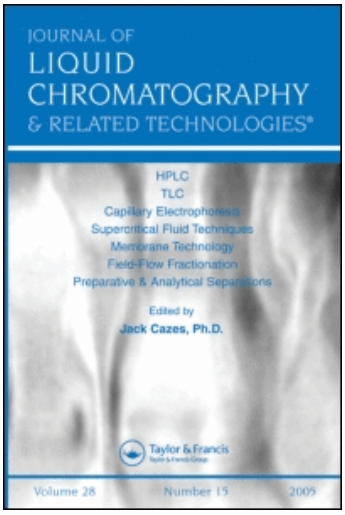

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Investigation of Retention Behavior for Racemate Drugs on Avidin- and Modified Avidin-Column

Y. Oda[a]; N. Mano[a]; N. Asakawa[a]; Y. Yoshida[a]; T. Sato[a]
[a] Tsukuba Research Laboratories, Eisai Co. Ltd., Ibaraki, Japan

To cite this Article Oda, Y. , Mano, N. , Asakawa, N. , Yoshida, Y. and Sato, T.(1994) 'Investigation of Retention Behavior for Racemate Drugs on Avidin- and Modified Avidin-Column', Journal of Liquid Chromatography & Related Technologies, 17: 16, 3393 — 3409
To link to this Article: DOI: 10.1080/10826079408013520
URL: http://dx.doi.org/10.1080/10826079408013520

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(16), 3393-3409 (1994)

INVESTIGATION OF RETENTION BEHAVIOR FOR RACEMATE DRUGS ON AVIDIN- AND MODIFIED AVIDIN-COLUMN

Y. ODA*, N. MANO, N. ASAKAWA, Y. YOSHIDA, AND T. SATO
Eisai Co. Ltd.
Tsukuba Research Laboratories
Tokodai 5-1-3
Tsukuba, Ibaraki 300-26, Japan

ABSTRACT

The binding properties of a native avidin were investigated by recording the fluorescence spectra of the native avidin, and by measuring the avidin-drug binding and the hydrophobicty of drugs. Some emission intensities were diminished by mixing avidin and drugs solutions compared with the native avidin solution. Also, the Scatchard plot analysis were performed to evaluate the avidin-drug binding. Then, modified avidin columns were prepared by acylation of amino groups and carboxyl groups to examine the retention and enantioselective properties of drugs. As a result, these functional groups contributed to chiral recognition of the avidin column for some drugs. Based on the results in this study, the avidin column have multiple binding sites for chiral separation.

INTRODUCTION

High-performance liquid chromatography (HPLC) has been usually considered as a tool for the evaluation of drug enantiomers, and many chiral stationary phases (CSPs) have been developed. While proteins are complex and high molecular weight polymers composed of L-amino acids, therefore

some protein- drug interactions can reflect the enantioselectivity of the drug. Protein-conjugated CSPs such as the albumin [1-3], α_1-acid glycoprotein [4], ovomucoid [5] and flavoprotein [6] have been applicable to the enantiomeric separation in a wide range of drugs. These columns are used under reversed-phase chromatographic conditions, which are suitable to analyze drug enantiomers in plasma [7]. We have previously developed an avidin-conjugated column which can allow to separate some drug enantiomers in plasma with direct injection method [8-10]. The retention and enantioselectivity on the avidin column were greatly affected by changing the mobile phase composition [11]. However, a native avidin has been little studied for the binding of drugs. In order to characterize the binding sites of the avidin column, some studies were performed.

EXPERIMENTAL

Apparatus

A chromatograph was set up with a Shimadzu model LC-9A system {two LC-9A pumps, a SIL-6B auto injector, a SCL-6B system controller, a SPD-6A UV monitor (254nm) and a C-R4AX integrating recorder}. Fluorescence spectra were measured with a Hitachi model F-2000 fluorescence spectrophotometer (excitation wave length 280 nm). A Shimadzu model CPR-005 (3000 rpm) as a centrifuge was used.

