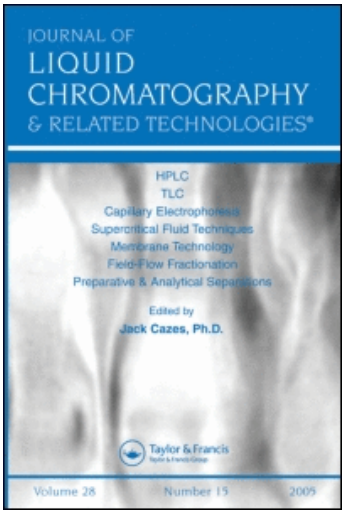

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Hiroko Itoh[a]; Tosfflo Kinoshita[a]; Noriyuki Nimura[a]
[a] School of Pharmaceutical Sciences Kitasato University, Tokyo, Japan

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REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF SMALL MOLECULES ON NONPOROUS C18 SILICA GEL COLUMN

HIROKO ITOH, TOSHIO KINOSHITA, AND NORIYUKI NIMURA*
School of Pharmaceutical Sciences
Kitasato University
9-1 Shirokane-5
Minato-ku, Tokyo 108, Japan

Dedicated to Professor S. Hara on the occasion of his 65th birthday

ABSTRACT

Reversed-phase high-performance liquid chromatographic (RP-HPLC) separation of small molecules on a nonporous octadecylsilyl (C18) spherical silica gel was compared with the separation on conventional porous packings. Various alkylbenzenes were separated on three different types, nonporous, macroporous (pore diameter; 30 nm), and microporous (pore diameter; 10 nm), C18 bonded silica columns under both isocratic and gradient elution conditions. Under the isocratic conditions, column efficiency of nonporous silica was inferior to that of others. In case of the gradient elution, the nonporous silica column gave the most favorable separation in a short time. Fast separation of small molecules on the nonporous column could be performed by using a conventional HPLC system with a steep gradient condition, because the void volume of the nonporous packing was extremely smaller than the porous ones. Various series compounds such as fatty acid derivatives, phenylthiohydantoin (PTH) amino acids, and vitamins were well separated within 1 or 2 min on the C18 nonporous silica column under steep gradient conditions.

INTRODUCTION

Since Unger's group pioneered[1-6] separations of proteins on a nonporous micro particle (d_p=1.5 μm) packing, some research groups[7-12] investigated the usability of the nonporous packing materials. However, these micro particle supports (dp=0.5 to 2.5 μm) are generally restricted by the concomitant decrease in column permeability due to excessive pressure. We also prepared[13,14] nonporous C18 silica gels of various particle sizes, and examined separation of proteins on these materials. As a result, it was found that an effect of particle size of the packing on the column efficiency was extremely small in the reversed-phase separation of proteins[13]. Therefore, the large-particle (20 μm) nonporous C18 silica was seemed to be useful for the purpose[14].

On the other hand, separation of low-molecular-weight compounds on nonporous packing materials has never been reported. In a previous report[7], it was described that the nonporous materials appeared to be less suitable for low-molecular-weight solutes, except for fundamental retention studies. And besides, no chromatographic data on small molecules has concretely been shown anywhere.

In the present study, we have evaluated the nonporous silica packing material for the reversed-phase separation of low-molecular-weight compounds. Separations of various small molecules such as alkylbenzenes on a nonporous C18 silica of particles of 5 μm were compared with those on conventional micro-

and macroporous C18 silica gels under both isocratic and gradient elution conditions. We found that the nonporous C18 silica column was useful for fast separation of small molecules using a steep gradient elution.

EXPERIMENTAL

Reagents

Alkylbenzenes were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Free fatty acids and lipophilic vitamins were purchased from Sigma (St. Louis, MO, U.S.A.). Phenylthiohydantoin (PTH) amino acids and phthalate esters were obtained from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). 9-Anthryldiazomethane (ADAM) was from Funakoshi (Japan), and the derivatization of fatty acid with ADAM was carried out as described previously[15]. HPLC-grade acetonitrile was from Kanto Chemical Co., Ltd. (Tokyo, Japan). Water was purified by passing through a Milli-R/Q system (Millipore Corp., Bedford, MA, U.S.A.).

Nonporous C18 silica gel (Develosil NP-ODS-5), microporous C18 silica gel (Develosil ODS-5), and macroporous C18 silica gel were manufactured by Nomura Chemical Co. Ltd. (Seto-city, Japan). Pore sizes of microporous and macroporous supports are 10 nm and 30 nm, respectively. Mean particle diameters of the all silicas were 5 μm. These materials were chemically bonded with n-

octadecyldimethylchlorosilane and end-capped with trimethylchlorosilane. Each of the C18 bonded phases was slurry packed into a stainless steel column tube (30 mm x 4.6 mm I.D.).

