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Journal of Chromatography A, 869 (2000) 151–157

JOURNAL OF CHROMATOGRAPHY A

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Characterization of an avidin-bonded column for direct injection in reversed-phase high-performance liquid chromatography

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Abstract

A denatured avidin-bonded column was suitable for use in the reversed-phase HPLC and complete recovery of serum protein over a wide range of pH. Retention property of the denatured avidin-bonded column was very nearly to a non-denatured avidin-bonded column already reported and, however, showed very high stability to organic solvent. A performance of the denatured avidin-bonded column was maintained even if 500 continuous injections of human serum (total 10 ml).

Keywords: Stationary phases, LC; Direct injection; Avidin; Proteins

1. Introduction

In recent years, some stationary phases consisted of biopolymer such as proteins were reported, almost of which were applied to chiral resolution of racemic compound [1–4]. In addition, an application of these packing materials into the direct injection analysis of biological samples was reported by Yoshida et al. who prepared a protein-coated ODS silica column by precipitating denatured bovine serum albumin and plasma proteins on a C_{18} silica [5]. Although plasma protein used as a sample matrix was well recovered from this column, there were still some problems with column efficiency and column lifetime.

A stationary phase consisting of avidin, glycoprotein in egg white, has been applied to chiral separation [6]. The column exhibited good enantio-selectivity in analysis of profens. Also, Oda et al. investigated a retention property of the avidin-modified column and found that injected human serum was excluded by the hydrophilic and bulky avidin surface phase without retention [7]. The column was applied to analysis of biological samples by direct injection [8]; however, there was a limitation in elution conditions. Organic solvent in the eluent gradually leads to irreversible change of the structure and extinction of the chiral recognition, although, the column still has a performance to exclude injected human serum. In the paper, chromatographic properties of a denatured avidin-bonded column were investigated. We found that a high stability of the column can be obtained by the purposeful denaturalization of avidin and the denatured avidin-bonded column can tolerate a high concentration of organic solvent. A column efficiency and lifetime are encouraging in comparison with other published non-denatured avidin-bonded column.

2. Experimental

2.1. Chemicals

Coomassie Brilliant Blue G-250 (CBB) was ob-

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0021-9673/00/$ – see front matter
PII: S0021-9673(99)00878-X

tained from Sigma Chemical (St. Louis, MO, USA) and human serum used was purchased from Nissui (Tokyo, Japan). Acetonitrile used for eluent was of HPLC grade from Kishida chemicals (Osaka, Japan). Water was purified with a Milli-Q system (Nihon Millipore Kogyo, Tokyo, Japan).

2.2. Chromatography

HPLC system used was LC-600 series (GL Sciences, Tokyo, Japan) consisted of PU-610 pumps, UV-620 UV–Vis detector, CO-630 column oven and AS-640 auto sample injector. Material of all tubing used in this system was PEEK (0.013 and 0.025 cm I.D.)

The denatured avidin-bonded column used was a commercially available column, Bioptic AV-2 (15× 0.46 cm I.D., GL Sciences, Tokyo, Japan), in which avidin was denatured by organic solvent after bonding it onto silica gel support (particle diameter 5 μm, surface area 450 cm^2/g, pore size 100 Å). A biotin binding activity was tested in advance under condition of 50 m*M* phosphate, 0.1 *M* sodium chloride (pH 7.0) and a loss of the activity owing to denaturing the avidin was confirmed.

Chlorpromazine, perphenazine, thioridazine, ethacrynic acid, diphenhydramine, lidocaine, *p*-hydroxybenzoic acid, *p*-hydroxybenzoic acid methyl, ethyl and propyl ester, benzylamine and carbamazepine were purchased from Sigma Chemical (St. Louis, MO, USA) and used as test solutes. All concentrations of the analytes without referring were 100 μg/ml.

Conditions of mobile phase are given in the figure captions. Void time t_o to calculate k' was measured with uracil (100 μg/ml) at the eluent of 50 m*M* phosphate (pH 2.5)–acetonitrile (90:10, w/w). Human serum samples were passed through a 0.45 μm membrane filter before being injected into the column. For the application of direct injection analysis of serum samples, the concentration of organic solvent in eluent was less than 20% (w/w) to avoid precipitating of serum proteins.

