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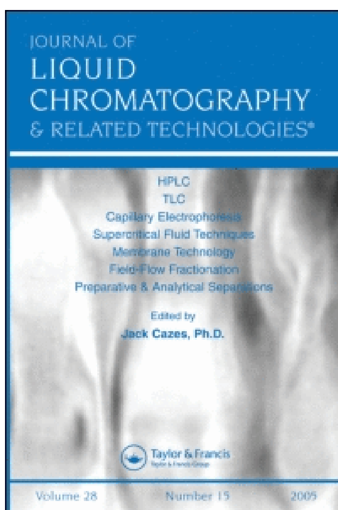

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Glycerylalkylsilylated Silica Gels for Direct Injection Analysis of Drugs in Serum by High-Performance Liquid Chromatography

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GLYCERYLALKYLSILYLATED SILICA GELS FOR DIRECT INJECTION ANALYSIS OF DRUGS IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A new packing material for direct injection analysis of drug in serum by high-performance liquid chromatography is described. It was found out that proteins are not adsorbed but low-molecular-weight compounds are analyzed by reversed-phase high-performance liquid chromatography using glycerylalkylsilylated silica gels with alkyl moiety ranging from C3 to C6. On chromatography using glycerylundecylsilylated silica gel, on the other hand, retention of low-molecular-weight compounds increased but recovery of proteins decreased from it. Reversed-phase separation of drugs on the new packing materials by direct injection of serum samples is demonstrated.

INTRODUCTION

Drugs in serum are analyzed commonly by reversed-phase high-performance liquid chromatography, in advance of which the samples need time-consuming pretreatment for removing protein

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ingredients. Direct injection of serum samples has long been unfavorable because of resulting in quick deterioration of HPLC columns caused by the denaturation and accumulation of proteins in serum.

Recently developed were new packing materials, in which serum samples can be injected directly without any pretreatment[1-7]. They are called restricted access packing materials[8,9]. The stationary phases consist of two parts. One part, to which proteins can access, are hydrophilic against adsorption of proteins. The other part, shielded from access of proteins, is hydrophobic for partition of low-molecular-weight compounds. Owing to this structure, these packing materials selectively retain low-molecular-weight compounds, while proteins are eluted in the void volume with high recovery. In general, preparation of these packing materials is not so simple and consists of at least two reaction steps; all surface of silica gel is covalently covered with hydrophobic compounds, then hydrophilic feature is introduced only the external surface.

It is known, on the other hand, that glycerylpropylsilylated silica gel can be used for size exclusion chromatography of proteins without denaturation nor adsorption[10]. We have found out that glycerylalkylsilylated silica gels have the function of restricted access packing materials[11]. Because the glycerylalkylsilyl group consists of hydrophilic and hydrophobic moieties, it is expected to possess both functions as a surface barrier against access of proteins and as a partition phase for low-molecular-weight compounds. The present results suggest that the glycerylalkylsilyl stationary phases form bilayer structure (Fig. 1). Further, these packing materials have an advantage over ISRP from the preparative point of view, because they are prepared with one-step silylation. Here we report the details of their preparation and characteristics in HPLC.

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EXPERIMENTAL

Reagents and Materials

3-Buten-1-ol, 3-methyl-3-buten-1-ol, 5-hexen-1-ol, 10-undecene-1-ol, epichlorohydrin and dicyclohexyl-18-crow-6 were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). $H_2PtCl_6 \cdot 6H_2O$, K_2PtCl_4, Bovine serum albumin, phenobarbital, phenytoin and carbamazepine were obtained from Wako Pure Chemical Industries (Osaka, Japan). Trimethylsilane and 3-glycidoxypropylsilane were form Shinetsu Chemical (Tokyo, Japan). Silica gel was Develosile (particle size 5 μm, pour size 60 Å and surface area 500 m^2/g) from Norura Chemicals (Seto, Japan).

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Preparation of 4-Glycidoxybutyltrimethoxysilane

4-(2-Hydroxy-3-chlorobutoxy)-1-butene: To a mixture of 72.1 g (1 mol) of 3-buten-1-ol and 9.3 g (0.1 mol) of epichlorohydrin were added 12 drops of conc.H_2SO_4 at room temperature. The mixture was refluxed for 4 h and cooled to room temperature, and 1.2 g of $BaCO_3$ was added to it. The mixture was stirred for 30 min and 1 mol of chloroform was added to it. The mixture was filtered, evaporated and distilled under vacuum to give 17.2 g (0.1 mol, 100 %) of 4-(2-hydroxy-3-chlorobutoxy)-1-butene[12].

