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Synthesis of mixed-functional-phase silica supports for liquid chromatography and their applications to assays of drugs in serum

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ABSTRACT

Mixed-functional-phase (MFP) silica supports were designed for the direct injection determination of drugs in serum. The MFP silicas were synthesized from porous silica materials in three or four steps: introduction of 3-glycidoxypropyl phases, introduction of phenyl, butyl or octyl phases and hydrolysis of the oxirane ring to diol phases, or these three steps plus further introduction of glycerylpropyl (*i.e.*, diol) phases. Although the further introduction of glycerylpropyl phases resulted in a reduction in the column efficiency, serum proteins were completely recovered in the first injection of serum samples. The prepared MFP packing materials can be used for the direct injection determination of hydrophobic and hydrophilic drugs in serum.

INTRODUCTION

Since the introduction of internal surface reversed-phase (ISRP) silica supports [1], various new stationary phases have been developed for direct injection assays of drugs and/or their metabolites in biological samples. The ISRP silica column, which is commercially available, designed by Hagestam and Pinkerton [1], involves binding glycyl-L-phenylalanyl-L- phenylalanine tripeptide and glycine residues bonded on glycerylpropyl-bonded porous silica particles as internal and external surfaces, respectively. These negatively charged and less hydrophobic phases resulted in low recoveries of serum proteins at eluent pH values below 6.0 and almost no retention of hydrophilic drugs such as amphoteric drugs [2]. We prepared a neutral ISRP silica support having N-octanoylaminopropyl and N-(2,3-dihydroxypropyl)aminopropyl phases as internal and external surfaces, respectively [2,3]. The neutral ISRP packing materials can be used for direct injection assays of hydrophilic or hydrophobic drugs in serum over the eluent pH range 3–7. However, the above ISRP packing materials were prepared by enzymatic cleavage of external hydrophobic phases or the enzymatic cleavage and attachment of diol phases. Hence, it was difficult to obtain batch-to-

batch reproducibility of the support properties, because enzymatic reactions were included in the preparation processes.

Kimata *et al.* [4] reported a new method for the preparation of ISRP packing materials, including partial decomposition of alkylsilylated silica stationary phases with aqueous acid followed by introduction of diol phases. Gisch *et al.* [5] prepared a shielded hydrophobic phase silica support which consists of a polymeric bonded phase containing a hydrophobic phenyl group in a hydrophilic polyoxyethylene network. Recently, Williams and Kabra [6] reported novel dual-zone material adsorbents having perfluorobutylethylenedimethylsilyl and octadecylsilyl groups on external and internal zones, respectively.

We have prepared and evaluated a novel chromatographic support, termed mixed-functional-phase (MFP) packings, for direct serum injection assays of achiral and chiral drugs [7,8]. The MFP supports have the properties that the pores are small enough to restrict the access of macromolecules such as serum proteins to the pores and the mixed functionality of hydrophilic and hydrophobic phases is introduced for avoiding the destructive accumulation of proteins and retaining small molecules such as drugs. Two MFP packing materials having phenyl–diol [7] and β-cyclodextrin–diol [8] phases have been developed for determinations of achiral and chiral drugs, respectively.

In this paper, we report the synthesis and characterization of MFP silica supports having phenyl, butyl or octyl groups as hydrophobic phases and diol groups as hydrophilic phases, and their applications to the direct injection determination of hydrophobic and hydrophilic drugs in serum.

EXPERIMENTAL

Reagents and materials

Theobromine was purchased from Nacalai Tesque (Kyoto, Japan), acetonitrile of HPLC grade from Kanto Chemical (Tokyo, Japan), 3-glycidoxypropyltrimethoxy-silane, phenyltrimethoxysilane, butyltrimethoxysilane and octyltriethoxysilane from Petrach Systems (Bristol, PA, U.S.A.) and N,N-diisopropylethylamine from Aldrich (Milwaukee, WI, U.S.A.). The other reagents, of analytical-reagent grade, and control human serum (Control Serum I) were purchased from Wako (Osaka, Japan). Phenobarbital, phenytoin, caffeine, carbamazepine, theophylline, cefotaxime, cef-menoxime and cefamandole were kindly donated by Sankyo (Tokyo, Japan), Nippon Ciba-Geigy (Takarazuka, Japn), Eisai (Tokyo, Japan), Hoechst Japan (Tokyo, Japan), Takeda Chemical Industry (Osaka, Japan) and Shionogi (Osaka, Japan). Develosil silica [particle diameter 5 μm; nominal pore size 55 Å (specified by the manufacturer); specific surface area 525 m^2/g] was obtained from Nomura Chemicals (Seto, Aichi, Japan).

