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Effect of stationary phase structure on retention and selectivity of restricted-access reversed-phase packing materials

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ABSTRACT

Various restricted-access reversed-phase (RARP) packing materials, including alkyl-, alkyl ether- and alkylamide-type stationary phases, were compared with a commercially available peptide-bonded internal surface reversed-phase material and a shielded-type RARP material. Alkyl-type RARP materials were found to be more hydrophobic than ether or amide-type phases, which in turn were more hydrophobic than the materials commercially available at present. The contribution of polar as well as ionic groups in the stationary phase structure to retention selectivity was noted with some materials. The advantages of having a variety of RARP materials in terms of retention and selectivity were shown for separation of various drugs with a limited range of mobile phases.

INTRODUCTION

Restricted-access reversed-phase (RARP) packing materials were developed for the high-performance liquid chromatographic (HPLC) analysis of body fluids, namely serum, in which proteins co-exist with the analytes. Pretreatment of biological samples can be simplified by using these materials, because high-molecular-weight solutes can be excluded from the pores of RARP materials which possess hydrophilic external and hydrophobic internal surfaces.

Two types of RARP materials have been reported in terms of the preparation procedure. The first method is a three-step procedure; (1) introduction of hydrophobic groups on all the surfaces of porous silica particles; (2) removal of hydrophobic groups from the external surface; (3) introduction of hydrophilic groups onto the external surface.

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Hagestam and Pinkerton [1] prepared a glycylphenylalanylphenylalanine (GFF)-bonded internal surface reversed-phase (ISRP) material [abbreviated to ISRP-peptide (GFF)] [1–6], and Haginaka and co-workers [7,8] prepared alkyl amide-bonded ISRP material (ISRP-amide) following this scheme by utilizing enzyme-catalyzed cleavage of external hydrophobic groups. The packing materials prepared by these methods necessarily include a functional group such as an amide group in the hydrophobic part of the stationary phase which can be cleaved by an enzyme.

RARP materials can also be prepared from ordinary alkylsilylated silica gels such as silica-C_{18}. Sudo *et al.* [9] used oxygen plasma, and Kimata *et al.* [10] used aqueous hydrochloric acid to remove alkylsilyl groups from the external surface of packing materials designed for reversed-phase liquid chromatography (RPLC).

The second type of RARP materials are mixed-type materials, which possess both hydrophobic and hydrophilic groups on all surfaces of the porous silica gel. Gisch and co-workers [11,12] reported an RARP material with phenyl groups bonded with polyoxyethylene chains, currently available as LC-HISEP. Haginaka and Wakai reported a C_{18} phase bonded together with diol groups [13]. They also reported an RARP material with chiral groups for the separation of enantiomers [14]. In these RARP materials, the hydrophobic properties of alkyl or aryl groups were controlled by the presence of hydrophilic groups so that proteins are not retained under the separation conditions for low-molecular-weight compounds.

The RARP materials prepared by different methods are expected to show different retention characteristics, depending upon the solute accessibility to and the chemical structure of the hydrophobic groups on the internal surface in relatively small pores of *ca.* 8 nm or less. In RPLC, a variety of packing materials, such as alkyl- or aryl-bonded silica gel [15], polymer-based materials [16] and carbon packing materials [17] have been used to meet various separation needs. The characterization of C_{18} packing materials from various manufacturers would allow one to select a proper column for a particular application [18]. Such characterization of RARP materials would be similarly useful for column selection as well as for designing new RARP materials, because a limited range of mobile phases can be used with RARP materials which should not denature proteins in a column.

Here we report the comparison of retention characteristics of various RARP materials with respect to the effect of hydrophobicity and the contribution of ionizable groups in the stationary phase.

EXPERIMENTAL

Equipment

The HPLC system consisted of an LC-6A pump, an SIL-6A automatic sample injector, an SPD-6A UV detector and a C-R5A data processor (all from Shimadzu, Kyoto, Japan). Column temperature was maintained at 30°C by using a thermostated water bath.

