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Synthesis of polymer-coated mixed-functional packing materials for direct analysis of drug-containing serum and plasma by high-performance liquid chromatography

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Abstract

Silicone polymer-coated mixed-functional (PCMF) silica packing materials were developed for the direct determination of drugs contained in serum or plasma. The new stationary phases were prepared by the following three-step procedure: (1) coating porous silica with a silicone polymer; (2) partial introduction of hydrophobic groups; and (3) introduction of hydrophilic groups. Two PCMFs were synthesized, one having polyoxyethylene groups as hydrophilic groups (PCMF-POE) and the other having oligoglyceryl groups (PCMF-OG). PCMF-POE showed higher recoveries for injected proteins and a greater overall retention for drug molecules than PCMF-OG. PCMF-POE did not show any column deterioration in 500 serum sample injections (10 ml in total).

1. Introduction

The determination of drugs in serum and plasma using high-performance liquid chromatography (HPLC) normally requires several sample preparation processes, such as filtration, deproteinization, solvent extraction and concentration. Although these steps are essential to prevent column clogging or other negative effects on separation efficiency, they are time consuming, and also generate possibilities of experimental errors in the final determination results.

Recently, packing materials that make it possible to inject directly serum and plasma containing drugs and their metabolites were developed in order to alleviate these problems. All of these packing materials are designed so that large protein molecules are quickly eluted from the system, and relatively small molecules, such as drug molecules, are retained longer. Hagestam and Pinkerton [1–3] designed a so-called "internal-surface reversed phase (ISRP)", which had hydrophobic tripeptide parts (glycyl-L-phenylalanyl-L-phenylalanine) on the internal surface of porous silica and hydrophilic diol–glycine residues on the external surface. Haginaka *et al.* [4] prepared another ISRP, having N-octanoylaminopropyl and N-(2,3-dihydroxypropyl)aminopropyl groups as internal and external groups, respectively. Kimata and co-workers [5,6] reported a simple method to prepare a "superficially hydrophilic reversed phase", which includes decomposition of alkylsilylated silica with aqueous acid, followed by introduction of diol groups. Gish *et al.* [7]

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prepared a "shield hydrophobic phase", which consists of a polymeric bonded phase containing hydrophobic phenyl groups enclosed by hydrophilic polyoxyethylene networks. Gluntz *et al.* [8] prepared a semipermeable surface, which has a hydrophilic polyoxyethylene polymer on external surface and hydrophobic phase on internal surface. Haginaka and co-workers [9–13] reported the use of another type of mixed-functional packing materials which possess hydrophobic groups and hydrophilic groups on both the external and internal surfaces, which showed advantageous features for direct analysis of serum.

A novel polymer-coating technique to form reactive polymethylsiloxane films on metal oxides by chemical vapour deposition of 1,3,5,7-tetramethylcyclotetrasiloxane (H4) was developed by Fukui *et al.* [14]. Ohtsu and co-workers [15,16] utilized this polymer coating technique to prepare a polymer-coated C_{18} silica packing.

Polymer-coated C_{18} silica seems to have a more homogeneous surface than another type of polymer-coated silica phase prepared with silicone oligomers [17]. Polymer-coated C_{18} showed an excellent performance in the determination of protonated amine and chelating compounds, which are normally considered difficult to determine owing to their undesirable secondary interactions with the silica surface [18].

In this paper, an attempt to combine the polymer coating technology mentioned above and the idea of a mixed-functional (MF) stationary phase is described. The MF phases here contain styrene groups as hydrophobic groups and oligoglycerol (OG) or polyoxyethylene (POE) moieties as hydrophilic groups, both of which are attached to the polymer-coated silica. The performance of the new stationary phases was evaluated by the direct analysis of serum and plasma containing drugs.

2. Experimental

2.1. Reagents and materials

High-purity silica (Shiseido, Tokyo, Japan) (particle diameter, 5 μm; pore size, 90 Å; specific surface area, 410 m^2/g; metal impurities, <5 ppm) was used as a starting material for polymer-coated mixed-functional (PCMF) packing materials. 1,3,5,7-Tetramethylcyclotetrasiloxane (H4), a silicone monomer used for the polymer coating, was purchased from Toshiba Silicone (Tokyo, Japan), calf bovine serum from Dainippon Pharmaceutical (Osaka, Japan), trimethoprim and chrolamphenicol from Nacalai Tesque (Kyoto, Japan), human serum, phenobarbital, carbamazepine, phenytoin, theophylline and indomethacin from Wako (Osaka, Japan), allyloligoglycerol (OG, degree of polymerization 3) from Nagase Chemicals (Hyogo, Japan) and polyoxyethylene allyl ether (POE, average degree of polymerization 16) from Nihonyushi (Tokyo, Japan). Other reagents and solvents used in the synthetic procedure were of special grade from Wako and were used as received. Acetonitrile used for mobile phases was of HPLC grade from Nacalai Tesque. Water was purified with a Milli-Q system (Nihon Millipore Kogyo, Tokyo, Japan).

