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# High-performance liquid chromatographic separation of nucleic acids on a fluorocarbon-bonded 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)*

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ABSTRACT

The separation of nucleic acids and related compounds was investigated using high-performance liquid chromatography on a new fluorocarbon-bonded silica gel column. The polyadenylate enzymic partial hydrolysate sample and a mixture of various polynucleotide samples were sufficiently separated in the reversed-phase mode using gradient elution with aqueous ammonium acetate–acetonitrile. A mixed-mode separation on the fluorinated phase coated with a tetraalkylammonium salt was also examined for the separation of various polynucleotides, including tRNAs.

## INTRODUCTION

Various types of material have been developed for column packings in HPLC. Some fluorinated packing materials [1,2], organic fluoropolymers and their derivatives, as well as silicas with fluorocarbon-bonded phases, have also been investigated. A fluoropolymer, Kel-F (polychlorotrifluoroethylene) [3–5], has been found to be a useful support for the preparation of alkyl- or aryl-bonded polymer beads as a packing material for liquid chromatography. RPC-5 [6] or Neosorb-LC-N [7], which is also based on a polychlorotrifluoroethylene bead coated with a tetraalkyl quaternary ammonium salt, has been used for mixed-mode LC separation of nucleic acids.

On the other hand, materials higher selective towards fluorine-containing solutes have been reported for fluorocarbon-bonded silica gel columns [8–10]. Reversed-phase separation of small molecules on the fluorinated silica columns has been compared with separation on conventional non-fluorine-containing alkylsilyl-bonded silica columns. The capacity factors ($k'$) of small molecules on the $C_{10}$ fluorocarbon-bonded phase, heptadecafluorodecylsilyl silica gel, were found to be approximately the same as those on the $C_3$ alkylsilyl-bonded silica column [8]. However, there have been few applications for the separation of polar biomolecules on fluorocarbon-bonded silicas. Reversed-phase separation of proteins on the $C_{10}$ fluorocarbon-bonded silica column was reported by Xindu and Carr [11].

Recently, a new fluorocarbonaceous packing material, 1H,1H,2H,2H,3H,3H-tridecafluoro-(4,4-dimethylheptyl)silyl ($F_{13}/C_9$) bonded spherical microporous silica gel, was prepared for HPLC by Konakahara *et al.* [12]. We have been evaluating this bonded phase for the HPLC analysis of biological substances.

In the present study, $F_{13}/C_9$ bonded macroporous (pore size 30 nm) silica gel (Fig. 1) was freshly prepared, and the separations of polynucleotides and tRNAs on the fluorinated silica column were investigated in the reversed-phase

* Corresponding author.

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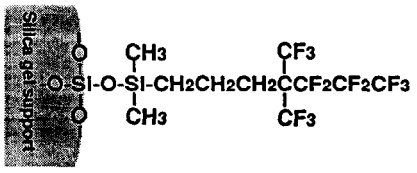


Fig. 1. Structural formula of the fluorocarbon-bonded phase.

mode as well as in the mixed mode. The mixed-mode separation included both an anion exchange mode and a reversed-phase mode on the trioctylmethylammonium chloride-coated fluorinated silica column.

## EXPERIMENTAL

### *Reagents*

Polyadenylate sodium salt [poly(A)] and nuclease P1 were purchased from Yamasa Shoyu (Chiba, Japan). Various tRNAs were obtained from Boehringer Mannheim Yamonouchi (Tokyo, Japan). HPLC-grade acetonitrile and other reagents were purchased form Kanto Chemical (Tokyo, Japan) and Wako (Osaka, Japan), respectively. Water was purified by passing it through a Milli-R/Q system (Millipore, Bedford, MA, U.S.A.).

### *Sample preparation*

Poly(A) (100 ml, 30 mg per ml of 0.3 *M* citrate buffer, pH 6) was digested by nuclease P1 (5 ml, 50 mg/ml) containing magnesium chloride (10 ml, 6 mg/ml) at 37°C for 60 min. Crude tRNA and other purified tRNAs were dissolved in concentrations of 1 mg/ml and 1 unit/200 $\mu$l, respectively. Both the polynucleotide sample and the tRNA solutions were stored at −18°C until they were used. They were diluted 10–20 times prior to use, and an aliquot of the mixture (10–20 $\mu$l) was injected into the HPLC system.

### *Packing materials*

The fluorinated silica gel used in the present study was prepared by NEOS (Shiga, Japan). Macroporous silica gel (mean particle diameter 5 $\mu$m; mean pore size 30 nm) was silylated with 1H,1H,2H,2H,3H,3H - tridecafluoro(4,4 - dimethylheptyl)dimethylchlorosilane (NEOS) and then end-capped with trimethylchlorosilane. The resulting material was slurry-packed into a 150 mm × 4.6 mm I.D. stainless-steel column tube for the reversed-phase separation.