Materials

An ISRP column (15 cm × 4.6 mm I.D.) of glycylphenylalanylphenylalanine-bonded phase [ISRP-peptide (GFF)], originally prepared by Hagestam and Pinkerton [1], was purchased from Kohken (Tokyo, Japan). An LC-HISEP column (15 cm × 4.6

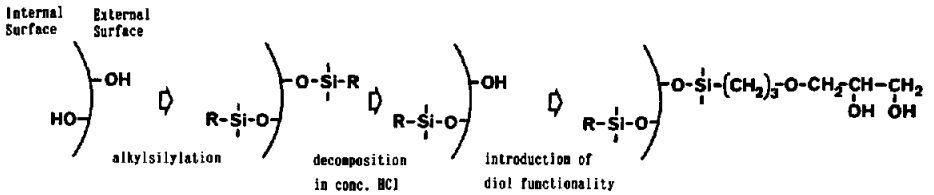

Fig. 1. Preparation method of SHRP packing materials by acid decomposition, and stationary phase structure. R: $C_{18}H_{37}$ (SHRP-C_{18}), C_8H_{17} (SHRP-C_8), $C_6H_5CH_2CH_2$ (SHRP-PE), $CH_3OCH_2CH_2OCH_2CH_2CH_2$ (SHRP-E2), $CH_3O(CH_2CH_2O)_2CH_2CH_2CH_2$ (SHRP-E3).

mm I.D.) was purchased from Supelco (Bellefonte, PA, USA). A size-exclusion chromatography column (30 cm × 7.5 mm I.D.), TSK G2000SW, was purchased from Tosoh (Tokyo, Japan). A Cosmosil 5-C_{18} column was obtained from Nacalai Tesque (Kyoto, Japan).

ISRP-amide packing material was prepared according to a previously reported procedure [7]. Superficially hydrophilic reversed-phase (SHRP) packing materials with alkyl groups, SHRP-C_{18}, SHRP-C_8 and SHRP-phenylethyl (SHRP-PE), as well as alkyl ether-type materials, SHRP-E2 and SHRP-E3 (see Fig. 1 for structures), were prepared by acid decomposition of bonded phases followed by the introduction of diol groups, as reported previously [10]. Acid decomposition of the precursor of SHRP-E2 and -E3 phases with concentrated hydrochloric acid was performed in the presence of stearyl alcohol (10% of the weight of packing material). The carbon contents of these packing materials at each stage of preparation are listed in Table I. The packing materials were packed into stainless-steel columns (150 mm × 4.6 mm I.D.).

Silica particles (Develosil; particle size = 5 μm; pore size = 6 nm; surface area = 500 m^2/g) were purchased from Nomura (Seto, Japan). Alkylsilylating reagents were either purchased from Petrarch System (Bristol, PA, USA) or prepared by standard methods. Bovine serum albumin (BSA) and drug standards were obtained commercially.

TABLE I

CARBON CONTENTS OF RARP PACKING MATERIALS

Packing material	Carbon content (%)		
	Before decomposition[a]	After decomposition	Alkyl/diol
SHRP-C_{18}	20.08	12.29	14.95
SHRP-C_8	14.01	6.79	9.06
SHRP-PE	14.33	5.85	8.62
SHRP-E2	5.76	4.38	5.37
SHRP-E3	5.76	4.05	7.29
ISRP-amide	–	–	5.44

[a] End-capped.

Chromatographic measurement

All chromatographic measurements were carried out at 30°C. The recovery of BSA was calculated based on the peak area by taking the area obtained without a column as 100%.

RESULTS AND DISCUSSION

Hydrophobicity

The hydrophobicity of RARP materials was compared in terms of the retention increase with the addition of one methylene group in the solute structure in 10% acetonitrile and in 60% methanol. The slopes of the plots of log k' (k' = capacity factor) values of phenylalkyl alcohols in 10% acetonitrile, and those of alkylbenzenes in 60% methanol, against the carbon number of the alkyl portion of the solutes were calculated according to eqn. 1 and are listed in Table II. The correlation coefficients were always greater than 0.99 for alkylbenzenes, and greater than 0.98 for phenylalkyl alcohols.

$$\log k' = aC_n + b \tag{1}$$

TABLE II

HYDROPHOBICITY OF RARP PACKING MATERIALS

Packing material	a value in 60% methanol[a]	k'[b]	a value in 10% acetonitrile[c]	k'[d]
SHRP-C_{18}	0.277	11.62	0.407	31.39
SHRP-C_8	0.255	9.19	0.376	29.74
SHRP-PE	0.201	5.92	0.322	14.24
SHRP-E2	0.164	3.65	0.292	11.56
SHRP-E3	0.158	2.95	0.270	8.58
ISRP-peptide (GFF)	0.031	0.95	0.113	1.70
LC-HISEP	0.076	1.22	0.187	2.17
ISRP-amide	0.168	2.29	0.262	5.66
C_{18}-60[e]	0.295	19.90	0.422	67.84
C_{18}-60-D[f]	0.282	10.31	0.418	39.20
C_{18}-100[g]	0.278	15.24	0.410	63.79
C_{18}-300[h]	0.266	4.34	0.397	23.54
C_{12}-300[h]	0.257	4.12	0.401	20.16
C_8-300[h]	0.236	3.45	0.386	16.31
C_6-300[h]	0.220	2.75	0.370	14.14
C_4-300[h]	0.196	1.78	0.336	8.57
C_3-300[h]	0.187	1.57	0.323	7.07
C_1-300[h]	0.170	1.35	0.293	3.53