2.2. Preparation of polymer-coated mixed-functional packing material

Preparation of polymer-coated silica

The H4-coated silica was prepared according to the method of Fukui *et al.* [14]. The H4 molecules were deposited on the silica surface, where they polymerized. The measured thickness of the homogeneous polymer layer was *ca.* 7 Å, which corresponds to that of a monolayer. The silicone polymer formed in this step had many reactive Si–H groups (2.15 mmol/g), which were to be utilized for the subsequent modifications.

Introduction of hydrophobic groups

H4-coated silica gels (30 g) were heated at 120°C for 2 h and then dispersed into 150 ml of dry toluene and well stirred. Portions of styrene (0.3359, 0.6718 or 1.3435 g), which are equivalent to 5, 10 or 20% of Si–H groups in the silicone polymer, respectively, were added to the mixture, in the presence of hexachloroplatinic acid (3 mg). After the mixture had been heated at refluxing temperature for 7 h, it was cooled at

room temperature. The solvent was filtered off and the silica was rinsed with toluene and acetone. The silica obtained here was heated for 2 h at 80°C. The silicas treated in this way were named 5Ph, 10Ph or 20Ph, according to the amount of styrene added.

Introduction of hydrophilic groups

The 5Ph, 10Ph and 20Ph materials (15 g) were dispersed into water (150 ml). Allyloligoglycerol (20 g) or polyoxyethylene allyl ether (25 g) was added to the dispersion. The mixture was refluxed for 3 h in the presence of hexachloroplatinic acid (1.5 mg) under a nitrogen atmosphere, then cooled at room temperature. The phases obtained here were named 5Ph-OG, 10Ph-OG, 20Ph-OG, or 5Ph-POE, 10Ph-POE and 20Ph-POE, according to the reagent used. The amounts of groups reacted were determined by elemental analysis (C,H,N) using a Model 2400 CHN elemental analyser (Perkin-Elmer). The micropore distribution, micropore volume and mean pore radius (surface area) were measured by the nitrogen adsorption method using Autosorb-1 (Quantachrom).

2.3. Instrumentation

The HPLC system consisted of an LC-6A pump, an SPD-6A UV detector, a CTO-6A column oven, a SIL-6B injector and a C-R3A data processor (Shimadzu, Kyoto, Japan). The column temperature was maintained at 40°C. The PCMF was packed into a stainless-steel column (100 or 150 mm × 4.6 mm I.D.). The flow-rate was maintained at 1.0 ml/min except for the determination of theophylline, for which it was 0.6 ml/min. The mobile phase conditions are given in the figure captions.

2.4. Preparation of human serum

The drugs were dissolved in human serum at known concentrations and 20 μl of human serum sample were passed through a 0.22-μm membrane filter (Nippon Millipore, Tokyo, Japan) before being injected into the column.

2.5. Recovery of serum proteins from polymer-coated mixed-functional packing materials

The recovery of human serum proteins from a column packed with the PCMF packing material was examined as follows. A 20-μl portion of a control human serum sample was injected into the column (100 mm × 4.6 mm I.D.) packed with the PCMF packing material using 100 m*M* phosphate buffer (pH 6.9)–acetonitrile (9:1, v/v) as the mobile phase. The eluate was collected for 9 min and diluted to 10 ml with the mobile phase. A 1.0-ml portion of the liquid was mixed with 5 ml of Coomassie Brilliant Blue G-250 reagent, and kept for 10 min. The absorbance of the mixture was measured at 595 nm with the mobile phase as a standard. The recovery was calculated as the absorbance ratio with and without the column.

3. Results and discussion

3.1. Preparation of polymer-coated mixed-functional packing material

The PCMF packing materials were prepared in three steps: (1) coating porous silica with a silicone polymer, (2) partial introduction of hydrophobic groups and (3) introduction of hydrophilic groups. Table 1 shows the carbon contents of the PCMF materials, surface coverage of hydrophobic and hydrophilic groups and the capacity factors (k') of naphthalene under the HPLC conditions given.

