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High-performance liquid chromatographic determination of drugs and metabolites in human serum and urine using direct injection and a unique molecular sieve

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Abstract

Silicalite is a molecular sieve that contains an intricate system of channels approximately 6 Å in diameter. These channels are hydrophobic and have been shown to retain relatively small hydrophobic and hydrophilic molecules from aqueous and biological samples. Silicalite is shown to be a restricted-access medium that permits the injection of biological fluids directly onto a HPLC column packed with Silicalite, eliminating the need for sample preparation. The sample macromolecules elute with high recovery mostly at the extraparticulate void. Simultaneously, Silicalite allows various drugs and metabolites to enter the channels and be retained. Recoveries >90% were generally obtained for a wide variety of drugs and their metabolites from human serum and urine. © 1998 Elsevier Science B.V.

Keywords: Molecular sieves; Silicalite

1. Introduction

The isolation and quantification of small molecules from biological samples using high-performance liquid chromatography (HPLC) presents many challenges. The major challenge is the removal of macromolecules, proteins, to avoid damage to chromatographic columns. Proteins in the biological fluids can precipitate or denature and adsorb onto the packing material, leading to back-pressure build-up, changes in retention time and decreased column efficiency and capacity. Chromatographic systems utilizing mobile phases containing organic modifiers are especially prone to protein precipitation and its effects.

Often, sample preparation is the most vital step in a HPLC clinical analysis. Sample preparation separates an analyte of interest from proteinaceous material, allowing the total amount of the analyte (protein bound and free) to be determined and preventing or reducing the adsorption of protein and other interferences onto the analytical column. At the same time, the analyte is concentrated to improve sensitivity and detection capabilities. Some of the most commonly used sample preparation techniques include liquid–liquid extraction, protein precipitation and solid-phase extraction. However, these methods are labor intensive, increase the total analysis time

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and reduce the total recovery of the analyte of interest.

One way to avoid protein adsorption and eliminate the need for sample preparation is to employ a direct injection technique. Reviews and descriptions of direct-injection techniques have been published [1–10], including a recent comprehensive review of restricted-access media by Rudolphi and Boos [5]. These authors point out that restricted-access columns can be used in coupled-column or column-switching mode as well as by direct injection into a single HPLC column. They concluded that development of new restricted-access media with different retention principles will make an even broader application spectrum available to chromatographers.

In the present work a unique molecular sieve known as Silicalite is used as a restricted-access column packing for HPLC. Silicalite, first synthesized in 1977 [11] has a framework of mostly five-member rings of silicon–oxygen tetrahedra. These tetrahedra form a three-dimensional system of intersecting channels, 6 Å in diameter, defined by rings of 10 oxygen atoms. The cavity in Silicalite is hydrophobic and able to retain organic compounds by hydrophobic interaction. The external surface appears to be relatively hydrophilic. The 6-Å cavity results in a sieving action. Large molecules are excluded from the cavity and pass rapidly through a column packed with Silicalite. However, smaller molecules are retained to varying degrees by Silicalite and can be separated by HPLC. Dumont and Fritz [12] found that many aromatic compounds and compounds with moderately long aliphatic chains are still retained by Silicalite. Mayer and Fritz have shown that Silicalite, when used as a sorbent for solid-phase extraction, successfully extracts a wide variety of organic compounds from aqueous [13] and biological samples [14]. In the present work, columns packed with Silicalite are used with direct injection for the HPLC determination of drugs and other small molecules in serum and urine samples.

2. Experimental

2.1. Reagents and chemicals

The drugs and metabolites of interest were obtained from Sigma (St. Louis, MO, USA). Potassium dihydrogenphosphate and acetonitrile were obtained from Fisher (Pittsburgh, PA, USA). Laboratory distilled water was further purified using a Barnstead Nanopure II system (Sybron Barnstead, Boston, MA, USA). Human serum and urine were personally donated. Silicalite from the UOP, were sieved to obtain 5–8 μm particles.

