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Separation of enantiomers on a pepsin-bonded column

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Abstract

Commercial crystalline pepsin from porcine stomach mucosa was characterized by high-performance capillary electrophoresis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. These results suggest that the average molecular mass of the pepsin is about 34 900, and that it contains heterogeneity due to the autodigestion. The pepsin was covalently bound to porous aminopropylsilica materials using an N,N′-disuccinimidyl carbonate reaction. By using a mixture of phosphate buffer and organic modifier as an eluent, basic and uncharged enantiomers were resolved, while no resolution of acidic enantiomers was observed.

1. Introduction

Many protein-bonded stationary phases have been developed for the resolution of enantiomers [1]. These include albumins such as bovine serum albumin [2] and human serum albumin [3], glycoproteins such as α_1-acid glycoprotein [4], ovomucoid [5] and avidin [6] and enzymes such as cellulase [7], trypsin [8], α-chymotrypsin [9] and lysozyme [10]. A wide range of compounds were separated on the protein-bonded columns because they can provide multiple interactions and/or multiple recognition sites. However, in general, all enantiomers cannot be separated by using only one column. In the process of surveying a new protein-bonded stationary phase, we found that pepsin-bonded silica materials could separate enantiomers of basic and uncharged solutes. It has been reported that the protein, whose molecular mass is 34 600, consists of 327 amino acids and contains three disulfide bonds [11]. This paper deals with characterization of commercial crystalline pepsin from porcine stomach mucosa by high-performance capillary electrophoresis (HPCE) and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and the chiral recognition properties of the pepsin-bonded silica stationary phases.

2. Experimental

2.1. Reagents and materials

Racemic compounds used in this study were kindly donated by pharmaceutical companies or purchased from Sigma (St. Louis, MO, USA). Pepsin from porcine stomach mucosa, purified by crystallization followed by chromatography, was obtained from Sigma and used without further purification. N,N′-Disuccinimidyl carbonate (DSC) was purchased from Wako (Osaka, Japan). The silica gels Ultron-120 (par-

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ticle diameter 5 μm, pore size 120 Å, surface area 300 m^2/g) and Ultron-300 (particle diameter 5 μm, pore size 300 Å, surface area 100 m^2/g) were obtained from Shinwa Chemical Industries (Kyoto, Japan). Capillary tubes coated with polyethylene glycol 20M (PEG-20M) was supplied by Shinwa Chemical Industries. Other reagents and solvent used were of analytical-reagent or HPLC grade. Water purified with a Nano-Pure II system (Barnstead, Boston, MA, USA) was used for the preparation of sample solutions and eluents.

2.2. Characterization of pepsin

High-performance capillary electrophoretic (HPCE) separation of pepsin was performed with a P/ACE system equipped with a photodiode-array detector (Beckman Instruments, Fullerton, CA, USA). PEG-20M-coated capillary tubes of 75 μm I.D. and 57 cm total length (effective length 50 cm) were thermostated with a liquid coolant. The applied voltage was held constant at −10 kV (175 V/cm). The detection wavelength was 214 nm. The running buffer solution was 50 m*M* sodium phosphate buffer (pH 8.5). Sample injection was performed with a pressurizing method (0.5 p.s.i., 1 s). The sample solution was 5 mg/ml.

MALDI-TOF mass spectra were obtained with a TOFSPEC linear-type TOF instrument (JASCO International, Tokyo, Japan) equipped with nitrogen laser operating at 337 nm. The ions generated were accelerated to a potential of 5 kV in the ion source and post-accelerated to a potential of 23 kV for detection with a secondary ion multiplier. The MALDI-TOF spectra were calibrated externally with a standard sample (bovine serum albumin, molecular mass 68 433). The matrix used was α-cyano-4-hydroxycinnamic acid, dissolved in 0.1% aqueous TFA–acetonitrile (2:1) at 50 m*M*. Samples were dissolved in water at 10^{-6} *M*. A 0.5-μl portion of the sample solution was mixed with an equal volume of the matrix solution on the target, resulting in a sample amount used of 500 fmol. After deposition on the stainless-steel target, the sample was air-dried and introduced into the mass spectrometer.

