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Synthesis and characterization of polymer-coated mixed-functional stationary phases with several different hydrophobic groups for direct analysis of biological samples by liquid chromatography

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Abstract

Three types of polymer-coated mixed-functional (PCMF) silica column-packing materials for the direct analysis of biological liquids have been synthesized. These packing materials all have polyoxyethylene groups as their hydrophilic part but differ in their hydrophobic part (methyl groups for Me-POE, phenyl groups for Ph-POE, and octyl groups for Oc-POE). Retention characteristics with respect to several drug molecules, and protein recovery, were studied for each of the three types with different amounts of hydrophobic groups attached. Of the PCMFs prepared, Oc-POE showed the greatest overall retention, and Me-POE displayed the greatest protein recovery.

1. Introduction

Various column-packing materials for the direct injection of serum and plasma containing drugs and their metabolites have been developed [1–5]. These packing materials are basically designed so that large molecules, such as proteins, are eluted from the system quickly, while relatively small molecules, such as drug molecules, are retained longer in the stationary phase. Pretreatments of biological samples (filtration, deproteinization, solvent extraction, concentration, etc.) are expected to be greatly simplified by the use of these packing materials.

A novel polymer-coating technique [6] has been applied to the preparation of polymer-coated reversed-phase packings [7,8]. The polymer-coated C_{18} columns showed excellent performance in the determination of protonated amines and chelating compounds by minimizing undesirable secondary interactions with the silica surface [9]. A combination of the polymer-coating technique and the idea of a mixed-functional (MF) phase was attempted in the work reported in the previous paper [10], where a synthesis procedure based on polymer-coated mixed-functional (PCMF) packing materials, and their application to the direct analysis of drug-containing serum and plasma, were described.

In the previous paper [10], oligoglyceryl and polyoxyethylene groups were compared as candidate hydrophilic groups for the formation of a mixed-functional phase. Polyoxyethylene groups were found to be the better protein repellent. PCMF column-packing material containing polyethylene and phenyl groups (Ph-POE) has found application in many drug analyses to date.

One problem with Ph-POE has been the

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restricted choice of mobile phase. The organic content of a mobile phase for Ph-POE has to be below 20% to avoid denaturation of proteins contained in biological samples. However, some drug molecules can only be determined at reasonable retention times if the organic content of the mobile phase exceeds this level. Conversely, drugs also exist which are not retained satisfactorily on Ph-POE even when the organic content of the mobile phase is zero.

The object of the research described in the present paper is to develop PCMF packing materials which display either larger or smaller overall drug-molecule retention than the previous Ph-POE phase. In addition to phenyl groups, methyl and octyl groups, common substituting groups for reversed-phase packing materials, have been considered as candidates for the hydrophobic groups of a new PCMF. The development of these stationary phases is expected to widen the range of drugs to which direct analysis based on a mixed-functional stationary phase can be applied.

2. Experimental

2.1. Reagents and materials

High-purity silica (Shiseido, Tokyo, Japan; particle diameter 5 μm; pore size 80 Å; specific surface area 420 m^2/g; metal impurities 5 ppm) was used as the starting material for the PCMF column-packing materials. 1,3,5,7-Tetramethylcyclotetrasiloxane (H4), a silicone monomer used for polymer coating, was purchased from Toshiba Silicone (Tokyo, Japan). Human serum, phenobarbital, carbamazepine, phenytoin, theophylline, caffeine and sulfadimethoxine were purchased from Wako (Osaka, Japan). Sulfamerazine was from Sigma (St. Louis, MO, USA). Polyoxyethylene allyl ether (POE with average degree of polymerization 16) was from Nihonyushi (Tokyo, Japan). Other reagents and solvents used in the synthesis 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

The PCMF phases were prepared by the following three-step procedure: (1) coating porous silica with a silicone polymer; (2) partial introduction of phenyl or octyl groups as hydrophobic groups; and (3) introduction of polyoxyethylene groups as hydrophilic groups. The second process was not performed to prepare Me-POE, the type having methyl groups. In this case the methyl groups present in the coating polymer were considered to be the hydrophobic groups for the PCMF phase.

2.3. Preparation of polymer-coated silica

The H4-coated silica was prepared according to the method described by Fukui and co-workers [6]. The thickness of the homogeneous polymer layer was measured as being 7 Å, which corresponds to that of a monolayer. The silicone polymer formed in this step had many reactive Si-H groups (2.09 mmol/g), which were to be utilized for the subsequent modification.

