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Review

Evaluation of vinylpyridine and vinylpyridinium polymers as column packings for high-performance liquid chromatography

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ABSTRACT

The versatility of cross-linked porous vinylpyridine and vinylpyridinium polymers as column packings in high-performance liquid chromatography is described. The proposed polymer columns were employed for the analysis of a variety of compounds, including carbohydrates and proteins. Among the polymers, an N-methylpyridinium polymer having a hydrophilic cross-linking structure is of interest for protein analysis. The resolution of serum albumin components is discussed and a wide range of applications including diagnosis are suggested.

CONTENTS

1. INTRODUCTION

A variety of synthetic polymer beads based on cross-linked polystyrene, polyacrylate, poly(vinyl acetate), polyacrylamide and poly(vinyl alcohol) have been widely applied as column packings in various modes of high-performance liquid chromatography (HPLC). Since 1975, we have been investigating the use of cross-linked 4-vinylpyridine [VP(III)] and quaternized vinylpyridinum [VP(IV)] polymers as separation materials such as chelating resins [1,2] and column packings for gas chromatography [3,4] and HPLC [5–10].

Applications as column packings for HPLC is of particular interest. The weak basicity of VP(III) polymer based on the pyridyl groups would delicately affect the retention of acidic/basic compounds in reversed-phase HPLC. In acidic media, the

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pyridyl nitrogen is protonated and the polymer shows ion-exchange character. It is also expected that the aromaticity of pyridyl group will favourably affect the retention of aromatic compounds. In addition, the pyridyl groups on the VP(III) polymer can be easily quaternized by a simple chemical modification, and the resulting VP(IV) polymer is applicable as a column packing in ion-exchange HPLC.

Although several stationary phases modified with pyridyl groups have been reported [11–14], only vinylpyridine polymers are dealt with here. In this paper, the characterization of VP(III) and N-methyl-VP(IV) polymers cross-linked with hydrophobic divinylbenzene (DVB) and hydrophilic ethylene glycol dimethacrylate (EG) (Fig. 1) in HPLC is reviewed.

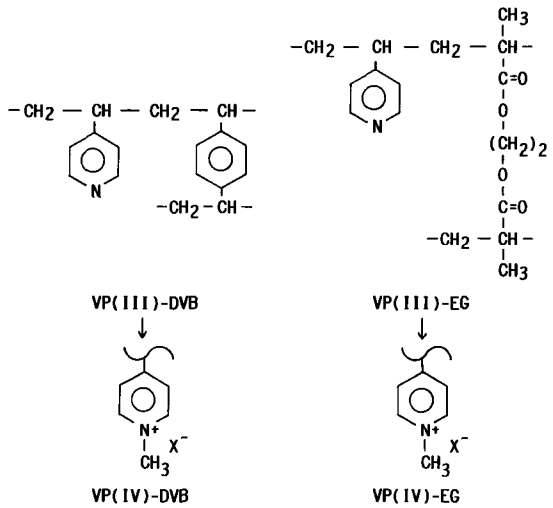

Fig. 1. Structures of polymers.

2. APPLICATION TO REVERSED-PHASE AND/OR ANION-EXCHANGE CHROMATOGRAPHY

Using reversed-phase HPLC using methanol as eluent, the retention behaviour of various aromatic compounds on VP(III)–DVB polymer cross-linked with 20% DVB was examined and compared with that on polystyrene cross-linked with DVB (St–DVB) polymer [5]. As shown in Table 1, the VP(III) DVB polymer, possessing lower hydrophobicity than the St–DVB polymer, tends to retain hydrophilic compounds, such as phenols and amines, more strongly. A characteristic of VP(III)–DVB polymer is particularly revealed in the high affinity for the phenolic hydroxyl group. The k' values of phenols on the VP(III)–DVB polymer increase with increase in the number of hydroxy groups in the phenols, but this tendency is reversed on the St–DVB polymer.

In the chromatography of a mixture of naphthalene, resorcinol and *p*-phenylenediamine, using methanol containing 10–20% of *n*-hexane–water as the mobile phase, the addition of *n*-hexane to methanol decreases the retention volume of naphthalene and increases in those of resorcinol and *p*-phenylenediamine. The heights equivalent to

TABLE 1

CAPACITY FACTORS OF VARIOUS COMPOUNDS ON VP(III)–DVB AND St–DVB POLYMER COLUMNS (FROM REF. 5)

Column: 50 × 0.26 cm I.D. Eluent: methanol at a flow-rate of 1 ml/min. Detection: UV (254 nm). Column temperature: ambient.

