compared with other protein columns such as α_1-acid glycoprotein, bovine serum albumin and human serum albumin. Also, it has been reported that OVM columns have greater flexibility of operating parameters and superior long-term stability [5].

In a previous paper [7], we compared the retention and enantioselective properties of racemic compounds on two modified OVM columns, one cross-linked with glutaraldehyde and the other further reduced with sodium tetrahydroborate, with those on an unmodified OVM column. The OVM column cross-linked with glutaraldehyde had much better stability against repetitive injections of samples and/or changes in eluent composition (eluent pH, type and content of organic modifier) than the unmodified OVM column. This paper deals with the retention and enantioselective properties of racemic compounds on OVM columns modified

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with glyceraldehyde, formaldehyde and glutaric anhydride.

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). Alprenolol, pindolol, tolperisone hydrochloride and benzoin were 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). 2-Propanol, ethanol, methanol and acetonitrile of HPLC grade were obtained from Wako (Osaka, Japan). Formaldehyde, DL-glyceraldehyde, glutaric anhydride, sodium cyanoborohydride and zinc sulphate 7-hydrate were obtained from Nacalai Tesque (Kyoto, Japan) and used without further purification.

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

Preparation of OVM, OVM-DIOL, OVM-ME and OVM-GLA materials

OVM was bonded to an aminopropylsilica gel (Ultrton-NH_2, 5 μm, 120 Å; Shinwa Chemical Industries, Kyoto, Japan) via the N,N-disuccinimidyl carbonate reaction as reported previously [7].

Six grams of OVM material were added to 100 ml of 20 m*M* phosphate buffer (pH 7.5), then 600 mg of sodium cyanoborohydride, 560 mg of zinc sulphate and 1.16 g of glyceraldehyde were added. After adjusting the pH to 7.5, the mixture was slowly rotated at 30°C for 15 h. The mixture was then filtered and washed with water and methanol. The