Materials

Native avidin was purified from chicken egg white according to the previously described method [12]. (±)-Trihexyphenidyl hydrochloride (TP), (±)-Cloperastine hydrochloride (CP) and solvents of HPLC grade were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). (±)-2-Phenylpropionic acid (PP) from Aldrich Chem. Co. (Milwaukee, WI, USA), (±)-Ibuprofen (IP) from Sigma chemical Co. (St. Louis, MO, USA) and (±)-chlormezanone (CM) from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) were used. Dissuccinimidyl suberate, sulfosuccinimidyl acetate and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride were purchased from Pierce (Rockford, IL, USA). All other chemicals were of analytical reagent grade. Centrifree micropartition systems (MPS-3) for ultrafiltration were

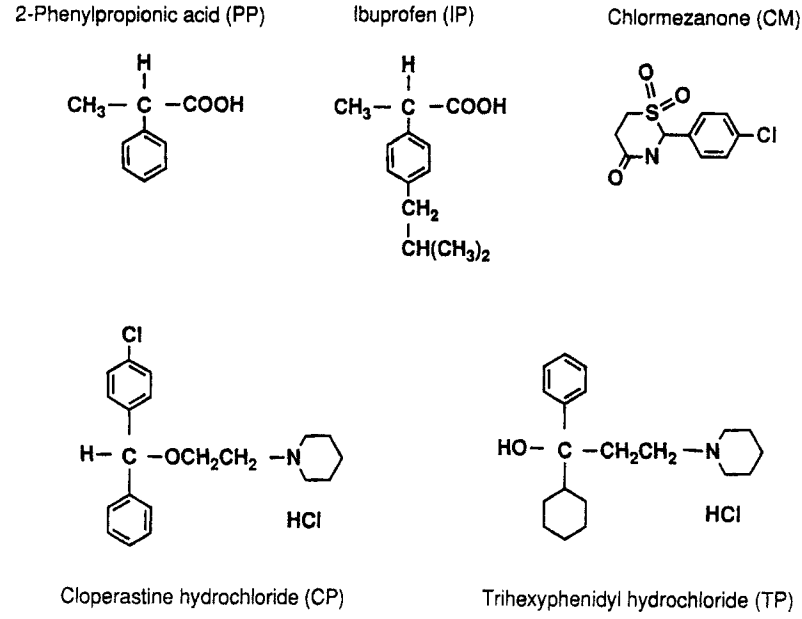

Fig.1 Structures of 2-phenylpropionic acid, ibuprofen, trihexyphenidyl hydrochloride, cloperastine hydrochloride and chlormezanone.

obtained from Amicon Inc. (Beverly, MA, USA). An ODS column used was Inertsil ODS-2 (5 μm, 15cm x 4.6mm i.d.) made by GL sciences Inc. (Tokyo). The structures of PP, IP, CM, CP and TP were shown in Fig.1.

Avidin column preparation

Nucleosil NH_2 (5μm, 2g) and dissuccimidyl suberate (2g) were allowed to react overnight in 30 ml of acetonitorile at room temperature. After the activated silica gel had been washed with 60 ml of acetonitorile and then suspended in 30 ml of 0.1 M sodium hydrogen carbonate aqueous solution with 1g of native avidin, this mixture was stirred for 2h at room temperature . Avidin-conjugated silica gel was packed into 15cm x 4.6mm i.d. stainless-steel column by conventional high-pressure slurry-packing procedure with 2-propanol - water mixture solvent (1/2 = v/v) at packing-pressure ca. 250kg / cm^2.

Chiral separation with avidin column

Samples were prepared by dissolving known amounts (0.08~40 mg / ml) of a drug in water - methanol and then a 25 μl portion of the sample was injected. Mobile phase was 20 mM potassium phosphate buffer - methanol mixed solution, and the flow rate was 1.0 ml / min. All operations were carried out at room temperature.

Measurement of free drug concentration

The binding of a drug to native avidin was determined as follows. A 1 ml portion of 1 μM native avidin or without avidin solution in the 20 mM potassium phosphate buffer (pH 7) was mixed with a 1 ml portion of drug solution in the 20 mM potassium phosphate buffer (pH 7) containing less than 20 % methanol. The drug concentration was varied to make the binding ratio, bound drug concentration / native avidin concentration, to be in a range 0.6 to 3.6. The 1 ml portion of the mixed solution was subjected to the ultrafiltration using MPS-3 at room temperature. After the centrifugation for 10 min, the drug concentration in the filtrate was determined by HPLC using an ODS column. The mobile phase was methanol - water - perchloric acid mixed solution and all operations were performed at ambient temperature with the flow rate of the mobile phase being 1.0 ml /min. The mobile phase composition was selected appropriately.