2.3. Preparation of pepsin-bonded materials

Ultron-120 or -300 silica gel (5 g) was dried in vacuo over P_2O_5 at 150°C for 6 h and the dry silica gel was added to 120 ml of dry toluene. The mixture was heated to reflux until all the water had been removed as an azeotrope into a Dean–Stark-type trap. Next, 3-aminopropyltriethoxysilane, corresponding to 10 $\mu mol/m^2$ of specific surface area, was added and reacted for 8 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.

The aminopropylsilica gels obtained were activated with DSC. A 5-g amount of gel was 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 and methanol. The silica gels obtained were dried in vacuo over P_2O_5 at 60°C for 2 h.

Pepsin was bound to DSC-activated aminopropylsilica gels as follow. A 1.2-g amount of the DSC-activated silica gel was slurried in 20 ml of 20 m*M* phosphate buffer (pH 4.5), then 600 mg of pepsin dissolved in 5 ml of the same buffer were added slowly at room temperature over 1 h by adjusting pH to 4.5, and further stirred for 15 h at 30°C. The reaction mixture was washed with water and further reacted with 2-aminoethanol–water (1:50) adjusted to pH 4.5 with hydrochloric acid at room temperature for 1 h. The reaction mixtures were filtered and washed with water and ethanol–water (5:95, v/v). The isolated materials were dried in vacuo over P_2O_5 at 40°C for 6 h.

The pepsin-bonded materials were packed into a 100 × 4.6 mm I.D. stainless-steel column by the slurry packing method using ethanol–water (5:95, v/v) as the slurry and packing solvent.

2.4. Elemental analysis

Elemental analysis of the pepsin-bonded materials was performed using ion chromatography combined with the oxygen-flask method for sulfur. The surface coverage ($\mu mol/g$) of pepsin was obtained from the sulfur content of the

material (%) and the number of sulfur atoms per pepsin molecule (n) as follows:

$$\text{surface coverage } (\mu\text{mol/g}) = \text{sulfur content } (\%) \cdot \frac{1}{100} \cdot \frac{1}{32.07n} \cdot 10^6$$

where 10 is used for n [11].

2.5. Chromatography

For the chiral resolution of solutes, the HPLC system used was composed of an LC-9A pump, an SPD-6A spectrophotometer, a SIL-6B auto-injector, 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 the elution times of retained and unretained solutes, respectively, and k'_1 and k'_2 are 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 $R_s = 2(t_2 - t_1)/(t_{w_1} + t_{w_2})$, where t_1 and t_2 are the retention times of the first- and second-eluted peaks, respectively, and t_{w_1} and t_{w_2} are the peak widths. 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 table and figure captions.

2.6. Sample preparation

A known amount of each 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 0.2–0.5 μg.

3. Results and discussion

3.1. Characterization of pepsin

Commercial crystalline pepsin from porcine stomach mucosa was characterized by HPCE and MALDI-TOF mass spectrometry. Fig. 1 shows an HPCE separation of commercial crystalline pepsin. It was reported that the glycoforms of commercial crystalline pepsin were separated by HPCE using a poly(vinyl alcohol)-coated capillary [12] or a bare capillary with putrescine as a buffer additive [13]. However, Rajagopalan et al. [14] reported that autodigestion occurred during the industrial preparation of 1:10 000 pepsin, the starting material from which crystalline pepsin has traditionally been prepared. Also, the heterogenous pepsin was separated into several peaks on a hydroxyapatite column. Hence, the HPCE separation of the pepsin is not a reflection of glycoforms but of autodigestive proteins.

Fig. 2 shows a MALDI-TOF mass spectrum of the pepsin. The average molecular mass is 34 918.7, estimated from an $[M + H]^+$ ion. This is in good agreement with the average molecular

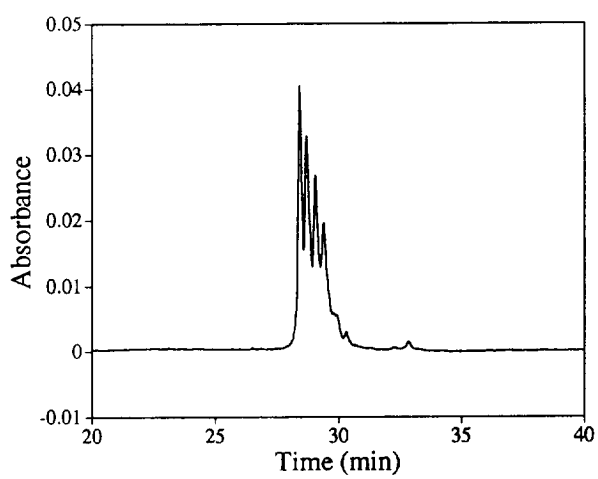

Fig. 1. HPCE separation of commercial crystalline pepsin.