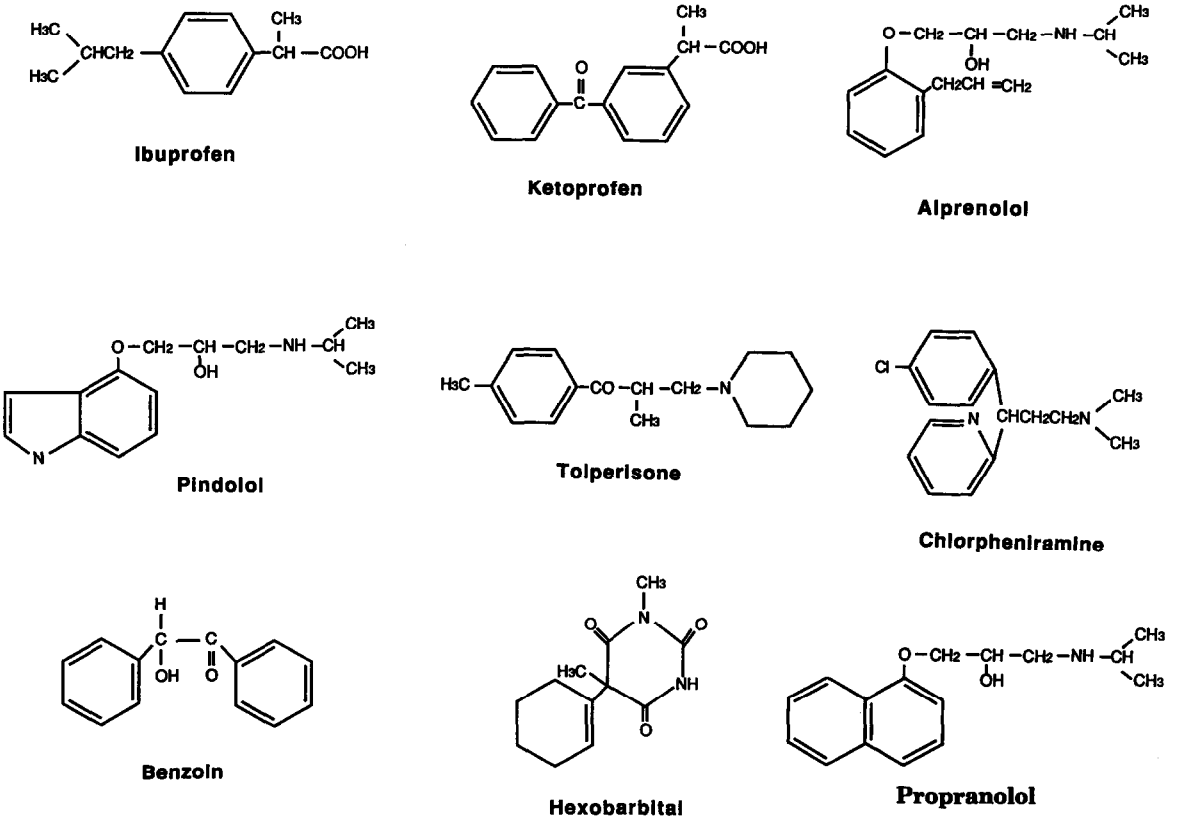

Fig. 1. Structures of the racemic compounds used.

isolated material (OVM-DIOL) was dried *in vacuo* over P_2O_5 at 40°C for 6 h.

Six grams of the OVM material were added to 100 ml of 20 m*M* phosphate buffer (pH 7.5), then 600 mg of sodium cyanoborohydride, 560 mg of zinc sulphate and 160 mg of formaldehyde were added. After adjusting the pH to 7.5, the mixture was slowly rotated at 30°C for 15 h. The mixture was then filtered and washed with water and methanol. The isolated material (OVM-ME) was dried *in vacuo* over P_2O_5 at 40°C for 6 h.

Six grams of the OVM material were added to 100 ml of 100 m*M* borate buffer (pH 8.5), then 490 mg of glutaric anhydride were added. After adjusting the pH to 8.5, the mixture was slowly rotated at room temperature for 1 h. The mixture was then filtered and washed with water and methanol. The isolated material (OVM-GLA) was dried *in vacuo* over P_2O_5 at 40°C for 6 h.

These materials were packed into a 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 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 elution times of retained and unretained solutes, respectively; k'_1 and k'_2 correspond to the capacity factors of the first- and second-eluted peaks, respectively. The retention time of an 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 peak 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 were prepared by using phosphoric acid–sodium dihydrogenphosphate or sodium dihydrogenphosphate–disodium hydrogenphosphate and organic modifier, are specified in the figure and table captions.

Sample preparation

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

RESULTS

Effect of eluent pH on retention and enantioselectivity of acidic, basic and uncharged compounds

Tables I–III show the effects of eluent pH on the retention and enantioselectivity of acidic, basic and uncharged compounds on OVM, OVM-DIOL, OVM-ME and OVM-GLA columns, with 20 m*M* phosphate buffer containing 10% ethanol as the eluent. The capacity factors (k'_1) of the first-eluted enantiomers of acidic compounds on the OVM-DIOL and OVM-ME columns were slightly higher than those on the OVM column. The k'_1 of acidic compounds was drastically decreased on the OVM-GLA column, especially when an eluent of pH > 4.0 was used. The enantioselectivity (α) of these compounds was slightly lower on the OVM-DIOL and OVM-ME columns and lower on the OVM-GLA column. On the OVM-DIOL and OVM-ME columns, the k'_1 of basic compounds was almost the same as that on the OVM column, whereas the k'_1 on the OVM-GLA column was considerably higher when an eluent of pH > 4.0 was used. The α values of these compounds were higher or approximately equal on all the modified columns except for separation of chlorpheniramine on the OVM-GLA column. The k'_1 values of uncharged compounds were slightly lower on the OVM-DIOL, OVM-ME and OVM-GLA columns, than on the OVM column. The α values of uncharged compounds were unchanged on the OVM-DIOL and OVM-ME columns, whereas those of benzoin and hexobarbital on the OVM-GLA column were lower (the latter is not resolved).