4-Glycidoxy-1-butene: To 500 ml of diethyl ether containing 22 g (0.55 mol) of powder NaOH was added gradually 55 g (0.33 mol) of 4-(2-hydroxy-3-chlorobutoxy)-1-buten and the suspenson was stirred for 15 h at room temperature. To the stirred suspension was added gradually 150 ml of water. The organic layer was washed by 300 ml of water, dried on $MgSO_4$ and evaporated. Vacuum distillation gave 28.6 g (0.22 mol, 67 %) of 4-glycidoxy-1-buten-e[13].

4-Glycidoxybutyltrimethoxysilane: To a solution of 15 g (0.12 mol) of 4-glycidoxy-1-butene in 10 ml of toluene was added 0.6 ml

of 1 % $H_2PtCl_6 \cdot 6H_2O$ in t-butanol at room temperature. A solution of 14.4 g (0.12 mol) of trimethoxysilane in 10 ml of toluene was added to the suspension. The suspension was refluxed for 1 h, evaporated and distilled under vacuum to give 11.9 g (0.05 mol, 42 %) of 4-glycidoxybutyltrimethoxysilane[14]. ^1H NMR (60 MHz) δ 0.67 (m, $SiCH_2$, 2H), 1.52 (m, CH_2, 4H), 2.65 (m, OCH_2, 2H), 3.05 (m, OCH, 1H), 3.47 (m, OCH_2, OCH_3, 13H).

Preparation of 4-Glycidoxy-2-methylbutyltrimethoxysilane

4-Glycidoxy-2-methylbutyltrimethoxysilane (13 %) was prepared form 3-methyl-3-buten-1-ol by the method similar to the preparation of 4-glycidoxybutyltrimethoxysilane. ^1H NMR (60 MHz) δ 0.66 (m, $SiCH_2$, 2H), 0.95 (d, CH3, 3H), 1.58 (m, CH, CH_2, 3H), 2.53 (m, OCH_2, 2H), 2.965 (m, OCH, 1H), 3.35 (m, OCH_2, OCH_3 13H).

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Preparation of 6-Glycidoxyhexyltrimethoxysilane

6-glycidoxy-1-hexene: 6-Glycidoxy-1-hexene (37 %) was prepared from 5-hexen-1-ol by the method similar to the preparation of 4-glycidoxy-1-butene

6-Glycidoxy-1-hexyltrimethoxysilane: To a mixture of 40 mg of K_2PtCl_4 and 200 mg of dicyclohexyl-18-crown-6 was added 20 ml of benzene. The mixture was refluxed for 2 h and allowed to stand for 16 h to give the catalyst. The catalyst was added to a mixture of 5 g (32 mmol) of 6-glycidoxy-1-hexene and 3.9 g (32 mmol) of trimethoxysilane at room temperature. The mixture was stirred at 100°C for 2 h, evaporated and distilled under vacuum to give 3.6 g (13 mmol, 41%) of 6-glycidoxyhexyltrimethoxysilane[15]. ^1H NMR (60 MHz) δ 0.65 (m, $SiCH_2$, 2H), 1.50 (m, CH_2, 8H), 2.67 (m, OCH_2, 2H), 3.08 (m, OCH, 1H), 3.50 (m, OCH_2, OCH_3, 13H).

Preparation of 11-Glycidoxyundecyltrimethoxysilane

11-Glycidoxy-1-undecyltrimethoxysilane (9.5 %) was prepared form 10-undecen-1-ol by the method similar to the preparation of 6-glycidoxyhexyltrimethoxysilane. ^1H NMR (60 MHz) δ 0.67 (m, $SiCH_2$, 2H), 1.40 (m, CH_2, 18H), 2.65 (m, OCH_2, 2H), 3.05 (m, OCH, 1H), 3.48 (m, OCH_2, OCH_3, 13H).

Preparation of Glycerylalkylsilylated Silica Gels

Silica gel (10 g) in a vial was dried under vacuum at 120°C for 2 h. To the silica gel was added 0.6 ml of water, the vial was sealed and allowed to stand for 24 h. To the silica gel was added 34 ml of toluene. To the stirred suspension was added 0.1 ml of 10 % p-toluenesulfonic acid in acetonitrile and 22 mmol of glycidoxyalkyltrimethoxysilane. The suspension was stirred at 120°C for 16 h, cooled, filtered and washed sequentially with 200 ml of toluene and 200 ml of acetone. To the silica gel was added 50 ml of 0.01 N H_2SO_4 in water. The suspension was refluxed for 1 h, cooled and washed with water. To the silica gel was added 50 ml of 10 mM phosphate buffer (pH 8.0). The suspension was refluxed for 1 h, cooled and washed sequentially with water, methanol and diethyl ether. The suspension was filtered and dried under vacuum at 60°C for 4 h to give glycerylalkylsilylated silica gel. In the case of glyceryhexcylslylation and glycerylundecylsilylation, the suspensions were refluxed for 3 h in each operation.