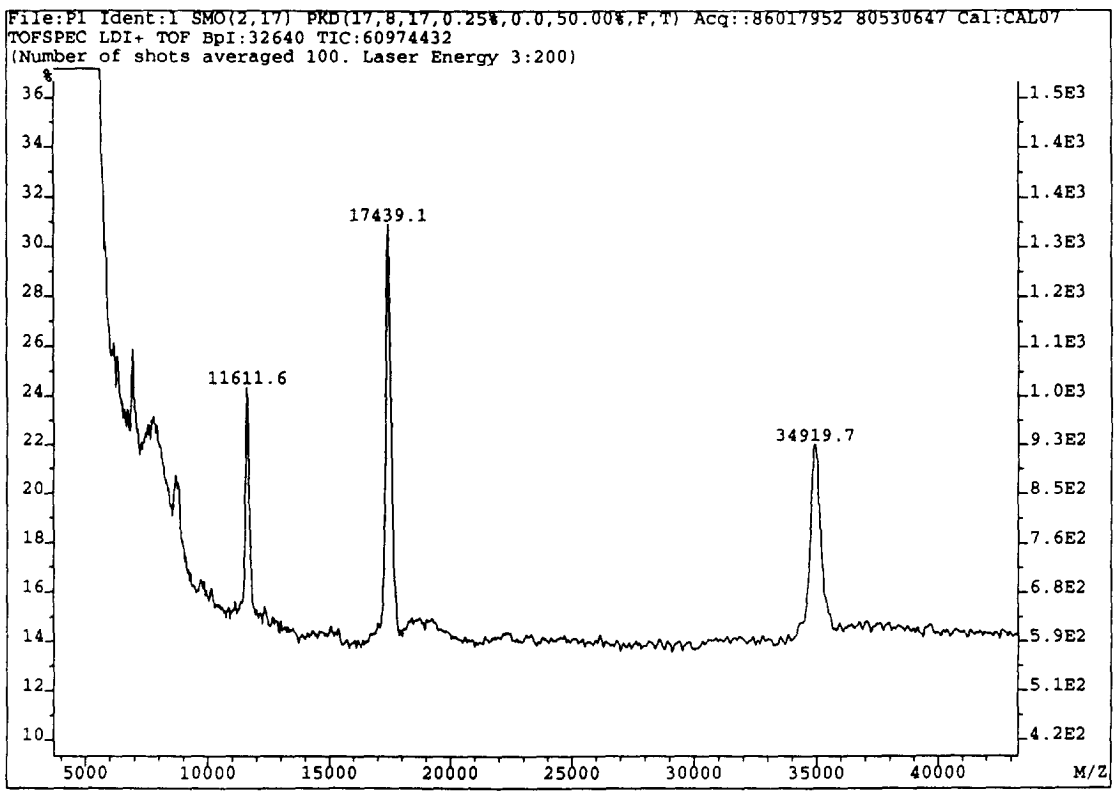

Fig. 2. MALDI-TOF mass spectrum of commercial crystalline pepsin.

mass of the pepsin of 34 644 reported by Tang et al. [11].

3.2. Comparison of base silica materials

Two pepsin-bonded materials, which are based on porous silica materials having average pore sizes of 120 and 300 Å, were prepared. The amounts of bonded pepsin were 6.9 and 2.5 μmol/g for the 120 and 300 Å materials, respectively. Table 1 illustrates the capacity factors and enantioseparation factors for atenolol, alprenolol and oxazepam on the two pepsin-bonded columns. The pepsin-bonded column prepared from the 120 Å base silica materials had higher amounts of bonded proteins and gave longer retentions for all solutes tested than that prepared from the 300 Å base silica materials, and

Table 1
Retention and enantioselectivity of solutes on pepsin-bonded columns different in pore size of base materials

Packing[a]	Solute					
	Atenolol		Alprenolol		Oxazepam	
	k_1	α	k_1	α	k_1	α
PP-120	0.78	1.17	2.71	1.32	4.44	1.16
PP-300	0.67	1.11	2.18	1.26	1.98	1.16

HPLC conditions: column, 100 × 4.6 mm I.D.; eluent, 20 m*M* phosphate buffer (pH 5.1) containing 5% and 3% ethanol for the PP-120 and PP-300 materials, respectively; flow-rate 0.8 ml/min; detection, 220 nm; column temperature, 25°C.