Fluorescence spectra of avidin and avidin - drug complexes

A native avidin solution (5 μg / ml) was prepared with 20 mM potassium phosphate buffer (pH 7) and a drug was dissolved at 35 ng / ml in 20 mM potassium phosphate buffer (pH 7) / methanol (5 / 1). The avidin - drug complex solution used was the mixture of the same volume of the native avidin solution and a drug solution, respectively. Fluorescence spectrum was measured by scanning 280 - 450 nm as emission wave length.

Measurement of drug hydrophobicity

The hydrophobicity of each drug is expressed as their calculated r-value on ODS column [13]. The mobile phase was the mixture of methanol - 10 mM potassium phosphate buffer (pH 7). The methanol concentration was varied from 25~80 % at an interval of 4 %. The flow rate was at 1.0 ml / min. The r-value was calculated by the least squares method as

$$\log k' = r \log (1 / [\text{methanol}]) + q$$

where k' is the capacity factor of a drug on ODS column.

RESULTS AND DISCUSSIONS

Loading capacity of avidin column and native avidin - drug binding

Loading capacity of the chiral separation column becomes an important consideration in scaling up separation procedures. In general, the chiral specific binding sites on protein-conjugated column are limited, therefore loading capacity is low. In addition, there are some nonspecific interactions between the chiral drugs and protein-conjugated stationary phases, which leads to the low column efficiency. Fig. 2 shows the representative chromatograms on the avidin column obtained by varying injection amounts. IP and CM were not optically resolved, when 20 ~ 30 μg of IP or CM was injected on the avidin column. While PP, CP and TP racemates could be separated up to 0.2 ~ 1 mg injection amounts.

The differences of the protein binding between drug enantiomers exert significant effect on the enantiomeric separation with an immobilized protein column [14]. Therefore, we examined the binding constants (K) and the binding sites (n) on a native avidin molecule. These results are shown in Table 1. The K value of these five drugs was in the following order: IP > CP > TP > CM > PP. The number of binding sites of PP was the minimum (n = 4.2) and that of IP was the maximum (n = 5.6) of these drugs. Although This result seemed to mean that the loading capacity was not so much different for each drug on the avidin column, PP was allowed to inject almost 1mg on the avidin column and 20 μg of IP was over loaded. Of course, although degree of overloading may rather be correlated to the retention, on the other hand, our studies may mean that the avidin column has nonspecific interactions for chiral discrimination with IP and CM, which leads to the low chromatographic efficiency [15].

Next we examined the effect of drug competitions for the binding sites on the native avidin by measuring free drug concentration of a drug in the presence of an other drug in avidin solution (data not shown). All drugs studied compete with the same binding sites on the native avidin ,or bind at different sites which can affect each other by inducing conformational changes in the native avidin, because the displacement phenomena were observed by competitions studies.