2.3. Recovery of serum protein from the denatured avidin-bonded column

CBB dye solution was prepared by dissolving 100 m mol of CBB in 50 ml of ethanol, adding 100 ml of phosphoric acid, and diluting the mixture to 1 L with purified water [9]. The recovery of human serum protein from the column was examined as follows [10]. A 20 μl of serum was injected the column and the eluate was collected for 10 min. A 0.5 ml portion of the eluate was mixed with 5 ml CBB reagent and after 3 min the absorbance was measured at 595 nm. Recovery was calculated from the absorbance ratio with and without the column.

3. Results and discussion

3.1. Recovery of human serum in a first injection

When using a high-performance column, it is very important not to inject the total sample until an acceptable recovery has been established. Therefore, the recovery of human serum from the column should establish in a first injection. Table 1 shows a result of measuring the recovery. Although serum proteins were not recovered at a water condition, completely recovered at a phosphate buffer condition. This indicated that a ''salting-out'' efficacy on the avidin is necessary for excluding serum proteins. The low recovery at pH 6.5 was due to the hydrophobicity of avidin that increased with increasing buffer pH (isoelectric point of avidin: 9.5–10.0). At a condition of phosphate buffer–acetonitrile, human serum was completely recovered over the range of pH 2.5–6.5. Oda et al. [7] reported that recoveries of serum proteins from a non-denatured avidin-bonded column were 97, 24 and 92% at eluent pH of 2.0, 4.5 and 7.0, respectively. The good recovery of the

Table 1
Recovery of serum proteins. HPLC conditions: column size, 15×0.46 cm I.D.; flow-rate, 1.0 ml/min; column temperature, ambient

Eluent	Recovery (%)		
	Buffer pH		
	2.5	4.5	6.5
Water	0		
Phosphate buffer[a]	102	105	90
Phosphate buffer–acetonitrile[b]	100	102	100

[a] 50 m*M* phosphate buffer.
[b] Buffer–acetonitrile (95:5, w/w).

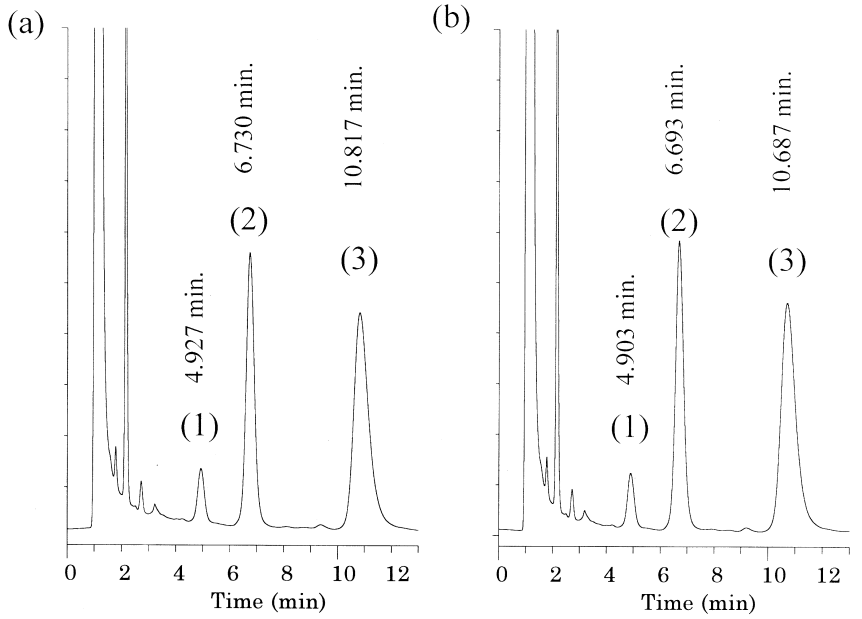

Fig. 1. Chromatograms of human serum spiked with (1) perphenazine (14 μg/ml), (2) chlorpromazine (17.5 μg/ml) and (3) thioridazine (27.5 μg/ml) on (a) before washing with methanol and (b) after washing. HPLC conditions: column size, 15×0.46 cm I.D.; eluent, 50 m*M* phosphate buffer (pH 3.0)–acetonitrile (95:5, w/w); detection, 254 nm; injection volume, 20 μl.

denatured avidin-bonded column suggested that the hydrophilicity of avidin was maintained over a wide range of chromatographic conditions because of the unchangeable conformation.