[a] Calculated according to the equation, $\log k' = a\,C_n + b$; solute: alkylbenzene, C_6H_5–C_nH_{2n+1}.
[b] Solute = methyl benzoate; mobile phase = 40% methanol.
[c] Solute = benzyl alcohol; 2-phenylethanol, 3-phenylpropanol; mobile phase = 10% acetonitrile.
[d] Solute = 3-phenylpropanol; mobile phase = 10% acetonitrile.
[e] Precursor of SHRP-C_{18}, before decomposition.
[f] Precursor of SHRP-C_{18}, after decomposition, before the introduction of diol groups.
[g] Prepared from silica gel with 10-nm pores.
[h] Prepared from silica gel with 30-nm pores.

Generally the longer the alkyl group of stationary phase, the greater the hydrophobicity. Commercially available ISRP-peptide (GFF) and LC-HISEP showed very low hydrophobicity. The *a* values obtained with the ISRP-amide phase were similar to those with SHRP-E2 and SHRP-E3 at an intermediate range. Alkyl-type SHRP materials gave *a* values which were closely related to the carbon contents of the packing materials prior to the introduction of the diol groups.

Also listed in Table II are the *a* values obtained with the precursor of SHRP-C_{18} and ordinary RPLC materials with end capping. The similar *a* values found with the precursors of SHRP-C_{18}, C_{18}-60 and C_{18}-60-D, as well as SHRP-C_{18} suggest that the acid decomposition of alkylsilylated phase proceeded preferentially at the external surface, leaving the C_{18} groups on the internal surface at relatively high surface densities [10]. C_{18} phases with lower surface coverages are known to give much smaller *a* values [10]. Note that the effect of pore size on *a* value, or on the hydrophobicity, is smaller than that of alkyl chain length.

Small-pore materials generally resulted in greater retention, because of their large surface areas, accompanied by slightly larger *a* values. A slight increase in retention was seen with the introduction of the diol function to the decomposed alkyl phase. These results suggest that the solute retention is determined not only by the hydrophobicity but also by the microscopic environment in the bonded phase composed of hydrophobic and hydrophillic groups, in addition to the availability of such surfaces, or the surface area of the packing materials.

Contribution of ionic groups in the stationary phase

Fig. 2 shows the dependence of retention of benzoic acid on the pH of the mobile phase. As shown in Fig. 3, the retention of the acid on LC-HISEP and ISRP-amide showed a clear decrease at low pH. The ISRP-amide phase possesses an alkylamine structure [7]. The pK_a of the amine structure in the stationary phase is expected to be around 9. As the protonation of the amino groups in the stationary phase increases from pH 8 to pH 6, the retention of ionized benzoic acid with a pK_a of *ca.* 4.2 [19]

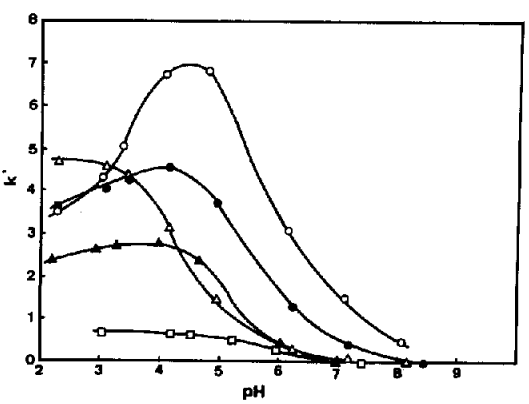

Fig. 2. The pH dependence of the *k'* value of benzoic acid. ○ = LC-HISEP; ● = ISRP-amide; △ = SHRP-E3; ▲ = ISRP-peptide (GFF); □ = SHRP-C_{18}. Methanol concentration of mobile phase: LC-HISEP = 10%; SHRP-amide and SHRP-E3 = 15%; SHRP-peptide (GFF) = 5%, SHRP-C_{18} = 60%; each mobile phase containing 0.02 *M* phosphate buffer.