2.4. Introduction of hydrophobic groups

H4-coated silica gels (20 g) were heated at 120°C for 2 h and then dispersed in 100 ml of dry toluene and well stirred. Portions of styrene (0.8707, 1.3060 or 1.7414 g) or 1-octene (0.9382, 1.4072 or 1.8763 g), which are equivalent to 20, 30 or 40% Si-H groups in the silicone polymer, respectively, were added to the mixture, in the presence of hexachloroplatinic acid (1 mg). After the mixture had been heated at reflux temperature for 5 h, it was cooled at room temperature. The solvent was filtered off and the silica was rinsed with toluene and acetone. The silica thus obtained was heated for 2 h at 80°C. The silicas treated in this way were named 20Ph, 30Ph or 40Ph and 20Oc, 30Oc or 40Oc, according to the amount of styrene or 1-octene added.

2.5. Introduction of hydrophilic groups

The H4-coated silica (having methyl groups as hydrophobic groups in a silicone polymer), and the 20Ph, 30Ph, 40Ph, 20Oc, 30Oc and 40Oc materials (10 g) were dispersed in water (100 ml). Polyoxyethylene allyl ether (20 g) was added to the dispersion. The mixture was refluxed for 3 h in the presence of hexachloroplatinic acid (0.5 mg) under a nitrogen blanket, then cooled at room temperature. The phases thus obtained were named Me-POE, 20Ph-POE, 30Ph-POE, 40Ph-POE, 20Oc-POE, 30Oc-POE and 40Oc-POE, according to the silica used. The numbers of groups that had reacted were determined by elemental analysis (C, H, N) using a Model 2400 CHN elemental analyser (Perkin-Elmer). The mean pore radius (surface area) was measured by the nitrogen adsorption method using an Autosorb-1 instrument (Quantachrom).

2.6. Instrumentation

The HPLC system consisted of an LC-10AD pump, an SPD-10AV UV detector, a CTO-10A column oven, an SIL-10A injector and a C-R4A 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 remaining conditions are given in the figure captions.

2.7. Preparation of human-serum samples

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

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

The recovery of human-serum proteins from a column packed with the PCMF packing materials was examined as follows. A 20- μl portion of a control human-serum sample was injected onto 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 rest of the procedure was as described in Ref. [10].

3. Results and discussion

3.1. Fundamental aspects of the three PCMFs

The structures of the three PCMFs are illustrated in Fig. 1. Since the coating polymer possesses methyl groups in its structure, methyl groups are expected to function as part of the hydrophobic moieties in Oc-POE and Ph-POE as well. Carbon content, surface coverage of hydro-

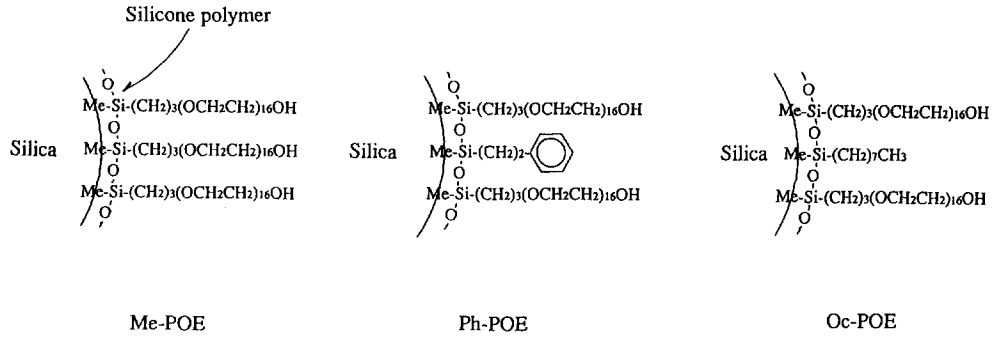

Fig. 1. Schematic diagrams of three PCMFs.

phobic and hydrophilic groups, and the naphthalene capacity factor (k') under the HPLC conditions given were measured for each stationary phase synthesized (Table 1).

The naphthalene capacity factor (k') in Ph-POEs or Oc-POEs increased with increasing density of the hydrophobic groups. The k' values of Oc-POEs were generally larger than those of Ph-POEs. While Me-POE showed the greatest carbon content and hydrophilic group density, the k' value of Me-POE was the smallest of all.