Sample	Capacity factor (k')	
	VP(III)–DVB	St–DVB[a]
Benzene	1.32	0.9
Toluene	1.34	1.20
Xylene	1.35	1.56
Naphthalene	2.37	3.33
Diphenyl	2.32	4.67
Anthracene	5.39	11.88
Phenanthrene	5.47	9.98
Triphenylene	18.00	38.07
Chlorobenzene	1.46	1.25
o-Dichlorobenzene	1.67	1.63
Nitrobenzene	1.79	1.27
Benzonitrile	1.35	0.86
Benzyl alcohol	1.26	0.27
p-Benzoquinone	1.35	0.65
Aniline	1.59	0.43
o-Phenylenediamine	1.55	0.38
m-Phenylenediamine	1.49	0.33
p-Phenylenediamine	0.81	1.23
Pyridine	0.85	0.47
α-Picoline	0.80	0.57
γ-Picoline	0.82	0.60
α-Aminopyridine	0.94	1.53
Quinoline	1.13	1.37
Quinaldine	1.07	1.47
Phenol	2.48	0.28
Hydroquinone	4.15	0.08
Resorcinol	6.24	0.06
Pyrogallol	10.41	0.04
o-Cresol	2.51	0.37
α-Naphthol	3.00	0.97
β-Naphthol	2.33	0.96
Methyl benzoate	1.35	1.42
Methyl salicylate	1.59	1.71

[a] Hitachi-3010.

a theoretical plate (HETP values) of all the compounds decrease slightly. The addition of water causes an appreciable increase in the retention volumes of naphthalene and resorcinol and a slight decrease in that of *p*-phenylenediamine, whereas the HETP values of these compounds increase. The selection of a mixed solvent system as a mobile phase is important for improving the peak resolution. The use of a suitable

buffer solution in the mixed solvent system may make possible the separation of a wide variety of compounds.

Ion-exchange HPLC of aromatic acids by using a VP(III)–DVB column has also been investigated [6], and the retention behaviour was compared with that on the VP(IV)–DVB column. The relationship between the eluent pH and the k' values for hydroxybenzoic and nitrobenzoic acids on these polymer columns are shown in Fig. 2. Over the pH range examined, 2-hydroxybenzoic acid and nitrobenzoic acids dissociate completely. Therefore, the decrease in the k' values of these acids with increasing eluent pH on the VP(III)–DVB polymer column is due to the decrease in the dissociation of the pyridyl group in the polymer. In spite of the apparent pK_a value (4.2) of this polymer, the k' values decreased over a broad pH range. This can probably be accounted for by the high density of pyridyl groups in the polymer. As the pH decreased, the pH profiles of the k' values for 3- and 4-hydroxybenzoic acids tended to flatten out because of a contribution from the undissociated acids. With VP(IV)–DVB polymer in which the pyridinium group is completely dissociated over a wide pH range, the k' values for all the acids tested increase with eluent pH up to their maximum dissociation. These results may be accounted for in terms of the extent of dissociation of both the solutes and the polymers. Organic solvents in the eluents also affect not only the dissociation of functional groups but also the hydrophobic interaction between the solute and the polymer. A linear relationship between log k' and the concentration of organic modifier in binary water–organic eluents was found for the VP(III)–DVB column.

The retention behaviour of various acids was investigated under chromatographic conditions such that the pyridyl groups of the VP(III)–DVB are slightly dissociated and the results are summarized in Table 2 together with those on the VP(IV)–DVB column. Benzenedicarboxylic acids and some aromatic acids with low pK_a values are strongly retained on the VP(IV)–DVB column, but only weakly

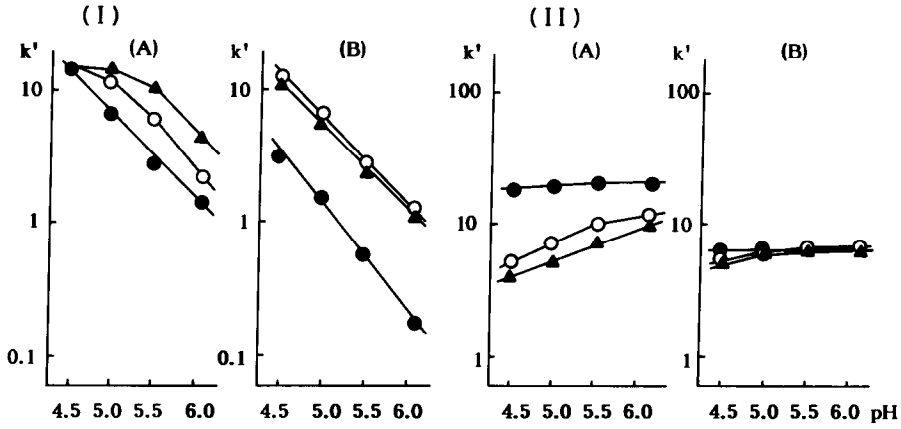

Fig. 2. Effect of pH on the capacity factor, k', of hydroxybenzoic acids (A) and nitrobenzoic acids (B) (from ref. 6). Column: I = VP(III)–DVB polymer; II = VP(IV)–DVB polymer (25 × 0.26 cm I.D.). ● = 2-, ○ = 3- and ▲ = 4-substituted benzoic acid. Eluent: methanol–water (1:1) containing 0.05 *M* phosphate buffer and 0.05 *M* sodium nitrate at a flow-rate of 0.5 ml/min. Column temperature: 55°C. Detection: UV (254 nm).