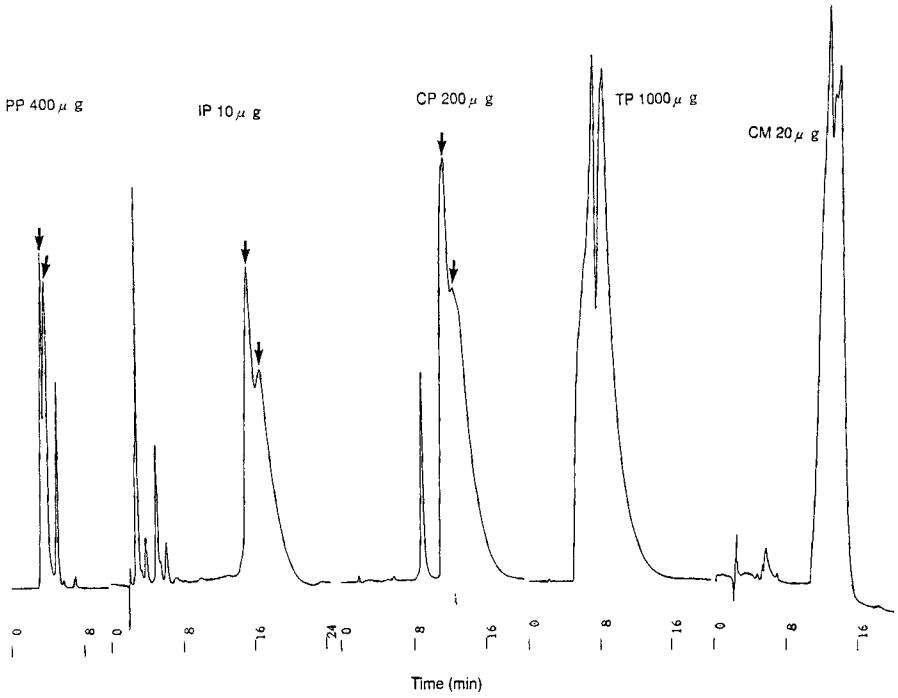

Fig.2 Representative chromatograms on the avidin column.
amounts injected, PP: 400 μg, IP: 10 μg, CP: 200 μg, TP: 1000 μg, CM: 20 μg
HPLC conditions, PP and CM: 20 mM phosphate buffer (pH 7) / methanol = 90 / 10, IP, CP and TP: 20 mM phosphate buffer (pH 7) / methanol = 70 / 30. See text for other conditions.

Table 1

Scatchard plot analysis of avidin - drug binding

	K	n	R	N
PP-avidin	0.55	4.2	-0.94	6
IP-avidin	1.49	5.6	-0.91	6
CP-avidin	1.20	5.1	-0.94	7
TP-avidin	0.93	5.2	-0.97	7
CM-avidin	0.61	5.0	-0.90	6

K: binding constant, n: binding site number

R: correlation coefficient, N: measurement number

See text for the conditions.

Fluorescence spectra of tryptophan residue on avidin

A native avidin molecule has 16 tryptophan residues [16], therefore fluorescence spectrum of the native avidin can be reflected differences of the native avidin conformation [17]. Fig. 3 shows one of the fluorescence spectra of the native avidin, drug and the native avidin-drug complex. The fluorescence intensity of avidin was weakened by the formation of avidin-PP, avidin-CM or avidin-TP complexes, although each drug has no fluorescence. IP and CP had no effects on fluorescence intensity of avidin. Therefore, some drugs can have some interactions with tryptophan residues on the surface of the native avidin molecule. It is known that the avidin has a very strong interaction with biotin which binds to tryptophan residues of the avidin [18] and the stereoselectivity of some drugs disappeared by the formation of avidin-biotin complex [11].

Effect of the pH on avidin column

Since the native avidin has many ionic amino acid residues [16], the properties of avidin column are changed by the pH of mobile phase. Table 2 shows the effect of the mobile phase on the avidin column. The k' values of acidic drugs (PP, IP) increased in the low-pH region, and the opposite results were obtained for basic drugs (CP, TP). However, there are different influences for the tryptophan residues of the native avidin between PP and IP, or CP and TP. While neutral drug, CM, showed the k' values obtained at pH 5 and pH 7 were almost 40% higher than that at pH 6 on the avidin column. The chiral separation of both acidic and basic drugs were not so affected the pH of the mobile phase, but as for neutral drug, CM enantiomers were not separated at pH 6.

Effect of zinc additive on avidin column

It is known that the metal ion such as zinc (II) is often an important structural components in some proteins to organize protein conformation [19]. Therefore, the effect of zinc additive on the avidin column was examined (Table 3). A tris buffer was used as a mobile phase instead of a phosphate buffer, because a phosphate is subject to form a the metal-phosphate chelate. The tris-buffer gave strong retentions to acidic drugs and weak retentions to basic drugs on the avidin column. Enantioselectivity of PP, CM and CP were lost by the change of the phosphate buffer to the tris buffer. But addition of zinc ions in the tris buffer was allowed to recover the enantioselectivity of CM and

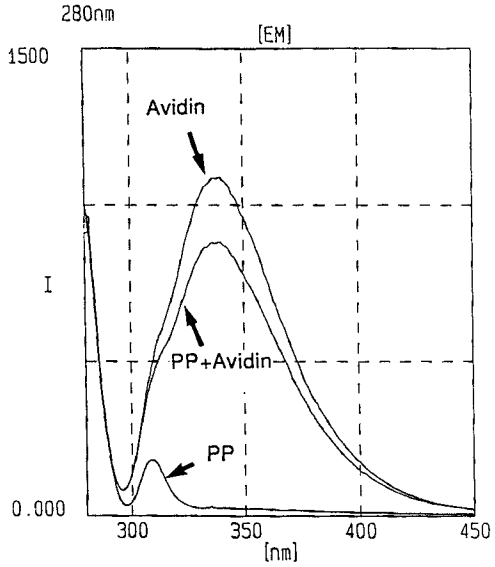

Fig. 3 Emission spectra of native avidin, PP and avidin-PP complex. See text for the measurement conditions.

