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Method for the preparation of internal-surface reversed-phase packing materials starting from alkylsilylated silica gels

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ABSTRACT

A simple method for the preparation of internal-surface reversed-phase (ISRP) packing materials was developed. Partial decomposition of alkylsilylated silica gel with an aqueous acid followed by the introduction of diol functionalities produced stationary phases possessing the properties of ISRP packing materials. The method is applicable to various types of reversed-phase packing materials irrespective of the structure of the bonded alkyl moieties. The alkyl/diol-type packing materials possess greater hydrophobic properties than other ISRP packing materials currently available for high-performance liquid chromatography. Reversed-phase separation of low-molecular-weight compounds on the alkyl/diol phase by direct injection of serum samples is demonstrated. The present packing materials prepared from relatively large particles with or without the introduction of the diol phase can also be used for open-column chromatography in the reversed-phase mode with a wide range of aqueous–organic mobile phases, as the external surfaces are wettable with water.

INTRODUCTION

Internal surface reversed-phase (ISRP) packing materials have been introduced to effect the analyses of drugs in serum or plasma by direct injection of samples[1,2]. The packing materials possess hydrophilic external surfaces and hydrophobic internal surfaces. High-molecular-weight proteins are eluted at the void volume from a column containing these packing materials without interaction with hydrophobic internal surfaces owing to steric exclusion from relatively small pores. Low-molecular-weight

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compounds permeable into the pores can be separated in a reversed-phase mode. These packing materials can therefore accept the direct injection of serum or plasma without deproteinization of samples.

Currently available ISRP packing materials are prepared by the cleavage of a hydrophobic stationary phase on the external surface of the particles by the use of enzymes[1-3] or an oxygen plasma[4], or by the introduction of a hydrophilic polymer network[5] or a protein[6] over the hydrophobic surfaces, to prevent hydrophobic interactions between proteins and the stationary phase.

Methods for the preparation of ISRP materials involving enzymatic cleavage of bonded groups at the external surfaces of the particles can be applied to silica particles with relatively small pores, typically 80 Å or less, and require certain functional groups such as an amide group in the hydrophobic stationary phase in order to be cleaved by enzymes. Methods of preparation using an oxygen plasma to remove the hydrophobic stationary phase from the external surface or the introduction of hydrophilic polymers over the hydrophobic surface are not limited with respect to the structure of bonded groups. As one of the advantages of reversed-phase liquid chromatography (RPLC) is the availability of a variety of packing materials, a simple preparation method that can afford various types of stationary phases would be of value.

We report here a simple method for the preparation of ISRP packing materials, including the partial decomposition of alkylsilylated silica stationary phases. The method permits the preparation of ISRP packing materials containing various alkyl chain lengths from C_1 to C_{18} and aromatic groups. The application of the resulting packing materials to the separation of drugs in serum and for open-column chromatography is demonstrated.

EXPERIMENTAL

Equipment

The HPLC system consisted of an LC-6A pump, SIL-6A autoinjector, SPD-6A UV detector and CR-5A data processor (all from Shimadzu, Kyoto, Japan). The column temperature was maintained at 30°C with a water-bath. A Model 101 UV detector (Nacalai Tesque, Kyoto, Japan) was used to monitor the effluent in open-column liquid chromatography.

Materials

Silica gel particles, Develosil (particle size 5 μm, pore size 55.7 Å, surface area 427 m^2/g) (Nomura Chemicals, Seto, Japan) and MS gel (75 μm, 120 Å, 296 m^2/g) (Dohkai Chemicals, Fukuoka, Japan), were used for the preparation of packing materials for HPLC and for open-column chromatography, respectively.

An ISRP column with bonded phenylalanine oligomers (abbreviated to ISRP-peptide), reported by Hagestam and Pinkerton[1], was purchased from Koken (Tokyo, Japan). Other chemicals, including glycyrrhizic acid (Nacalai Tesque) and bovine serum albumin (Sigma, St. Louis, MO, U.S.A.), and LC-grade solvents were obtained commercially.

Preparation of ISRP packing materials

Silica gel particles were treated with 6 *M* hydrochloric acid at reflux temperature

for 4 h prior to a bonding reaction to remove metal impurities[7,8]. Alkylsilylation was achieved by reaction of the silica particles with alkyldimethylchlorosilanes, such as octadecyldimethylchlorosilane or octyldimethylchlorosilane, in toluene at reflux temperature for 6 h in the presence of pyridine. The details of the bonding and end-capping were given previously[9].