TABLE 2

CAPACITY FACTORS OF AROMATIC ACIDS ON VP(III)–DVB AND VP(IV)–DVB POLYMER COLUMNS (FROM REF. 6)

Eluent: methanol–water (1:1) containing 0.05 *M* phosphate buffer and 0.05 *M* sodium nitrate (pH 5.5) at a flow-rate of 0.5 ml/min. Column: 25 × 0.26 cm I.D. Detection: UV (254 nm). Column temperature: 55°C.

Compound	pK_a	k'	
		VP(III)–DVB	VP(IV)–DVB
2-Aminobenzoic acid	2.05, 4.95	8.08	6.55
3-Aminobenzoic acid	3.07, 4.74	2.06	4.64
4-Aminobenzoic acid	2.38, 4.89	4.78	4.06
2-Hydroxybenzoic acid	3.00, 13.40	2.75	20.27
3-Hydroxybenzoic acid	4.08, 9.93	5.94	9.89
4-Hydroxybenzoic acid	4.58, 9.23	10.11	7.19
2-Nitrobenzoic acid	2.17	0.56	6.36
3-Nitrobenzoic acid	3.49	2.67	6.66
4-Nitrobenzoic acid	3.44	2.33	6.23
2-Chlorobenzoic acid	2.94	1.11	4.05
3-Chlorobenzoic acid	3.82	4.69	5.96
4-Chlorobenzoic acid	3.99	5.25	5.13
1,2-Benzenedicarboxylic acid	2.95, 5.41	2.52	19.47
1,3-Benzenedicarboxylic acid	3.62, 4.60	2.25	35.23
1,4-Benzenedicarboxylic acid	3.54, 4.46	1.97	29.03
2-Methylbenzoic acid	3.91	3.84	2.09
3-Methylbenzoic acid	4.27	5.28	2.71
4-Methylbenzoic acid	4.37	5.31	2.55
Benzoic acid	4.21	3.31	3.17
Nicotinic acid	4.82, 12.00	0.25	1.30
Isonicotinic acid	4.84, 12.33	0.17	1.34
4-Methylbenzenesulphonic acid	1.70	0.39	10.07

retained on the VP(III)–DVB column. From these results, it is presumed that the contribution of ion exchange to the retention on the VP(III)–DVB column is small. Both ionic and non-ionic interactions between the acids and the pyridyl groups were analysed by a dual binding model [15], and the conclusion drawn is that the solute retention on VP(III)–DVB is due predominantly to non-ionic interactions under the experimental conditions. However, it is almost certain that ionic interactions do exist in this chromatographic system, as the dissociation of the pyridyl group affected the retention of the acids. On the other hand, considerable ionic interaction must be involved in solute retention on VP(IV)–DVB under the same conditions. Recently, a column packed with a similar type of VP(III)–DVB polymer has been available commercially (ACT-2 from Interaction Chemicals or Polysher RPCI from Merck), and a wide variety of applications with control of the pH of the mobile phase are to be expected.

3. APPLICATION TO ANALYSIS OF CARBOHYDRATES

In the analysis of carbohydrates by HPLC, anion- and cation-exchange resins [16,17] and silica gel bearing amino [18] and hydroxy [19] groups have been used as

column packings. In this section, the characterization of the VP(IV)–DVB polymer in the analysis of carbohydrates is described [7]. It is expected that the difference in functional groups between conventional anion-exchange resins and vinylpyridine polymers may lead to improvements in the retention behaviour of carbohydrates.

The retentions of carbohydrates on the different ionic forms of VP(IV)–DVB columns were examined. Among the ionic forms of VP(IV)–DVB columns, the order of retention of carbohydrates is phosphate > sulphate > bromide > nitrate. The elution order of carbohydrates is deoxyhexose < aldopentose ⩽ ketohexose < aldohexose < disaccharide. These are essentially identical with those reported by D'Amboise *et al.* [20] using an anion-exchange column (Hitachi 3013N, phosphate form), except for aldopentose. Although the peaks of fucose and ribose were not separated on the Hitachi 3013N column, complete separation of both compounds was achieved on the phosphate form of the VP(IV)–DVB column. As shown in Table 3, among the various ionic forms of the VP(IV)–DVB polymers, the phosphate form gives the best resolution of carbohydrates.