Apparatus

The prepared glycerylalkylsilylated silica gels were packed into stainless steel tubes (150 mm x 4.6 mm I.D.) by conventional high-pressure slurry-packing procedures.

The HPLC system consisted of a pump (LC-6A, Shimadzu, Kyoto, Japan), a UV detector (SPD-6A, Shimadzu), a data processor (CR-4A, Shimadzu) and a injector (model 7125, Rheodyne, U.S.A).

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RESULTS AND DISCUSSION

Surface Coverage of Glycerylalkylsilylated Silica Gel

Table 1 shows the surface coverage of the packing materials estimated from the value of carbon contents. The values of surface coverage fall between 2.03 and 3.00 $\mu mol/m^2$. These values suggest that the stationary phases on these silica gels are not polymeric[16] but are monomeric layers of glycerylalkylsilyl groups composed of hydrophilic and hydrophobic moieties.

Chromatography of Low-molecular-weight Compounds

Tables 2 and 3 show the capacity factors of aromatics (benzene and naphthalene) and drugs (phenobarbital and carbamazepine), respectively, on the glycerylalkylsilylated silica gels. The packing materials with a longer alkyl moiety gave larger capacity factor, and more hydrophobic compounds gave larger capacity factor. This result indicates that the low-molecular-weight compounds can be analyzed by reversed-phase chromatography.

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Recovery of BSA

Kimatas' method[6] was referred to for testing recovery of proteins from the packing materials. A dilute solution of BSA was used to detect slight differences among the packing materials. As the recovery of BSA from the columns increased with repeat number of injection, a newly prepared column was used in each run. Table 4 shows the results. The recovery was high from glycerylalkylsilylated silica gels with an alkyl moiety of C3 to C6. On the other hand, the recovery was very low from the packing material with an alkyl moiety of C11, indicating that there was a strong interaction between the hydrophobic undecyl moiety and BSA. When in contact with a highly aqueous mobile phase, the stationary phase with an alkyl moiety of C3 to C6 is

TABLE 1

Surface Coverage of Glycerylalkylsilylated Silica Gels

Stationary phase	Carbon contents (%)	Surface coverage (μmol/m^2)
Glycerylpropyl	8.35	3.00
Glycerylbutyl	8.82	2.69
Glyceryl-2-methylbutyl	8.86	2.32
Glycerylhexyl	11.1	2.72
Glycerylundecyl	13.0	2.03

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TABLE 2

Capacity Factor of Benzene and Naphthalene on Glycerylalkylsilylated Silica Gels

Stationary phase	Capacity factor k'	
	Benzene	Naphthalene
Glycerylpropyl[a]	0.702	3.96
Glycerylbutyl[a]	0.994	6.51
Glyceryl-2-methylbutyl[a]	1.03	6.57
Glycerylhexyl[a]	4.40	-
Glycerylhexyl[b]	1.82	6.21
Glycerylundecyl[b]	4.83	-

[a]Mobile phase: acetonitrile-water (1:9)
[b]Mobile phase: acetonitrile-water (3:7)

TABLE 3

Capacity Factor of Drugs on Glycerylalkylsilylated Silica Gels

Stationary phase	Capacity factor k'	
	Phenobalbital	Carbamazepine
Glycerylpropyl	0.162	0.675
Glycerylbutyl	0.310	1.22
Glyceryl-2-methylbutyl	0.418	1.43
Glycerylhexyl	1.55	4.53

Mobile phase: acetonitrile-100mM phosphate buffer (pH 6.9) (2:8)

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TABLE 4

Recovery of BSA from the Glycerylalkylsilylated Silica Gels

Stationary phase	Recovery of BSA (%)
Glycerylpropyl	102
Glycerylbutyl	98.0
Glyceryl-2-methylbutyl	97.0
Glycerylhexyl	94.9
Glycerylundecyl	5.7

HPLC conditions: mobile phase, acetonitrile-100mM phosphate buffer (pH 6.9) (2:8); Flow rate, 1ml/min; UV detection, 295 nm; sample, 10mg/ml; injection volum, 20 μl

in a "bristle" state, whereas with a C11 moiety it is in a "folded" state[17-19]. Hence, it is suggested that the stationary phases with an alkyl moiety of C3 to C6 form bilayer structure (Fig. 1) but that with an alkyl moiety of C11 does not in the present condition.

