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CHARACTERISTICS OF AN AVIDIN-CONJUGATED COLUMN IN DIRECT LIQUID CHROMATOGRAPHIC RESOLUTION OF RACEMIC COMPOUNDS

TOSHINOBU MIWA* and TAKESHI MIYAKAWA
Eisai Co. Ltd., Pharmaceutical Research Laboratory, 1 Takehaya-machi, Kawashima-cho, Hashima-gun, Gifu 483 (Japan)
and
YASUO MIYAKE
Eisai Co. Ltd., Tsukuba Laboratory, 5-1-3 Tokodai, Toyosato-cho, Tsukuba-shi, Ibaragi 300-26 (Japan)
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SUMMARY

An avidin-conjugated column exhibited chiral recognition of acidic compounds such as profen derivatives. Amine enantiomers were not resolved by this column, of which biotin acted as a potent modifier. Avidin showed hydrophobic interaction with the solutes. A differential scanning calorimetry study showed that thermodynamically stable avidin is inapplicable as a chiral recognition ligand.

INTRODUCTION

Avidin, present in small amounts in egg protein, has a number of interesting characteristics. It is a rare basic protein having a p*I* of between 9.5 and 10.0. Avidin is composed of four identical subunits, the sequence of which has been determined, and each of which has a molecular weight of 16 400 daltons[1,2]. Each subunit bears a glycosidic chain linked to asparagine 17, and binds one molecule of biotin[3]. The avidin–biotin interaction, having an association constant of 10^{15} M^{-1}, is utilized in many fields of molecular biology including immunochemistry[4].

Proteins utilized as column ligands for direct optical resolution are almost all acidic. We have reported that chicken egg-white ovomucoid is a useful column ligand for the resolution of racemic amines[5]. This column also separated racemic profen derivatives, but the chromatograms obtained were not satisfactory[6]. It may be concluded empirically that a column ligand effectively retains and resolves oppositely charged solutes. We therefore selected avidin as a column ligand and employed it in the resolution of racemic acids. We used the succinimide activation method for the conjugation of avidin to the stationary phase, as reported previously. We also investigated the chiral recognition principle of avidin by measuring the thermodynamic stability of this protein.

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EXPERIMENTAL

Apparatus

A Shimadzu LC-6A pump equipped with an SPD-6A variable-wavelength UV monitor and with an SCL-6A automatic sample injector was used. Circular dichroism (CD) spectra were measured with a Jasco J-20 spectropolarimeter. A Seiko instrument DSC-100 calorimetric apparatus was used for the differential thermal analysis of protein solutes.

Chemicals

Ibuprofen, ketoprofen, and atropine from Nihon Balk Yakuhin, flurbiprofen from Kaken, chlorprenaline from Eisai, chlorpheniramine from Kowa, and pindolol from Shiratori Seiyaku, all of pharmaceutical grade, were used. Oxprenolol was extracted from a pharmaceutical (Trasacor® from Ciba-Geigy) with ethanol. CM-Sephadex C-25 was obtained from Pharmacia Fine Chemicals and Duolite C-464 from Diamond Shamrock. 4-Hydroxyazobenzene 2′-carboxylic acid (HABA) was obtained from Tokyo-Kasei. All other chemicals were of reagent grade or higher quality.

Assay and preparation of chicken avidin

Avidin was assayed according to the spectrophotometric titration with HABA described by Green[7]. All steps of the preparation process were performed at 4°C. The buffer used in all cases in the assay and preparation was 0.1 *M* phosphate buffer pH 6.8 which is referred to as "the buffer" in this section.

Powdered egg white (20 kg) was dissolved in 80 l of the buffer and passed through a Duolite C-464 column (volume 30 l) equilibrated with the buffer. The column was washed with the buffer and subsequently eluted with 0.1 *M* phosphate buffer containing 1 *M* sodium chloride. The active fractions were dialyzed against the buffer, then applied to a CM-Sephadex C-25 column equilibrated and washed with the same buffer. Avidin was eluted with a linear gradient of 0–1 *M* sodium chloride and active fractions were collected. The pH of these fractions was adjusted to 3.5 with hydrochloric acid, and they were centrifuged at 8000 *g* for 20 min. Sodium ammonium sulphate was added to the supernatant to 50% saturation, and the resulting liquid was allowed to stand for 1 h and centrifuged at 8000 *g* for 20 min. The supernatant was 80% saturated with ammonium sulphate, and the resulting precipitate was collected by centrifugation. The precipitate was dissolved in a small volume of water and dialyzed against water.