Water prepared with a Nanopure II unit (Barnstead, Boston, MA, U.S.A.) was used for the preparation of the eluent and the sample solution.

Preparation of the MFP silica

Preparation of 3-glycidoxypropylsilica. Develosil silica gel (2 g) was dried *in vacuo* over P$_2$O$_5$ at 150°C for 6 h and the dry silica gel was added to 120 ml of dry toluene. The mixture was heated to reflux until all the water had been removed as an

azeotrope into a Dean–Stark-type trap. Next, 3.8 ml of 3-glycidoxypropyltrimethoxy-silane, which is equivalent to 16 μmol per square metre of surface area, were added to the mixture, which was then reacted at 95°C for 3 h. The reaction mixture was cooled to room temperature, filtered and washed with toluene and methanol. The isolated silica gel was then dried *in vacuo* over P_2O_5 at 60°C for 2 h. The silica support thus obtained is abbreviated to 16G, derived from the number of micromoles of 3-glycidoxy-propyltrimethoxysilane per square metre of surface area used for the reaction.

Preparation of phenyl-, butyl- or octylsilica. 3-Glycidoxypropylsilica (3 g) was added to 80 ml of dry toluene and stirred. To the mixture, 2.0, 2.0 or 3.3 ml of phenyl- or butyltrimethoxysilane or octyltriethoxysilane and 6.0 ml of N,N-diisopropyl-ethylamine as a basic catalyst were added under a nitrogen atmosphere and the mixture was refluxed for 9 h. The reaction mixture was cooled to room temperature, filtered and washed with toluene and methanol. The isolated silica gel was dried *in vacuo* over P_2O_5 at 60°C for 2 h.

Hydrolysis of the oxirane ring to diol phases. To 2 g of the phenyl-, butyl- or octylsilica, 80 ml of perchloric acid (pH 3.0) were added and the mixture was refluxed for 4 h. The mixture was filtered and washed with water and methanol. The isolated silica was dried *in vacuo* over P_2O_5 at 60°C for 2 h. The MFP silica supports thus obtained are abbreviated to 16G-Ph, 16G-Bu and 16G-Oc, respectively, derived from the organosilanes used for the reaction.

Further introduction of diol phases. To 2 g of 16G-Ph silicas, 30 ml of aqueous solution containing 2.3 ml of 3-glycidoxypropyltrimethoxysilane, whose pH was adjusted to 3.5 by addition of perchloric acid, were added and the mixture was reacted at 90°C for 4 h. The mixture was filtered and washed with water and methanol. The isolated silica was dried *in vacuo* over P_2O_5 at 60°C for 2 h. The MFP silica support thus obtained is abbreviated to 16G-Ph-10G.

Intrumentation

The amounts of 3-glycidoxypropyl-, phenyl- or butyltrimethoxysilane or octyltriethoxysilane reacted were determined by elemental analysis using Type MT-3 CHN analyser (Yanagimoto, Kyoto, Japan).

The prepared MFP silica support was packed into a 50 × 4.6 mm I.D. or 100 × 4.6 mm I.D. stainless-steel tube by conventional high-pressure slurry-packing procedures [9].

The chromatographic system consisted of a Model 880 pump (Japan Spectro-scopic, Tokyo, Japan) equipped with a a Model 875-UV variable-wavelength detector (Japan Spectroscopic). The eluents used are specified in the captions of tables and figures. Detection was performed at 254 or 275 nm. The precolumn (50 × 4.6 mm I.D.) packed with LC-sorb Sp-A-ODS (particle size 25–40 μm; Chemco Scientific, Osaka, Japan) was inserted between the pump and injector to protect the analytical column from microparticles in the eluent. Samples were injected with a Sil-9A Auto Injector (Shimadzu, Kyoto, Japan). The chromatograms were recorded and integrated using a Chromatopac CR-6A (Shimadzu). All separations were carried out at ambient temperature.

Preparation of human serum samples

Drugs were dissolved in human serum at a known concentration and an

appropriate volume of the serum sample was applied to the MFP silica support after filtration through a 0.22-μm membrane filter (Nippon Millipore, Tokyo, Japan).

Recovery of serum proteins from the MFP silica

The recovery of serum proteins from the MFP silicas was measured as described previously [2].