Alkylsilylated silica gels (10 g) were hydrolysed with 200 ml of concentrated hydrochloric acid at 100°C with rapid mechanical stirring (*ca.* 1000 rpm) for 5 h. The surface coverage of the resulting packing materials or the extent of the decomposition was examined chromatographically, by regularly removing the particles during the reaction and testing the retention characteristics of packed columns. After the reaction, the particles were washed successively with distilled water, tetrahydrofuran (THF) and chloroform.

In one instance, 0.8 g of octadecanol was loaded onto 4 g of C_{18} phase prior to the decomposition. In another instance, the particles were washed with THF at 5-h intervals during the decomposition process in order to wash out the hydrophobic decomposition products from the stationary phase.

Introduction of a diol functionality in the partially decomposed stationary phases was achieved by reaction of the partially hydrolysed RPLC packing materials with glycidylpropyltrimethoxysilane in water[10].

Bonding of the diol phase is not necessary for the packing materials to be used for open-column chromatography, unless samples which are expected to interact with silanols, such as polypeptides, are to be separated. Therefore, partially decomposed C_{18} phase (C_{18}-CLC) was used for open-column chromatography without bonding the diol phase.

A slurry method was used to pack stainless-steel columns (150 mm × 4.6 mm I.D.) for HPLC. Glass columns of 20 mm I.D. were packed with 75-μm particles by a gravity method using a slurry containing 30 g of packing material in 300 ml of mobile phase solvents.

Chromatographic measurements

UV detection at 280 nm was used for bovine serum albumin and at 254 nm for other compounds. Recoveries of proteins were examined by measuring the UV absorption, taking the recovery in the absence of a column as 100%.

RESULTS AND DISCUSSION

Preparation of ISRP packing materials

The proposed method for the preparation of ISRP packing materials includes the reaction of chemically bonded phases such as octadecyl- or octylsilylated silica gels with aqueous hydrochloric acid at reflux temperature for several hours. Fig. 1 shows the decomposition of a C_{18} stationary phase in 70% methanol in the presence of acids. The packing materials are wettable by the reaction medium in this instance. Decomposition of the stationary phase is achieved more effectively by using hydrochloric acid than with other acids. The hydrophobicity of the packing materials, expressed by the retention of toluene in 60% methanol on the resulting stationary phase, decreased rapidly in the early part of the reaction, then levelled off.

Similar results were obtained in the decomposition reaction in concentrated

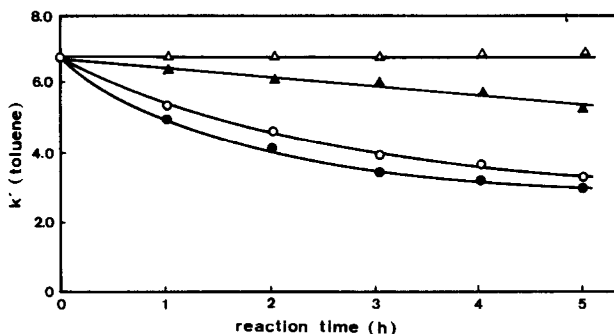

Fig. 1. Decomposition of stationary phase with acid in 70% methanol. Remaining retentivity is expressed by the k' value of toluene with each packing material in 60% methanol. Reaction mixtures contained (△) phosphoric acid, (▲) sulphuric acid, (○) nitric acid or (●) hydrochloric acid, at a concentration of 6 *N*.

hydrochloric acid in the absence of methanol, as shown in Fig. 2. The heterogeneous decomposition reaction gave a C_{18} phase with about 60% of the original carbon content at 5 h, without much decrease in carbon content thereafter. Washing of the reacting C_{18} phase with THF at 5-h intervals during the decomposition resulted in stationary phases with much lower carbon contents. These results indicate that the decomposition product in an aqueous reaction medium can protect the remainder of the stationary phase from further decomposition. The slower hydrolysis reaction in the presence of octadecanol supports this interpretation. This implies that it is possible to produce stationary phases with various surface coverages. It is known that many factors, including how the silicas are made, influence the stability of bonded phases[11]. Assuming similar surface chemistries of silicas at external and internal surfaces, the alkylsilyl groups at the external surfaces appeared to undergo decomposition more