Table 2

Effect of Mobile Phase PH on Avidin Column

	PP[1]		CM[1]		TP[2]		CP[2]		IP[2]	
	k_1	α	k_1	α	k_1	α	k_1	α	k_1	α
pH 7	0.69	1.13	5.54	1.09	4.77	1.56	9.68	1.13	7.13	1.13
pH 6	2.44	1.22	4.07	1.00	2.35	1.60	4.57	1.15	11.2	1.14
pH 5	5.36	1.10	5.59	1.13	2.05	1.51	3.02	1.14	15.3	1.14

HPLC condition: avidin column (150 mm X 4.6 mm), Flow Rate (1.0 ml / min), UV254nm

Mobile Phase, 1) 20 mM Phosphate Buffer / Methanol = 10 / 90

2) 20 mM Phosphate Buffer / Methanol = 30 / 70

* k_1 means the capacity factor of the first eluted enantiomer.

** α means the separation factor ($\alpha = k_2 / k_1$).

Table 3

Effect of Zinc Additive

	PP[1)]		CM[1)]		TP[2)]		CP[2)]		IP[2)]	
	k_1	α	k_1	α	k_1	α	k_1	α	k_1	α
0 mM	1.43	1.00	5.69	1.00	2.05	1.48	4.34	1.00	9.95	1.13
5mM $ZnCl_2$	2.51	1.00	5.16	1.17	1.14	1.61	2.20	1.15	10.9	1.00

HPLC condition: avidin column (150 mm X 4.6 mm), Flow Rate (1.0 ml / min), UV254nm

1), Mobile Phase, 50 mM Tris buffer (pH 7) / Methanol = 90 / 10

2), Mobile Phase, 50 mM Tris buffer (pH 7) / Methanol = 70 / 30

* k_1 means the capacity factor of the first eluted enantiomer.

** α means the separation factor ($\alpha = k_2 / k_1$).

CP and to lose that of IP. Thus, adding zinc ions to the mobile phase may be sometimes an useful procedure for chiral separation of drug enantiomers. But these experiments gave the avidin column strong damages, so retention properties had not recovered to initial states with the using phosphate buffer. The tris buffer is a primary amine which is bound to the carboxyl groups of avidin column, and / or to other high affinity sites on the silica matrix. Thus, these results mean that both the retention and enantioselectivity are affected by small residues of tris(hydroxymethyl) aminomethane and metal ions on the avidin column, and then it is difficult to remove completely the trace level of tris buffer and metal ions from the avidin column.

Hydrophobicity of drug

The r-value is well correlated to the hydrophobic properties of the solute such as non-polar surface area and the 1-octanol / water partition coefficient [13]. Therefore, the hydrophobicity of each drug was expressed as r-value given in Table 6. The hydrophobicity order was CP > TP > IP > CM > PP. This order is similar to that of the binding constant value (K) of drug-avidin: IP > CP > TP > CM > PP (Table 1) except IP. The native avidin has a moderate hydrophobicity as it contains many hydrophobic amino acid residues [16]. Lindner et al. reported that a correlation was found between retention

behaviour and hydrophobicity for the cellulase CSP [20]. In our study, the hydrophobic interaction plays an important role in the binding drugs on the native avidin. Then, because the native avidin is a basic protein (pI: 10.5) [21], the avidin column is expected to retain electrostatically acidic drugs (such as IP) much strongly than basic drugs. The elution order from the avidin column was CP > IP > TP > CM > PP at pH 7 of the mobile phase (Table 2). A correlation was found between the retention behaviour and the hydrophobicity of drugs (except IP), therefore the hydrophobic interaction is important for retention of drugs on the avidin colunm, although the electrostatic interaction is also necessary to the enantioselectivity.

Acylation of amino group on avidin column

Acylation of a primary amino group (such as a lysine residue) was performed *in situ* by recycling sulfosuccinimidyl acetate (100 mg) in 20 mM sodium hydrogen carbonate buffer / acetonitrile for 5h [22] at room temperature (amino acylate avidin column). Fig. 4 shows the chromatograms obtained with this amino acylate avidin column. Comparison of the avidin column, the k' values of acidic drugs (PP, IP) markedly decreased and enantioselectivity disappeared. As for basic drugs (CP, TP), the k' values increased and enantioselectivity improved. Table 4 shows the effect of mobile phase pH on the amino acylate avidin column. The retention properties for varying pH were almost the same as those on the avidin column. Both the k' value and enantioselectivity of neutral drug (CM) were not so affected by acylation of amino groups.