Effect of organic modifier on enantioselectivity of acidic, basic and uncharged compounds

Tables IV–VI show the effects of the organic modifier (ethanol, 2-propanol, methanol and acetonitrile) on the enantioselectivity (α) of acidic, basic and uncharged compounds on the OVM, OVM-DIOL, OVM-ME and OVM-GLA columns. For

TABLE I

EFFECT OF pH ON RETENTION AND ENANTIOSELECTIVITY OF ACIDIC COMPOUNDS ON OVM, OVM-DIOL, OVM-ME AND OVM-GLA COLUMNS

Eluent: 20 m*M* phosphate buffer–ethanol (90:10, v/v).

Column	Compound	pH 3.2[a]		pH 4.0		pH 5.1		pH 6.0		pH 6.9	
		k'_1	α	k'_1	α	k'_1	α	k'_1	α	k'_1	α
OVM	Ibuprofen	4.79	1.29	7.35	1.29	4.62	1.15	1.48	1.06	0.39	1.00
	Ketoprofen	13.6	1.32	20.0	1.20	8.03	1.08	2.32	1.00	0.66	1.00
OVM-DIOL	Ibuprofen	4.82	1.25	7.39	1.22	5.12	1.12	1.53	1.00	0.45	1.00
	Ketoprofen	13.2	1.21	18.0	1.13	8.92	1.06	2.43	1.00	0.67	1.00
OVM-ME	Ibuprofen	5.09	1.24	7.85	1.21	5.30	1.10	1.77	1.00	0.52	1.00
	Ketoprofen	12.7	1.24	17.4	1.15	8.31	1.06	2.61	1.00	0.79	1.00
OVM-GLA	Ibuprofen	4.20	1.15	4.60	1.20	1.43	1.19	0.17	1.00		
	Ketoprofen	8.00	1.14	9.31	1.13	2.28	1.12	0.35	1.00		

[a] Buffer pH.

TABLE II

EFFECT OF pH ON RETENTION AND ENANTIOSELECTIVITY OF BASIC COMPOUNDS ON OVM, OVM-DIOL, OVM-ME AND OVM-GLA COLUMNS

Eluent: 20 m*M* phosphate buffer–ethanol (90:10, v/v).

Column	Compound	pH 4.0[a]		pH 5.1		pH 6.0		pH 6.9	
		k'_1	α	k'_1	α	k'_1	α	k'_1	α
OVM	Alprenolol	0.18	1.00	2.84	1.00	13.4	1.12	59.4	1.14
	Pindolol			0.29	1.00	1.13	1.22	3.80	1.20
	Tolperisone	0.06	3.29	0.52	1.57	2.28	1.33	9.30	1.24
	Chlorpheniramine	0.22	1.00	1.58	1.54	6.89	1.74	29.3	1.80
OVM-DIOL	Alprenolol	0.13	1.00	2.38	1.00	13.6	1.09	50.9	1.08
	Pindolol	0.01	1.00	0.24	1.36	1.20	1.34	3.83	1.34
	Tolperisone			0.35	2.19	1.87	1.59	7.19	1.44
	Chlorpheniramine	0.41	1.55	1.29	1.63	6.07	1.76	21.8	1.76
OVM-ME	Alprenolol	0.28	1.00	2.35	1.00	12.5	1.10	49.5	1.14
	Pindolol			0.22	1.25	1.07	1.23	3.47	1.20
	Tolperisone			0.35	1.70	1.76	1.41	6.57	1.27
	Clorpheniramine	0.16	1.69	1.23	1.63	6.52	1.89	21.7	1.97
OVM-GLA	Alprenolol	0.59	1.00	5.53	1.20	27.3	1.17	97.3	1.14
	Pindolol	0.32	1.00	2.39	1.00	6.13	1.11	10.4	1.21
	Tolperisone	0.48	1.53	3.31	1.70	9.37	2.08	18.0	2.01
	Chlorpheniramine	0.98	1.00	6.00	1.16	18.1	1.37	42.8	1.59

[a] Buffer pH.

TABLE III

EFFECT OF pH ON RETENTION AND ENANTIOSELECTIVITY OF UNCHARGED COMPOUNDS ON OVM, OVM-DIOL, OVM-ME AND OVM-GLA COLUMNS

Eluent: 20 m*M* phosphate buffer–ethanol (90:10, v/v).