[a] PP-120 and PP-300 materials were prepared from 120 and 300 Å base silica materials, respectively.

Table 2
Protein converges of pepsin-bonded columns

Packing	Pore size of base silica (Å)	Protein phase	
		Sulfur content (%)[a]	Surface coverage (μmol/g)
PP-2.5	120	0.20	6.2
PP-3.5	120	0.25	7.8
PP-4.5	120	0.22	6.9
PP-5.5	120	0.17	5.3
PP-6.6	120	0.20	6.2

[a] Calculated from the sulfur elemental analysis data.

the former column had higher enantioselectivity. Thus, the pepsin-bonded materials prepared from 120 Å base silica materials were used in the experiments described below.

3.3. Reaction pH for bonding of pepsin

The reaction pH for bonding of the pepsin to DSC-activated aminopropylsilica gels was examined by varying the pH at 2.5, 3.5, 4.5, 5.5 and 6.6. The materials were termed PP-2.5, PP-3.5, PP-4.5, PP-5.5 and PP-6.6, respectively. Table 2 illustrates the sulfur content and surface coverage of pepsin-bonded materials. Table 3 gives the capacity factors and enantioseparation factors of atenolol, alprenolol and oxazepam on the pepsin-bonded columns prepared at the different reaction pH values. The surface coverages of pepsin-bonded materials are almost the same at the different reaction pH values tested. However, the PP-2.5, PP-3.5 and PP-4.5 materials gave higher enantioselectivity than the PP-5.5 and PP-6.6 materials. The PP-6.6 materials had no chiral recognition properties. It has been reported that loss of the enzymatic activity of pepsin was observed at ca. pH 6 [15]. These results suggest that the loss of enzymatic activity might result in no chiral recognition properties, and that the conformational change might occur at above pH 6. With regard to the PP-2.5 and

Table 3
Retention and enantioselectivity of solutes on pepsin-bonded columns differing in reaction pH for bonding

Packing	Solute					
	Atenolol		Alprenolol		Oxazepam	
	k_1	α	k_1	α	k_1	α
PP-2.5	1.02	1.21	3.43	1.35	3.37	1.20
PP-3.5	1.47	1.19	5.21	1.36	4.06	1.18
PP-4.5	0.78	1.17	2.71	1.32	4.44	1.16
PP-5.5	1.70	1.13	4.89	1.19	2.98	1.10
PP-6.6			1.17	1.00	4.01	1.00

HPLC conditions: column, 100 × 4.6 mm I.D.; eluent, 20 m*M* phosphate buffer (pH 5.1) containing 5% ethanol; flow-rate, 0.8 ml/min; detection, 220 nm; column temperature, 25°C.

PP-3.5 materials, it took 1 day to attain a flat baseline. In addition, the capacity factors of the first-eluted enantiomer of alprenolol changed from 3.43 to 1.61 for the PP-2.5 materials and from 5.21 to 3.91 for the PP-3.5 materials after 72 h of continuous flow of the eluent, whereas for the PP-4.5 materials the capacity factor remained unchanged (from 2.71 to 2.76). We assumed that for the PP-2.5 and PP-3.5 materials pepsin would adsorb on the surface of the materials without being chemically bound. Hence the reaction pH for bonding of the pepsin was determined to 4.5.

3.4. Effect of eluent pH on retention and enantioselectivity of basic and uncharged solutes

Chiral resolution of acidic, basic and uncharged enentiomers was examined on a pepsin-bonded material. Basic and uncharged enantiomers were resolved. Acidic enantiomers such as 2-arylpropionic acid derivatives were not resolved, despite the attempt at optimization of the eluent conditions. Next, we examined effects of eluent pH on the retention and enantioselectivity of basic and uncharged solutes on the pepsin-bonded column (Table 4). When an eluent of pH 7 was continuously delivered, the pepsin-bonded column lost its chiral recognition properties. It has been reported that pepsin was denatured at pH 8.5 [16]. These results reveal that the immobilized pepsin should be irreversibly denatured above pH 7. Therefore, the eluent pH was varied between 4 and 6. The capacity factors of basic solutes increased with increase in eluent pH, whereas those of uncharged solutes remained almost unchanged. The enantioseparation factor of alprenolol decreased with increase in eluent pH, whereas that of atenolol increased. Oxazepam gave the highest enantioselectivity at