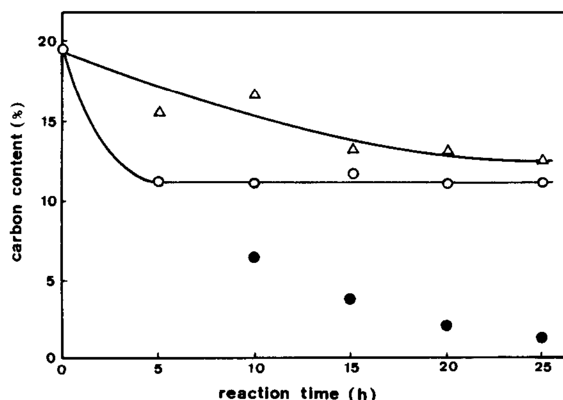

Fig. 2. Decrease in carbon content of C_{18} packing materials with decomposition in concentrated hydrochloric acid, (○) without washing and (●) with THF washing at 5 h intervals and (△) in the presence of octadecanol without washing.

TABLE I

EFFECT OF SURFACE COVERAGE AND SILICA PORE SIZE ON THE PLANARITY RECOGNITION OF THE C_{18} PHASE

Stationary phase	*Surface coverage ($\mu mol/m^2$) [carbon content, C(%), in parentheses]*	*Planarity recognition, $\alpha_{T/O}$[a]*	*Decomposed stationary phase*	*Surface coverage, C(%)*	*Planarity recognition, $\alpha_{T/O}$[a]*
C_{18}-1[b]	3.2(19.17)	1.58	A-5[e]	15.33	1.50
C_{18}-2[b]	3.0(18.30)	1.52	A-10[e]	16.60	1.57
C_{18}-3[b]	2.9(17.79)	1.44	A-15[e]	13.18	1.59
C_{18}-4[b]	2.2(14.26)	1.33	A-20[e]	13.04	1.60
			A-25[e]	12.66	1.50
C_{18}-50[c]	2.0(17.06)	1.61	B-5[f]	11.19	1.54
C_{18}-60[d]	2.2(19.39)	1.58	B-10[f]	11.14	1.55
C_{18}-100[c]	2.6(16.94)	1.51	B-15[f]	11.73	1.59
C_{18}-300[c]	3.4(7.37)	1.45	B-20[f]	11.15	1.57
C_{18}-500[c]	2.6(2.09)	1.42	B-25[f]	11.11	1.58
			C-5[g]	11.19	1.54
			C-10[g]	6.39	1.58
			C-15[g]	3.58	1.64
			C-20[g]	2.13	1.72
			C-25[g]	1.32	1.93

[a] Retention ratio, $k'_{triphenylene}/k'_{terphenyl}$, indicating the planarity recognition of the stationary phases.
[b] Prepared by alkylsilylation of silica gel of 100 Å pore size to give the surface coverages listed.
[c] Nucleosil particles of different pore size were used for alkylsilylation to produce C_{18} phase with maximum coverage.
[d] Starting C_{18} phase prepared from silica of 60 Å pore size for decomposition reaction in concentrated HCl.
[e] Decomposition product in the presence of octadecanol shown in Fig. 2. The numbers indicate the reaction time (h).
[f] Decomposition product with HCl without other additives.
[g] The decomposition product was washed with THF at 5-h intervals starting from B-5.

easily than those at the internal surfaces. Note that the products having 60% of the original carbon content are wettable by water, whereas a C_{18} phase prepared with carbon contents of about 10–12% were not wettable by water or 40% methanol.