TABLE 3

PEAK RESOLUTION OF SELECTED CARBOHYDRATES ON VARIOUS FORMS OF VP(IV)–DVB POLYMER COLUMNS (FROM REF. 7)

Peak resolution (R_s) is defined as the ratio of the distance between the maxima (retention times, t_R) of two adjacent peaks and arithmetic mean of their base widths (w): $R_s = 2(t_{R2} - t_{R1})/(w_1 + w_2)$. Column: 50 × 0.26 cm I.D. Eluent: acetonitrile–water (4:1) at a flow-rate of 1 ml/min. Detection: refractive index. Column temperature: 70°C.

Compounds	VP(III)–DVB			
	Bromide	Phosphate	Nitrate	Sulphate
Fucose–ribose	0.24	1.46	0.23	0.46
Ribose–arabinose	0.86	2.30	0.60	1.51
Arabinose–xylose	0.15	1.55	0.05	0.96
Xylose–mannose	0.73	1.63	0.31	1.07
Mannose–galactose	0.33	1.80	0.29	1.06
Fructose–glucose	1.14	4.89	0.79	3.00
Glucose–sucrose	0.05	1.36	0.22	0.63

Mopper and Degens [21] stated that the chromatographic separation of carbohydrates was attributable to the partition of water between the polymer and the mobile phase. This indicates that the hydrophilicity of the polymer may affect the retention of carbohydrates. As shown in Fig. 3, a linear relationship exists between the water regain, which is used as an indication of the hydrophilicity of each ionic form of VP(IV)–DVB, and the k' value per gram of packing material.

4. APPLICATION TO ANALYSIS OF PROTEINS

Among the column packings for the ion-exchange HPLC of proteins, organic polymer-based supports have the advantages over surface-modified silica gel of chemical stability in alkaline solutions, higher sample loading capacities and longer

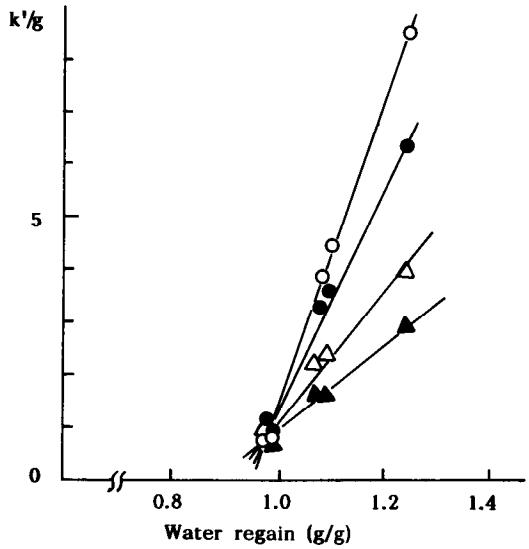

Fig. 3. Relationship between the water regain of VP(IV)–DVB polymer and the k' value per gram of packing material. (From ref. 7). Carbohydrates: ● = arabinose, ○ = fructose, ▲ = fucose, △ = ribose. Eluent: acetonitrile–water (8:2) at a flow-rate of 1 ml/min. Column: 50 × 0.26 cm I.D. Column temperature: 70°C. Detection: refractive index.

column lifetimes. In general, improvements in these matrices occur with hydrophilic polymers such as poly(vinyl alcohol) gel [22–24] and poly(glycol methacrylate) gel [25]. Quaternized VP polymers having a hydrophilic nature are also expected to be useful supports for protein analysis. The applicability of VP(IV)–DVB polymer and a similar type of N-methyl-4-vinylpyridinum polymer cross-linked with 50% ethylene glycol dimethacrylate [VP(IV)–EG] in the ion-exchange HPLC of proteins have been investigated [8].

It is generally desirable that the ion exchangers are macroporous in order to permit the penetration of proteins into the ion-exchange group. It has been reported that the pore size of the support markedly affects the resolution and recovery of proteins in ion-exchange HPLC [26,27] and in reversed-phase HPLC [28–30].

Although the most common pore radius of VP(IV)–DVB polymer determined in the dry state is larger than that of VP(IV)–EG, as shown in Table 4, the exclusion limits

TABLE 4

CHARACTERIZATION OF N-METHYLPYRIDINIUM BROMIDE POLYMERS IN THE DRY STATE (FROM REF. 8)

Polymer	Elemental analysis (%)		Specific surface area (m^2/g)	Pore volume (ml/g)	Most frequent pore radius (nm)
	N	Br			
VP(III)–DVB	4.62	27.90	23.90	0.29	27.5
VP(IV)–EG	3.87	22.28	9.49	0.08	8.7

of both polymers for carbohydrates are similar and small (about 500 dalton). The pore volumes (ml/ml of polymer) in the wet state determined by the method of Duncan *et al.* [31] were 0.38 ml [VP(IV)–DVB] and 0.34 ml [VP(IV)–EG], *i.e.*, nearly identical. This indicates that the pore size distribution of both polymers in the wet state is different from that in the dry state. This may suggest that the proteins scarcely penetrate into the pores and interact only on the limited surface of the polymers as well as non-porous-type polymer packings [31–33].