3.2. Analysis of drugs in serum by direct injections

Fig. 1(a) shows a chromatogram of direct injection analysis of human serum including chlorpromazine, perphenazine and thioridazine. After the analysis of (a), the column was washed with methanol of 100 ml. Then, chromatogram (b) was obtained with the column under same conditions. Recoveries of these drugs before and after the washing were measured and relative standard deviation (RSD) of their values are shown in Table 2. High recovery and stability were obtained with the column. Although, there was a negligibly small decrease in retention time of each drug when using the column washed with methanol; this conformed that a denatured avidin-bonded column has enough stability to organic solvent.

Table 2
Recovery and RSD of retention time and peak-area before and after washing with methanol of 100 ml

Analyte	Before washing			After washing		
	Recovery[a] (%)	RSD[b] (%)		Recovery (%)	RSD (%)	
		Retention time	Peak-area		Retention time	Peak-area
Perphenazine (14.4 μg/ml)	102.3	0.064	1.02	108.9	0.114	0.54
Chlorpromazine (17.5 μg/ml)	99.3	0.069	0.98	100.3	0.184	0.526
Thioridazine (27.5 μg/ml)	98.4	0.117	1.13	100.1	0.269	0.923

[a] The recovery was calculated from the peak-area ratio for a given concentration of analyte dissolved in human serum and water.
[b] $n=5$.

3.3. Retention properties of the denatured avidin-bonded column

3.3.1. Effect of organic solvent

Fig. 2 shows k' values of ethacrynic acid, naphthalene, imipramine and carbamazepin under conditions of different acetonitrile contents. The k' values diminished with increasing a content of acetonitrile; therefore, that is the same behavior to reversed-phase mode. A same result was also obtained in the precious paper reported by Oda with native avidin-phase [7]. Fig. 3 gives a result of log k' values plotted to acetonitrile content and the plotted are not given a straight line, whereas straight line is obtained in reversed-phase mode, therefore, this graph suggests that a retention is not only hydrophobic interaction but also any other interactions, for example ion-exchange.

3.3.2. Effect of buffer pH

Fig. 4 shows a behavior of lidocaine (pK_a=7.9), chlorpromazine (9.3), diphenhydramine (9.0) and ethacrynic acid (3.5) under condition of different pHs. Capacity factors of lidocaine, chlorpromazine and diphenhydramine increased with increasing buffer pH because of increase in both hydrophobicities of the solutes and avidin (pI=9.5–10.0).

Ethacrynic acid was strongly retained at pH 3.5. Oda reported a same phenomenon with a native avidin-bonded column and Mano et al. [3] considered that the phenomenon is owing to alteration of the conformation of avidin itself based on a dissociation of an amino acid residue. The consideration, however, is unbecoming for the denatured avidin-bonded column because uncovalently bonding to maintain the conformation is already broken with organic solvent, which lead to unchangeable conformation. Therefore, alternation of an inclusive electric charge on avidin owing to dissociation of an amino acid residue might be influential in the

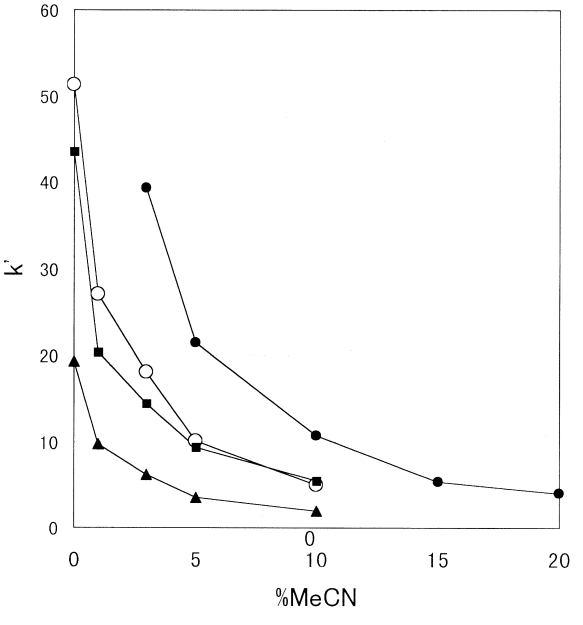

Fig. 2. Dependence of k' value on content of acetonitrile. ● ethacrynic acid, ○ carbamazepine, ■ naphthalene and ▲ imipramine. HPLC conditions: column size, 15×0.46 cm I.D.; eluent, 50 mM phosphate buffer (pH 3.0)–acetonitrile (w/w); detection, 254 nm.