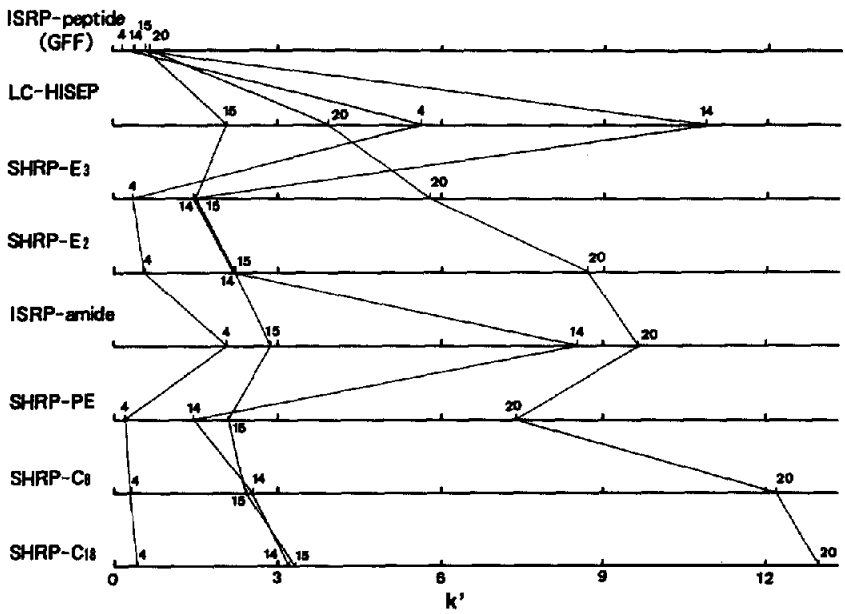

Fig. 3. Difference in selectivity of various RARP materials. Mobile phase: 30% acetonitrile–0.02 *M* phosphate buffer containing 0.1 *M* sodium sulfate (pH 7). Solutes: 4 = furosemide; 14 = indomethacin; 15 = carbamazepine; 20 = nifedipine.

increases. With the decrease in the extent of ionization of benzoic acid below pH 5, the retention decreases at relatively constant ionization state of the amine. A similar observation with LC-HISEP suggests the presence of an amine function on this stationary phase. Other packing materials showed normal retention behavior for benzoic acid for the pH range studied.

Recovery of BSA from RARP materials

Table III shows the recovery of BSA from the RARP columns. All the stationary phases gave nearly complete recovery of BSA with less than 40% acetonitrile in the

TABLE III

RECOVERY OF BSA FROM RARP COLUMNS

Packing material	Acetonitrile content (%) of mobile phase[a]					
	0	10	20	30	40	50
ISRP-peptide (GFF)	94	89	103	100	89	50
SHRP-C_{18}	85	85	96	96	93	64
LC-HISEP	101	94	100	103	115	73
ISRP-amide	98	91	90	84	89	47
TSK gel G2000SW	107	99	97	102	75	47

[a] Mobile phase = acetonitrile–0.02 *M* phosphate buffer + 0.1 *M* sodium sulfate (pH 7).