The avidin obtained was electrophoretically homogeneous and lyophilized.

Column preparation

LiChrosorb NH_2 (2 g) and N,N-disuccinimidyl carbonate (3 g) were allowed to react overnight in 60 ml of coupling buffer (0.1 *M* sodium bicarbonate, pH 6.8) at room temperature, using a rotary evaporator without aspiration. After the activated silica gel had been washed with 50 ml of coupling buffer and then suspended in 30 ml of the same buffer, 30 ml of avidin solution (2 g in 30 ml of coupling buffer) were added gradually over a period of 30 min with shaking. This mixture was stirred for 2 h and the avidin-conjugated silica gel obtained was washed with coupling buffer. Stainless-steel columns (15 cm × 4.6 mm I.D.) were slurry-packed with avidin-conjugated silica gel

by using coupling buffer. The amount of avidin conjugated was determined by subtracting the amount recovered by washing from the amount used for conjugation.

Differential scanning calorimetry (DSC) of avidin, and of avidin–biotin and avidin–ketoprofen complexes

A 3-ml sample of 5% avidin solution was mixed with 220 mg of biotin or 230 mg of ketoprofen and left to stand for 2 h at 4°C. Each solution was dialyzed against water for 48 h at 4°C. Free ketoprofen was absent from the dialyzed solution, as was confirmed by high-performance liquid chromatography (HPLC). A 70-μl volume of dialyzed solution was sealed in a silver beaker and its thermal absorption was measured using the same amount of water as a control.

RESULTS AND DISCUSSION

A 1-g amount of LiChrosorb NH_2 adsorbed 120–163 mg of avidin, according to our method. Fig. 1 shows the resolution of profen-derivatized racemates using a column of silica gel conjugated with 163 mg of avidin per g. The effect of adding ethanol to the mobile phase when the same column is used is shown in Table I. Ethanol concentrations of 6% and below decreased the retention and resolution of profen derivatives, though the separation factors of ketoprofen and flurbiprofen were not changed. The separation factor of ibuprofen decreased between 0 and 3% ethanol, which resulted in a substantial decrease in resolution (R_s). The decrease in retention between 0 and 3% ethanol was very marked for ketoprofen (more than seven-fold), whereas a decrease in retention of only 20% was observed between 3 and 6% ethanol. The behaviour of ketoprofen may be attributed to the fact that it has the lowest solubility in water of the three profen derivatives, and also to the existence of a second hydrogen bonding group (carbonyl).

Table II shows the effect of mobile phase pH on the retention and resolution of solutes. In this experiment, a column of silica gel conjugated with 120 mg/g of avidin

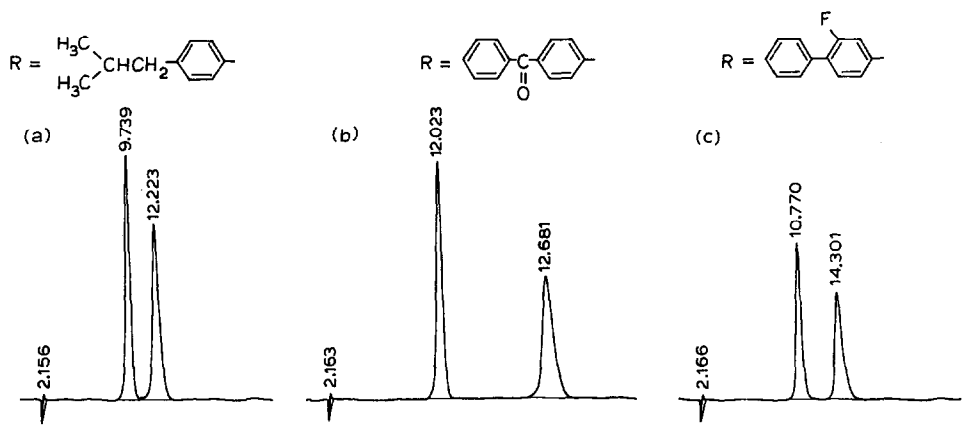

Fig. 1. Separation of the enantiomers of profens, $RCH(CH_3)COOH$, on an avidin-conjugated column. (a) Ibuprofen; (b) ketoprofen; (c) flurbiprofen (compound structures shown above figures). Mobile phase: 20 m*M* potassium phosphate (pH 6.5) containing 6% ethanol. UV detection at 220 nm. Column temperature: 20°C. Flow-rate: 1.2 ml/min. Numbers at peaks indicate retention times in min.