The results in Table I also indicate that the remaining alkylsilyl groups exist in relatively small pores. The planarity recognition by the C_{18} chains in the stationary phase, which can be indicated by the separation factor, α, between planar triphenylene and non-planar *o*-terphenyl[12,13], increases with increase in the surface coverage and with decrease in the pore size with ordinary C_{18} phases prepared by the alkylsilylation of silica gel, as shown in Table I. If the decomposition of a bonded phase takes place at similar rates at both the external and internal surfaces, the $\alpha_{T/O}$ values would decrease as the carbon contents decrease. However, the partially decomposed C_{18} phases obtained by acid treatment of the bonded phases gave $\alpha_{T/O}$ values much larger than C_{18} phases prepared with similar carbon contents. This tendency became pronounced as the decomposition reaction proceeded further. The results imply that the decomposition of a bonded phase takes place more slowly in smaller pores, probably owing to the

TABLE II

CARBON CONTENTS OF THE ISRP PACKING MATERIALS AND THE INTERMEDIATES

Stationary phase	*Carbon content, C(%)*				
	Before end-capping[a]	*(Surface coverage, μmol/m²)*	*End-capped*[b]	*Decomposed*[c]	*C_{18}/diol*
ISRP C_{18}/diol	18.81	(2.4)	19.39	11.19	14.95
ISRP C_8/diol	13.27	(3.2)	14.01	6.79	9.06
ISRP C_1/diol	5.37	(3.9)	–	0.20	5.34
ISRP PE/diol	13.77	(3.3)	14.33	5.85	8.62
ISRP CLC	–	–	18.64	9.95	–

[a] Alkylsilylated silica gel.
[b] After end-capping, prior to decomposition.
[c] After decomposition, prior to bonding diol phase.

protection of the bonded phase by the presence of the decomposition product during the preparation of the present ISRP packing material.

Another possible explanation is the presence of air bubbles in small pores that hinder the contact between the alkylsilylated silica surface and the reaction medium. These factors seemed to lead to a faster decomposition of the stationary phases in larger pores.

Table II lists the carbon contents at each stage of the preparation of the ISRP packing materials. The original C_{18} phase (C_{18}-60) showed a lower surface coverage than stationary phases with shorter alkyl groups owing to the small pore size of 60 Å. The packing materials with smaller alkyl groups, however, lost more alkylsilyl groups on acid treatment, presumably owing to the lower hydrophobicity of the stationary phase.

TABLE III

RETENTION OF BENZENE DERIVATIVES ON ISRP PACKING MATERIALS

Stationary phase	*k' ($\alpha = k'_x/k'_{benzene}$)*			
	Acetophenone	*Methyl benzoate*	*Benzene*	*Toluene*
ISRP C_1/diol	0.44 (1.02)	0.45 (1.04)	0.43	0.46 (1.07)
ISRP C_8/diol	4.03 (0.15)	9.19 (1.39)	6.62	15.29 (2.31)
ISRP PE/diol	3.38 (0.31)	5.92 (1.86)	3.18	6.38 (2.01)
ISRP C_{18}/diol	4.52 (0.15)	11.62 (0.39)	29.49	49.26 (1.67)
ISRP-peptide[a]	0.74 (0.80)	0.95 (1.02)	0.93	1.16 (1.26)

[a] ISRP-peptide phase described by Hagestam and Pinkerton[1]. Mobile phase: 40% methanol.

TABLE IV

EFFECT OF MOBILE PHASE COMPOSITION ON THE RECOVERY OF BSA[a]

Mobile phase	*ISRP C_{18}/diol*	*ISRP-peptide*[b]
Organic solvent[c]:		
None	87.4	97.7
CH_3CN, 10%	91.4	95.5
CH_3CN, 20%	96.2	97.0
CH_3CN, 30%	95.9	96.8
CH_3OH, 10%	93.6	96.7
CH_3OH, 20%	98.4	97.1
THF, 10%	99.5	97.5
THF, 20%	98.4	97.7
10% isopropanol + 6% THF	99.7	97.5
10% isopropanol + 6% THF[d]	100.2	98.4
pH[e]:		
2.76	81.1	0.6
3.77	75.6	0.9
4.41	86.2	0.9
4.91	86.9	1.1
6.02	93.7	67.7
7.00	95.3	96.8
Na_2SO_4(mol/l)[f]:		
0.05	98.8	98.2
0.1	97.7	98.7
0.2	98.7	99.2
0.3	97.3	97.5

[a] Determined by UV absorbance at 280 nm. Sample: BSA (20 mg/ml), 100-μl injection.
[b] ISRP-peptide phase described by Hagestam and Pinkerton[1].
[c] Mobile phase: organic solvent–0.02 *M* phosphate buffer + 0.1 *M* Na_2SO_4 (pH 7).
[d] Mobile phase: organic solvent–0.1 *M* phosphate buffer (pH 7).
[e] Mobile phase: 15% acetonitrile–0.02 *M* phosphate buffer + 0.1 *M* Na_2SO_4.
[f] Mobile phase: 15% acetonitrile–0.02 *M* phosphate buffer + Na_2SO_4 (pH 7).