RESULTS AND DISCUSSION

Characterization of the MFP silica support

Previously [7], we reported a method for the preparation of an MFP silica support having phenyl and diol phases. The MFP silica support could be used for the direct injection determination of hydrophilic and hydrophobic drugs in serum. The MFP packing material was prepared by three steps: introduction of 3-glycidoxypropyl phases, introduction of phenyl phases and hydrolysis of the oxirane ring to diol phases. In this study, we tried to introduce phenyl, butyl or octyl groups as hydrophobic phases into silica matrices and to compare the three MFP silicas with respect to physical and chromatographic properties.

The introduction of diol phases was kept constant by reaction with 16 μmol/m^2 of 3-glycidoxypropyltrimethoxysilane without the addition of a basic catalyst. The introduction of phenyl, butyl or octyl phases was intended to the maximum by adding N,N-diisopropylethylamine as a basic catalyst. Table I gives the carbon contents and surface coverages of the MFP packing materials. The ligand density of octyl phases introduced was lower than that of phenyl or butyl phases, as steric hindrance impedes diffusion of octyltriethoxysilane into the pores during silanization, because the reactivity of ethoxysilane (used for the introduction of octyl phases) is almost comparable to that of methoxysilane (used for the introduction of phenyl or butyl phases) [10].

Table II gives the retention properties of anticonvulsant drugs, methylxanthine derivatives and cephalosporins on the MFP silicas. The solutes tested were well retained on the 16G-Ph silica compared with the 16G-Bu and 16G-Oc silicas. This might be due to the low ligand density of the 16G-Oc silica and the low hydrophobicity of the 16G-Bu silica. Anticonvulsant drugs were well separated on the three MFP silicas, as shown in Table II. Methylxanthine derivatives, theophylline and theo-

TABLE I

CARBON CONTENTS AND SURFACE COVERAGES OF THE MFP PACKING MATERIALS

Silica	Carbon content (%)	Surface coverage (μmol/m^2)	
		Diol phase	Hydrophobic phase
16G	3.69	0.75	—
16G-Ph	8.40	0.75	1.94
16G-BU	5.50	0.75	1.42
16G-Oc	4.57	0.75	0.45
16G-Ph-10G	12.60	—	1.94

TABLE II

RETENTION PROPERTIES OF VARIOUS COMPOUNDS ON THE MFP PACKING MATERIALS
(100 × 4.6 mm I.D. COLUMN)

Compound	Capacity factor (k')			
	16G-Ph	16G-Bu	16G-Oc	16G-Ph-10G
Anticonvulsant drugs[a]				
Phenobarbital	2.24	1.10	0.61	3.16
Phenytoin	9.56	4.21	2.85	16.59
Carbamazepine	12.86	5.98	4.21	13.34
Methylxanthine derivatives[b]				
Theophylline	5.06	1.10	0.73	2.28
Theobromine	7.39	1.16	0.74	2.69
Caffeine	15.15	1.97	1.97	5.55
Cephalosporins[c]				
Cefotaxime	1.42	2.12	1.64	1.00
Cefmenoxime	3.30	4.01	3.26	1.86
Cefamandole	3.31	4.06	4.71	2.23

[a] Capacity factors were measured under the following chromatographic conditions: eluent, 50 mM NaH$_2$PO$_4$ + 50 mM Na$_2$HPO$_4$–CH$_3$CN (9:1, v/v); flow-rate, 0.8 ml/min.
[b] Capacity factors were measured under the following chromatographic conditions: eluent, 50 mM NaH$_2$PO$_4$ + 50 mM Na$_2$HPO$_4$–CH$_3$CN (23:1, v/v); flow-rate, 0.8 ml/min.
[c] Capacity factors were measured under the following chromatographic conditions: eluent, 90 mM NaH$_2$PO$_4$ + 10 mM Na$_2$HPO$_4$–CH$_3$CN (12:1, v/v) for the 16G-Ph and 16G-Ph-10G silicas and 90 mM NaH$_2$PO$_4$ + 10 mM Na$_2$HPO$_4$–CH$_3$CN (100:1, v/v) for 16G-Bu and 16G-Oc silicas; flow-rate, 0.8 ml/min.

bromine were not separated on the 16G-Bu and 16G-Oc silicas but were separated on the 16G-Ph silica. The separation of cephalosporins, cefmenoxime and cefamandole was not achieved on the 16G-Ph and 16G-Bu silicas with a short run time, but was successful on the 16G-Oc silica. These results indicate that the introduction of different functionalities could be useful for selective separations of various solutes. Almost the same column efficiency was obtained in spite of differences in hydrophobic phases introduced; the number of theoretical plates (N) was 2600, 2400 and 2200 for carbamazepine for the 16G-Ph, 16G-Bu and 16G-Oc silicas, respectively, packed into a 100 × 4.0 mm I.D. column.