2.2. Instrumentation

Approximately 1.5 g of Silicalite was slurry packed into a 100×4.6 mm I.D. stainless steel column. The following packing procedure was followed. Silicalite was added to degassed 2-propanol and sonicated under vacuum for 30 min. This step removed any trapped air from within the Silicalite and produced a slurry. A Shandon HPLC packing pump (Shandon Southern, Sewichley, PA, USA) was used at a pressure of 3000 p.s.i. (1 p.s.i.=6894.76 Pa) to pack the slurry into the column. The chromatographic system for the quantitation studies and isocratic elution consisted of an Isco syringe pump equipped with a Kratos 783 UV–Vis detector (Applied Biosystems, Ramsey, NJ, USA). Gradient elution was achieved using a chromatographic system consisting of a Waters Model 600E pump controller, Model 610 pump and valve station. A Waters Model 996 photodiode array was used for identifying the eluting compounds. Samples were manually injected using a Rheodyne system fitted with a 5-μl injection loop.

2.3. Recovery of serum and urine components from Silicalite

The recovery of serum and urine, including proteins and endogenous compounds, was calculated by comparing the peak areas obtained using a column packed with Silicalite with those obtained with an empty 100×4.6 mm I.D. column. The effects of mobile phase conditions, acetonitrile concentration and pH, on the recovery of the components of the biological fluids were studied. The effluent from the columns was monitored at 236 nm and all recoveries were calculated as an average of three injections.

2.4. Recovery of drugs and metabolites from serum and urine

Aqueous standards were prepared by adding ace-

tonitrile solutions of the drugs or metabolites to 1.0 ml of deionized water. Biological samples were prepared by adding the same acetonitrile solutions of the drugs or metabolites to 1.0 ml of serum or urine. The final concentration of each drug was in its therapeutic range. The recovery of each drug and metabolite was calculated from the peak area ratio of the analytes dissolved in the biological fluids and in deionized water.

2.5. Determination of caffeine metabolites in human urine

Caffeine for oral administration was in the form of a diet cola, 2 l. A 2-l dose (180 mg caffeine) was administered over a 24-h period, after which urine was collected and pooled. Concentrations of methylxanthines and methyluric acids in urine were determined using direct injection and isocratic elution. Calibration curves for the determination of caffeine metabolites in urine were derived as follows. To a 500-μl aliquot of urine was added 10 μl of known concentrations of a caffeine metabolite.

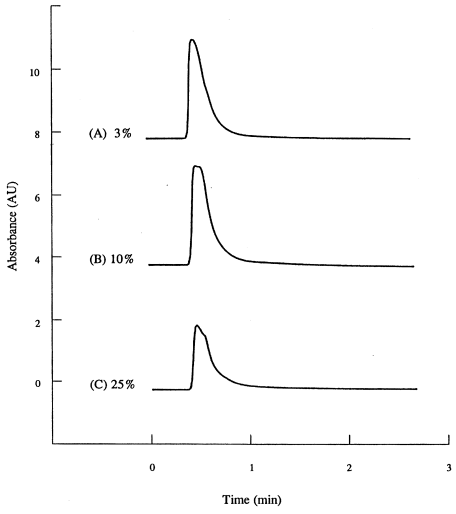

Fig. 1. Effect of acetonitrile concentration on the elution of human serum. Conditions: column, Silicate (5–10 μm) 10 cm×4.6 mm I.D.; mobile phase, aqueous 20 m*M* phosphate buffer, pH 6.9; (A) 3% (v/v) acetonitrile, (B) 10% (v/v) acetonitrile, (C) 25% (v/v) acetonitrile flow-rate, 1.0 ml/min; detection 236 nm; injection volume, 5 μl.

3. Results and discussion

3.1. HPLC of serum samples

The smallest major protein in serum is serum albumin [15] which constitutes nearly 60% of the total serum protein. Serum albumin has a molecular mass of approximately 66 000 Da and its effective spherical radius has been estimated to be 40 Å [16]. Therefore, serum albumin should be excluded from the channels of Silicalite and not be retained by a column packed with Silicalite.