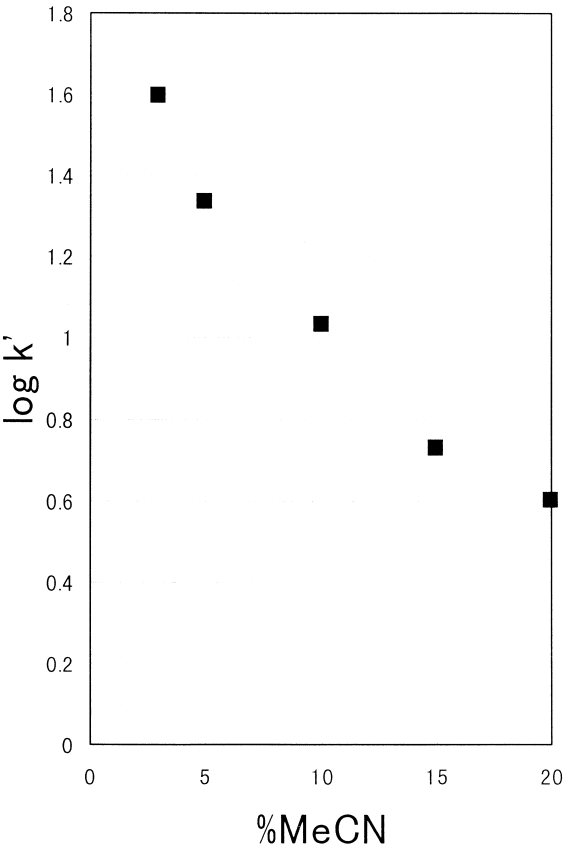

Fig. 3. Dependence of log k' value of ethacrynic acid on a content of acetonitrile. HPLC conditions are the same to Fig. 2.

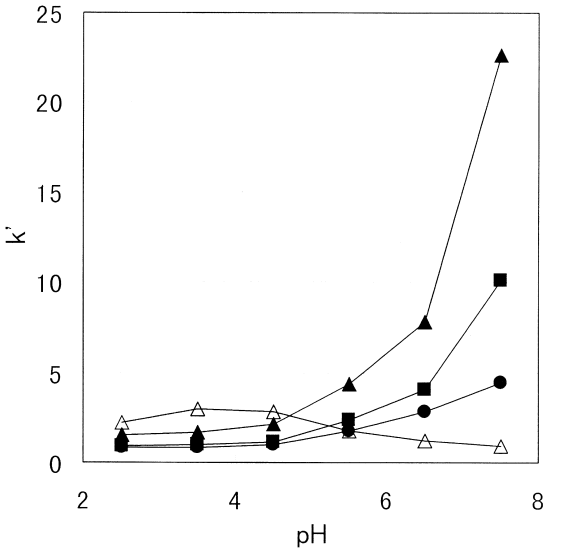

Fig. 4. Dependence of k' value on phosphate buffer pH. ▲ diphenhydramine, ■ chlorpromazine, ● lidocaine and △ ethacrynic acid. HPLC conditions: column size, 15×0.46 cm I.D.; eluent, 50 m*M* phosphate buffer–acetonitrile (95:5, w/w); detection, 254 nm.

