Journal of Chromatography, 592 (1992) 301–307
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2413

Retention and enantioselectivity of racemic solutes on a modified ovomucoid-bonded column

I. Cross-linking with glutaraldehyde

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ABSTRACT

Ovomucoid (OVM)- and cross-linked OVM-bonded materials were prepared for the chiral resolution of racemic solutes. OVM was immobilized to aminopropylsilica via an N,N-disuccinimidyl carbonate coupling reaction. The OVM materials were cross-linked with glutaraldehyde (OVM-GA) and further reduced with sodium borohydride (OVM-GA-R). The retentive and enantioselective properties of acidic, basic and uncharged solutes on OVM, OVM-GA and OVM-GA-R materials were investigated with respect to eluent pH and organic modifier. The retention properties of all solutes tested on these three materials were similar except that they were less retained on the OVM-GA and OVM-GA-R than the OVM material. The enantioselectivity of various solutes decreased in the order OVM, OVM-GA and OVM-GA-R. However, the OVM materials whose primary amino groups were cross-linked with glutaraldehyde retained a chiral recognition ability.

INTRODUCTION

Recently, several protein-bonded stationary phases have been developed for the chiral resolution of racemic solutes by high-performance liquid chromatography (HPLC) [1]. Protein-bonded stationary phases include albumins such as bovine serum albumin [2] and human serum albumin [3], glycoproteins such as α_1-acid glycoprotein (AGP) [4] and ovomucoid (OVM) [5] and enzymes such as α-chymotrypsin [6], trypsin [7] and fungal cellulase (an acid glycoprotein) [8]. Above all, glycoprotein-bonded phases are more stable and have wider chiral recognition properties owing to the presence of proteins and carbohydrates, which are chiral molecules. It is suggested that carbohydrates are essential for the chiral recognition of racemic solutes on glycoprotein-bonded columns [9,10]. OVM, which is an acid glycoprotein from egg white, was bonded to aminopropylsilica via N,N-disuccinimidyl carbonate activation reaction by Miwa *et al.* [5], and an OVM-bonded column is now commercially available. The OVM-bonded column has been utilized for the chiral resolution of acidic, basic and uncharged solutes [11–14], and has been applied to the chiral resolution of basic drugs in biological fluids [15–17].

Recently, Kirkland *et al.* [18] compared commercially available glycoprotein-bonded columns based on OVM and AGP. They reported that the former column showed generally higher enantioselectivity and column efficiency and better long-term stability than the latter. However, our preliminary results suggest that the OVM column is unstable against

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repetitive injections of solutes and/or changes in eluent composition (eluent pH, type and content of organic modifier) (see Results and Discussion) [19]. In order to improve the stability of the OVM column, and to expand the applicability of the OVM column, we tried to cross-link the OVM-bonded column. Cross-linking of the OVM protein might result in improvement of column stability owing to its greater conformational rigidity. In this study, we prepared OVM and cross-linked OVM columns: OVM was immobilized to aminopropylsilica via an N,N-disuccinimidyl carbonate coupling reaction [5]. The OVM materials were cross-linked with glutaraldehyde (OVM-GA) and further reduced with sodium borohydride (OVM-GA-R). This paper deals with the comparison of the retentive and enantioselective properties of acidic, basic and uncharged solutes on OVM, OVM-GA and OVM-GA-R columns with respect to eluent pH and organic modifier. Also, the stabilities of OVM and OVM-GA columns were preliminarily compared.

EXPERIMENTAL

Reagents and materials

Ibuprofen, ketoprofen, flurbiprofen, chlorpheniramine maleate, oxprenolol hydrochloride, tolperisone hydrochloride and hexobarbital were kindly donated by Kaken Pharmaceutical (Tokyo, Japan), Chugai Pharmaceutical (Tokyo, Japan), Essex Nippon (Osaka, Japan), Nippon Ciba-Geigy (Takarazuka, Japan), Nippon Kayaku (Tokyo, Japan) and Teikoku Chemicals (Osaka, Japan). The structures of racemic solutes used in this study are shown in Fig. 1. Methanol, ethanol and acetonitrile of HPLC grade and 20% glutaraldehyde solution were obtained from Wako (Osaka, Japan). OVM from egg white was kindly donated by Dr. T. Miwa (Eisai, Kawashima, Gifu, Japan). Other reagents were of analytical-reagent grade and were used as received.