Column	Compound	pH 3.2[a]		pH 4.0		pH 5.1		pH 6.0		pH 6.9	
		k'_1	α	k'_1	α	k'_1	α	k'_1	α	k'_1	α
OVM	Benzoin	1.69	1.63	2.29	1.92	2.55	2.40	2.59	2.25	2.95	1.96
	Hexobarbital	0.47	1.00	0.52	1.14	0.62	1.00	0.56	1.22	0.63	1.36
OVM-DIOL	Benzoin	1.51	1.59	2.09	2.12	2.47	2.30	2.59	2.22	2.61	1.93
	Hexobarbital	0.38	1.00	0.47	1.00	0.52	1.15	0.52	1.25	0.53	1.34
OVM-ME	Benzoin	1.58	2.29	2.12	2.16	2.34	2.21	2.42	2.09	2.74	1.86
	Hexobarbital	0.40	1.00	0.49	1.00	0.50	1.12	0.52	1.23	0.58	1.37
OVM-GLA	Benzoin	1.61	1.26	1.93	1.61	2.12	1.78	2.12	1.90	2.22	1.82
	Hexobarbital	0.44	1.00	0.49	1.00	0.51	1.00	0.47	1.00	0.42	1.00

[a] Buffer pH.

acidic compounds, the eluents used were 20 m*M* phosphate buffer (pH 3.2) containing 10% ethanol, 7% 2-propanol, 15% methanol and 8% acetonitrile, whereas for basic and uncharged compounds, the eluents were 20 m*M* phosphate buffer (pH 6.0) containing the same percentage of each organic modifier. Almost the same retentions of the solutes tested were observed with use of eluents containing 10% ethanol, 7% 2-propanol, 15% methanol and 8% acetonitrile. In addition, acidic compounds gave better separations with use of acidic eluents, whereas basic and uncharged compounds gave higher enantioselectivities with the use of an eluent of pH 6 or 6.9. Therefore, we checked the enantioselectivity under the conditions as shown in Tables IV–VI. The use of methanol as an organic modifier gave the highest enantioselectivity for ibuprofen, ketoprofen and hexobarbital on the unmodified and modified OVM columns. For the separation of benzoin, 2-propanol gave the highest enantioselectivity on the OVM, OVM-DIOL and OVM-ME columns, whereas methanol gave the highest enantioselectivity on the OVM-GLA column. On the other hand, for the separation of basic compounds, the highest enantioselectivity was obtained with ethanol, 2-propanol or methanol, depending on the solute or column used. However, acetonitrile was not a good organic modifier for the chiral separation of the compounds tested.

Separation of racemic propranolol on unmodified and modified OVM columns

Fig. 2A, B, C and D show the separations of racemic propranolol on the OVM, OVM-DIOL, OVM-ME and OVM-GLA columns, respectively, with 20 m*M* phosphate buffer (pH 5.1)–ethanol (90:1, v/v) as the eluent. The capacity factor of propranolol was lower on the OVM-DIOL and OVM-ME columns than on the unmodified OVM column, whereas on the OVM-GLA column the capacity factor was slightly higher. All the modified columns gave higher enantioselectivity and better resolution than the unmodified OVM column under the conditions employed.

DISCUSSION

The OVM-DIOL and OVM-ME columns gave similar retentions and enantioselectivities for acidic, basic and uncharged compounds, whereas the OVM-GLA column gave very different values. By taking into account the pK_1 value of glutaric acid (4.3) and the isoelectric point of ovomucoid (p*I* = 3.8–4.3), the retention properties of acidic and basic compounds on the OVM-GLA column were easily elucidated. With an eluent of pH > 4–5 glutaric acid residues were negatively charged, so the OVM-GLA material was much more negatively charged than the unmodified OVM. Hence the retentions of

TABLE IV

EFFECT OF ETHANOL (EtOH), 2-PROPANOL (2-PrOH), METHANOL (MeOH) AND ACETONITRILE (ACN) ORGANIC MODIFIERS ON ENANTIOSELECTIVITY (α) OF ACIDIC COMPOUNDS ON OVM AND MODIFIED OVM COLUMNS

Eluents used were a mixture of 20 m*M* phosphate buffer (pH 3.2) and 10% EtOH, 7% 2-PrOH, 15% MeOH and 8% ACN.