Instruments

A gradient HPLC system used in this study consisted of two 880-PU HPLC pumps (Jasco,Tokyo, Japan) equipped with an ERC-3510 degasser (Erma, Tokyo, Japan), an 880-30 solvent mixing module (Jasco), a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.), and an 875-UV spectrophotometric detector (Jasco). Chromatograms were recorded and processed by C-R6A Chromatopac integrator (Shimadzu, Kyoto, Japan.).

Chromatographic conditions

Mobile phases, gradient conditions and other chromatographic conditions used are given in figure legends. All separations were performed at room temperature at a constant flow rate of 2.0 ml/min. Sample injections were made to coincide with commencement of the gradient, and actual gradient delay was previously determined by tracer technique with acetone containing eluent.

In order to evaluate the column efficiency, theoretical plate number (N) was calculated according to

$$N = 16 \cdot (t_R/W)^2$$

where t_R is the retention time and W is peak width at base line.

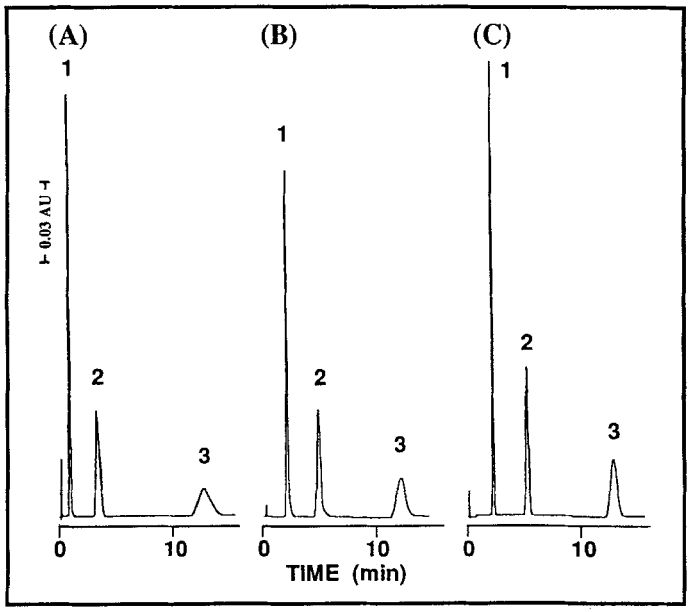

FIGURE 1. Isocratic chromatographic profiles of alkylbenzenes on (A) nonporous, (B) macroporous and (C) microporous C18 silica gel columns.
Conditions; flow rate, 2 ml/min. column temperature, ambient. detection, UV 215 nm. mobile phase, (A) 35 % aqueous acetonitrile, (B) 60 % aqueous acetonitrile, (C) 70 % aqueous acetonitrile. peaks, (1) n-hexylbenzene, (2) n-octylbenzene, (3) n-decylbenzene. Each alkylbenzene was dissolved in acetonitrile in a concentration of 5 mg/ml. And the sample solutions were mixed and diluted prior to the injection. An aliquot of the mixture (5 μl) was injected into HPLC system.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatograms of alkylbenzenes on nonporous, macroporous and microporous C18 silica columns using an isocratic elution. The surface areas of the nonporous, macroporous and microporous silica gels were approximately 0.6,

TABLE 1
Theoretical Plate Number (N) of Alkylbenzenes Separated on C18 Bonded Nonporous, Macroporous, and Microporous Silica Gel Columns

Sample \ Column	Theoretical Plate Number (N)		
	Nonporous	Macroporous	Microporous
n-hexylbenzene	215	1108	2239
n-octylbenzene	400	1119	2090
n-decylbenzene	440	1057	1994

100 and 340 m^2/g, respectively. So the different concentration of organic modifier in the mobile phase should be prepared to give approximate similar retention time of the samples. Actually, two-fold concentration of acetonitrile was required in the microporous column system compared with the nonporous column system. Theoretical plate number of the each peak of alkylbenzenes was calculated and listed in Table 1. In the nonporous column system, since the nonporous material had only a low retention capacity, it was obliged to use a mobile phase having notably poor solubility to give adequate retentions of the solutes. The peaks on the nonporous material were, therefore, broadened compared with those on the porous materials. In contrast, symmetry of the former was rather satisfactory than that of the latter porous columns, because the surface of the nonporous material might eminently be smooth. These results prompted us to apply the nonporous packing to the separation by using a gradient elution.