Acylation of carboxyl group on avidin column

The avidin column has several carboxyl groups, such as aspartic acid residues and glutamic acid residues [15]. A carboxyl acylate avidin column was obtained by recycling 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (200mg) and methylamine (200mg) in 20 mM potassium dihydrogen phosphate buffer (pH 4.8) for 12 h *in situ* at room temperature [23]. Fig. 5 shows the chromatograms obtained with the carboxyl acylate avidin column. The k' values of acidic drugs increased compared with those on the avidin column, while those of basic drugs were reversed. The enantioselectivity of CP and IP disappeared and that of TP became very weak. Table 5 shows the effect of mobile phase pH on the carboxyl acylate avidin column. The

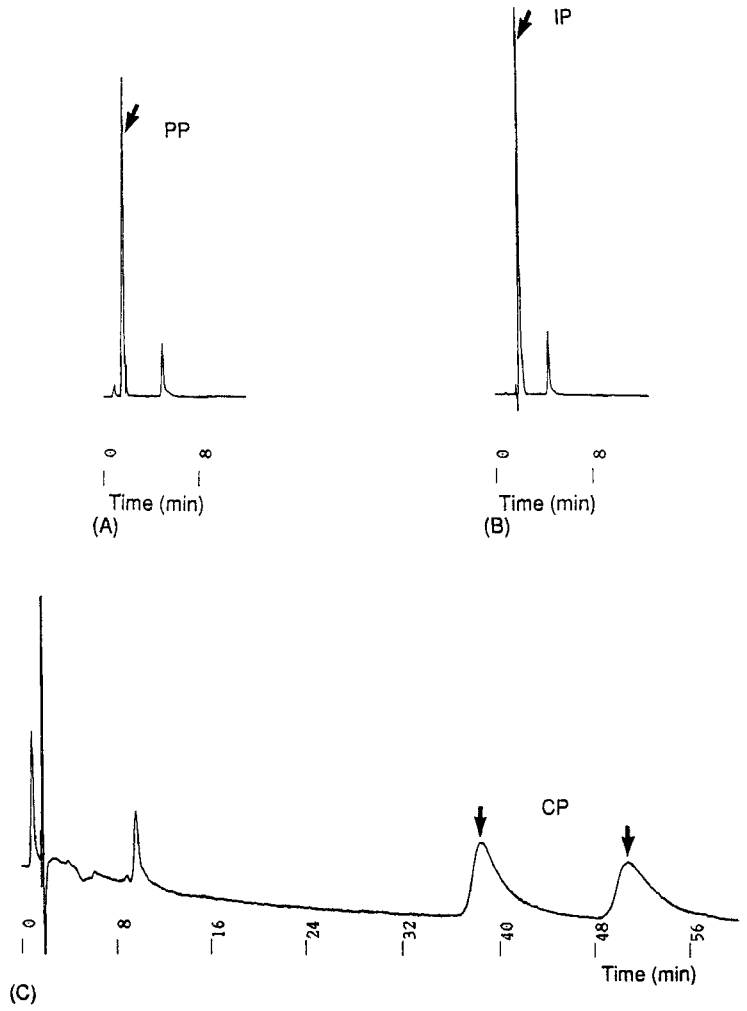

Fig. 4 Representative chromatograms on the amino acylate avidin column. (A) PP, (B) IP, (C) CP, (D) TP and (E) CM
HPLC conditions, (A) and (E): 20 mM phosphate buffer (pH 7) / methanol = 90 / 10, (B), (C) and (D): 20 mM phosphate buffer (pH 7) / methanol = 70 / 30. See text for other conditions.

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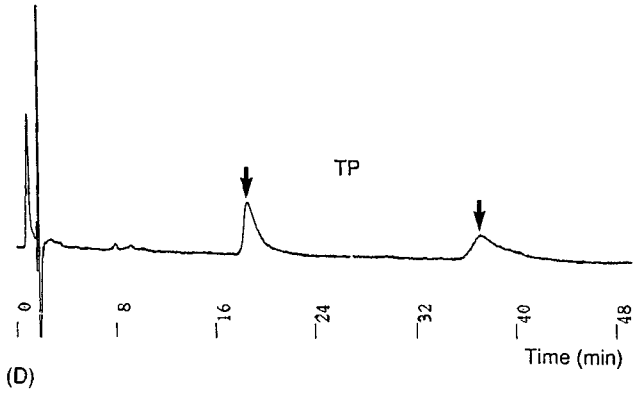