Column	Compound	EtOH	2-PrOH	MeOH	ACN
OVM	Ibuprofen	1.29	1.17	1.37	1.13
	Ketoprofen	1.32	1.26	1.43	1.23
OVM-DIOL	Ibuprofen	1.25	1.16	1.37	1.16
	Ketoprofen	1.21	1.22	1.36	1.20
OVM-ME	Ibuprofen	1.24	1.15	1.38	1.15
	Ketoprofen	1.24	1.24	1.41	1.22
OVM-GLA	Ibuprofen	1.15	1.09	1.23	1.10
	Ketoprofen	1.14	1.10	1.24	1.11

the positively charged compounds (basic compounds) on the OVM-GLA column were increased and those of the negatively charged compounds (acidic compounds) were decreased. Also, a slight increase in the retentions of acidic compounds on the OVM-DIOL and OVM-ME columns could be ascribed to hydrophobic interactions caused by suppression of the dissociation of amino groups on

TABLE V

EFFECT OF ETHANOL (EtOH), 2-PROPANOL (2-PrOH), METHANOL (MeOH) AND ACETONITRILE (ACN) ORGANIC MODIFIERS ON ENANTIOSELECTIVITY (α) OF BASIC COMPOUNDS ON OVM AND MODIFIED OVM COLUMNS

Eluents used were a mixture of 20 m*M* phosphate buffer (pH 6.0) and 10% EtOH, 7% 2-PrOH, 15% MeOH and 8% ACN.

Column	Compound	EtOH	2-PrOH	MeOH	ACN
OVM	Alprenolol	1.12	1.10	1.16	1.11
	Pindolol	1.22	1.00	1.24	1.00
	Tolperisone	1.33	1.67	1.33	1.10
	Chlorpheniramine	1.74	2.02	1.87	1.84
OVM-DIOL	Alprenolol	1.09	1.08	1.15	1.00
	Pindolol	1.34	1.13	1.27	1.00
	Tolperisone	1.59	1.47	1.41	1.00
	Chlorpheniramine	1.76	1.60	1.71	1.61
OVM-ME	Alprenolol	1.10	1.08	1.18	1.13
	Pindolol	1.23	1.09	1.21	1.00
	Tolperisone	1.41	1.44	1.37	1.00
	Chlorpheniramine	1.89	1.84	1.95	1.74
OVM-GLA	Alprenolol	1.17	1.04	1.11	1.08
	Pindolol	1.11	1.06	1.15	1.00
	Tolperisone	2.08	1.86	2.58	1.33
	Chlorpheniramine	1.37	1.27	1.51	1.26

TABLE VI

EFFECT OF ETHANOL (EtOH), 2-PROPANOL (2-PrOH), METHANOL (MeOH) AND ACETONITRILE (ACN) ORGANIC MODIFIERS ON ENANTIOSELECTIVITY (α) OF UNCHARGED COMPOUNDS ON OVM AND MODIFIED OVM COLUMNS

Eluents used were a mixture of 20 m*M* phosphate buffer (pH 6.0) and 10% EtOH, 7% 2-PrOH, 15% MeOH and 8% ACN.

Column	Compound	EtOH	2-PrOH	MeOH	ACN
OVM	Benzoin	2.25	2.61	2.15	1.52
	Hexobarbital	1.22	1.26	1.37	1.14
OVM-DIOL	Benzoin	2.22	2.30	2.20	1.46
	Hexobarbital	1.25	1.21	1.31	1.00
OVM-ME	Benzoin	2.09	2.36	2.20	1.43
	Ketoprofen	1.23	1.18	1.31	1.00
OVM-GLA	Benzoin	1.90	1.91	2.08	1.40
	Hexobarbital	1.00	1.00	1.17	1.00

OVM proteins as a result of the modification. We previously reported [7] that hydrophobic and electrostatic interactions play an important role in the retention of these compounds on both cross-linked and unmodified OVM columns. This is true with the OVM columns modified with glyceraldehyde, formaldehyde and glutaric anhydride.