The capacity factor (k') of naphthalene increased as the amount of hydrophobic groups increased. The k' values with Ph-POEs are generally larger than those with Ph-OGs with the same amount of surface coverage. With introduction of OG groups, the group density remained constant even when the surface coverage of hydrophobic groups was altered, whereas the group density decreased slightly with increasing amount of the hydrophobic groups on introduction of POE groups. These results suggest that the final density of hydrophilic groups is not greatly affected by the amount of hydrophobic groups within the tested range (5Ph to 20Ph),

Table 1
Carbon content and surface coverage of packing materials and capacity factor of naphthalene

Packing material	Carbon content (%)	Surface coverage (μmol/m^2)		k' [a]
		Hydrophobic phase	Hydrophilic phase	
5Ph-OG	10.29	0.22	1.03	3.38
10Ph-OG	10.65	0.31	1.03	3.61
20Ph-OG	10.78	0.37	1.01	3.89
5Ph-POE	14.40	0.22	0.57	5.42
10Ph-POE	14.34	0.31	0.55	5.71
20Ph-POE	14.33	0.37	0.53	5.93

[a] Capacity factors were measured under the following HPLC conditions: column size, 100 mm × 4.6 mm I.D.; mobile phase, acetonitrile–water (30:70, v/v); detection, 254 nm.

but rather is determined by the steric hindrance of the hydrophilic groups themselves, which are assumed to occupy significant parts of micropores.

Fig. 1 shows the pore distribution in each preparation step of 10Ph stationary phases. The pore radius decreased from 91.6 to 83.8 Å during the initial polymer coating and then from 83.8 to 82.0 Å on introduction of styrene groups. The final introduction of OG or POE groups further lowered the pore radius to 76.4 or 76.2 Å, respectively. The shifts of the distribution curves, which were not accompanied by major changes in their shapes, indicate that the introductions of hydrophobic and hydrophilic groups were performed highly homogeneously, not only on the external but also on the internal surface. It should be noted that the amount of phenyl groups hardly affected the pore radius and distribution curve.

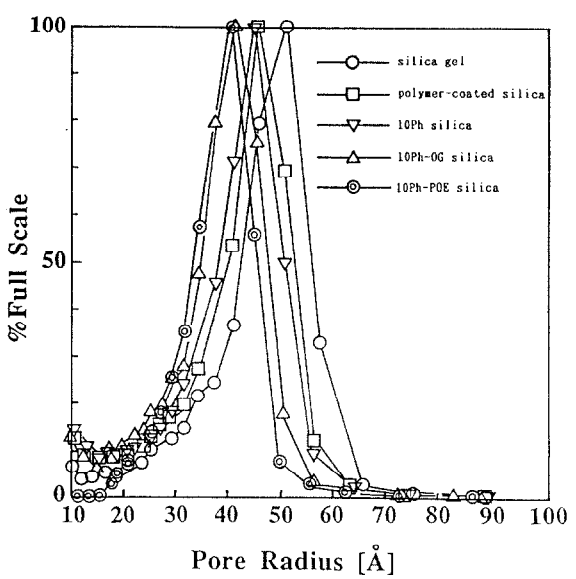

Fig. 1. Pore distribution at each step of the prepared 10Ph-OG and 10Ph-POE packing materials.

3.2. Recovery of serum proteins in first injection and column efficiency

Table 2 shows the recoveries of human serum proteins in the first injection and the plate

Table 2
Recovery of proteins in first injection and efficiency of PCMF packing material

Packing material	Recovery of proteins in first injection[a] (%)	Column efficiency[b] (plates per 100 mm)
5Ph-OG	35	3600
10Ph-OG	30	3900
20Ph-OG	20	3900
5Ph-POE	100	4300
10Ph-POE	100	4400
20Ph-POE	100	4600

[a] The recovery of serum proteins was measured using Coomassie Brilliant Blue G-250 reagent under the following HPLC conditions: column size, 100 mm × 4.6 mm I.D.; mobile phase, 100 m*M* phosphate buffer (pH 6.9)–acetonitrile (90:10, v/v); injection volume, 20 μl.