Properties of alkyl/diol-type ISRP packing materials

Whereas ordinary stationary phases are not wettable by methanol–water mixtures with low methanol contents, the alkyl/diol-type ISRP packing materials and also partially decomposed alkylsilylated silica gels are completely wettable by water. This property is required when RPLC packing materials are to be applied in open-column chromatography, because non-wettable packing materials have problems with regard to bed stability in highly aqueous mobile phases.

The hydrophobicities of the present alkyl/diol-type ISRP packing materials are much greater than that of an ISRP packing material with bonded phenylalanine oligomers (ISRP-peptide), as indicated by the retention of benzene derivatives in Table III. The retention of these benzene derivatives on the ISRP packing materials are about 50% of those on ordinary stationary phases with maximum surface coverage when measured in 60% methanol, in fair agreement with the carbon contents after the decomposition reaction.

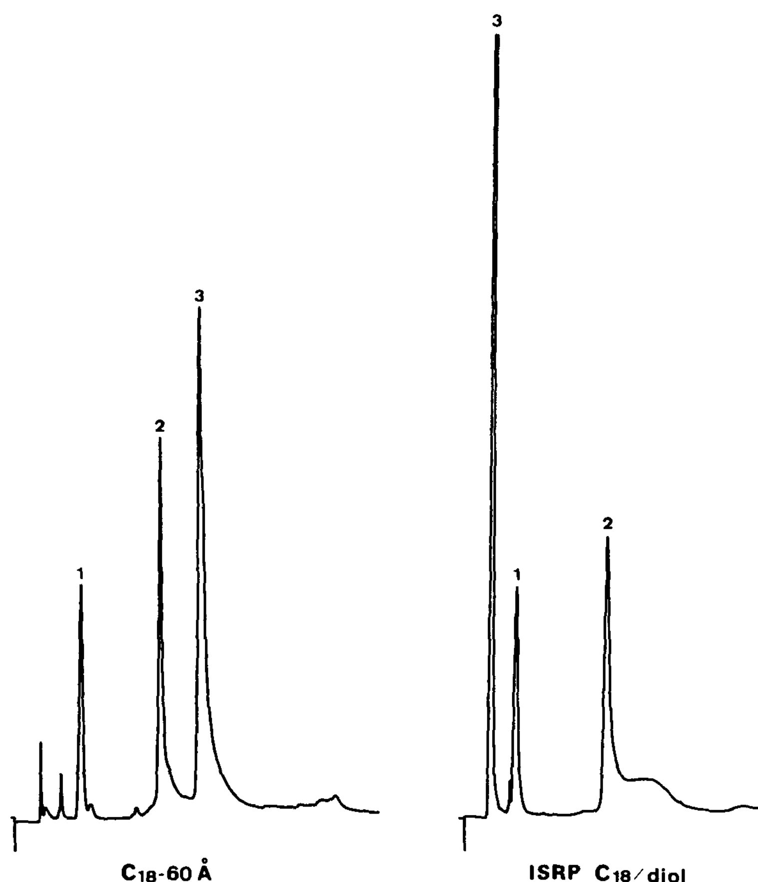

Fig. 3. Gradient elution of polypeptides with ISRP C_{18}/diol and ordinary C_{18} phase prepared from silica gel of 60 Å pore size. 1, α-Endorphin (1 mg/ml, 8 μg); 2, insulin (3 mg/ml, 9 μg); 3, bovine serum albumin (20 mg/ml, 200 μg). HPLC conditions: mobile phase, (A) 20% acetonitrile–0.02 *M* phosphate buffer containing 0.05 *M* sodium sulphate (pH 6), (B) 60% acetonitrile–0.02 *M* phosphate buffer containing 0.05 *M* sodium sulphate (pH 6) with a linear gradient from A to B in 10 min. Flow-rate, 1.0 ml/min; detection, UV (280 nm).