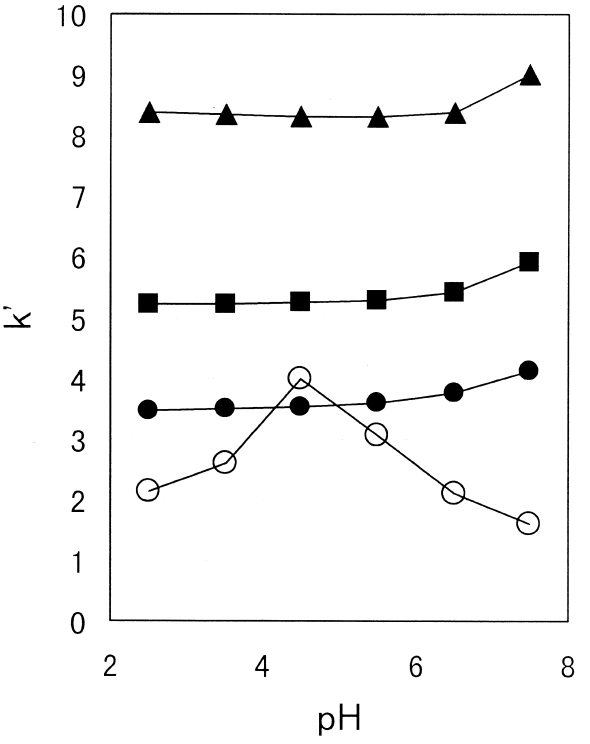

Fig. 5. Dependence of k' values on phosphate buffer pH. ○ *p*-hydroxybenzoic acid, ● methyl ester, □ ethyl ester and ▲ propyl ester. HPLC conditions: column size, 15×0.46 cm I.D.; eluent, 50 m*M* phosphate buffer–acetonitrile (98:2, w/w); detection, 254 nm.

retention behavior. Carboxylic acid group (pK_a: 4.2–4.9) from aspartic acid and glutamic acid including 5 and 7 per avidin subunit, respectively (one subunit including 128 residues) seems to be effective on the ethacrynic acid behavior under mobile phase condition of pH 3.5–4.5.

Fig. 5 shows capacity factors of *p*-hydroxybenzoic acid and their esters. The strength of retention was in order of carbon number in the esters. As buffer pH increase up, a increase in the hydrophobicity of avidin (p*I*=9.5–10.0) seems to lead capacity factors of the esters into increasing.

3.3.3. Effect of buffer concentration

Fig. 6 shows capacity factors at varying phosphate buffer concentration under no organic solvent.

A capacity factor of *p*-hydroxybenzoic acid decreased with increasing the buffer concentration in Fig. 6(a), and a capacity factor of benzylamine increased with increasing the buffer concentration in Fig. 6(b). Avidin has positive charge at an available pH range of silicagel support because isoelectric point of avidin is 9.5–10.0. Therefore, negative charge in the eluent gathers on avidin molecules depending on a buffer concentration. It seems that *p*-hydroxybenzoic acid having negative charge is retained weakly at higher concentration of phosphate buffer and benzylamine having positive charges is retained strongly owing to ionic repulsion and attraction.

Because a dissociation of *p*-hydroxybenzoic acid dose not occur at pH 2.5, the capacity factors did not change comparing to at pH 6.5.

3.4. Durability

Fig. 7 shows a chromatographic result of (a) first and (b) 500th injections (a total volume of serum injected was 10 ml) chromatograms in a continuous injection of human serum to evaluate a durability of the denatured avidin-bonded column. Within 500 repetitive injections, the chromatograms appeared to