For the mixed-mode separation, the fluorinated silica gel was coated with triotylmethylammonium chloride (TOMAC) as follows. The fluorinated silica gel (4 g) was suspended in a chloroform solution of TOMAC (500 mg/50 ml) *in vacuo* in an ultrasonic bath, and then the chloroform was evaporated to dryness. The resulting TOMAC-coated silica material (*ca.* 1.5 g) was suspended with aqueous ammonium acetate as a slurry solvent, and then slurry-packed into a 50 mm × 6.0 mm I.D. stainless-steel column tube at a constant pressure of 300 kg/cm$^2$.

### *HPLC apparatus*

The high-pressure 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) and a Model 7125 injector (Rheodyne, Cotati, CA, USA). All the samples were detected with an 875-UV spectrophotometric detector (Jasco) operated at 260 nm. The chromatograms were recorded and processed by a C-R6A Chromatopac integrator (Shimadzu, Kyoto, Japan.).

### *Chromatographic conditions*

The mobile phases, gradient conditions and other chromatographic conditions that were used are given in the text and figure legends. All HPLC separations were performed at room temperature. Since a liquid chromatograph generally contains some dead volumes, a lag time occurs during the running of a gradient. Sample injections, therefore, were made to coincide with commencement of the gradient. The actual gradient delay was previously determined by a tracer technique to draw a gradient curve using a mobile phase B containing a UV-absorbing solvent, such as acetone.

## RESULTS AND DISCUSSION

Reversed-phase systems have been used for the separation of oligonucleotides [13,14].

Chemically bonded alkylsilyl silica gels, such as ODS (octadecylsilyl) silica, with an acetonitrile gradient were mainly used for the purpose. A column packed with uncoated Kel-F82 (polychlorotrifluoroethylene) particles was used for the reversed-phase separation of oligonucleotides by Usher [15]. In the field of LC separation of nucleic acids, the separation is not so efficient in the reversed-phase mode as in the ion-exchange mode [16]. However, reversed-phase separation is suitable for preparative use because volatile solvents are used as the mobile phase.

We first examined the reversed-phase separation of various nucleotides on the present fluorinated macroporous silica gel column. A linear gradient elution system with aqueous ammonium acetate and acetonitrile was used. Fig. 2 shows a reversed-phase chromatographic profile of oligoadenylates, which were prepared by the partial digestion of poly(A) with nuclease P1. Oligoadenylates up to the 30-mer were separated sufficiently within 30 min. The separation was satisfactory compared with the previous separation data obtained on a conventional alkylsilyl-bonded silica column. In 1980, Berendsen *et al.* [8] investigated the reversed-phase separation of a number of fluorinated and non-fluorinated solutes on a heptadecafluorodecyl-bonded silica column. Although separation could be carried out with mobile phases containing over 40% (v/v) methanol, the column's performance deteriorated rapidly when the methanol content decrease below this point. Mobile phases with a high aqueous content were completely unusable because they did not wet the bonded phase. Regenerating the column required pure methanol. In contrast, the present separations carried out under highly aqueous conditions containing only a few percent of acetonitrile provided good results. In appears that the fluorocarbon-bonded phase is useful for the separation of polar compounds, such as nucleotides, even if an aqueous mobile phase is used.

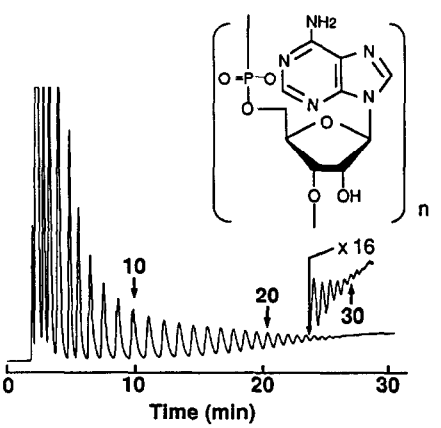


Fig. 2. Reversed-phase HPLC separation of poly(A) enzymic partial hydrolysate on a fluorinated silica column. Eluent A, 0.1 *M* ammonium acetate; eluent B, 10% aqueous acetonitrile; linear gradient from 5% B to 30% B in 60 min; flow-rate, 1.0 ml/min; sample volume, 20 μl.

Next we investigated mixed-mode separation on the fluorocarbon-bonded silica gel. Mixed-mode separations [6,7,17] of nucleic acids and related compounds have previously been performed either on a fluoropolymer gel coated with tetraalkylammonium salt or on an alkylsilyl-bonded silica gel. The separation was based on both an anion-exchange mode and a reversed-phase mode [17]. TOMAC and aqueous sodium perchlorate containing ethylenediamine tetraacetate have generally been used as the coating cationic salt and the mobile phase, respectively. Accordingly, the present coating and separation procedures described in the Experimental section referred to the previous methods.