Injection of proteins

Table IV shows the recovery of BSA from the ISRP C_{18} and the ISRP-peptide phases. As the newly prepared ISRP alkyl/diol-type phases showed a low recovery of BSA, 10 mg of BSA were applied to the column prior to testing the recovery. Although the recovery from ISRP C_{18} was slightly lower than those from the ISRP-peptide phase with mobile phases with low contents of acetonitrile or methanol at pH 7, comparable results were obtained with mobile phases containing 10–20% or more organic solvents. Injection of BSA, 2 mg at a time, in 0.1 *M* phosphate buffer–isopropanol–THF (84:10:6) at pH 7.4, onto an ordinary C_{18} phase (C_{18}-60 in Table I) resulted in a protein recovery of less than 2% until 50 mg of BSA had been injected. The recovery increased slightly thereafter, with a simultaneous increase in the column back-pressure.

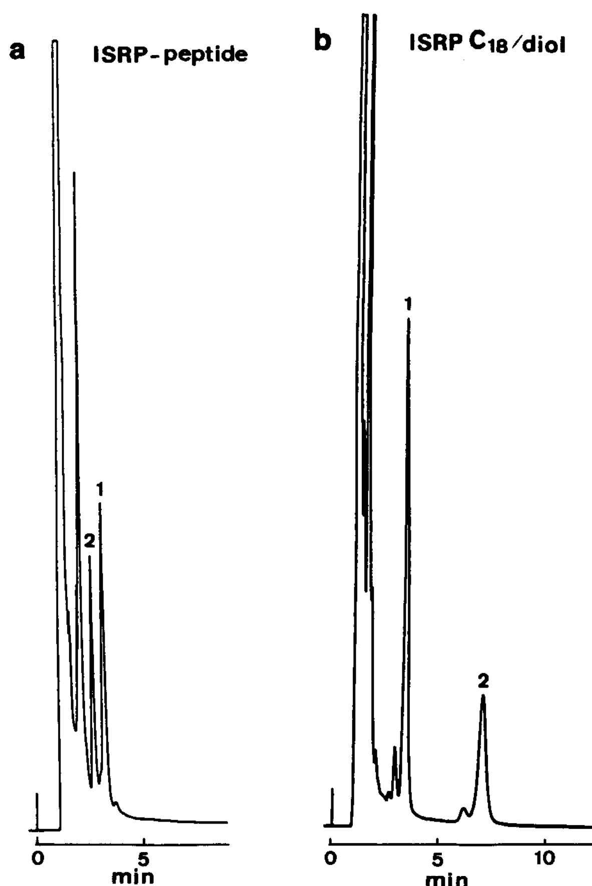

Fig. 4. Elution of human serum spiked with hydrophilic drugs using (a) ISRP-peptide phase[1] and (b) ISRP C_{18}/diol. 1, Theophylline (1 mg/ml, 1 μg); 2, barbital (1 mg/ml, 3 μg) with human serum (20 μl). HPLC conditions: (a) mobile phase 0.02 *M* phosphate buffer containing 0.1 *M* sodium sulphate (pH 7), flow-rate 1.0 ml/min, UV detection at 254 nm; (b) mobile phase 10% acetonitrile–0.02 *M* phosphate buffer containing 0.1 *M* sodium sulphate (pH 7).

At acidic pH the present ISRP materials showed much higher protein recoveries. This is due to the presence of carboxyl groups in the stationary phase in the phenylalanine oligomer-bonded phase[3]. The ionic strength of the mobile phase did not affect the protein recovery.

Fig. 3 shows that most BSA (85%) eluted from ISRP C_{18} without interacting with the hydrophobic part of the stationary phase, while smaller peptides were chromatographed in the reversed-phase mode. On the ordinary C_{18} phase, the three peptides eluted according to the molecular weight of the solutes under an acetonitrile gradient.