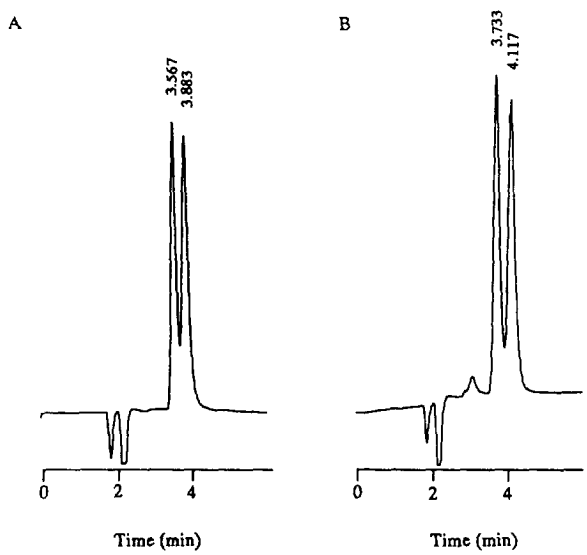

Fig. 3. Chiral resolution of salbutamol (A) and atenolol (B) on a pepsin-bonded column. HPLC conditions: eluent, 20 m*M* phosphate buffer (pH 5.1) containing 5% ethanol; flow-rate, 0.8 ml/min; detection, 220 nm; injection volume, 20 μl (20 μg/ml each).

Table 4
Effect of eluent pH on retention and enantioselectivity of solutes on pepsin-bonded columns

Eluent pH[a]	Solute					
	Atenolol		Alprenolol		Oxazepam	
	k_1	α	k_1	α	k_1	α
4.0	–	–	0.34	1.40	3.75	1.16
5.1	0.78	1.17	2.71	1.32	4.44	1.16
6.0	2.20	1.21	7.47	1.29	3.72	1.18

HPLC conditions: column, 100 × 4.6 mm I.D.; eluent, 20 m*M* phosphate buffer (pH 5.1) containing 5% ethanol; flow-rate, 0.8 ml/min; detection, 220 nm; column temperature, 25°C.
[a] Buffer pH.

Table 5
Retention, enantioselectivity and resolution of various solutes on a pepsin-bonded column

Solute[a]	k_1	α	R_s
β-Blockers			
Alprenolol	2.71	1.32	1.49
Atenolol	0.80	1.17	0.67
Oxprenolol	2.09	1.23	0.83
Pindolol	1.92	1.13	1.01
Propranolol	5.64	1.17	1.12
Befunolol[b,c]	2.42	1.16	1.17
Sympathomimetic agents			
Isopreterenol	0.77	1.11	0.46
Salbutamol	0.69	1.16	0.49
Antihistaminics			
Chlorpheniramine	5.70	1.10	0.54
Azelastin[b,d]	13.0	1.20	1.63
Alimemazine[b,e]	12.9	1.38	3.14
Homochlorcyclizine[b]	13.1	1.35	2.66
Promethazine[b]	8.17	1.07	0.45
Dimetindene[f]	6.88	1.05	<0.3
Skeltal muscle relaxants			
Eperisone[d]	4.49	1.10	0.49
Tolperison	2.65	1.03	<0.3
Benzodiazepines			
Lorazepam	5.99	1.13	0.40
Oxazepam	4.44	1.16	0.46
Lormetazepam	5.69	1.18	<0.3
Temazepam	4.15	1.08	<0.3
Others			
Trimipramine	13.5	2.94	7.14
Trihexyphenidyl	5.33	1.08	<0.3
Verapamil	15.0	1.34	2.42
Bupivacaine[b]	1.56	1.25	0.63
Cloperastin[b]	9.23	1.12	0.92

The solutes resolved were positively charged in the eluent used, except for benzodiazepines. HPLC conditions: column, 100 × 4.6 mm I.D.; eluent, 20 m*M* phosphate buffer (pH 5.1) containing 5% ethanol, except where indicated otherwise; flow-rate, 0.8 ml/min; column temperature, 25°C.