Table 5 shows the retention times and recoveries of proteins obtained using 30-min linear gradient elution from 0 to 0.5 *M* sodium chloride in Tris–HCl buffer (pH 7.0). The retention times of the proteins in both polymer columns are approximate. The retention times of the proteins varied with the pH of the buffer, and considerable changes in the retentions of proteins were observed at pH values near the p*I* values of the proteins. The major retention process of a protein having an overall negative charge may be governed by ionic interactions. However, it is also necessary to take into account the effect of other interactions, such as hydrophobic and π–π interactions, on the retention of proteins.

Although both polymers show similar physical properties and retentions of proteins, a clear difference was found in the recoveries of proteins, *i.e.*, the recovery on the VP(IV)–EG column was almost quantitative for all the proteins tested, whereas that on the VP(IV)–DVB column was poor. A hydrophobic interaction between the protein and the cross-linking part of the polymer may affect the non-specific adsorption of the protein. Therefore, the VP(IV)–EG polymer having a hydrophilic cross-linking structure is suitable as a column packing for protein analysis.

TABLE 5

RETENTION TIMES AND RECOVERY OF PROTEINS ON VP(IV)–DVB (I) AND VP(IV)–EG (II) COLUMNS IN TRIS–HCl BUFFER SYSTEM (FROM REF. 8)

The proteins were eluted using a 30-min linear gradient of sodium chloride from 0 to 0.5 *M* in 0.05 *M* Tris–HCl (pH 7.0) at a flow-rate of 0.5 ml/min. Sample loading: 60 μg. Column: 25 cm × 4 mm I.D. Detection: UV (280 nm). Column temperature: ambient. BSA = Bovine serum albumin; β-LG = bovine milk β-lactoglobulin; CEA = chicken egg albumin; STI = soybean trypsin inhibitor; γ-G = bovine γ-globulin; LY = chicken egg lysozyme; CYC = horse heart cytochrome *c*; α-CHT = bovine pancreatic α-chymotrypsin.

Protein	p*I*	t_R (min)		Recovery (%)[a]	
		I	II	I	II
BSA	4.7–4.9	15.82, 16.50, 16.89	17.20, 17.98, 18.40	85.9	101.2
β-LGA	5.2	21.40	23.34	74.6	92.5
β-LGB	5.1	19.12	21.32		
CEA	4.6	15.63	17.1	70.1	93.8
STI	4.3	20.62	21.44	63.6	89.3
γ-G	8.0	2.42	2.40	84.3	104.6
LY	11.4	2.45	2.40	97.8	103.1
CYC	11.0	2.42	2.40	95.1	102.2
α-CHT	8.1	2.57	2.48	95.9	100.9

[a] Recovery was obtained by injection of proteins (0.2 mg) into the column equilibrated with initial eluent and by eluting with final eluent of the gradient elution. Spectrophotometric determination at 225 nm of proteins in the column effluent, which was pooled for 20 min after the protein injection, gave the recovery.

The column lifetime is sufficient under the conditions used for the protein analysis. No significant deterioration of the column was observed even after continuous use for 3 months. In addition, the resolution of proteins on columns did not change after a regeneration treatment in which 30 ml of dilute sodium hydroxide solution containing 0.2 *M* potassium chloride (pH 12) was passed through the column at a flow-rate of 0.5 ml/ml.

5. RESOLUTION OF SERUM ALBUMIN COMPONENTS

An interesting feature of the hydrophilic VP(IV)–EG column has been demonstrated in the resolution of serum albumin components [9]. Serum albumins, *e.g.*, human serum albumin (HSA) and bovine serum albumin (BSA), are a mixture of mercaptoalbumin and non-mercaptoalbumin. Mercaptoalbumin has one sulphydryl group based on cysteine moiety at the 34-position in HSA and BSA. Mercaptoalbumin in serum is equilibrated with its mixed disulphide with cysteine or glutathione (non-mercaptoalbumin). The equilibrium is of interest in connection with physiology and diagnosis, as the mercaptoalbumin content changes in the elderly [34] and in some diseases [35].