TABLE I

EFFECTS OF ETHANOL ON THE RETENTION AND RESOLUTION OF PROFEN DERIVATIVES

Mobile phase: 20 m*M* potassium phosphate (pH 6.5). Column: avidin bound to LiChrosorb NH_2 (163 mg/g), 150 mm × 4.6 mm I.D.

Ethanol (%)	*Derivative*	k'_1	k'_2	α	R_s
0	Ibuprofen	14.92	27.5	1.84	2.39
	Ketoprofen	>50			
	Flurbiprofen	23.55	37.79	1.61	1.89
3	Ibuprofen	7.42	9.59	1.29	0.82
	Ketoprofen	7.29	14.34	1.97	2.67
	Flurbiprofen	7.79	10.94	1.41	1.17
6	Ibuprofen	4.41	5.79	1.31	0.64
	Ketoprofen	5.68	11.04	1.95	1.93
	Flurbiprofen	4.98	6.94	1.39	0.84

was used. Amine racemates were not resolved by this column. Acidic compounds were strongly retained by the column at a low pH, and higher capacity factors (k') for amines were obtained at higher pH values. This behaviour is the same as that with an acidic protein-immobilized column such as an orosomucoid-conjugated Enantiopak® (ref. 8) or an ovomucoid column[6], though avidin has the opposite charge. As for chiral selectivity, the mobile phase pH did not alter the separation factor, α, of acidic solutes, and this phenomenon also accords with the result obtained with Enantiopak, but it must be emphasized that profen enantiomers were better resolved by an avidin-conjugated column than by an acidic protein column.

The amount of avidin immobilized on the supports affected the retention and separation of solutes (Tables I and II). The decreased amount of avidin (the column

TABLE II

EFFECTS OF MOBILE PHASE pH ON THE RETENTION AND SEPARATION OF RACEMIC COMPOUNDS

Mobile phase: 20 m*M* potassium phosphate. Column: avidin bound to LiChrosorb NH_2 (120 mg/g), 150 mm × 4.6 mm I.D.

Compound	*pH 5.5*		*pH 6.0*		*pH 6.5*		*pH 7.0*	
	k'_1	α	k'_1	α	k'_1	α	k'_1	α
Ibuprofen	14.1	1.28	7.64	1.38	4.46	1.53	2.7	1.53
Flurbiprofen	21.2	1.32	12.5	1.29	6.96	1.39	4.78	1.37
Ketoprofen							4.74	2.34
Chlorprenaline	0	1	0	1	0.34	1	0.38	1
Chlorpheniramine					2.30	1	3.49	1
Oxprenolol	0	1	0.37	1	0.45	1	0.69	1
Pindolol	0	1	0.23	1	0.36	1	0.49	1
Atropine	0	1	0.19	1	0.61	1	0.95	1

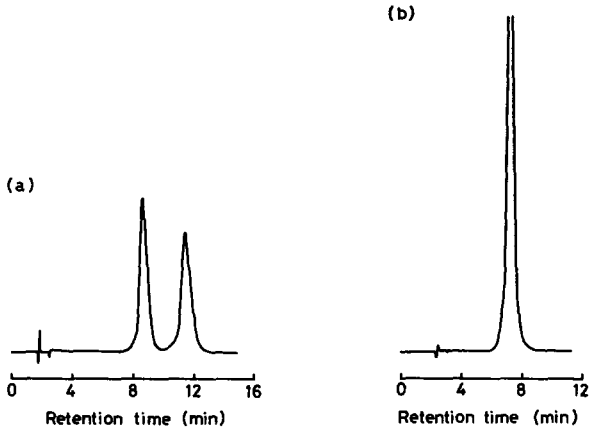

Fig. 2. Chromatogram of ketoprofen on an avidin-conjugated column. (a) Free avidin-conjugated column: mobile phase, 20 m*M* potassium phosphate buffer (pH 6.0); UV detection at 220 nm; column temperature, 20°C; amount of sample, 5 μg. (b) After 20 mg biotin had been passed through the column: the same amount of ketoprofen as for (a) was injected.