Fig. 3 shows the mixed-mode separation of poly(A) enzymic hydrolysate on the TOMAC-coated fluorinated silica column. Oligoadenylates

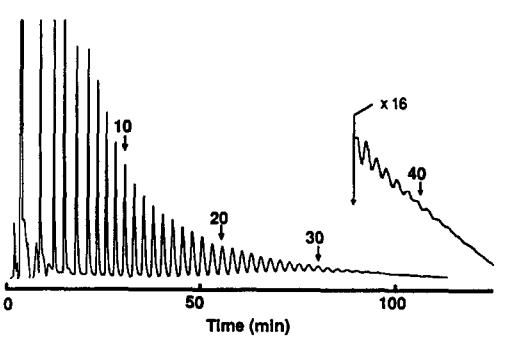


Fig. 3. Mixed-mode HPLC separation of poly(A) enzymic partial hydrolysate on a TOMAC-coated fluorinated silica column. Eluent A, 10 m*M* sodium perchlorate +10 m*M* Tris–acetate buffer (pH 7.5) +1 m*M* $Na_2EDTA$; eluent B, 0.5 *M* sodium perchlorate +10 m*M* Tris–acetate buffer (pH 7.5) +1 m*M* $Na_2EDTA$; linear gradient from 0% B to 100% B in 150 min; flow-rate, 1.0 ml/min; sample volume, 15 μl.

up to the 40-mer that were eluted with the perchlorite gradient were efficiently separated within 120 min. Other types of oligonucleotide, such as the oligocytidylates and oligodeoxynucleotides, were also well separated in the mixed mode. Furthermore, tRNAs were also satisfactorily separated on a TOMAC-coated fluorinated silica column by mixed-mode chromatography. Fig. 4 shows the chromatographic profiles of the crude mixture of tRNAs from *Escherichia coli* and purified tRNAs that are specific for amino acids. The present separation of tRNAs with this mixed-mode material equals or surpasses those obtained with RPC-5-like or TOMAC-coated ODS silica gel material [18,19].

The column performance of the TOMAC-coated fluorinated silica was good, and the coating was found to be extremely stable. The lifetime of the fluoropolymer column coated with alkylammonium salt was generally not so long. It has been reported that the polymer-based mixed-mode columns could be used up to several tens of times, but their lifetimes were shorter than those of alkylsilyl-bonded phase columns [6,7]. On the other hand, we have continuously used one of the TOMAC-coated columns for more than a year, for several hundred gradient runs,

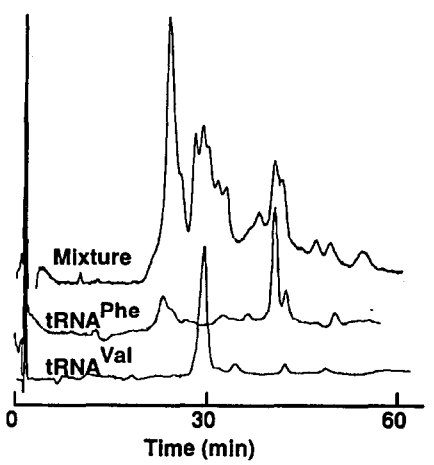


Fig. 4. Mixed-mode HPLC profiles of tRNAs (*E. coli*) on a TOMAC-coated fluorinated silica column. Eluent A, 10 m*M* sodium perchlorate +10 m*M* Tris–acetate buffer (pH 7.5) +1 m*M* $Na_2EDTA$; eluent B, 0.2 *M* sodium perchlorate +10 m*M* Tris–acetate buffer (pH 7.5) +1 m*M* $Na_2EDTA$; linear gradient from 15% B to 100% B in 60 min; flow-rate, 1.0 ml/min; sample volume, 10 $\mu$l.

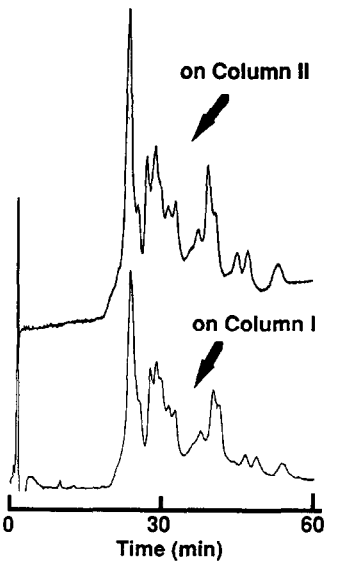


Fig. 5. Mixed-mode HPLC profiles of tRNAs (crude mixture form *E. coli*) on two TOMAC-coated fluorinated silica columns prepared separately. All the conditions were same as in Fig. 4.

with no decrease in the efficiency of the column. It appears that some attracting force other than hydrophobic interaction is occurring between the fluorocarbon phase and the TOMAC, possibly some ionic adsorption of TOMAC on the exposed silanol groups; however, we currently cannot explain this observation. The reproducibility of the coating procedure was also sufficient (Fig. 5); several TOMAC-coated columns showed equal performance and life-time.

The present separation system using the new fluorinated silica gel column may be useful for the separation of nucleic acids in the fields of organic synthesis and biochemistry.

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