The resolution of both components of HSA by HPLC was first reported by Sogami *et al.* [36], who used a Asahipak GS 520/520H column intended for use in the aqueous gel permeation mode. However, this column is useful only for HSA and the retention mechanism is not clear. Subsequently, it was reported that Asahipak ES 502N, having diethylaminoethyl as a functional group, was also applicable to the analysis of HSA [34]. With VP(IV)–EG polymer, which is a strong anion-exchange resin, it is assumed that the retention/resolution mechanism of albumins is different from that on Asahipak GS 520/520H and ES 502N columns. Typical chromatograms of BSA and HSA with linear gradient elution are shown in Fig. 4. The four main peaks observed in the chromatograms were characterized according to the method of Sogami *et al.* [36], that is, mercaptoalbumin was converted into non-mercaptoalbumin by

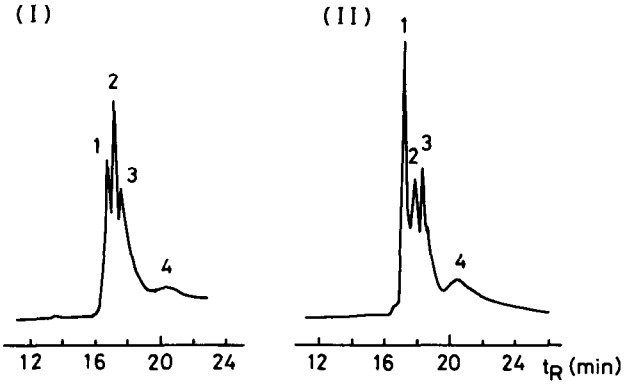

Fig. 4. Chromatograms of HSA (I) and BSA (II). (From ref. 9) Peaks: 1 = mercaptoalbumin; 2 = non-mercaptoalbumin (Cys); 3 = oxidized form; 4 = oligomeric albumins. Column: VP(IV)–EG (25 × 0.4 cm I.D.). Elution: 30-min linear gradient of sodium chloride from 0 to 0.5 *M* in 0.05 *M* Tris–HCl (pH 7.0) at a flow-rate of 0.5 ml/min. Detection: UV (280 nm). Column temperature: ambient.

treatment with cystine or oxidized glutathione and non-mercaptoalbumins led to mercaptoalbumin by a selective reduction with dithiothreitol. In addition, the peaks coincided exactly with those of the proteins prepared by an authentic method [37,38]. The resolution of mercaptoalbumin and non-mercaptoalbumin in HSA and BSA is strongly affected by the pH of the mobile phase. The best resolution is obtained at pH 6.5–7.0. On the other hand, with the Asahipak ES 502N column the resolution of mercaptoalbumin and non-mercaptoalbumin is achieved by gradient elution with ethanol in a sodium acetate buffer containing sodium sulphate (pH 4.8). It is assumed that below pH 5 albumin has insufficient negative charge for retention on the VP(IV)–EG column. The retention time of albumin increases with increasing pH of the mobile phase, and the resolution was poor at higher pH. The retention behaviour of these albumins is probably influenced by conformation changes of the protein caused by alteration of the eluent pH. A change in the buffer system used in the eluent markedly affects the resolution of mercaptoalbumin and non-mercaptoalbumin.

Various sources of albumin (human, bovine, rabbit and rat) were chromatographed by using three buffer systems as eluents, and the results are shown in Fig. 5. HSA, BSA and rabbit serum albumin (RSA) could be resolved into their components in all the buffer systems used. However, the resolution of each component of rat serum albumin (RtSA) is not clear. In the case of phosphate buffer, the peaks of HSA and RtSA are broad in comparison with those of BSA and RSA, and all the albumins

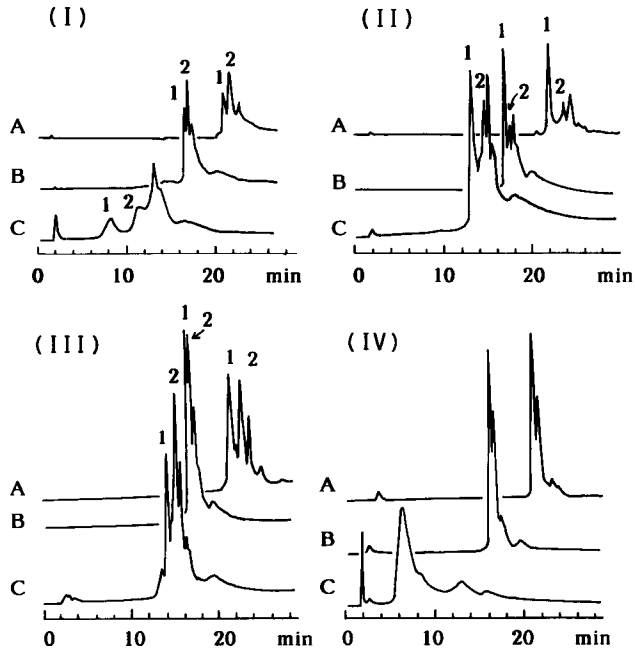

Fig. 5. Chromatograms of HSA (I), BSA (II), RSA (III) and RtSA (IV). Eluent: (A) 0.05 *M* Tris–acetic acid (pH 7.0) (gradient, CH_3COONa 0 to 0.5 *M*); (B) 0.05 *M* HCl (pH 7.0) (gradient, NaCl 0 to 0.5 *M*); (C) phosphate buffer (pH 7.0) 0.05 to 0.5 *M*. Gradient time: 30 min linear. Peaks: 1 = mercaptoalbumin; 2 = non-mercaptoalbumin. Other conditions as in Fig. 4.