Portions of human serum (5 μl) were injected onto a 10 cm×4.6 mm I.D. column packed with Silicalite. A large serum peak eluted from the column spanning approximately 0.5 to 1.2 min, followed by a steady baseline with no additional

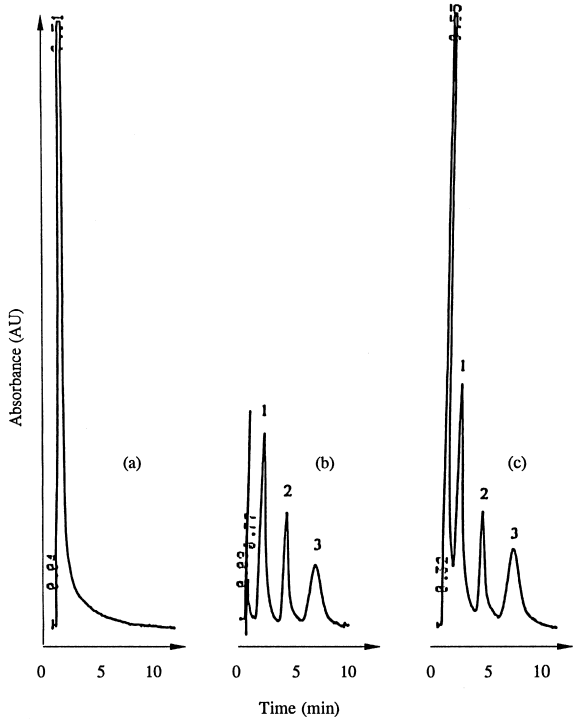

Fig. 2. Chromatogram of human serum blank (a) chromatogram of aqueous standard containing 1. sulfapyridine (8 ppm), 2. 1-naphthol (16 ppm) and 3. carbamazepine (8 ppm) (b); and chromatogram of human serum spiked with the same three analytes (c). Conditions: column, Silicate (5–10 μm) 10 cm×4.6 mm I.D.; mobile phase, aqueous 20 m*M* phosphate buffer–acetonitrile (80:20) (pH 6.9); flow-rate, 1.0 ml/min; detection 254 nm; injection volume, 5 μl.

peaks. Additional experiments showed the serum peak to depend somewhat on the pH and solvent composition of the mobile phase. The effect of acetonitrile concentration was studied by using an aqueous mobile phase containing 20 m*M* potassium phosphate buffer (pH 6.9) with varying percentages of acetonitrile. Serum blanks are shown in Fig. 1. The area of the serum peak remained approximately constant up to 20% acetonitrile but decreased in 25% acetonitrile, suggesting possible denaturation and precipitation. These findings agree with Pinkerton et al. [17] who determined that protein precipitation begins in solutions containing approximately 25% acetonitrile. Subsequent injections of serum showed no build-up in the area of the serum peak. This shows that virtually all of the serum protein passes through the Silicalite column.

The mobile phase pH is another concern when using direct injection of serum samples. Proteins are more prone to precipitate at their isoelectric point (p*I*), which is 4.7 for serum albumin [2]. The effect of pH on the elution of serum components was investigated using mobile phases in the pH range from 2.5 to 6.9 containing 5% and 20% acetonitrile in each pH series. Based on the serum peak area, recoveries decreased somewhat at pH 4.0 and below. At pH lower than 4.7 serum albumin will have a net positive charge which could result in some loss by attraction to negatively charged silanol groups on the Silicalite surface. Based on these experiments, it is recommended that the mobile phase pH be higher than 4.7 and should contain no more than 20% acetonitrile.

3.2. Recovery of drugs from serum

Most drugs in blood are bound to serum proteins to a different extent. For example, phenytoin, theophylline and acetaminophen are 90%, 55% and 30%

Table 1
Percentage recoveries of various drugs from human serum

Drug	ACN (%, v/v) in mobile phase	Retention time (min)	Concentration of drug (μg/ml)	Recovery (%)
Cardiac				
Nifedipine	7	1.35	12	94
Antibacterial				
Sulfapyridine	20	2.06	3	100
Sulfamethoxazole	10	7.42	2	95
Septra	10	5.63	8	92
Anorexic				
Deoxyephedrine	10	3.79	25	94
Anti-inflammatory				
Acetaminophen	5	4.99	10	99
Ibuprofen	3	5.29	15	96
Anticonvulsant				
Ethosuximide	10	2.45	60	99
Theophylline	5	2.33	10	96
Primidone	10	2.16	10	95
Phenobarbital	13	3.58	5	91
Carbamazepine	20	7.61	7	90
Phenytoin	19	2.88	16	90
Sedatives				
Barbital	8	7.60	5	99
Stimulants				
Caffeine	11	2.99	20	92

Mobile phase: 20 m*M* phosphate buffer (pH 6.9), flow-rate: 1.0 ml/min, column: 10 cm×4.6 mm I.D., detection: 254 nm, injection: 5 μl.

bound, respectively [18]. Our analytical goal was to separate and recover the total amounts of drugs present (bound+unbound).