Total carbon content is thought to decrease with increasing numbers of hydrophobic groups, as a long polyoxyethylene chain is replaced by the eight-carbon hydrophobic groups. The density of hydrophilic groups in Ph-POEs and Oc-POEs decreased as the number of hydrophobic groups increased. However, densities of hydrophilic groups were found to be different in Oc-POEs from those in Ph-POEs, even when the same quantities of the corresponding olefins (1-octene and styrene) had been used for the hydrosilylation. Their structural difference (aliphatic as against aromatic) seemed to be the cause of the difference in reactivity. The hydrosilylation time was set at five hours, which corresponds to approximately two hours plus the time required to reach the reaction plateau in both types. For both types of PCMF the number of hydrophobic groups was highly reproducible in replicate experiments (data not shown).

3.2. Recovery of serum proteins from the first injection, pore radius and column efficiency

The recoveries of human-serum proteins from the first injection, together with the pore radius, capacity factor (k') and plate number (phenytoin peak), were measured for each of the PCMF packing materials (Table 2). The recovery of human-serum proteins in Oc-POEs significantly decreased as the number of hydrophobic groups increased. The same tendency was observed for Ph-POEs, but the extent of the decrease was not as drastic as that observed in Oc-POEs. As shown in Table 1, there are differences in hydrophobic-group density between Oc-POEs and Ph-POEs. In the synthetic format of these bifunctional phases, a higher hydrophobic-group density also leads to a lower hydrophilic-group (or 'protein-repellent') density. The general relationship between the density of hydrophobic groups and strength of interaction with proteins in reversed-phase chromatography [11,12] was probably more pronounced in these bifunctional phases.

There was no significant difference in pore radius values. All the PCMFs had a pore radius

Table 1
Carbon content, surface coverage of substituting groups, and naphthalene capacity factor k'

Packing material	Carbon content (%)	Surface coverage (μmol/m^2)		k'[b]
		Hydrophobic phase[a]	Hydrophilic phase	
Me-POE	14.13	4.98[c]	0.66	4.81
20Ph-POE	13.16	0.32	0.53	5.35
30Ph-POE	12.91	0.33	0.51	5.46
40Ph-POE	12.78	0.38	0.49	5.54
20Oc-POE	13.31	0.51	0.49	7.97
30Oc-POE	13.12	0.55	0.47	8.36
40Oc-POE	12.87	0.58	0.45	8.78

[a] Surface coverage values for hydrophobic groups in Ph-POEs and Oc-POEs correspond to the reacted styrene or 1-octene.
[b] Capacity factors 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.
[c] The value for Me-POE corresponds to the number of methyl groups in the coating polymer. This is the same for all the PCMFs.

Table 2
Recovery of proteins from first injection, and pore radius and efficiency of PCMF packing materials

Packing material	Recovery of proteins from first injection[a] (%)	Pore radius[b] (Å)	Column efficiency[c] (plates per 100 mm)	k'[d]
Me-POE	100.0	70.1	4200	10.6
20Ph-POE	99.3	70.7	5000	12.8
30Ph-POE	99.7	70.2	5100	12.9
40Ph-POE	97.6	70.8	5200	13.1
20Oc-POE	93.0	69.7	5500	17.3
30Oc-POE	62.8	69.5	5700	17.6
40Oc-POE	45.8	69.5	5900	18.3

[a] 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] Mean pore radius was measured by the nitrogen adsorption method.

[c] Number of theoretical plates and [d] capacity factors for phenytoin peaks were determined 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.

of around 70 Å. It seems that a pore radius is basically determined by the large hydrophilic groups. Pore radius is one of the very important factors in protein separation by liquid chromatography. In this study, 80-Å silica was chosen, as the size-exclusion effect it provided was thought to be sufficient for serum proteins.

The peak efficiency for phenytoin, an anti-epileptic, increased with increasing density of hydrophobic groups in both Ph-POEs and Oc-POEs. Oc-POEs showed better column efficiency than Me-POE and Ph-POEs. These results do not contradict the fact that common octyl and phenyl columns (reversed-phase columns), which can be considered as 100% hydrophobic and 0% POE in this context, show approximately 10 000 and 8000 plates for a phenytoin peak, respectively.