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

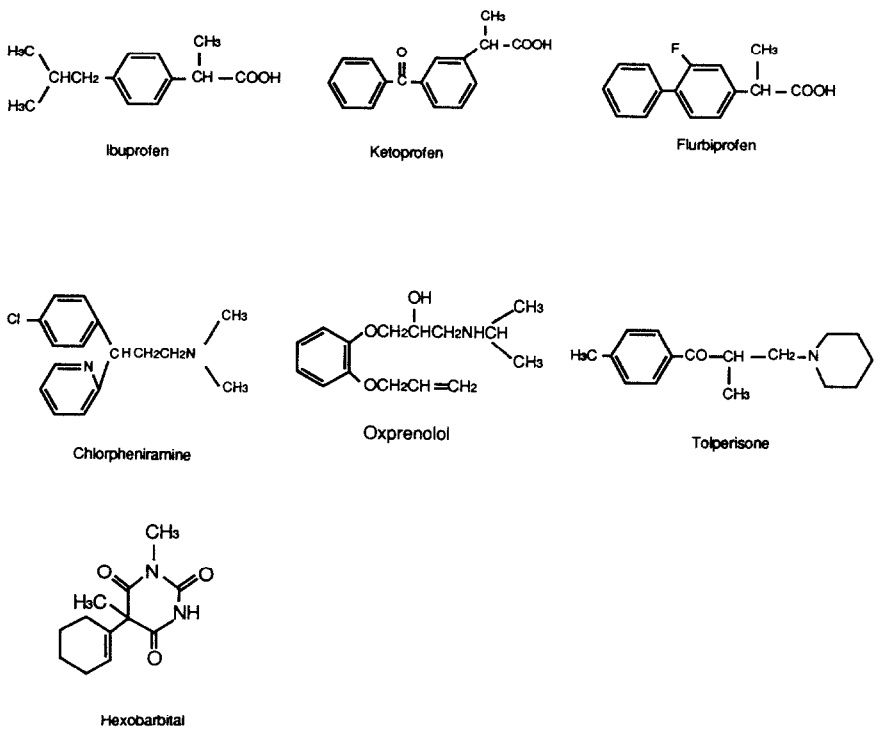

Fig. 1. Structures of racemic solutes used.

Preparation of OVM, OVM-GA and OVM-GA-R materials

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

Five grams of the OVM materials were added to 50 ml of 100 m*M* ammonium dihydrogenphosphate buffer solution (pH 4.4) and sonicated for 5 min, then 500 μl of 5% glutaraldehyde solution were slowly added. The mixture was slowly rotated at 30°C for 15 h, filtered and washed with 100 m*M* ammonium dihydrogenphosphate buffer solution (pH 4.4), 50 m*M* sodium phosphate buffer (pH 7.0), water and methanol. The isolated materials (OVM-GA) were dried *in vacuo* over P_2O_5 at 40°C for 6 h.

Five grams of the OVM-GA materials were added to 50 ml of sodium phosphate buffer (pH 7.0) and sonicated for 5 min, then 60 ml of 10% sodium borohydride solution were added. The mixture was slowly rotated for 4 h at 10°C, filtered and washed with 50 m*M* sodium phosphate buffer (pH 7.0), water and methanol. The isolated materials (OVM-GA-R) were dried *in vacuo* over P_2O_5 at 40°C for 6 h.

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

Chromatography

The HPLC system was composed of a Model 880-PU pump (Japan Spectroscopic, Tokyo, Japan), a Rheodyne (Cotati, CA, USA) Model 7125 injector equipped with 100-μl loop and an SPD-6A spectrophotometer (Shimadzu, Kyoto, Japan). The flow-rate was maintained at 1.0 ml/min. Chromatograms were recorded and integrated with a Chromatpac C-R6A integrator (Shimadzu).

Capacity factors were calculated from the equation of $k' = (t_R - t_0)/t_0$, where t_R and t_0 are elution times of retarded and unretarded 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 unretarded 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 is calculated from the equation $\alpha = k'_2/k'_1$. All separations were performed 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 legends.

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 onto the column. The on-column weight was 0.2 μg, except when measuring retention times above 60 min, for which it was 0.5 μg.