Fig. 4 shows the results of the injection of serum samples spiked with theophylline and barbital. Whereas the ISRP-peptide phase showed a small retention for these compounds possessing relatively small hydrophobicities, with a mobile phase not containing an organic solvent, ISRP C_{18}/diol provided an adequate separation

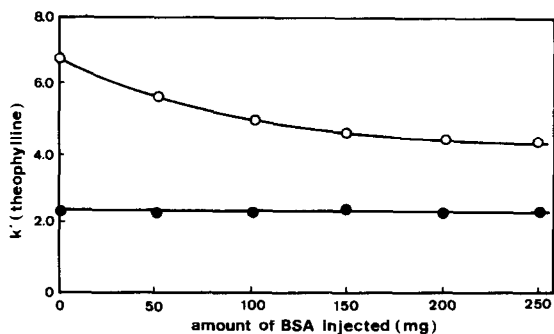

Fig. 5. Effect of BSA injection on retention of theophylline. The k' value of theophylline in 10% methanol is compared for (●) ISRP C_{18} and (○) C_{18}-60. The k' value was measured following each injection of 50 mg of BSA dissolved in 2.5 ml of distilled water onto each column.

with much longer retention with a mobile phase containing 10% acetonitrile. The greater retentivity of the alkyl/diol phases can be an advantage in such an instance.

Fig. 5. shows the variation of the retention of low-molecular-weight solutes with the injection of BSA. Whereas the ordinary C_{18} phase showed a gradual decrease in retention, the ISRP C_{18} phase showed a constant retention with up to 250 mg of BSA injected, which corresponds roughly to the total injection of 5 ml of serum. Constant k' values for theophylline and barbital were observed on ISRP C_{18}/diol in 20% acetonitrile with the injection of up to a total of 2.5 g of BSA. The experimental conditions are different from actual repeated injections of serum samples in small

TABLE V

WETTABILITY OF REVERSED-PHASE PACKING MATERIALS BY METHANOL–WATER MIXTURES

+ = Completely wettable; − = non-wettable; ± = partially wettable.

Methanol content (%)	C_{18}-CLC	C_{18}[a]	C_{18}-P[b]	C_8[a]	C_1[a]
0	+	−	−	−	−
10	+	−	−	−	−
20	+	−	−	−	−
30	+	−	−	−	−
40	+	−	−	−	−
50	+	−	±	−	−
60	+	−	+	−	±
70	+	+	+	+	+
80	+	+	+	+	+
90	+	+	+	+	+
100	+	+	+	+	+

[a] Maximum surface coverage.
[b] Carbon content *ca.* 12% with end-capping.

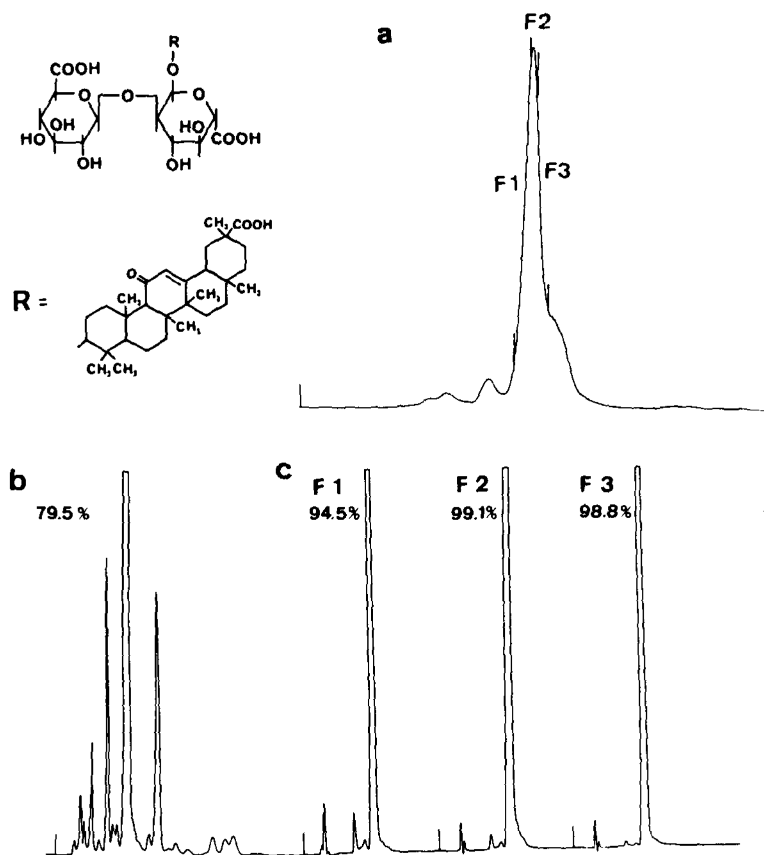

Fig. 6. Purification of glycyrrhizic acid using C_{18}-CLC packing material. (a) 20 mg of glycyrrhizic acid in 0.5 ml of mobile phase were charged. The three fractions, F1, F2 and F3, were collected from the effluent, then examined for purity by injecting into a Cosmosil C_{18} RPLC column (Nacalai Tesque) with 40% acetonitrile containing 0.1% trifluoroacetic acid as mobile phase at a flow-rate of 1.0 ml/min. (b) Prior to purification. (c) Elution of fractions F1, F2 and F3. Purity was based on the peak area with UV detection at 254 nm.