3.3. Direct-injection analysis of serum containing drugs and retention properties of PCMF packing materials containing methyl, phenyl or octyl groups

Fig. 2 shows the results of direct-injection analysis of human serum containing anti-epileptics (phenobarbital, carbamazepine and phenytoin) at therapeutic levels on Me-POE, 20Ph-POE and 20Oc-POE column packings. The three drugs were well separated from the serum proteins. Also, all the PCMFs gave complete separation between the three drugs. Figs. 3 and 4 show results obtained from calf bovine serum containing two antibiotics (sulfamerazine and sulfadimethoxine) and from human serum containing xanthine derivatives (theophylline and caffeine) on Me-POE, 20Ph-POE and 20Oc-POE

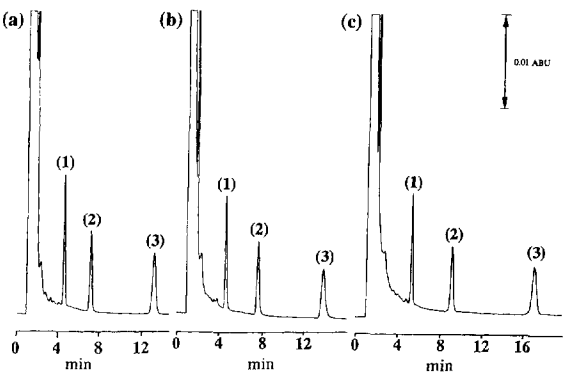

Fig. 2. Chromatograms of human serum spiked with (1) phenobarbital (20 μg/ml), (2) carbamazepine (5 μg/ml) and (3) phenytoin (40 μg/ml) on (a) Me-POE, (b) 20Ph-POE and (c) 20Oc-POE packing materials. HPLC conditions: column size 150 mm × 4.6 mm I.D.; mobile phase 100 m*M* phosphate buffer (pH 6.9)–acetonitrile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

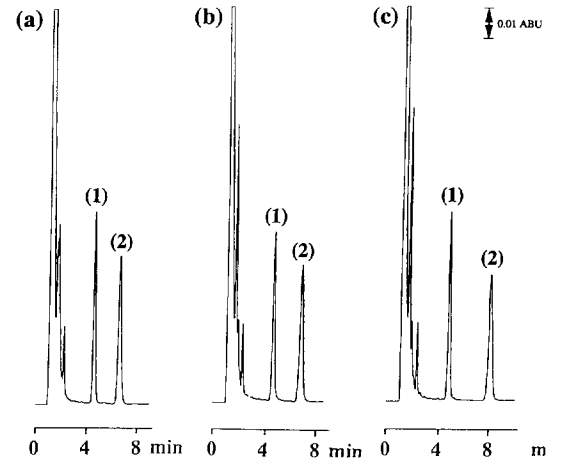

Fig. 3. Chromatograms of calf bovine serum spiked with (1) sulfamerazine (10 μg/ml) and (2) sulfadimethoxine (10 μg/ml) on (a) Me-POE, (b) 20Ph-POE and (c) 20Oc-POE packing materials. HPLC conditions: column size 150 mm × 4.6 mm I.D.; mobile phase 100 m*M* phosphate buffer (pH 6.9)–acetonitrile (90:10, v/v); detection 254 nm; injection volume 20 μl.

column packings. With the exception of the xanthine derivatives, most of the drugs were separated satisfactorily from proteins.

Capacity factors of all the drugs are listed in Table 3. The anti-epileptics and antibiotics were retained satisfactorily on all the PCMFs. The overall retention of the analytes decreased in the order: 20Oc-POE > 20Ph-POE > Me-POE. Although the retentions of xanthine derivatives were not sufficient in any of the PCMFs under the HPLC conditions used, the same tendency in order of overall retention was observed.

It is known that xanthine derivatives, theophylline and caffeine are generally retained not only by hydrophobic interaction but also by hydrogen bonding with silanol groups left on the silica surface [9,13]. It seems that the low retention of xanthine derivatives were caused by the absence of silanol groups in the PCMFs due to their initial polymer coating process.