[a] Solutes obtained from sigma unless indicated otherwise.

[b] Eluent, 20 m*M* phosphate buffer (pH 5.1) containing 10% ethanol.

[c] From Kaken Pharmaceutical.

[d] From Eisai.

[e] From Daiichi Seiyaku.

[f] From Ciba-Geigy.

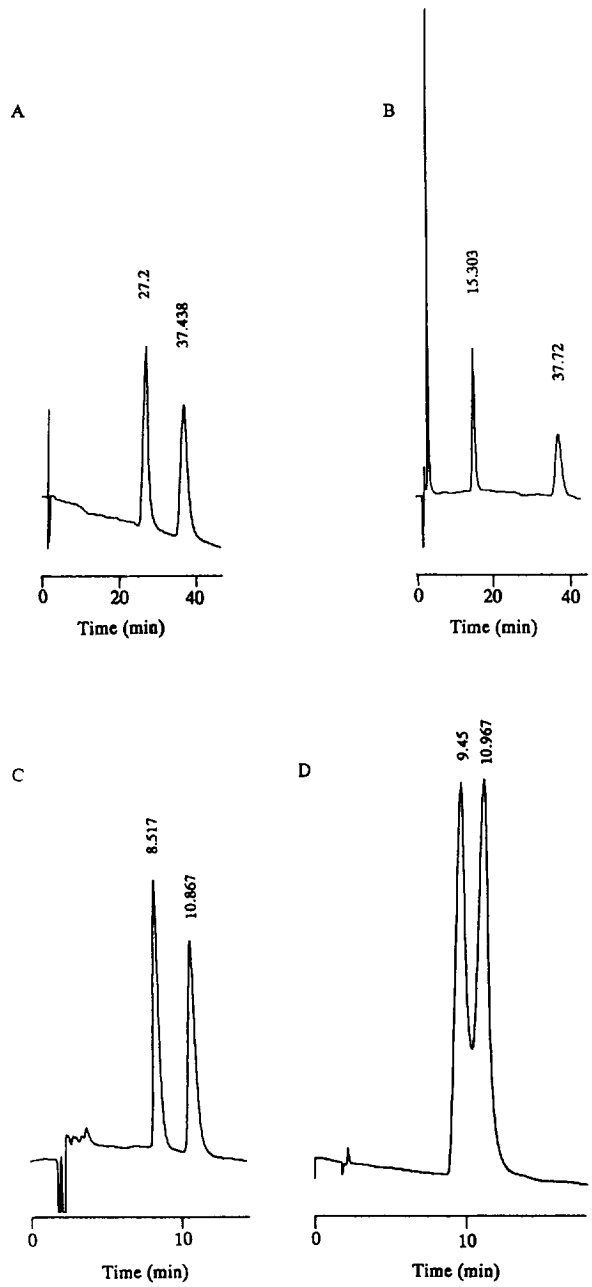

Fig. 4. Chiral resolution of (A) homochlorcyclizine, (B) verapamil, (C) alprenolol and (D) oxazepam on a pepsin-bonded column. HPLC conditions: eluent, 20 m*M* phosphate buffer (pH 5.1) containing (A) 5 and (B) 10% acetonitrile and (C) and (D) 5% ethanol; flow-rate, 0.8 ml/min; detection, 220 nm, except (D) 254 nm; injection volume, (A) and (B) 20 μl (40 μg/ml), (C) 20 μl (20 μg/ml) and (D) 1 μl (200 μg/ml).

an eluent pH of 6. With an increase in the content of organic modifier, the capacity factors of all the solutes tested decreased. These results reveal that hydrophobic, electrostatic and hydrogen bonding interactions should play an important role in the retentive and enantioselective properties of a pepsin-bonded column.

3.5. Chiral resolution of solutes on a pepsin-bonded column

Table 5 shows the chiral resolution of different classes of compounds, β-blockers, synpathomimetic agents, antihistaminics, skeltal muscle relaxants, benzodiazepines and others, on the pepsin-bonded column. Figs. 3 and 4 show typical chromatograms of basic and uncharged solutes on the pepsin-bonded column. The pepsin-bonded silica column has chiral recognition properties for basic solutes (isoproterenol, salbutamol and atenolol), which cannot be resolved on an ovomucoid-bonded column [17].

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