Each of 15 drugs was added individually to serum sample so that the concentration of each drug was within its therapeutic range. The conditions for effective separation of the drugs by isocratic elution varied considerably. A range of acetonitrile concentrations from 3 to 20% (v/v) was used in the mobile phase which was buffered in all cases to an apparent pH of 6.9 with 20 m*M* potassium phosphate. In some cases, several drugs could be separated isocratically at the same time. This is illustrated in Fig. 2 which compares chromatograms for three drugs separated in aqueous and in serum samples. Quantitative data (average of three runs) for direct-injection chromatographic determination of individual drugs in serum are shown in Table 1. Excellent recoveries were obtained for all of the 15 drugs, the average being 95%. The average relative standard deviation (R.S.D.) was 3%. These results indicate that the direct-injection method gives essentially quantitative results for these drugs in serum regardless of individual differences in their degrees of binding to serum proteins.

Gradient elution can be used to separate several drugs in a single run. Fig. 3 shows the separation of seven drugs in serum using gradient elution. It was possible to use acetonitrile concentrations at least as high as 50% without protein denaturization or adsorption; the proteins were eluted from the column at much lower concentrations of acetonitrile. Quantification of these seven drugs was accomplished by comparing the peak areas of the serum sample with a standard mixture of drugs run under the same gradient conditions. The average recovery of the seven drugs in serum was 97% with an average R.S.D. of 4%.

Table 2
Percentage recoveries of various metabolites from human urine

Drug and metabolite(s)	Concentration of drug or metabolite (μg/ml)	Recovery from human urine (%)
Caffeine	10	95
1-Methyluric acid	8	81
1-Methylxanthine	8	97
1,7-Dimethylxanthine	7	100
7-Methylxanthine	6	95
1,3-Dimethylxanthine	5	97
3-Methylxanthine	6	100
3,7-Dimethylxanthine	2	100
1,3-Dimethyluric acid	5	96
Phenytoin	3	91
5-(*p*-Hydroxyphenyl)-5-phenylhydantoin	1	99
Primidone	4	95
Phenobarbital	4	98
Acetylsalicylic acid	4	95
Salicylic acid	8	85
O-Hydroxyhippuric acid	9	90
Acetaminophen	3	94
p-Acetamidophenyl β-D-glucuronide	5	95
Phenylalanine	5	100
Phenylpyruvic acid	8	87
Carbamazepine	0.5	100
Carbamazepine-10,11-epoxide	10	96

Mobile phase: 20 m*M* phosphate buffer (pH 2.5), flow-rate: 1.0 ml/min. Column: 10 cm×4.6 mm I.D., detection: 262 nm, injection: 5 μl.

3.3. Determination of drugs in urine

Unlike serum, urine contains little protein but it does contain major amounts of various salts, uric acid, creatinine and urea. With a mobile phase at pH 4.5 (phosphate buffer) containing 5% acetonitrile, a human urine blank shows several partially resolved peaks between about 0.6 min and 1.2 min. These peaks sharpen and appear as a single peak when the acetonitrile concentration is increased to 15% (v/v). Increasing the acetonitrile concentration to 25% (v/v) sharpens the urine peak even more. Experiments with the urine blank as a function of acetonitrile in the mobile phase indicated rapid, complete elution of urine components between 10% and 55% acetonitrile, both at pH 2.5 and pH 4.5.

Each of seven analytes (six drugs and one nutrient) and some of their metabolites were quantitated individually from urine. The acetonitrile content of the isocratic mobile phase varied from 1 to 30% (v/v). In all determinations, the mobile phase consisted of 20 m*M* potassium phosphate buffer (pH 2.5) and was adjusted at a flow-rate of 1.0 ml/min. The percentage recoveries of the analytes are given in Table 2. The average percentage recoveries of the parent drugs and metabolites were 97% and 95%, respectively with an average R.S.D. of 3%.