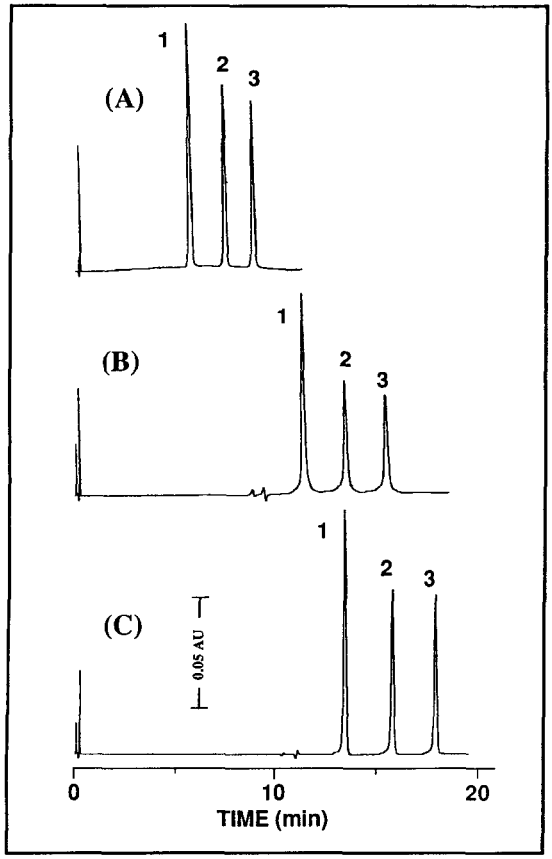

FIGURE 2. Gradient chromatographic profiles of alkylbenzenes on (A) nonporous, (B) macroporous and (C) microporous C18 silica columns.
Conditions; mobile phase, A=10 % aqueous acetonitrile, B=100 % acetonitrile. linear gradient, from 0 % eluent B to 100 % B for 20 min. flow rate, 2 ml/min. column temperature, ambient. peaks, (1) n-hexylbenzene, (2) n-octylbenzene, (3) n-decylbenzene.

TABLE 2
Peak Width and Peak Symmetry of The Peaks of Alkylbenzenes Separated on C18 Bonded Nonporous, Macroporous, and Microporous Silica Gel Columns

	Nonporous			Macroporous			Microporous		
Peaks	a	b	(a+b)/2a	a	b	(a+b)/2a	a	b	(a+b)/2a
1	37.9	41.1	1.04	94.7	107.4	1.07	69.5	44.2	0.82
2	50.5	50.5	1.00	94.7	82.1	0.93	75.8	44.2	0.79
3	44.2	44.2	1.00	94.7	101.1	1.03	69.5	56.8	0.91

a, b; peak width in second indicating as below illustration

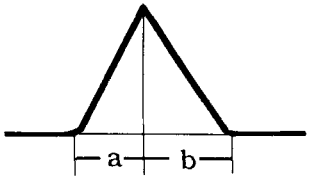

Fig. 2 shows chromatograms of alkylbenzenes on the nonporous and the porous columns using a conventional gradient condition. The nonporous column was the equal of the porous ones in the separation efficiency, and it gave a curtailment of the analysis time. Considering from the peak symmetry, the nonporous column was the best of all. Peak widths and peak symmetry factors at the base line of the alkylbenzenes in Fig. 2 were listed in Table 2. The nonporous column gave the lowest value for the peak width and the most sufficient peak symmetry factor. In addition, void volume of the nonporous column is smaller than those of the porous columns as the nonporous silica has no pore volume. Consequently, actual retention time on the nonporous column can be reduced without rising the flow rate. Furthermore, nonporous supports might be favorable for the