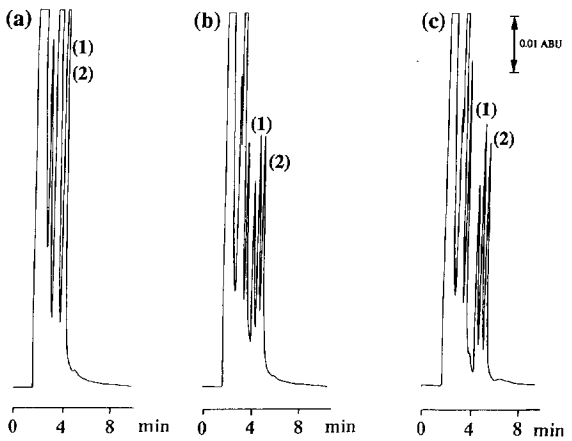

Fig. 4. Chromatograms of human serum spiked with (1) theophylline (10 μg/ml) and (2) caffeine (10 μg/ml) on (a) Me-POE, (b) 20Ph-POE and (c) 20Oc-POE packing materials. HPLC conditions: column size 150 mm × 4.6 mm I.D.; mobile phase 200 m*M* phosphate buffer (pH 6.9)–acetonitrile (98:2, v/v); detection 254 nm; injection volume 20 μl.

3.4. Durability of PCMF column packing

In the previous paper [10], 20Ph-POE packing material showed a durability of 500 injections. Durability was studied for Me-POE and 20Oc-POE, which had shown higher protein recoveries among the phases discussed in this paper. Fig. 5 shows chromatograms of (a) the first and (b) the 500th injection of 20 μl (total 10 ml) of calf bovine serum spiked with trimethoprim (25 μg/ml) using Me-POE packing material. The trimethoprim capacity factors for the first and the 500th injection were 4.11 and 4.28, respectively. The same measurement was carried out for 20Oc-POE (chromatogram not shown). The trimethoprim capacity factors for the first and the 400th injection were 5.34 and 5.15, respectively. There was no noticeable decrease in peak efficiency. These results suggest that 20Oc-POE and Me-POE are highly stable in the analysis of serum samples.

4. Conclusions

PCMFs displaying stronger and weaker drug-molecule retentions than the Ph-POE already in use could be prepared by employing octyl and methyl groups as hydrophobic groups, respectively. Although the recovery of proteins decreased in the order: PCMF-Me ≥ PCMF-Ph > PCMF-Oc, a high durability was maintained for

Table 3
Retention of anti-epileptics, antibiotics and xanthine derivatives on the PCMF packing materials

Drug type	Compound	Capacity factor k'		
		Me-POE	20Ph-POE	20Oc-POE
Anti-epileptics[a]	Phenobarbital	1.81	1.96	2.40
	Carbamazepine	3.41	3.90	4.86
	Phenytoin	7.22	7.86	9.95
Antibiotics[b]	Sulfamerazine	1.67	1.68	1.82
	Sulfadimethoxine	2.85	2.95	3.74
Xanthine derivatives[c]	Theophylline	0.72	0.93	1.06
	Caffeine	0.72	1.08	1.21

[a] Capacity factors were measured under the following 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.
[b] Capacity factors were measured under the following HPLC conditions: column size 150 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.
[c] Capacity factors were measured under the following 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 20 μl.

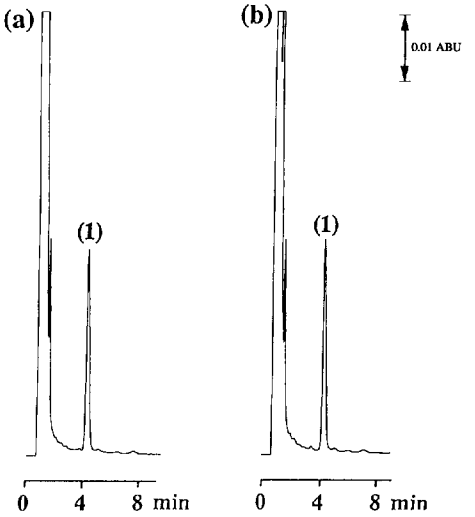

Fig. 5. Chromatograms for (a) the first and (b) the 500th replicate injection of calf bovine serum spiked with (1) trimethoprim (25 μg/ml) on Me-POE packing materials. HPLC conditions: column size 100 mm × 4.6 mm I.D.; mobile phase 100 m*M* phosphate buffer (pH 6.9)–acetonitrile (90:10, v/v); detection 254 nm; injection volume 20 μl.

all three types at 20% hydrophobic groups. Oc-POE and Me-POE are expected to widen the range of analytes applicable in the direct analysis of biological liquids.

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