RESULTS AND DISCUSSION

Effect of eluent pH on retention and enantioselectivity of charged and uncharged solutes

Tables I–III show the effects of eluent pH on the retention and enantioselectivity of acidic, basic and uncharged solutes on OVM, OVM-GA and OVM-GA-R materials, with 20 m*M* phosphate buffer containing 10% ethanol as the eluent. The trends of the capacity factors (k'_1) and enantioseparation factors (α) oserved with these three columns were similar except that they decreased in the order OVM, OVM-GA and OVM-GA-R materials. The capacity factors of acidic solutes (ibuprofen, ketoprofen and flurbiprofen) gave maximum values at an eluent pH of *ca.* 4. This could be elucidated by taking account into the pK_a values of 2-arylpropionic acid derivatives (4.0–4.5) and the isoelectric point of ovomucoid (pI = 3.8–4.3) [20]. The decrease in the capacity factors of 2-arylpropionic acid derivatives is ascribable to ion exclusion in addition to ionic repulsion between the carboxyl groups of 2-arylpropionic acid derivatives and negatively charged OVM with increase in eluent pH. As at eluent pH values below the pI of OVM the OVM became positively charged, the capacity factors of undissociated 2-arylpropionic acid derivatives could be decreased by decreasing the eluent pH.

Taking into account the same trends in the capacity factors of 2-arylpropionic acid derivatives on the OVM-GA and OVM-GA-R as on the OVM material, the changes in the pI values of these materials due to cross-linking should be small. On the other

TABLE I

EFFECT OF pH ON RETENTION AND ENANTIOSELECTIVITY OF ACIDIC DRUGS ON OVM, OVM-GA AND OVM-GA-R COLUMNS[a]

Column	Compound	pH 3.2[b]		pH 3.9		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	7.17	1.42	10.3	1.44	5.14	1.28	1.38	1.16	0.39	1.00
	Ketoprofen	24.6	1.36	35.6	1.27	11.5	1.15	2.50	1.07	0.67	1.00
	Flurbiprofen	32.7	1.39	46.5	1.39	16.1	1.21	3.67	1.10	1.10	1.00
OVM-GA	Ibuprofen	6.45	1.26	10.3	1.29	5.34	1.15	1.66	1.07	0.48	1.00
	Ketoprofen	18.2	1.23	29.9	1.17	9.86	1.05	2.73	1.00	0.76	1.00
	Flurbiprofen	30.4	1.29	44.2	1.26	15.9	1.09	4.57	1.00	1.41	1.00
OVM-GA-R	Ibuprofen	5.25	1.19	8.30	1.17	6.13	1.09	2.14	1.00	0.68	1.00
	Ketoprofen	13.2	1.20	20.1	1.13	10.1	1.02	3.13	1.00	1.05	1.00
	Flurbiprofen	21.2	1.19	33.5	1.19	17.1	1.09	5.26	1.00	1.74	1.00

[a] 20 m*M* phosphate buffer–ethanol (90:10, v/v) was used as the eluent.
[b] Buffer pH.

hand, the capacity factors of basic solutes (chlorpheniramine, oxprenolol and tolperisone) increased with increase in eluent pH. Because of the pK_a values of these solutes (9.0–9.5), the retention of basic solutes should be due to electrostatic interactions with the positively charged solutes and the negatively charged protein, in addition to hydrophobic interactions. At eluent pH value below the p*I* of OVM, these solutes were eluted rapidly because of electrostatic repulsion between positively charged solutes and the protein. Hence, the capacity factors of these solutes at low eluent pH were not measured. Although the capacity factor of an uncharged solute (hexobarbital) showed almost no influence of eluent pH, a slight increase was observed with increasing eluent pH. This increase might be

TABLE II

EFFECT OF pH ON RETENTION AND ENANTIOSELECTIVITY OF BASIC DRUGS ON OVM, OVM-GA AND OVM-GA-R COLUMNS[a]

Column	Compound	pH 3.9[b]		pH 5.1		pH 6.0		pH 6.9	
		k'_1	α	k'_1	α	k'_1	α	k'_1	α
OVM	Chlorpheniramine	0.23	1.46	2.65	1.75	12.9	1.95		
	Oxprenolol	0.20	1.00	3.04	1.18	15.1	1.30		
	Tolperisone			0.90	1.77	4.04	1.42	16.7	1.21
OVM-GA	Chlorpheniramine	0.42	1.00	1.50	1.58	7.14	1.81	28.6	1.92
	Oxprenolol	0.25	1.00	1.03	1.29	7.16	1.25	29.4	1.32
	Tolperisone			0.49	1.72	2.29	1.39	8.67	1.28
OVM-GA-R	Chlorpheniramine			0.95	1.49	4.63	1.73	20.7	1.87
	Oxprenolol			0.71	1.15	4.44	1.20	18.4	1.31
	Tolperisone			0.29	1.70	1.57	1.34	6.32	1.25

[a] 20 m*M* phosphate buffer–ethanol (90:10, v/v) was used as the eluent.
[b] Buffer pH.