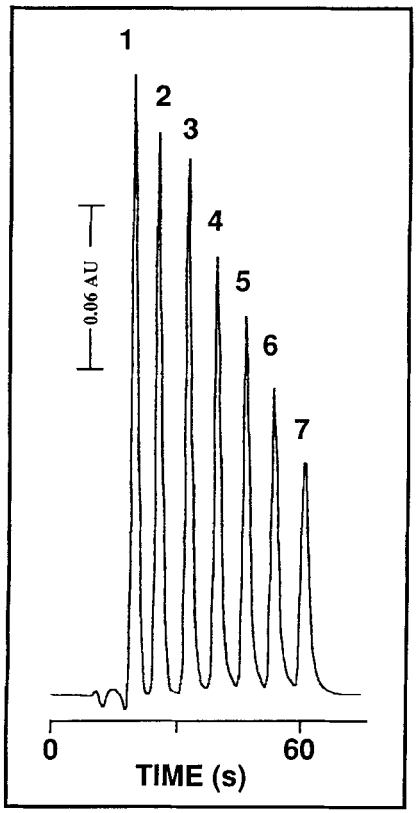

FIGURE 3. Fast separation of alkylbenzenes on nonporous C18 silica column.
Conditions; linear gradient, from 30 % eluent B to 100 % B for 1 min. peaks, (1) n-hexylbenzene, (2) n-octylbenzene, (3) n-decylbenzene, (4) n-dodecylbenzene, (5) n-tetradecylbenzene, (6) n-hexadecylbenzene, (7) n-octadecylbenzene. Other conditions are same as in Fig. 2.

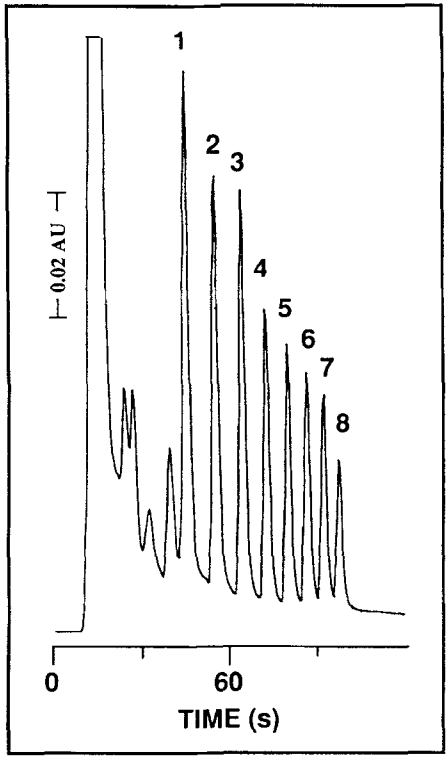

FIGURE 4. Chromatogram of ADAM derivatives of free fatty acids on nonporous C18 silica column.
Conditions; mobile phase; A=10 % aqueous acetonitrile, B=50 % tetrahydrofuran in acetonitrile. gradient condition; 30 % B for 0.25 min, and then from 30 % B to 100 % B for 3 min. detection; UV-250 nm peaks; (1) C10:0, (2) C12:0, (3) C14:0, (4) C16:0, (5) C18:0, (6) C20:0, (7) C22:0, (8) C24:0. Each free fatty acid was dissolved in methanol in a concentration of 20 μg/ml, and the each solution was mixed as equivalent volume. ADAM was dissolved in methanol in a concentration of 1 mg/ml. Fatty acids solution and ADAM reagent was mixed and stored for 1 hr, and then an aliquot of the mixture (1 μl) was injected into HPLC system.

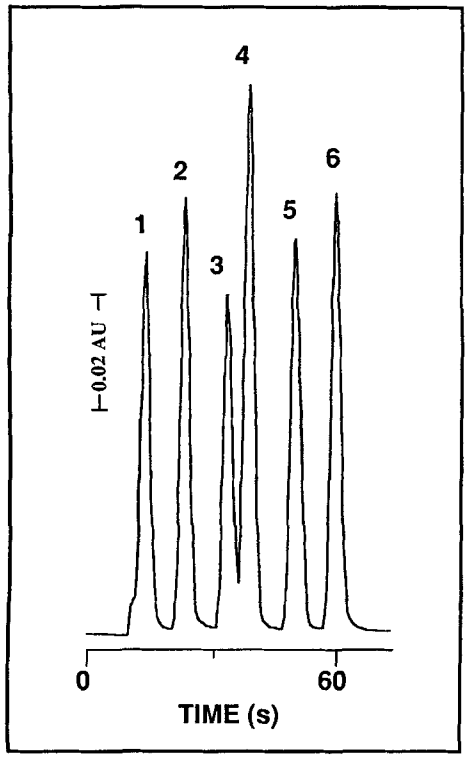

FIGURE 5. Chromatogram of PTH-amino acids on nonporous C18 silica column.
Conditions: mobile phase; A=10 mM phosphate buffer (pH 4.5), B=90 % aqueous acetonitrile. gradient condition; from 0 % B to 100 % B for 3 min. detection; UV-268 nm. peaks; (1) Asp, (2) Ala, (3) Tyr, (4) Val, (5) Leu, (6) Lys. Each amino acid was dissolved in acetonitrile in a concentration of 500 μg/ml, and the each solution was mixed as equivalent volume prior to injection. An aliquot of the mixture (1 μl) was injected into HPLC system.