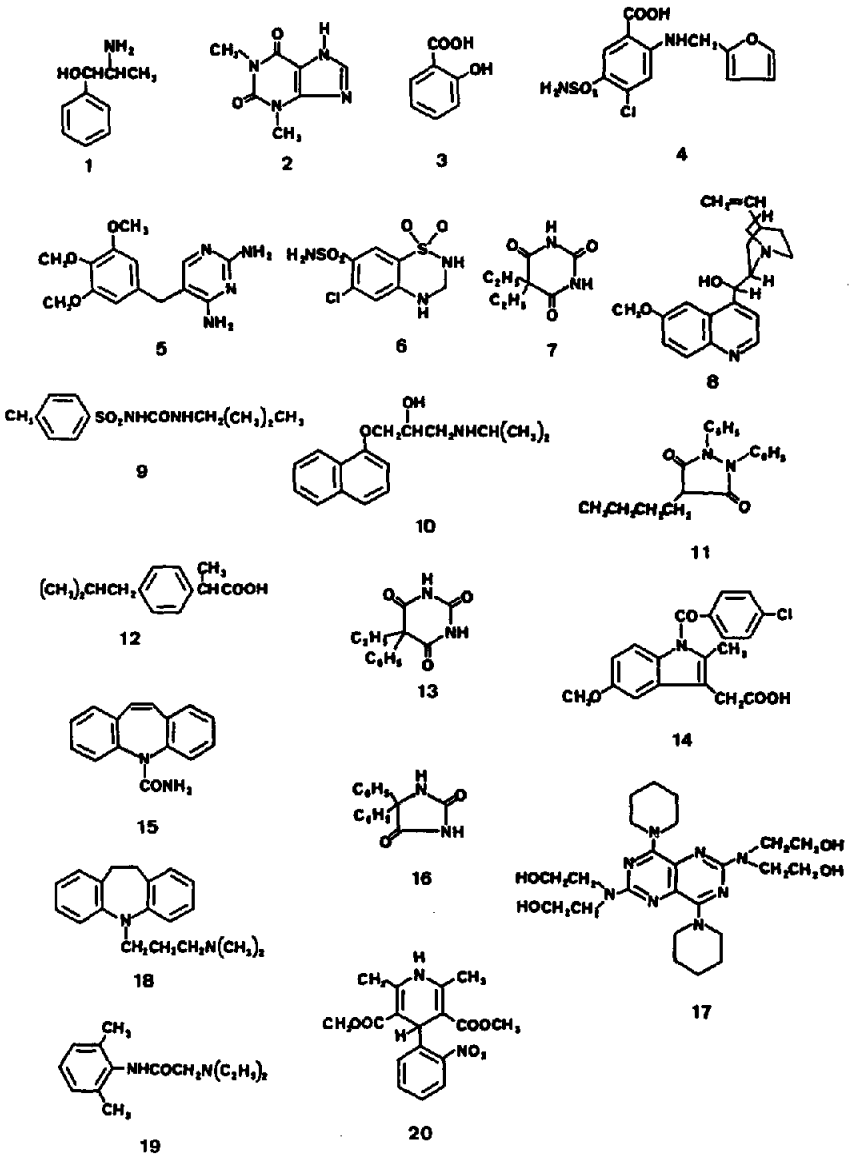

Fig. 4. Structure of drugs. 1 = Phenylpropanolamine; 2 = theophylline; 3 = salicylic acid; 4 = furosemide; 5 = trimethoprim; 6 = hydrochlorothiazide; 7 = barbital; 8 = quinidine; 9 = tolbutamide; 10 = propranolol; 11 = phenylbutazone; 12 = ibuprofen; 13 = phenobarbital; 14 = indomethacin; 15 = carbamazepine; 16 = phenytoin; 17 = dipyridamole; 18 = imipramine; 19 = lidocaine; 20 = nifedipine.

mobile phase. The recovery of BSA in 40% acetonitrile was noticeably lower, and the column for aqueous size-exclusion chromatography, TSK G20000SW, was not an exception. This suggests that the denaturation of BSA was not caused by a particular stationary phase structure, but by the mobile phase. The results indicate that the organic solvent content in mobile phase should be limited to be 30% or less, if

acetonitrile is to be used as an organic solvent. Pinkerton *et al.* [2] suggested that the organic solvent content should not exceed 25% acetonitrile, 20% isopropanol and 10% tetrahydrofuran. The mobile phase composition is limited when RARP materials are to be used for serum analysis.

Retention selectivity for drugs

Fig. 3 shows the retention of drugs on the RARP columns (The structures of the drugs are shown in Fig. 4). The stationary phases are arranged in order of increasing hydrophobicity. The stationary phase with greater hydrophobicity generally gives the larger retention for neutral compounds (Nos. 15 and 20), with SHRP-PE being an exception. Compounds with a carboxyl group (Nos. 4 and 14), however, were retained on LC-HISEP and on ISRP-amide much more than expected on the basis of hydrophobicity.