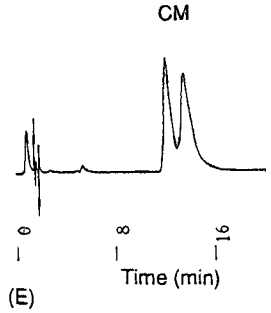

Fig. 4 (Continued)

Table 4

Effect of Mobile Phase PH on Amino Acylate Avidin Column

	PP[1]		CM[1]		TP[2]		CP[2]		IP[2]	
	k_1	α	k_1	α	k_1	α	k_1	α	k_1	α
pH 7	0.09	1.00	6.67	1.14	10.6	2.09	23.1	1.33	0.31	1.00
pH 6	0.30	1.00	5.10	1.00	7.58	2.45	18.0	1.45	1.82	1.00
pH 5	1.41	1.00	6.74	1.02	4.07	2.41	7.95	1.44	3.75	1.00

HPLC condition: amino methylate avidin column (150 mm X 4.6 mm)

Flow Rate (1.0 ml / min), UV254nm

Mobile Phase, 1) 20 mM Phosphate Buffer / Methanol = 10 / 90

2) 20 mM Phosphate Buffer / Methanol = 30 / 70

* k_1 means the capacity factor of the first eluted enantiomer.

** α means the separation factor ($\alpha = k_2 / k_1$).

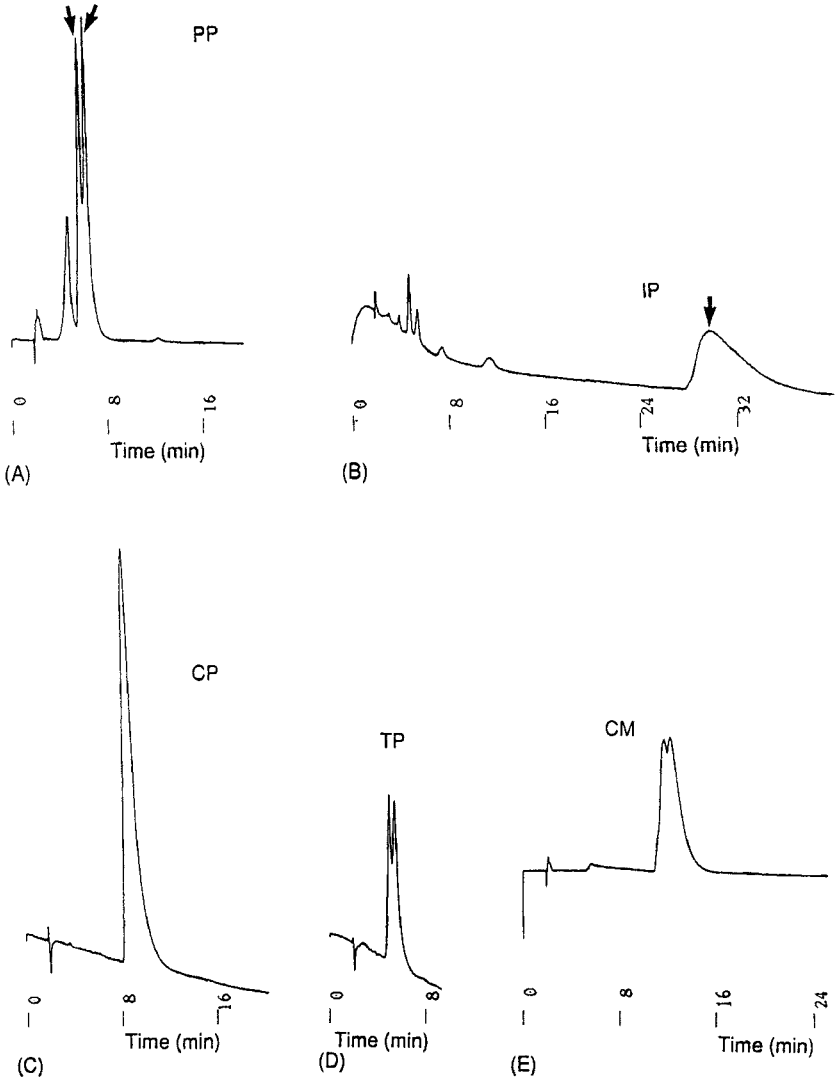

Fig. 5 Representative chromatograms on the carboxyl acylate avidin column. (A) PP, (B) IP, (C) CP, (D) TP and (E) CM
HPLC conditions, (A) and (E): 20 mM phosphate buffer (pH 7) / methanol = 90 / 10, (B), (C) and (D): 20 mM phosphate buffer (pH 7) / methanol = 70 / 30. See text for other conditions.