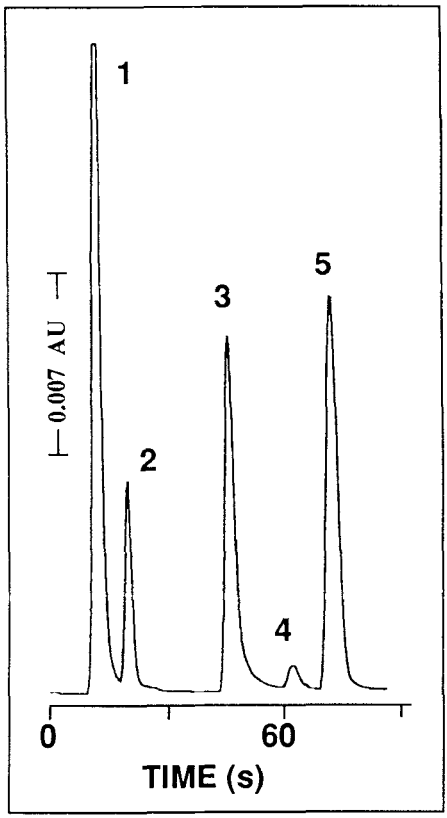

FIGURE 6. Chromatogram of lipophilic vitamins on nonporous C18 silica column.
Conditions: mobile phase; A=Water, B=90 % aqueous acetonitrile. gradient condition; from 50 % B to 100 % B for 1 min. detection; UV-266 nm. peaks; (1) Vitamin K_3, (2) Vitamin A, (3) Vitamin D_2, (4) Vitamin E, (5) Vitamin K_1. Each vitamin was dissolved in acetonitrile in a concentration of 1 mg/ml, and the each solution was mixed and diluted with acetonitrile prior to injection. An aliquot of the mixture (1 µl) was injected into HPLC system.

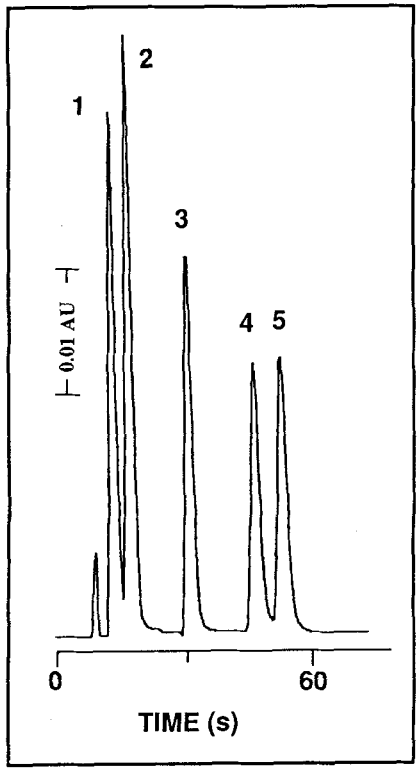

FIGURE 7. Chromatogram of phthalate esters on nonporous C18 silica column.
Conditions: mobile phase; A=Water, B=90 % aqueous acetonitrile. gradient condition; from 10 % B to 100 % B for 1 min. detection; UV-254 nm. peaks; (1) dimethylphthalate (DMP), (2) diethylphthalate (DEP), (3) dibutylphthalate (DBP), (4) dihexylphthalate (DHP), (5) dioctylphthalate (DOP). Each phthalate ester was dissolved in acetonitrile in a concentration of 6.5 mg/ml, and the each solution was mixed and diluted with acetonitrile prior to the injection. An aliquot of the mixture (1 μl) was injected into HPLC system.

separation with a gradient elution, because the stream of the mobile phase should not become stagnant in pores observed on porous supports. As mentioned above, it seemed that a rapid separation using a steep gradient elution on a conventional HPLC apparatus would demonstrate the efficiency of the nonporous packing.

Fig. 3 shows a chromatogram of alkylbenzenes on the nonporous C18 silica column using a steep gradient with acetonitrile. The seven alkylbenzenes are completely separated within 1 min. Since such rapid separation was performed at the constant flow rate of 1 to 2 ml/min, conventional detector equipped with a flow cell of which volume is normal, 8 to 12 μl, could be used. Fast separations of ADAM derivatives of fatty acids, PTH-amino acids, lipophilic vitamins, and phthalate esters on the nonporous C18 column were also achieved, and their chromatographic profiles were shown in Fig. 4 to 7, respectively. Each series of compounds was satisfactorily separated within 1 or 2 min.

As a result, it was found that nonporous column packings was useful for the separation of not only biopolymers such as proteins but also low-molecular-weight compounds.

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