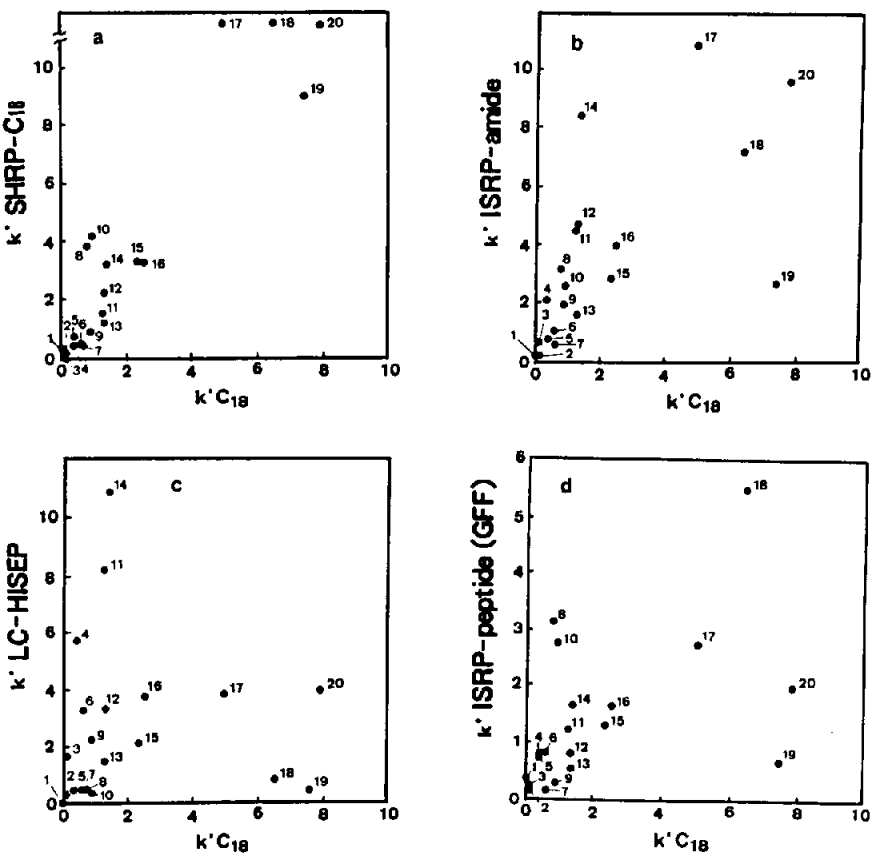

Fig. 5. Selectivity of RARP materials relative to C_{18} packing material. Acetonitrile concentration of mobile phase: C_{18} = 40%; ISRP-peptide (GFF) = 20%; other RARP packing materials = 30%. The mobile phase contained 0.02 *M* phosphate buffer and 0.1 *M* sodium sulfate (pH 7). Solute number as indicated in Fig. 4.

The log k' values on SHRP-C_{18}, ISRP-peptide (GFF), LC-HISEP and ISRP-amide are compared with those on an ordinary C_{18} column in Fig. 5. The retention selectivities of RARP materials prepared via acid decomposition of the bonded phase, SHRP-C_{18}, as well as C_8, E2 and E3, were relatively similar to that of ordinary C_{18}, as shown in Fig. 5a.

ISRP-amide and LC-HISEP showed preferential retention of solutes with a carboxyl group (Nos. 14 and 4), as shown in Fig. 5b and c, while relatively little retention was seen with amino compounds (Nos. 8, 10, 18 and 19). The contribution of amino groups in the stationary phase should be responsible for these results. In contrast, the ISRP-peptide (GFF) phase showed selective retention of amino compounds (Nos. 8 and 10). This packing material is known to contain a carboxyl group in the peptide-bonded stationary phase, which exists as an anion at this pH [4–6]. These RARP materials showed widely different retention selectivity indicated by the scattered plots in Fig. 5b–d from ordinary C_{18}, presumably due to the presence of hydrophilic groups in the stationary phase. The difference in the range of k' value as well as the difference in selectivity provided by these RARP materials can increase the capability of RARP materials, because a limited range of mobile phases can be used with these packing materials to retain the native state of proteins in a sample, as mentioned earlier.

It has been pointed out that hydrophilic compounds were not well resolved from the early-eluting protein peak by using commercial ISRP-peptide (GFF) [7]. Fig. 6 shows the chromatograms for theophylline, phenylpropanolamine and barbital on ISRP-peptide (GFF), LC-HISEP, SHRP-C_{18} and C_8. ISRP-peptide (GFF) and LC-HISEP gave very small retention for these drugs under the present conditions. Although the quantification of theophylline and caffeine has been successfully achieved by using ISRP-peptide (GFF) [20,21], the optimization of the separation conditions would be easier with stationary phases showing greater retention. A newer peptide-bonded ISRP phase, ISRP-peptide (GFF)-II, has been reported to be more retentive than ISRP-peptide (GFF) [22]. As shown in Fig. 6, SHRP-C_8 and C_{18} gave adequate retention for these compounds in the presence of 5% acetonitrile, although the peaks were somewhat broader on these phases.