Table 5

Effect of Mobile Phase PH on Carboxy Acylate Avidin Column

	PP[1)]		CM[1)]		TP[2)]		CP[2)]		IP[2)]	
	k_1	α	k_1	α	k_1	α	k_1	α	k_1	α
pH 7	2.13	1.13	5.20	1.05	1.61	1.15	3.62	1.00	14.7	1.00
pH 6	5.21	1.11	4.95	1.12	0.40	1.34	1.09	1.00	30.0	1.00
pH 5	8.71	1.10	4.73	1.15	0.06	1.00	0.16	1.00	45.8	1.00

HPLC condition: carboxy methylate avidin column (150 mm X 4.6 mm)

Flow Rate (1.0 ml / min), UV254nm

Mobile Phase, 1) 20 mM Phosphate Buffer / Methanol = 10 / 90

2) 20 mM Phosphate Buffer / Methanol = 30 / 70

* k_1 means the capacity factor of the first eluted enantiomer.

** α means the separation factor ($\alpha = k_2 / k_1$).

Table 6

r-values of drugs on ODS column

	r	q
PP	1.94	3.29
IP	7.48	13.8
CP	8.17	16.2
TP	7.74	15.5
CM	4.12	7.29

$\log k' = r \log (1 / [MeOH]) + q$

tendency of retention for acidic drugs or basic drugs were the similarity that on the avidin column. However, CM enantiomers were separated at pH 6 of mobile phase, which was a different result observed on the avidin column and the acylate amino avidin column.

We modified these functional groups on the avidin column to examine their contribution to the chiral separation. Whereas, the specific modification of

the native avidin may affect a remote binding site by conformational changes, because the native avidin is flexible and its tertiary structure is not fixed. While, the avidin conformation seems to be stabilized (not completely) by immobilization on a silica support with a succinimide linker compared with not immobilized avidin [11]. Therefore, acylations of amino groups or carboxyl groups of the avidin column were performed *in situ* to minimize an alteration of tertiary structure caused by these modifications. Also, Haginaka et al. reported that the blocking of primary amino groups on an ovomucoid (OVM) column gave higher or almost equal enantioselectivities for basic drugs compared with the unmodified OVM column [24, 25]. These results mean that the modifications of amino groups of avidin play a key role for the chiral separation of acidic drugs, and the accessibility of basic drugs to the chiral recognition sites may be hindered by the electrostatic repulsion between drugs and the primary amino groups. While the retention of acidic drugs became strong and that of basic drugs became weak by the acylation of carboxyl groups of the avidin column, so these results were vice versa those obtained with the amino acylate avidin column. There are two possible explanations, the first is that the Coulombic interactions between the charged drugs and the charged avidin molecules exist in the retention mechanism on the avidin column, and the second is that the secondary structure of the avidin is affected by the modifications from ionic groups to uncharged groups on the avidin column despite the fact the avidin is immobilized. As for the chiral separation, the carboxyl groups of the avidin column are necessary to separate IP enantiomers and TP enantiomers, although those do not participate the enantioselectivity of PP.

The k' value and the enantioselectivity of CM were little affected by the acylation of amino and carboxyl groups of the avidin column. However, CM enantiomers were not separated at pH 6 of the mobile phase buffer on the avidin column (Table 2), therefore histidine residues (imidazolium group: pK = 6) of avidin may contribute to the enantioselectivity of CM.

CONCLUSION

Changes in the properties of the mobile phase (pH, metal ion modifier) and acylation of amino and carboxyl groups on avidin column can alter the binding ability for drug enantiomers on the avidin column. The change of the

microenvironment on the avidin column altered the ability to retain and discriminate drug enantiomers [26]. Our results also suggest that the retaining phase contains multiple chiral and non-chiral binding sites on the avidin column. The binding of the drugs on the avidin column may be mainly due to hydrophobic interactions. Then, the avidin column has many different binding sites giving the possibility of enantioselective interactions with a broad range of drugs. Mechanistic studies for chiral recognition on the avidin column are of interest in prediction of the optimum HPLC conditions and the increments of the column efficiency.

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Received: February 16, 1994
Accepted: March 2, 1994