[b] Number of theoretical plates for carbamazepine under the following HPLC conditions: column size, 100 mm × 4.6 mm I.D.; mobile phase, 100 m*M* phosphate buffer (pH 6.9)–acetonitrile (85:15, v/v); detection, 254 nm.

number for each PCMF packing material. The recovery of human serum proteins with Ph-OGs decreased with increasing density of hydrophobic groups, whereas those measured with Ph-POEs were almost 100% in spite of the low density of hydrophilic groups. It is suggested that OG groups do not hinder the interaction between the hydrophobic groups and proteins, whereas POE groups are more effective in limiting the contact between them owing to their bulky structure. Ph-POEs also showed a better column efficiency than Ph-OGs for small molecules, probably because the molar-based density of hydrophilic groups is lower in Ph-POEs than in Ph-OGs, and mass transfer of small molecules is expected to be easier in Ph-POEs.

3.3. Direct injection analysis of serum containing drugs

Fig. 2 shows the results of direct injection analysis of human serum containing antiepileptics drugs (phenobarbital, carbamazepine and phenytoin) at therapeutic levels. Although these drugs were eluted after the serum proteins in the void volume, 20Ph-OG could not separate phenobarbital and serum proteins sufficiently. In contrast, 20Ph-POE showed a complete separation for phenobarbirtal and also carbamazepine and phenytoin. Figs. 3–5 show chromatograms for the direct injection analysis of human serum containing chloramphenicol, an antibacterial drug, indomethacin, an anti-inflamatory, and theophylline, a xanthine derivative, at therapeutic levels. These drugs were well separated from serum proteins. Thus, the prepared PCMF packing material having POE groups as hydrophilic phase seems to have advantageous features as a stationary phase for the direct injection analysis.

Table 3 gives the relative standard deviation (R.S.D.) values in the determination of the drugs in serum samples and the recoveries of these small drug molecules. The recovery of the

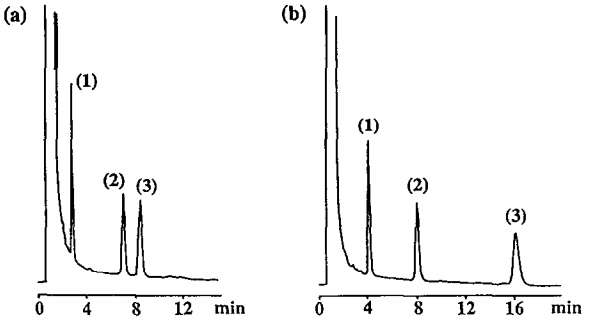

Fig. 2. Chromatograms of human serum spiked with (1) phenobarbital (20 μg/ml), (2) carbamazepine (5 μg/ml) and (3) phenytoin (40 μg/ml) on (a) 20Ph-OG and (b) 20Ph-POE packing materials. HPLC conditions: column size, 150 mm × 4.6 mm I.D.; mobile phase, 100 m*M* phosphate buffer (pH 6.9)–acetonitrile (80:20, v/v); detection, 254 nm; injection volume, 20 μl.

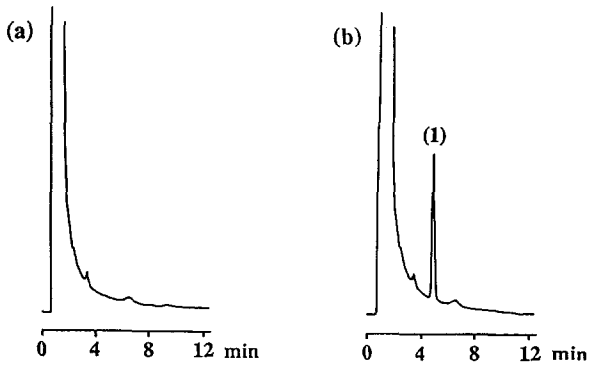

Fig. 3. Chromatograms of (a) human serum and (b) human serum spiked with (1) chloramphenicol (10 μg/ml) on 20Ph-POE packing material. HPLC conditions: column size, 100 mm × 4.6 mm I.D.; mobile phase, 100 m*M* phosphate buffer (pH 6.9)–acetonitrile (90:10, v/v); detection, 280 nm; injection volume, 20 μl.

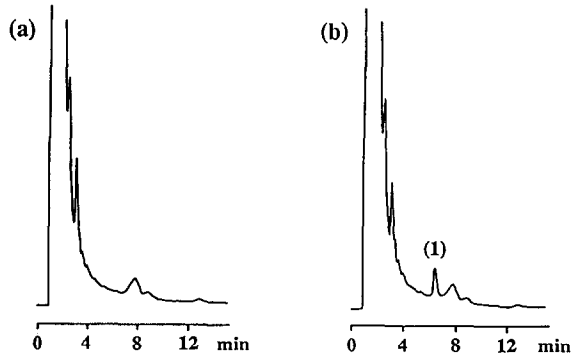

Fig. 4. Chromatograms of (a) human serum and (b) human serum spiked with (1) indomethacin (500 ng/ml) on 20Ph-POE packing material. HPLC conditions: column size, 150 mm × 4.6 mm I.D.; mobile phase, 100 m*M* phosphate buffer (pH 6.9)–acetonitrile (80:20, v/v); detection, 254 nm; injection volume, 20 μl.