When hydrophobic drugs were chromatographed, the ISRP-peptide (GFF) column gave fast separation of dipyridamole, nifedipine and imipramine in 20% acetonitrile, while SHRP-C_{18} showed prolonged retention, as shown in Fig. 7. Because the recovery of proteins tend to decrease with acetonitrile content at 40% or higher, the RARP materials with high hydrophobicity cannot conveniently be used for these drugs. These are just a few examples of the advantages of having a variety of RARP materials with different retention properties.

Fig. 8 shows another example to illustrate the advantage of the availability of various types of stationary phases. The two RARP columns with similar hydrophobicity can provide different selectivity. When one phase fails to give separation, still another phase is available. These RARP materials with intermediate hydrophobicity seem to be applicable for a wide range of compounds. The acid decomposition method for the preparation of RARP materials can produce a variety of phases from RPLC phases currently available.

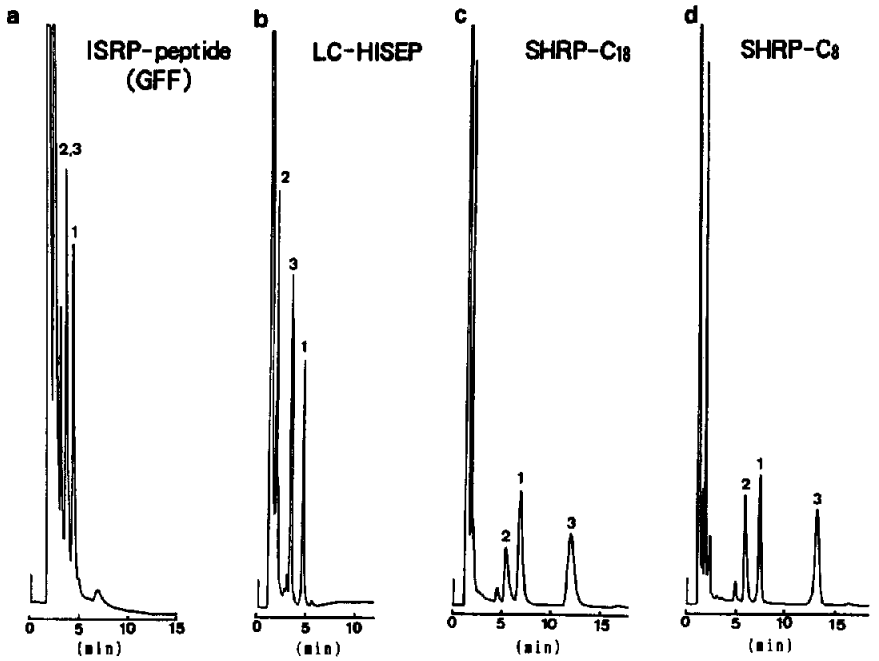

Fig. 6. Chromatograms of hydrophilic drugs injected with 20 μl of human serum. (a) ISRP-peptide (GFF), (b) LC-HISEP, (c) SHRP-C_{18}, (d) SHRP-C_8. Solute: 1 = theophylline; 2 = phenylpropanolamine; 3 = barbital. Mobile phase: (a) and (b), 0.02 *M* phosphate buffer + 0.1 *M* sodium sulfate (pH 7); (c) and (d) mobile phase for (a) and (b) + 5% acetonitrile. Flow-rate: 1.0 ml/min. Wavelength: 254 nm.