The optimized separation of primidone, phenytoin and carbamazepine and their metabolites in urine is shown in Fig. 4. The six analytes were well resolved

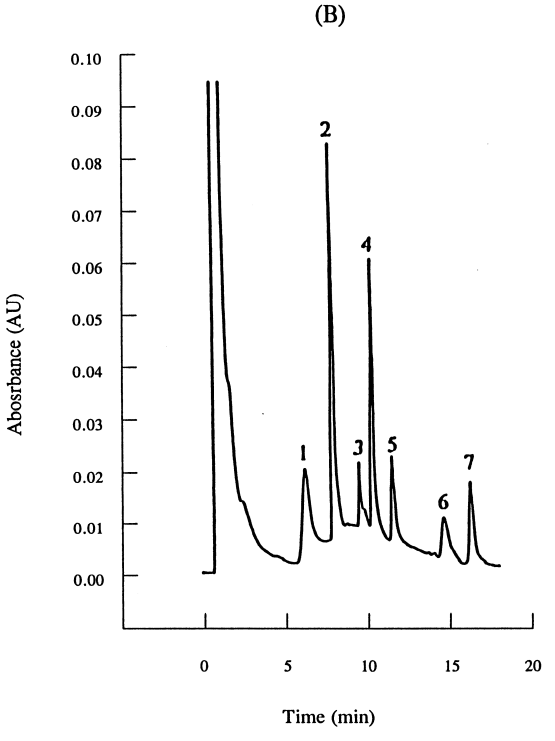

Fig. 3. Chromatogram of human serum spiked with (1) acetaminophen (2 μg/ml), (2) barbital (25 ppm), (3) primidone (15 ppm), (4) phenobarbital (20 ppm), (5) phenytoin (20 ppm), (6) sulfapyridine (2 ppm), (7) carbamazepine (2 ppm) (B). Conditions: column, Silicate (5–10 μm) 10 cm×4.6 mm I.D.; mobile phase, aqueous 20 m*M* phosphate buffer (pH 6.9); gradient elution steps: 5–20% (v/v) acetonitrile in 2 min, 20–25% (v/v) acetonitrile in 2 min, 25–30% (v/v) acetonitrile in 4 min, 30–50% (v/v) acetonitrile in 2 min, and 50% (v/v) acetonitrile for 10 min; flow-rate, 1.0 ml/min; detection 254 nm; injection volume, 20 μl.

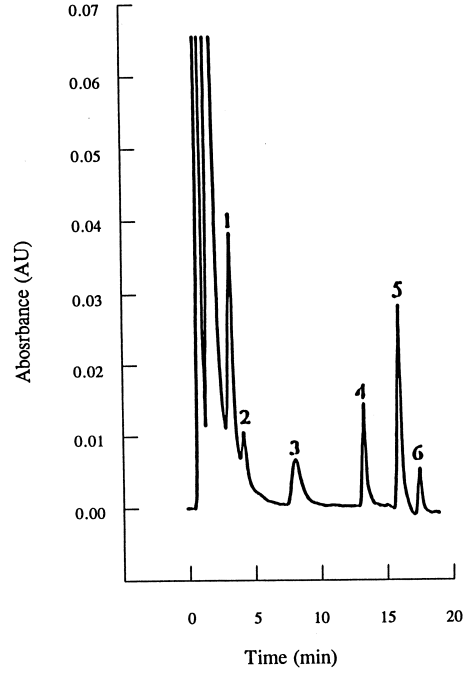

Fig. 4. Chromatogram of human urine spiked with (1) 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (4 ppm), (2) primidone (8 ppm), (3) phenobarbital (12 ppm), (4) phenytoin (12 ppm), (5) carbamazepine-10,11-epoxide (10 ppm) and (6) carbamazepine (2 ppm) (B). Gradient elution: 14% (v/v) acetonitrile for 5 min, 14–25% (v/v) acetonitrile in 1 min, 25–30% (v/v) acetonitrile in 2 min, 30–50% (v/v) acetonitrile in 3 min and 50% (v/v) acetonitrile for 6 min; flow-rate, 1.0 ml/min; detection 230 nm; injection volume, 5 μl.