The average pore diameters of the prepared MFP supports were 50, 51 and 58 Å for 16G-Ph, 16G-Bu and 16G-Oc silicas, respectively, when measured by the inverse size-exclusion chromatographic method reported by Cook and Pinkerton [11]. The recovery of serum proteins for the first injection increased in the sequence 16G-Oc < 16G-Ph < 16G-Bu by 30, 50 and 80%, respectively. The serum proteins were completely recovered with the second injection of a 20-μl human serum sample for the 16G-Bu silica or with the third injection for the 16G-Ph and 16G-Oc silicas, when MFP silicas packed into a 50 × 4.6 mm I.D. stainless-steel column were used. It has been reported that the recovery of ovalbumin on a reversed-phase column decreased with increasing alkyl chain length [12] and proteins were less completely recovered from silica-bonded alkyl silanes with low surface coverage [13]. Hence it is plausible that the

16G-Oc silica gives the lowest recovery of serum proteins in the first injection. These results reveal that the 16G-Bu and 16G-Oc silicas in addition to the 16G-Ph silica [7] can be used for direct serum injection assays of drugs.

Further introduction of diol phases to the 16G-Ph silica

In order to recover serum proteins completely from the MFP silica with the first injection, we tried to introduce further hydrophilic phases (*i.e.*, diol phases) into the 16G-Ph silica. The further introduction of diol phases to the 16G-Ph silica was achieved by reaction with 3-glycidoxypropyltrimethoxysilane in aqueous media [14]. The serum proteins were completely recovered from the obtained MFP silica, 16G-Ph-10G, which has an average pore diameter of 39 Å. However, the column efficiency of the 16G-Ph-10G silica was decreased to about half of that of the 16G-Ph
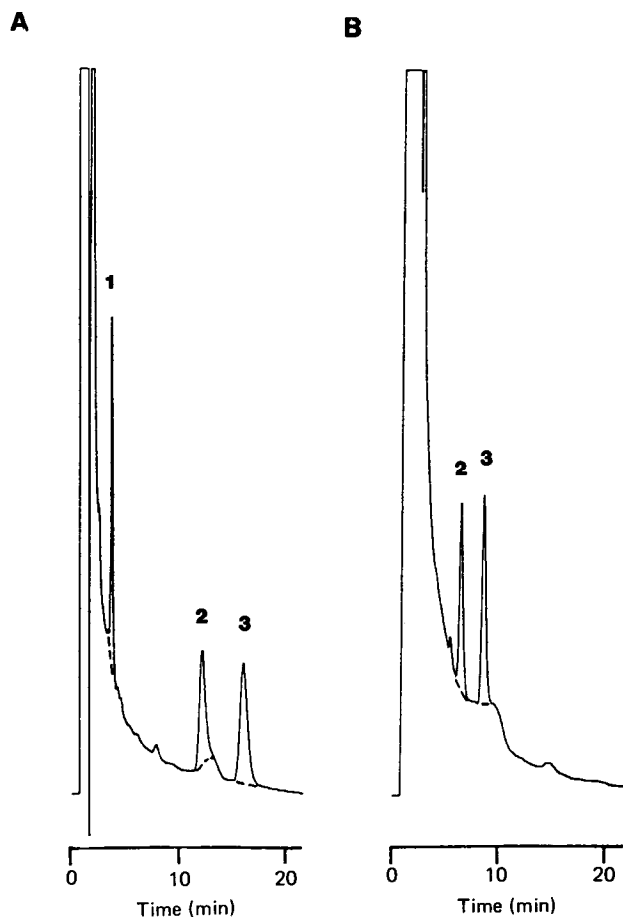

Fig. 1. Chromatograms of control serum spiked with (1) phenobarbital (20 μg/ml), (2) phenytoin (40 μg/ml) and (3) carbamazepine (8 μg/ml) on (A) 16G-Ph and (B) 16G-Bu silica. Chromatographic conditions: column, MFP silicas packed into a 100 × 4.0 mm I.D. column; eluent, 50 mM NaH$_2$PO$_4$ + 50 mM Na$_2$HPO$_4$–CH$_3$CN (9:1, v/v); flow-rate, 0.8 ml/min; detection, 254 nm; sensitivity, 0.032 a.u.f.s.; injection volume, 10 μl. Dashed lines indicate serum blank.

silica. The 16G-Ph-10G silica showed excellent chromatographic selectivity, that is, separations of cefmenoxime and cefamandole and also theophylline and theobromine were achieved, as shown in Table II. Also, it should be noted that the elution order of phenytoin and carbamazepine should be reversed on the 16G-Ph and 16G-Ph-10G silicas.