With respect to enantioselectivity, it is interesting that the OVM column gave the highest enantioselectivity against the acidic and uncharged com-

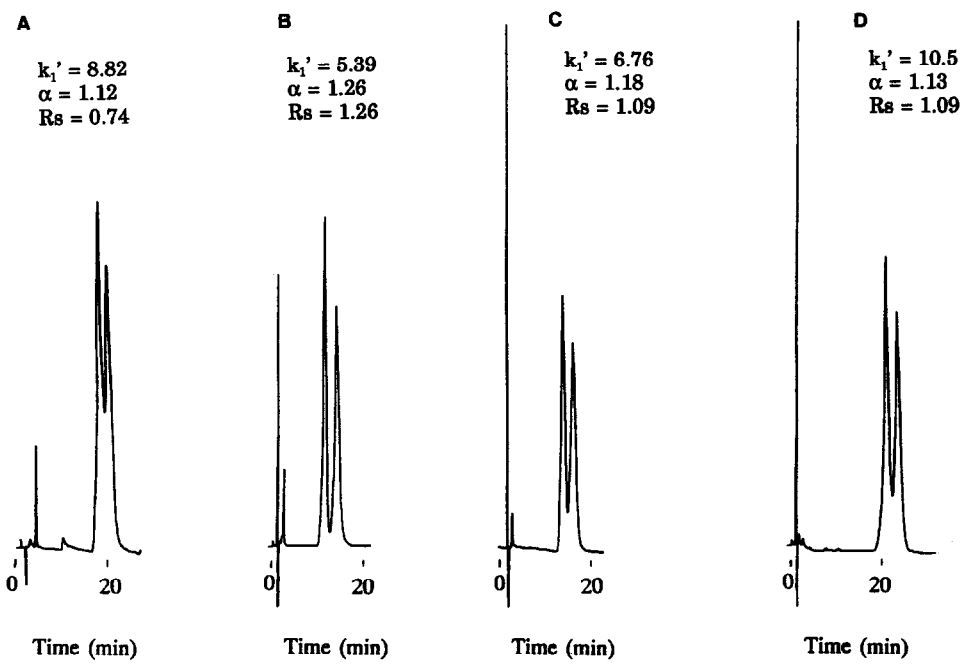

Fig. 2. Separation of racemic propranolol on (A) OVM, (B) OVM-DIOL, (C) OVM-ME and (D) OVM-GLA columns. Eluent, 20 m*M* phosphate buffer (pH 5.1) containing 10% ethanol; injection volume, 20 μl (concentration, 10 μg/ml); detection, 220 nm. k_1', α and R_s are the capacity factor of the first-eluted enantiomer, enantioseparation factor and resolution, respectively.

pounds tested, whereas the modified columns gave better separations against the basic compounds. Also, chiral recognition of basic compounds on the unmodified and modified OVM columns was affected by the organic modifier used. As reported by Iredale *et al.* [4], these results suggest that competition between the organic modifier and the solutes for the chiral binding site(s) might occur. Hence, it is important to select the most suitable organic modifier and eluent pH for the chiral separation of a compound of interest.

Although the blocking of amino groups of ovomucoid proteins with methyl or glyceryl group(s) (*i.e.*, OVM-ME or OVM-DIOL column) did not affect the retentions of acidic, basic and uncharged compounds so much, the OVM-DIOL and OVM-ME columns gave higher or almost equal enantioselectivities for β-blockers (alprenolol, pindolol and propranolol) compared with the unmodified OVM column. The OVM-GLA column gave much longer retentions for basic compounds, whereas it gave a much higher enantioselectivity for tolperisone and a lower enantioselectivity for chlorpheniramine. These results may be mainly attributed to changes in protein conformation, especially changes in chiral recognition site(s) as a result of modification. Also, the carboxylate ion of glutaric acid residue might contribute to the chiral recognition of basic compounds. These results indicate that modification of OVM proteins may be effective for chiral separations of basic compounds.

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