used in Table II bound 74% of the avidin that bound to the column used in Table I) caused 17% and 14% decreases in α, for ibuprofen and flurbiprofen, respectively. On the other hand, the capacity factors of these solutes obtained from a column conjugated with 120 mg/g avidin were 30% of that from the column conjugated with 163 mg/g of avidin, which may indicate that avidin has more than two sites in one molecule for interaction with solutes. Though Schill *et al.*[8] reported a plate height, *H*,

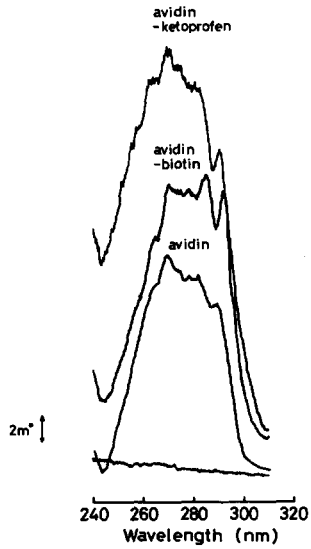

Fig. 3. Circular dichroism of avidin, avidin–biotin complex and avidin–ketoprofen complex. The dialyzed solutions obtained in experiments for DSC analysis were diluted in water to 1 mg/ml. Cell length: 0.5 cm. $2m^\circ = 2^\circ \cdot 10^{-3}$.

of 0.1 mm for carboxylic acids using Enantiopak, an avidin-conjugated column showed $H = 0.3$ mm for profens. The best way to improve the resolution is to increase the α value of protein-immobilized columns, because these columns are limited in relation to increasing the theoretical number of plates. Though α increases in proportion to the amount of protein immobilized, this operation causes increased retention. It is important to determine the appropriate amount of protein which results in high efficiency.

The effect of biotion on this column is shown in Fig. 2. A biotin-bound avidin-conjugated column did not exhibit chiral recognition of profens. This was also confirmed by conjugating a biotin–avidin complex with silica gel. A column packed with this silica gel showed a chromatogram of ketoprofen similar to that seen in Fig. 2b, and the chiral recognition effect of these columns could not be restored by washing with 50% 2-propanol or 0.5 *M* sodium chloride. The biotin–avidin binding could be separated at 132°C[9], but this temperature is not practical for protein-conjugated silica gel.

We attempted spectroscopic and thermodynamic studies to elucidate the effect of biotin on the chiral recognition ability of avidin. The molecule of avidin contains no α-helix[2], as we have confirmed by examination of its CD spectrum. The effects of biotin and ketoprofen are recognized around 280 nm (Fig. 3), which reflects a π–π transition of aromatic amino acid residues in proteins. Biotin altered the shape of the spectrum, while ketoprofen affected its magnitude. We consider that biotin modifies the structure of avidin, whereas ketoprofen does not, forming a π–π bond with avidin.

The DSC study yielded similar results. Fig. 4 shows the changes in the thermal absorption temperature of avidin caused by biotin and ketoprofen. Biotin-bound avidin has no transition temperature under 100°C, while biotin-free avidin is

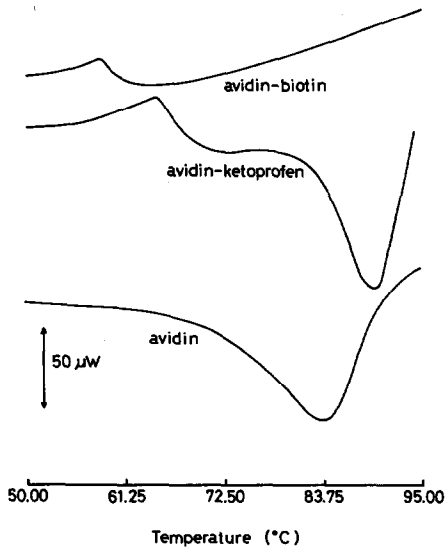

Fig. 4. Differential thermal analysis of avidin, avidin–biotin complex and avidin–ketoprofen complex. Details as in the Experimental section.

denaturated at 85°C. The ketoprofen–avidin complex showed a higher absorption temperature than did free avidin, but this temperature change may not reflect a structural change in the protein, such as is seen in the biotin–avidin complex. The result of integration of the DSC curve for the ketoprofen–avidin complex between 70 and 95°C is half of that for free avidin. This thermal stability may be related to the absence of chiral selectivity, and also to the decrease in capacity factor for ketoprofen-complexed avidin.

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