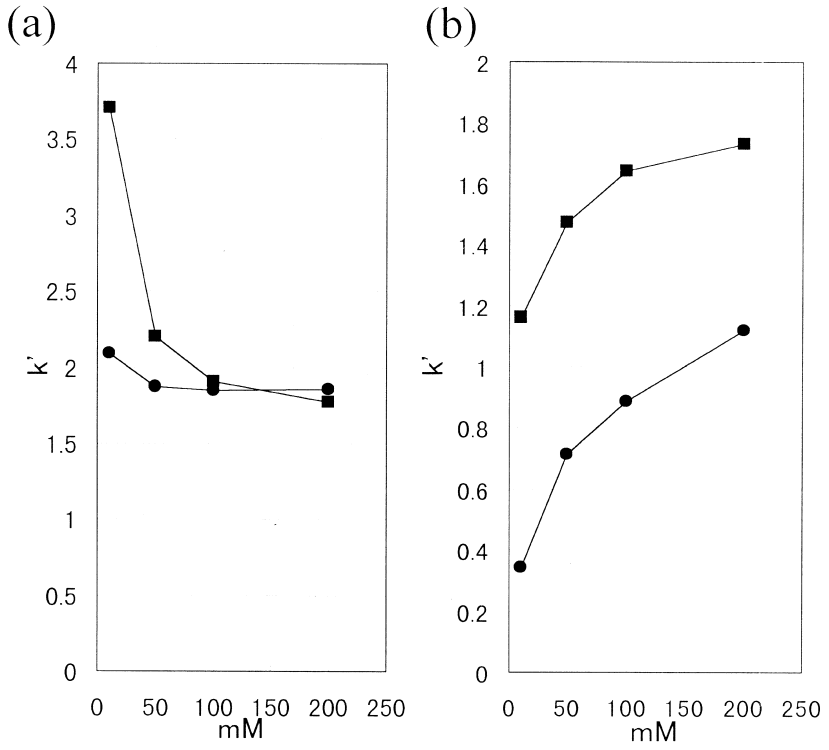

Fig. 6. Dependence of k' values of (a) *p*-hydroxybenzoic acid and (b) benzylamine on phosphate buffer concentration. ● pH 2.5 and ■ pH 6.5. HPLC conditions: column size, 15×0.46 cm I.D.; eluent, phosphate buffer; detection, 254 nm.

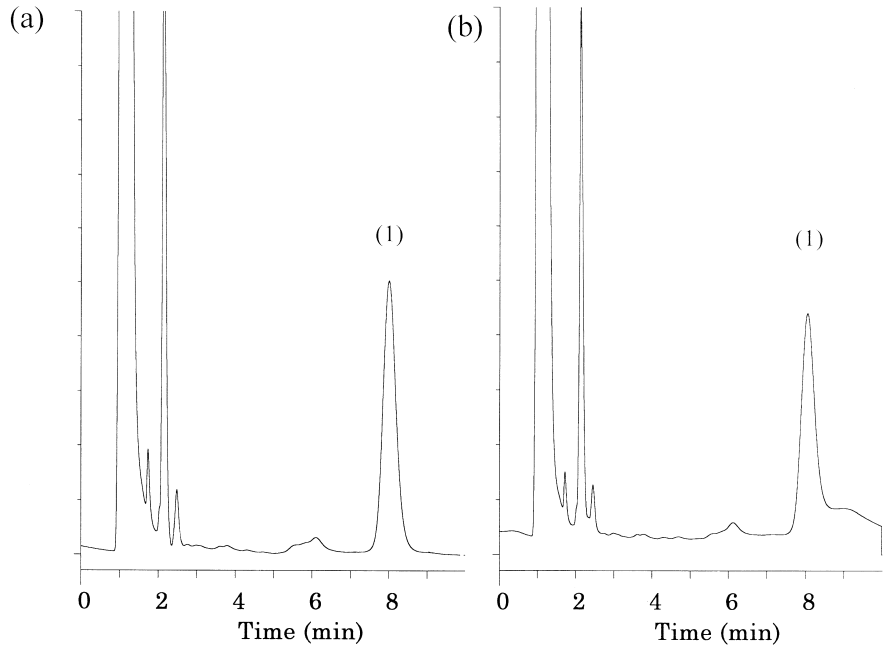

Fig. 7. Chromatograms of (a) the first and (b) the 500th repetitive injection of human serum spiked with (1) carbamazepine (29 μg/ml). HPLC conditions: column size, 15×0.46 cm I.D.; eluent, 50 m*M* phosphate buffer (pH 2.5)–acetonitrile (90:10, w/w); detection, 254 nm; injection volume, 20 μl.

be well reproducible retention time of carbamazepin added into human serum. Although there was a little change in the peak shape, the change was not influential on a biological assay. A back-pressure of the column was between 40–45 kg/cm^2 through the continuous injection. These results indicated that the denatured avidin-bonded column could be successfully applied in analysis of drugs by direct injection of serum.

4. Conclusions

A denatured avidin-bonded column was suggested to use for direct injection analysis of serum sample at over the range of pH because of the completely recoveries of serum protein injected and a durability of the column. A retention property of the column was investigated, that property was stable to organic solvent.

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