Direct injection determination of drugs in serum

Fig. 1A and B show chromatograms of the direct injection determination of anticonvulsant drugs, phenobarbital, phenytoin and carbamazepine, in human serum on the 16G-Ph and 16G-Bu silicas, respectively. Under these conditions, phenobarital was not separated from the background components of serum on the 16G-Bu silica. Fig. 2 shows a chromatogram of the direct injection determination of theophylline, theobromine and caffeine in serum on the 16G-Ph silica. Fig. 3 shows separation of cefmenoxime and cefamandole by direct serum injection on the 16G-Oc silica. Fig.
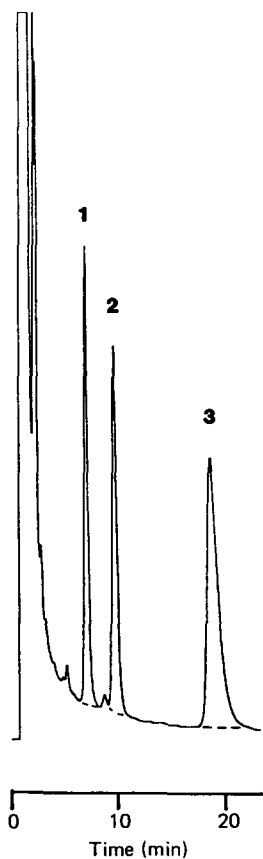

Time (min)

Fig. 2. Chromatogram of control serum spiked with (1) theophylline (10 μg/ml), (2) theobromine (10 μg/ml) and (3) caffeine (20 μg/ml). Chromatographic conditions: column, 16G-Ph silica packed into a 100 × 4.0 mm I.D. column; eluent, 50 mM NaH$_2$PO$_4$ + 50 mM Na$_2$HPO$_4$–CH$_3$CN (23:1, v/v); fow-rate, 0.8 ml/min; detection, 275 nm; sensitivity, 0.032 a.u.f.s.; injection volume, 10 μl. Dashed line indicates serum blank.

4 shows a chromatogram of anticonvulsant drugs in serum on the 16G-Ph-10G silica.

As shown in Figs. 1–4, these hydrophobic or hydrophilic drugs were eluted following the elution of serum proteins in the void volume, and were well separated from the background components of serum.

Durability of the MFP silicas

In a previous paper [7], we reported that the 16G-Ph silica could tolerate up to about 500 repetitive injections of a 10-μl serum sample (total serum sample of 5 ml) with no change in the column efficiency and back-pressure. However, previously [7] the column was washed with the stronger eluent after about 100–150 injections of a 10-μl serum sample. In this study, repetitive injections of serum samples onto the