tested showed sharp peaks in buffer systems other than phosphate. The best resolution of mercaptoalbumin and non-mercaptoalbumin of HSA, BSA and RSA was achieved using Tris–acetic acid buffer.

Another characteristic is the resolution of two kinds of non-mercaptoalbumin, namely mixed disulphides with cysteine [non-mercaptoalbumin(cys)] and glutathione [non-mercaptoalbumin(glu)] [39,40]. Satisfactory resolution of the two non-mercaptoalbumins in HSA and BSA on the VP(IV)–EG column is shown in Fig. 6. The good resolution of the mixed disulphides, which was not observed on Asahipak GS 520H and ES 502N columns, was obtained with the VP(IV)–EG column. This shows that the retention may be influenced by small changes in the albumin structure. The alteration of the charge at the surface of the protein molecule is probably the main factor in this peak resolution.

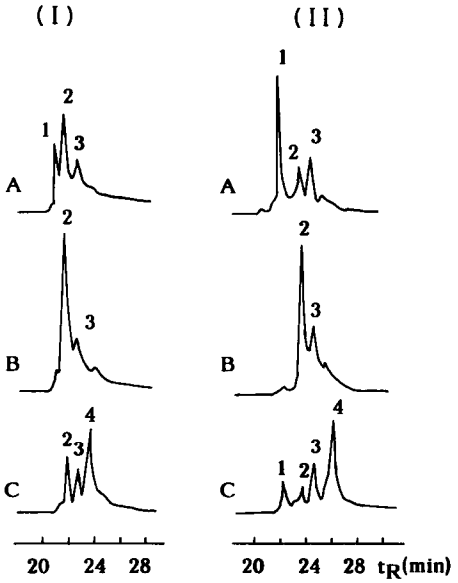

Fig. 6. Chromatograms of non-mercaptoalbumin (Cys) and non-mercaptoalbumin (Glu) of HSA (I) and BSA (II). (From ref. 9). (A) Non-treated albumin; (B) albumin treated with cystine; (C) albumin treated with oxidized glutathione. Peaks: 1 = mercaptoalbumin; 2 = non-mercaptoalbumin (Cys); 3 = oxidized form; 4 = non-mercaptoalbumin (Glu). Albumins were eluted using a 30-min linear gradient of sodium acetate from 0 to 0.5 *M* in 0.05 *M* Tris–acetic acid (pH 7.0) at a flow-rate of 0.5 ml/min. Other conditions as in Fig. 4.

As an application of the VP(IV)–EG column, the analysis of human sera from haemodialysis patients was carried out. The albumin components in serum could be analyzed by direct injection of the sample. The amount of mercaptoalbumin in serum from haemodialysis patients increased after haemodialysis, as reported by Sogami *et al.* [35].

Further, the VP(IV)–EG column was applied to a study of the reaction of cisplatin (DDP), an antineoplastic agent, with BSA used as a typical serum albumin [10]. Gonias and Pizzo [41] suggested that DDP had strong affinity for the sulphydryl

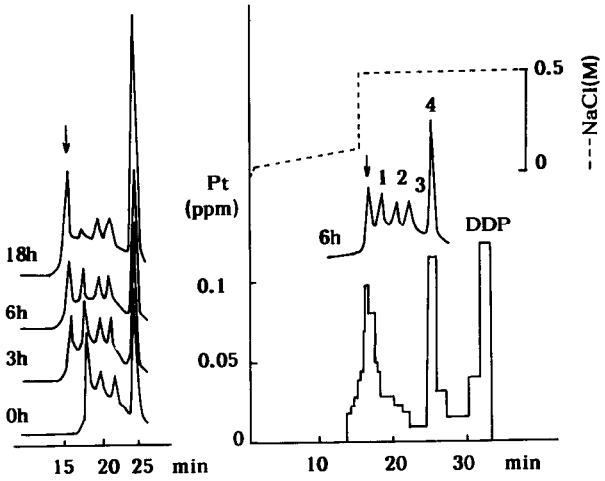

Fig. 7. Chromatograms of BSA incubated with cisplatin (DDP) for various periods of time at 37°C. Peaks: 1 = mercaptoalbumin; 2 = non-mercaptoalbumin (Cys); 3 = oxidized form; 4 = oligomeric albumin. Albumin was eluted with a gradient from 0 to 0.5 *M* sodium chloride in 0.05 *M* Tris–HCl buffer (pH 7.0). Other conditions as in Fig. 4. Determination of platinum fractions of eluate was carried out by flameless atomic absorption spectrophotometry.

group in mercaptoalbumin. In another investigation [42], an albumin–DDP complex was analysed by reversed-phase HPLC, but the resolution between the complex and the unreacted albumin did not appear to be satisfactory.