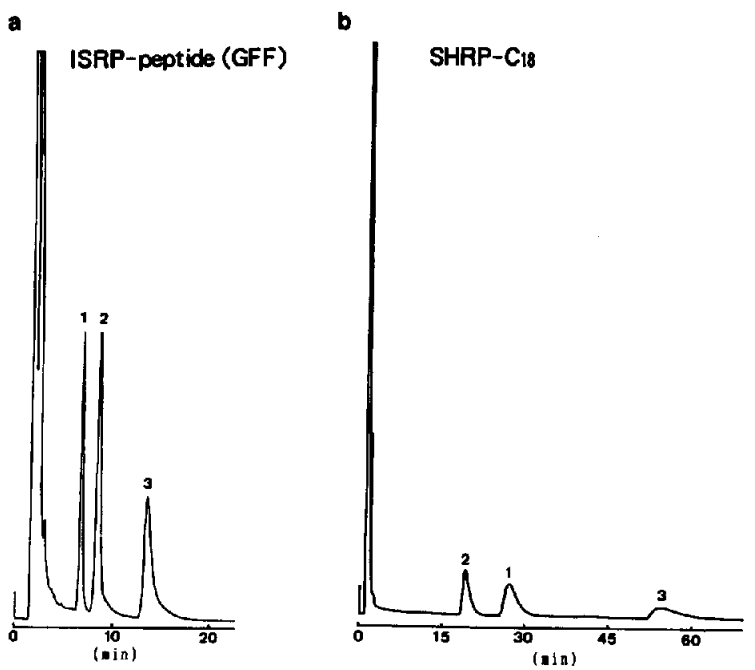

Fig. 7. Chromatograms of hydrophobic drugs injected with 20 μl of human serum. (a) ISRP-peptide (GFF), (b) SHRP-C_{18}. Mobile phase: (a) = 20% acetonitrile–0.02 *M* phosphate buffer + 0.1 *M* sodium sulfate; (b) = 30% acetonitrile–0.02 *M* phosphate buffer + 0.1 *M* sodium sulfate. Solute: 1 = dipyridamole; 2 = nifedipine; 3 = imipramine. Flow-rate: 1.0 ml/min. Wavelength: 254 nm.

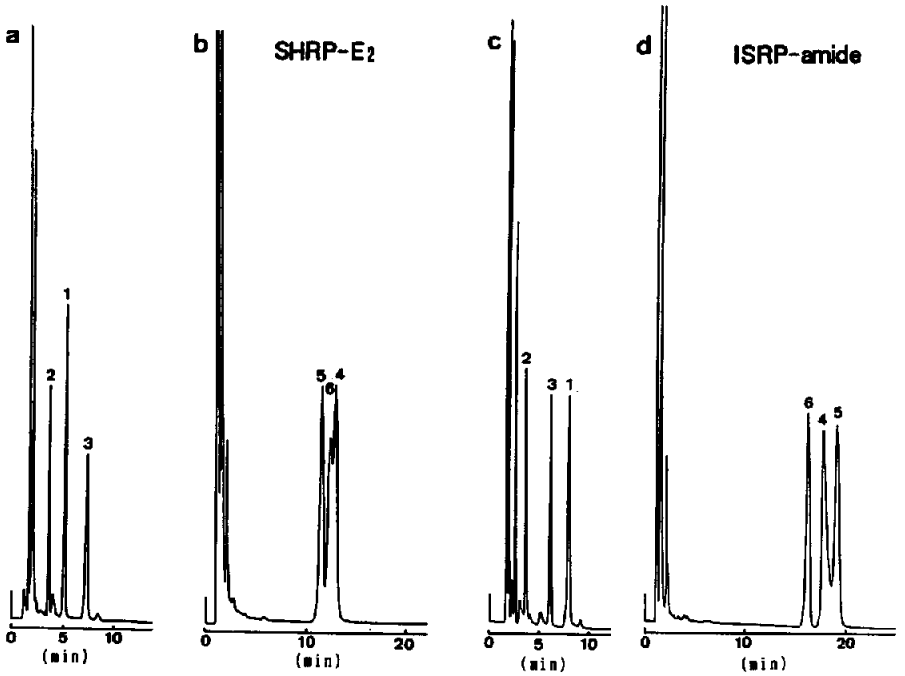

Fig. 8. Drug separation using (a and b) SHRP-E2 and (c and d) ISRP-amide. Mobile phase: (a) 5% acetonitrile–0.02 M phosphate buffer + 0.1 M sodium sulfate, (b and d) 30% acetonitrile–0.02 *M* phosphate buffer + 0.1 *M* sodium sulfate, (c) 0.02 *M* phosphate buffer + 0.1 *M* sodium sulfate. Solutes (injected with 20 μl of human serum): 1 = theophylline; 2 = phenylpropanolamine; 3 = barbital; 4 = dipyridamole; 5 = nifedipine; 6 = imipramine. Flow-rate: 1.0 ml/min. Wavelength: 254 nm.

CONCLUSIONS

The hydrophobicity of RARP materials was found to be as follows: SHRP-C_{18} > SHRP-C_8 > SHRP-E2 ≈ E3 ≈ ISRP-amide > LC-HISEP > ISRP-peptide (GFF). The order of extent of retention was roughly the same. In order to achieve complete recovery of proteins, and also to elute analytes in a proper k' range, hydrophobic RARP materials should be used for the separation of hydrophilic drugs, and hydrophilic stationary phases for hydrophobic drug analysis. Those with intermediate hydrophobicity can be used for both hydrophobic and hydrophilic compounds. The availability of RARP materials with a wide range of hydrophobicity and with different selectivity will increase the separation capability of RARP stationary phases in the analysis of biological samples. Further addition of RARP materials is expected [22].

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