TABLE III

EFFECT OF pH ON RETENTION AND ENANTIOSELECTIVITY OF HEXOBARBITAL ON OVM, OVM-GA AND OVM-GA-R COLUMNS[a]

Column	pH 3.2[b]		pH 3.9		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	1.10	1.40	1.46	1.51	1.79	1.51	2.13	1.56	2.59	1.78
OVM-GA	0.85	1.25	1.04	1.40	1.14	1.40	1.19	1.48	1.54	1.71
OVM-GA-R	0.81	1.24	0.84	1.31	0.90	1.33	1.09	1.41	1.14	1.56

[a] 20 m*M* phosphate buffer–ethanol (95:5, v/v) was used as the eluent.
[b] Buffer pH.

due to changes in the binding properties of the protein resulting from changes in the eluent pH. The above results suggest that hydrophobic and electrostatic interactions should play an important role in the retention and enantioselectivity of racemic solutes on OVM, OVM-GA and OVM-GA-R columns.

The maximum enantioseparation factors of 2-arylpropionic acid derivatives were obtained with the use of an eluent pH of 3.2–3.9. The enantioseparation factors of chlorpheniramine and oxprenolol increased with increase in eluent pH, whereas that of tolperisone decreased with increase in eluent pH. However, when acetonitrile was used as an organic modifier, the enantioseparation factor of tolperisone increased with increasing eluent pH. These results might be correlated with the enantiomeric elution order of tolperisone, as reported previously [21]. Generally, acidic solutes gave higher enantioselectivities with decrease in eluent pH, whereas basic solutes were well resolved with increase in eluent pH. The enantioseparation factor of hexobarbital increased with increasing eluent pH.

Almost the same retention of these solutes as obtained with the use of eluents containing 10% ethanol were observed with the use of eluents containing 15% methanol or 8% acetonitrile on OVM, OVM-GA and OVM-GA-R materials. This indicates that the solvent strength decreases in the order acetonitrile, ethanol and methanol on OVM, OVM-GA and OVM-GA-R columns. However, the enantioselectivity of these solutes was dependent on the organic modifier used, as described below.

Effect of organic modifier on enantioselectivity of charged and uncharged solutes

Table IV shows effects of organic modifier on the enantioseparation factors of acidic, basic and uncharged drugs on OVM, OVM-GA and OVM-GA-R materials. For acidic drugs, the eluents used were 20 m*M* phosphate buffer (pH 3.9) containing 10% ethanol, 15% methanol and 8% acetonitrile. The results show that the use of methanol as an organic modifier gives the highest enantioselectivity of ibuprofen and ketoprofen, and that for the chiral resolution of flurbiprofen a higher enantioselectivity is obtained with the use of acetonitrile. For basic and unchanged drugs, 20 m*M* phosphate buffer (pH 6.0) containing ethanol, methanol and acetonitrile was used. It was found that almost the same enantioseparation factors of chlorpheniramine and tolperisone were obtained with the use of ethanol and methanol, and that of oxprenolol was independent of the organic modifier used. Racemic hexobarbital gave the highest enantioselectivity with the use of methanol. Fig. 2 shows typical chromatograms of 2-arylproprionic acid derivatives on OVM-GA and OVM-GA-R materials.