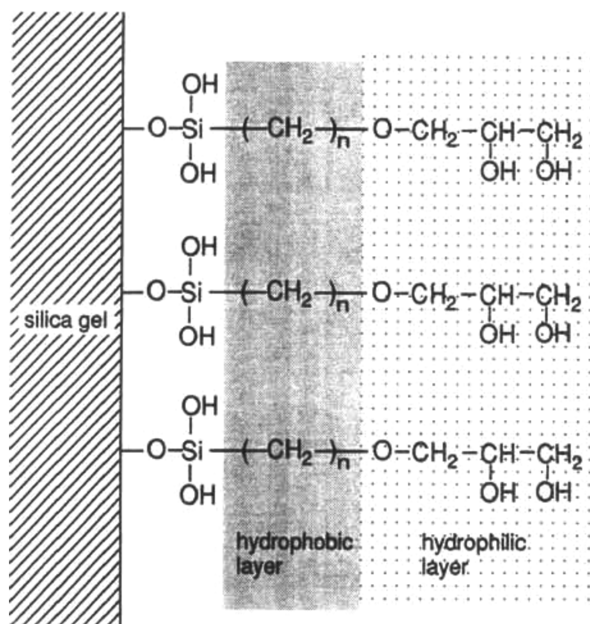

FIGURE 1. Structure of glycerylalkylsilylated silica gel.

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Analysis of Drugs in Derum by Direct Injection

Fig. 2 shows separation of drugs in serum by direct injection on the glycerylalkylsilylated silica gels. The serum proteins are eluted in the void volume, and the drugs can be analyzed quantitatively.

In the case of direct injection analysis of serum, in general, concentration of organic solvent in mobile phase is limited to low in order to avoid denaturation of proteins[20]. This is a strong restriction when only one kind of packing materials can be available, because it is quite probable that hydrophobic drugs hardly elute whereas hydrophilic ones elute too easily. This difficulty is to be solved by changing the nature of the stationary phase. The present packing material is one of

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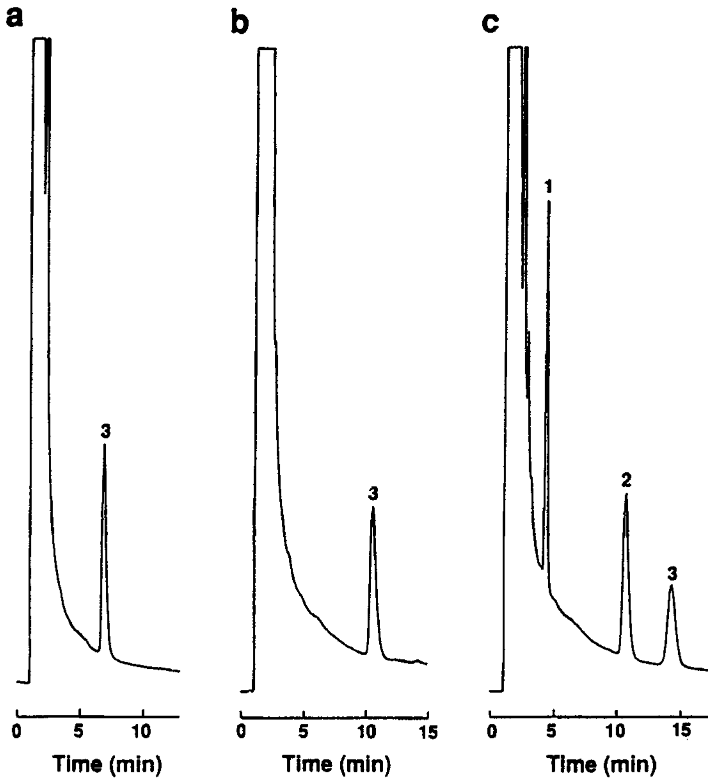

FIGURE 2. Chromatograms of human serum spiked with drugs by direct injection on (a) glycerylpropylsilylated silica gel, (b) glycerylhexylsilylated silica gel and (c) glyceryl-2-methylbutylsilylated silica gel. Solutes: 1, phenobarbital (40 µg/ml); 2, phenytoin (20 µg/ml); 3, carbamazepine (10 µg/ml). HPLC conditions: mobile phase, (a) acetonitrile-100mM phosphate buffer (pH 6.9) (1:9), (b) acetonitrile-100 mM phosphate buffer (pH 6.9) (2:8), (c) THF-100mM phosphate buffer (pH 6.9) (8:92); flow rate, 1 ml/min; UV detection, 220nm; injection volume, 20 µl.

such examples; if the glycerylalkylsilylated silica gel with an alkyl moiety of suitable length to the drugs of consideration is selected, the drugs in serum with various hydrophobicity or hydrophilicity can be analyzed quantitatively.

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