In order to improve the resolution of albumin components, various salt gradients were investigated, and the conditions shown in Fig. 7 were adopted. In the chromatograms of BSA incubated with DDP, a new peak appeared before the mercaptoalbumin peak (arrow in Fig. 7). The height of this peak increases with decreasing peak height of mercaptoalbumin, and a high concentration of platinum was present in this peak. These chromatographic changes suggest that DDP has a high reactivity for mercaptoalbumin. Consequently, it is considered that the new peak is that of a mercaptoalbumin–DDP complex. The molar ratio of albumin to platinum in the peak fraction was determined to be 1:1. Non-mercaptoalbumin (mixed disulphide with cysteine) showed a poor reactivity for DDP, but a disulphide-type albumin dimer prepared separately incorporated platinum.

The VP(IV)–EG column was further applied to study the reaction of HSA and sodium aurothiomalate (AuTS), which is an anti-arthritic drug [43]. Tris–acetic acid buffer was used as the eluent. As shown in Fig. 8, a new peak containing gold which corresponds to albumin–AuTS is observed at a different retention time from those of albumin components, and the peak increases with increasing reaction time accompanying a decrease in the peak height of mercaptoalbumin. The reactivity of non-mercaptoalbumin with AuTS is poor, as with DDP. Hence the VP(IV)–EG column could be successfully applied to the investigation of interactions between serum albumin and compounds having a high affinity for the sulphydryl group.

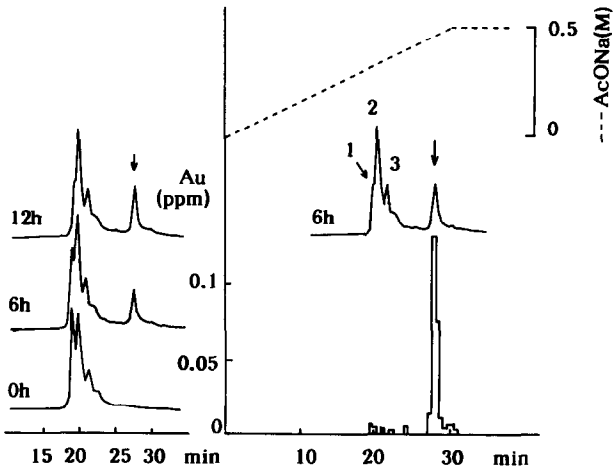

Fig. 8. Chromatograms of HSA incubated with sodium aurothiomalate for various periods of time at 37°C. Peaks: 1 = mercaptoalbumin; 2 = non-mercaptoalbumin (Cys); 3 = oxidized form. Albumin was eluted with a gradient from 0 to 0.5 *M* sodium acetate in 0.05 *M* Tris–acetic acid buffer (pH 7.0). Other conditions as in Fig. 4. Determination of gold in the fractions of eluate was performed by flameless atomic absorption spectrophotometry.

6. CONCLUSIONS

Porous VP(III)–DVB, VP(IV)–DVB and VP(IV)–EG polymers were investigated as column packing materials in HPLC. In a reversed-phase chromatographic system, the VP(III)–DVB polymer column shows a characteristic retention behaviour for aromatic compounds. Hydrophobic and ionic interactions favourably affect the retention of the compounds. VP(III)–DVB and VP(IV)–DVB polymer columns were applied to the separation of aromatic acids in the ion-exchange mode, where the extends of dissociation of both the solute and the polymer affect the retention/separation of the compounds. The VP(IV)–DVB polymer column is also useful for carbohydrate analysis in the partition mode.

The VP(IV)–DVB and VP(IV)–EG polymer columns can be applied to the analysis of proteins by ion-exchange HPLC, despite their low-molecular-weight exclusion limit. Although both columns show similar retention behaviour for the proteins tested, the hydrophilic VP(IV)–EG column is superior for the recovery of proteins. An interesting feature of the VP(IV)–EG column was found in the resolution of serum albumin components, namely, mercaptoalbumin, non-mercaptoalbumin conjugated cysteine or glutathione and oligomeric albumins. This column is applicable to studies on the reaction of albumin with pharmaceuticals reactive to the sulphydryl group. Applications in diagnosis appear promising.

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