These results indicate that for chiral resolution of solutes it is necessary to select the most suitable eluent pH and organic modifier to give the maximum enantioselectivity on OVM, OVM-GA and OVM-GA-R materials. Also, methanol might be a suitable organic modifier for the chiral resolution of solutes. The enantioselectivity of the solutes on the materials prepared decreased in the order OVM, OVM-GA and OVM-GA-R. OVM-GA and OVM-

TABLE IV

EFFECT OF ETHANOL, METHANOL AND ACETONITRILE (ACN) ORGANIC MODIFIERS ON ENANTIOSELECTIVITY OF ACIDIC, BASIC AND UNCHARGED DRUGS ON OVM, OVM-GA AND OVM-GA-R COLUMNS

Type of drug	Compound	OVM column			OVM-GA column			OVM-GA-R column		
		Ethanol	Methanol	ACN	Ethanol	Methanol	ACN	Ethanol	Methanol	ACN
Acidic[a]	Ibuprofen	1.44	1.54	1.19	1.29	1.43	1.13	1.17	1.22	1.09
	Ketoprofen	1.27	1.39	1.27	1.17	1.29	1.17	1.13	1.20	1.13
	Flurbiprofen	1.39	1.44	1.49	1.26	1.34	1.35	1.19	1.33	1.26
Basic[b]	Chlorpheniramine	1.95	1.97	1.82	1.81	1.84	1.64	1.73	1.68	1.52
	Oxprenolol	1.30	1.27	1.32	1.25	1.25	1.28	1.20	1.25	1.25
	Tolperisone	1.42	1.41	1.11	1.39	1.33	1.00	1.34	1.25	1.00
Uncharged[c]	Hexobarbital	1.56	1.70	1.38	1.48	1.63	1.40	1.41	1.50	1.30

[a] Eluents used were a mixture of 20 m*M* phosphate buffer (pH 6.0) and 10% ethanol, 15% methanol or 8% acetonitrile.
[b] Eluents used were a mixture of 20 m*M* phosphate buffer (pH 3.9) and 10% ethanol, 15% methanol or 8% acetonitrile.
[c] Eluents used were a mixture of 20 m*M* phosphate buffer (pH 6.0) and 5% ethanol, 7.5% methanol or 4% acetonitrile.

GA-R materials were slightly positive against the ninhydrin reaction, indicating that the OVM materials whose primary amino groups were cross-linked with glutaraldehyde retained their chiral recognition ability.

One of the disadvantage of protein-bonded phases is low long-term stability. In preliminary studies, we compared the long-term stability of OVM and OVM-GA columns: 20 m*M* phosphate buffer (pH 3.9)–acetonitrile (90:10, v/v) was pumped at 1.0 ml/min for 60 min and then 20 m*M* phosphate buffer (pH 6.9)–ethanol (90:10, v/v) was passed through for 60 min. Hexobarbital was loaded under the latter eluent conditions. This cycle was repeated for about 200 times. For the OVM column the decreases in the capacity factor and enantioseparation factor were of the order of 17 and 5.7%, respectively, after 204 injections, whereas for the OVM-GA column the decreases were 7.7 and 1.6%, respectively, after 217 injections. These results suggest that the OVM-GA materials are more stable towards repetitive injections and/or changes in eluent composition (eluent pH, type of organic modifier) than the OVM materials. Hence the OVM-GA materials might be a promising candidate for the chiral resolution of racemic solutes. This aspect is now being investigated.

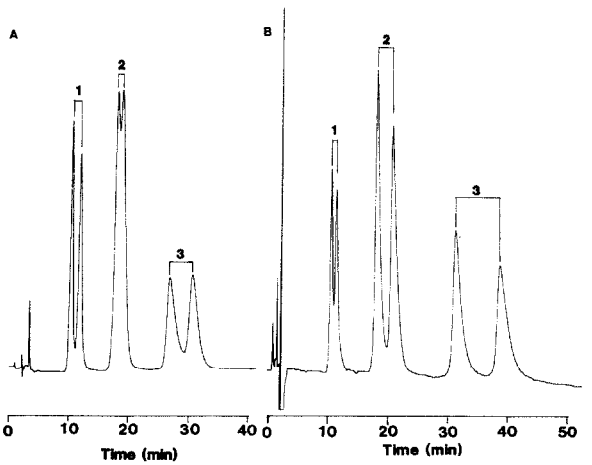

Fig. 2. Typical chromatograms of racemic 2-arylpropionic acid derivatives on (A) OVM-GA and (B) OVM-GA-R materials. Peaks: 1 = ibuprofen; 2 = ketoprofen; 3 = flurbiprofen. Eluents: (A) 20 m*M* phosphate buffer (pH 5.1) containing 10% ethanol; (B) 20 m*M* phosphate buffer (pH 3.2) containing 8% acetonitrile. Injection volume: 20 μl (each solute concentration, 10 μg/ml). Sensitivity, 0.016 a.u.f.s.

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