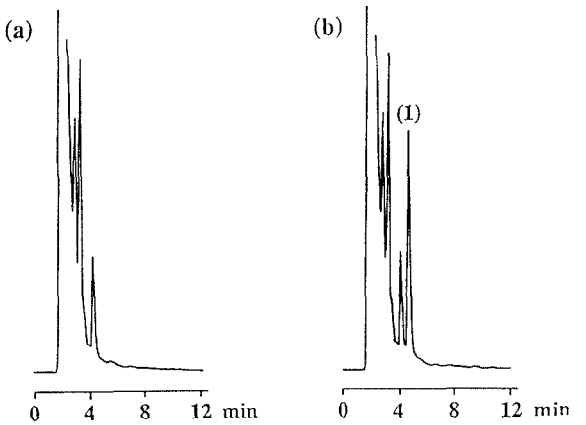

Fig. 5. Chromatograms of (a) human serum and (b) human serum spiked with (1) theophylline (10 μg/ml) on 20Ph-POE packing material. HPLC conditions: column size, 150 mm × 4.6 mm I.D.; mobile phase, 200 m*M* phosphate buffer (pH 6.9)–acetonitrile (98:2, v/v); detection, 254 nm; injection volume, 10 μl.

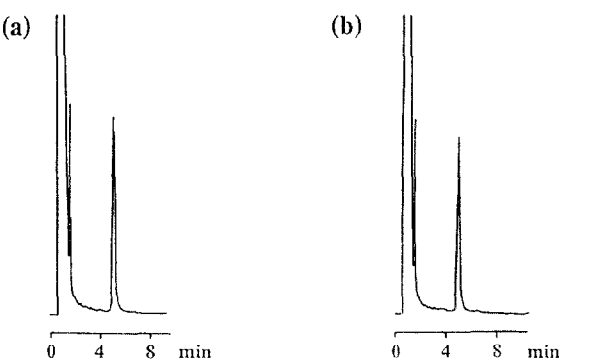

Fig. 6. Chromatograms for (a) the first and (b) the 500th repetitive injection of calf bovine serum spiked with trimethoprim (25 μg/ml) on 20Ph-POE packing material. HPLC conditions: column size, 100 mm × 4.6 mm I.D.; mobile phase, 100 m*M* phosphate buffer (pH 6.9)–acetonitrile (90:10, v/v); detection, 254 nm; injection volume, 20 μl.

drugs in human serum was almost 100% with good reproducibility. This was to be expected from a previous report [19], where drug molecules were found to be released from binding proteins in an organic-containing mobile phase.

3.4. Durability of polymer-coated mixed-functional packing material

Fig. 6 shows chromatograms of (a) the first and (b) the 500th injection of 20 μl (total 10ml) of calf bovine serum spiked with trimethoprim (25 μg/ml) and using 20Ph-POE. The capacity factors of trimethoprim for the first and 500th injection were 5.19 and 5.20, respectively. There was no noticeable decrease in peak efficiency. These results suggest that 20Ph-POE is highly stable at least during 500 analyses. The durability under alkaline conditions (pH 9) was also examined. The initial capacity factor ($k' = 5.21$) of lidocaine on 20Ph-POE, which was injected together with human serum (10 μg/ml), was slightly decreased to 5.01 after 50 h. The 20Ph-POE seems to be less stable against alkaline conditions than more hydrophobic C_{18} polymer-coated phases are [20].

Table 3
Recovery of drug molecules and reproducibility of analysis

Drug	Concentration (μg/ml)	Recovery[a] (%)	R.S.D.[b] (%)
Phenobarbital	20	97.9	1.06
Carbamazepine	5	101.6	0.69
Phenytoin	40	98.8	0.80
Chloramphenicol	5	96.5	0.14
Indomethacin	0.5	97.3	0.65
Theophylline	10	98.5	0.13

[a] The recovery was calculated from the peak-area ratio for a given concentration of the drug dissolved in human serum and mobile phase.
[b] Relative standard deviation of five analyses.

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