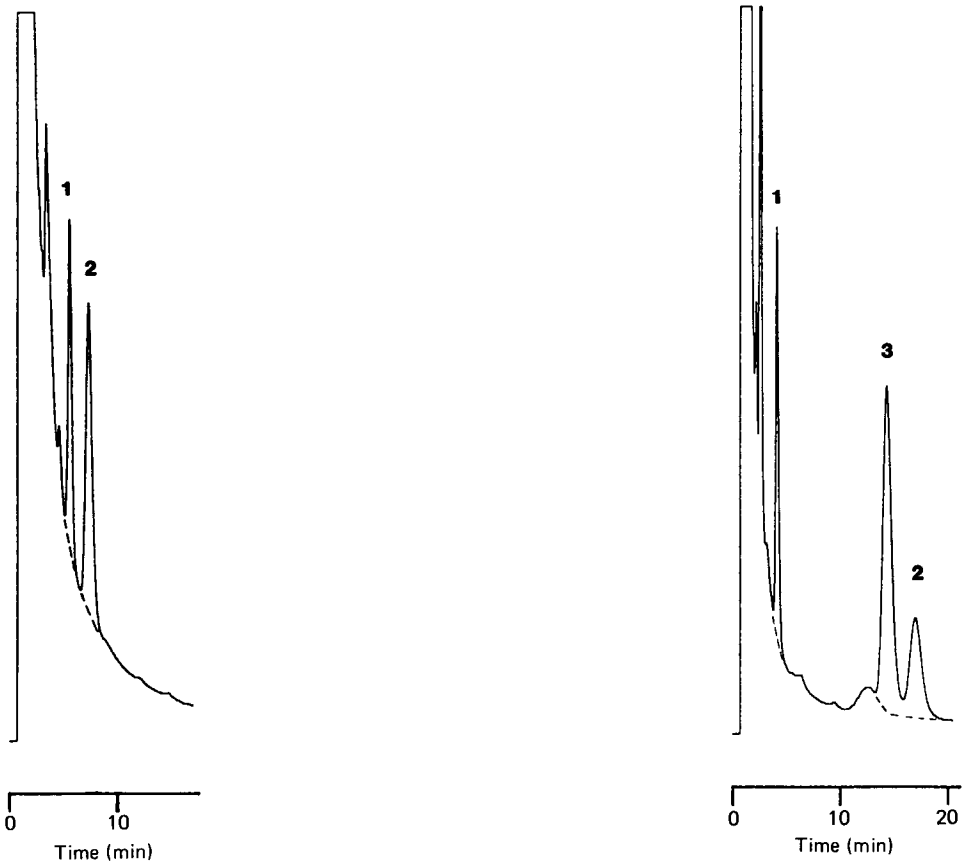

Fig. 3. Chromatogram of control serum spiked with (1) cefmenoxime (20 μg/ml) and (2) cefamandole (40 μg/ml). Chromatographic conditions: column, 16G-Oc silica packed into a 100 × 4.0 mm I.D. column; eluent, 90 mM NaH$_2$PO$_4$ + 10 mM Na$_2$HPO$_4$–CH$_3$CN (100:1, v/v); flow-rate, 0.8 ml/min; detection, 254 nm; sensitivity, 0.032 a.u.f.s.; injection volume, 10 μl. Dashed line indicates serum blank.

Fig. 4. Chromatogram of control serum spiked with (1) phenobarbital (20 μg/ml), (2) phenytoin (40 μg/ml) and (3) carbamazepine (20 μg/ml) on the 16G-Ph-10G silica. Chromatographic conditions as in Fig. 1 except sensitivity, 0.016 a.u.f.s. Dashed line indicates serum blank.
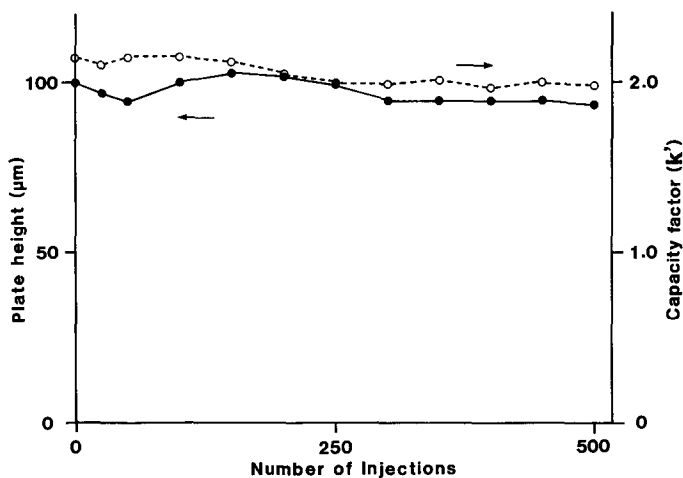
Fig. 5. Dependence of the plate height and capacity factor measured for phenobarbital on the number of injections of a 20-μl serum sample. Chromatographic conditions as in Fig. 1.

16G-Ph-10G support were tried without washing the column and without using the in-line filter and the guard column. As shown in Fig. 5, no changes in the column efficiency and capacity factor for phenobarbital were observed after about 500 repetitive injections of a 20-μl serum sample (total serum sample of 10 ml) without an increase in back-pressure. The more hydrophilic MFP silica 16G-Ph-10G, could be more suitable than the 16G-Ph silica for direct serum injection assays of drugs.

MFP silica supports having hydrophobic (phenyl, butyl or octyl) and diol phases were prepared for direct serum injecton assays of drugs. These supports could be used for the assays of drugs in serum either alone or in combination with conventional reversed-phase supports such as C_{18} or C_8. The preparation method can be applied to the preparation of MFP packing materials having various functionalities for a wider range of applications.

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