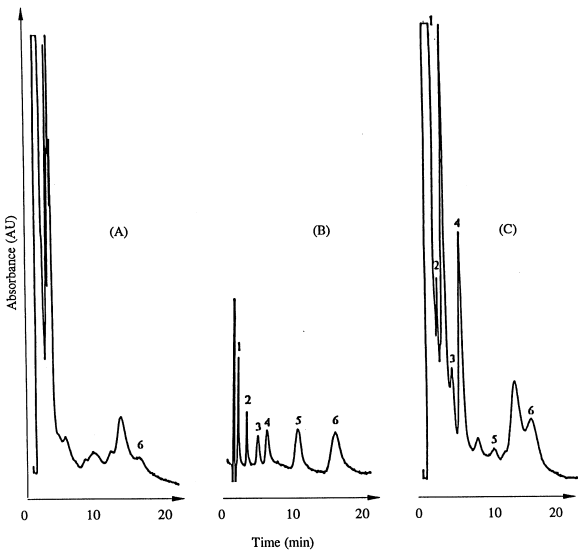

Fig. 5. Caffeine metabolites. (A) Human urine blank, (B) standard caffeine metabolites, (C) human urine after consumption of a caffeine cola. Peak identification: 1=1-methyluric acid, 2=1,3-dimethyluric acid, 3=3=methylxanthine, 4=1-methylxanthine, 5=3,7-dimethylxanthine, 6=1,7-dimethylxanthine. Mobile phase: acetonitrile at pH 2.5.

and eluted in less than 16 min using gradient elution. The three drugs had absolute limits of detection of 1 to 3 ng.

3.4. Analysis of urine for caffeine metabolites

The adsorption of caffeine from the gastrointestinal tract is rapid but irregular [19,20] and its disposition in the human body is variable. Caffeine is extensively metabolized with only 2% of a dose excreted unchanged in the urine. The primary degradation of caffeine is *N*-demethylation and/or oxidation to theophylline (1,3-dimethylxanthine), paraxanthine (1,7-dimethylxanthine), theobromine (3,7-dimethylxanthine) and 1,3,7-trimethyluric acid. These compounds can degrade further to dimethylated uric acids, monomethylxanthines and monomethyluric acid.

Human urine samples were collected and pooled both after caffeinated cola consumption and after abstaining from caffeine products for several days. The urine blank, with no caffeine ingestion (Fig. 5A) shows only a single peak other than the initial urine peaks. Fig. 5B is the chromatogram of a standard aqueous mixture of six caffeine metabolites. The chromatogram in Fig. 5C shows peaks for all of these metabolites in urine after consumption of a caffeine cola drink. The amount of each metabolite in urine, given in Table 3, was obtained by comparing the peak area in the urine with those in the standard mixture.

3.5. Durability and stability of silicalite

The durability of the Silicalite column was not exhaustively tested. The same column was used in analyzing both urine and serum. The back pressure remained constant and urine recovery from Silicalite did not decrease after at least 200 (5 μl) injections of urine using a pH range of 2.5 to 7.8 and an acetonitrile concentration range of 3 to 70% (v/v). The back pressure increased slightly when serum was injected under some experimental conditions. The stainless steel frit was more prone to clogging when using a mobile phase pH of 5 or lower. After approximately 100 injections of serum samples the front frit was replaced, decreasing the pressure. Prolonged use with a mobile phase of pH 4 and lower containing 20% acetonitrile resulted in a yellowish film at the head of the column. After

Table 3
Caffeine metabolites in urine

Caffeine metabolite	Amount of metabolite found in urine (μg/ml)
1-Methyluric acid	50
1,3-Dimethyluric acid	9
3-Methylxanthine	1
1-Methylxanthine	6
3,7-Dimethylxanthine	1
1,7-Dimethylxanthine	4

removal of this film and replacement of the frit, the back pressure decreased and the column again behaved normally.

4. Conclusions

Silicalite is an excellent stationary phase for the HPLC determination of drugs in biological fluids using direct injection. Macromolecules are excluded from the Silicalite and elute in the void volume from the column but drugs and other relatively small molecules can be separated chromatographically. The use of a Silicalite of smaller, more uniform particles should permit even sharper separations. The stability and durability of the Silicate columns with continued use was found to be very good. Some adsorption of macromolecules on to the stainless steel frit was noted, but replacement of the frit restored the column to its original performance level.

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