amounts. Nonetheless, the results indicate that the present ISRP materials can accept the direct injection of proteins.

Use of ISRP packing materials in open-column chromatography

Conventional RPLC packing materials are not wettable by methanol–water mixtures containing less than 60–70% of methanol. Such mobile phases cannot be used in open-column chromatography. The present ISRP packing materials with or without a diol phase is wettable by a wide range of water–organic solvent mixtures, as shown in Table V. This permits their use in open-column chromatography without the problem of disintegration of the column bed. Open-column chromatography in the reversed-phase mode will be useful when the normal-phase mode is not effective. An example is shown in Fig. 6 for the purification of glycyrrhizic acid.

By using a 20 mm I.D. column with a 30 cm bed height packed with 75-μm particles of C_{18}-CLC, the purity of glycyrrhizic acid was increased from 80% to higher than 99%. The diol bonding reaction after the decomposition of the alkyl phase is not necessary, unless solutes that are sensitive to the presence of silanols such as polypeptides and hydrogen-bond acceptors are to be chromatographed.

CONCLUSION

A simple method for the preparation of ISRP packing materials was developed by utilizing the partial decomposition of alkylsilylated stationary phases in an acidic medium followed by the introduction of a diol phase. The acid decomposition proceeded rapidly at the external surface and in the larger pores of silica particles than in smaller pores owing to the difference in the accessibility of the reagent. The method can be applied to the preparation of various types of stationary phases for a wide range Sof applications. The resulting ISRP phases can be used for the determination of drugs in serum by eluting large proteins at the void volume. The simple method also permits the preparation of packing materials for open-column reversed-phase chromatography and possibly for thin-layer chromatography with a wide range of water–organic solvent mixtures.

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REFERENCES

1 I. H. Hagestam and T. C. Pinkerton, *Anal. Chem.*, 57 (1985) 1757.
2 I. H. Hagestam and T. C. Pinkerton, *J. Chromatogr.*, 351 (1986) 239.
3 J. Haginaka, N. Yasuda, J. Wakai, H. Matsunaga, H. Yasuda and Y. Kimura, *Anal. Chem.*, 61 (1989) 2445.
4 Y. Sudo, R. Miyagawa and Y. Takahata, *Chromatography*, 9, No. 2 (1988) 179.
5 H. Yoshida, I. Morita, G. Tamai, T. Masujima, T. Tsuru, N Takai and H. Imai, *Chromatographia*, 19 (1984) 466.
6 D. J. Gisch, B. T. Hunter and B. Feibush, *J. Chromatogr.*, 433 (1988) 264.
7 M. Verzele, M. De. Potter and J. Ghysels, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 2 (1979) 151.
8 Y. Ohtsu, Y. Shiojima, T. Okumura, J. Koyama, K. Nakamura, O. Nakata, K. Kimata and N. Tanaka, *J. Chromatogr.*, 481 (1989) 147.
9 K. Jinno, S. Shimura, N. Tanaka, K. Kimata, J. C. Fetzer and W. R. Biggs, *Chromatographia*, 27 (1989) 285.
10 F. E. Regnier and R. Noel, *J. Chromatogr. Sci.*, 14 (1976) 316.
11 N. Sagliano, Jr., T. R. Floyd, R. A. Hartwick, J. M. Dibussolo and N. T. Miller, *J. Chromatogr.*, 443 (1988) 155.
12 N. Tanaka, Y. Tokuda, K. Iwaguchi and M. Araki, *J. Chromatogr.*, 239 (1982) 761.
13 K. Jinno, T. Nagoshi, N. Tanaka, M. Okamoto, J. C. Fetzer and W. R. Biggs, *J. Chromatogr.*, 392 (1987) 75.