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Methylcellulose-immobilized Reversed-phase Precolumn for Direct Analysis of Drugs in Plasma by HPLC

Eiichi YAMAMOTO,† Kaoru MURATA, Yasushi ISHIHAMA, and Naoki ASAKAWA

Analytical Research Laboratories, Eisai Co., Ltd., 5-1-3 Tokodai, Tsukuba, Ibaraki 300–2635, Japan

We evaluated a new restricted access media (RAM) precolumn for direct analysis of drugs in plasma using a column switching HPLC system. The new RAM material was prepared by the modification of the external surface of porous silica with hydrophilic methylcellulose (MC), followed by modification of the internal surface with octadecylsilane (ODS). The external surface of the MC-immobilized ODS silica material (MC-ODS) suppressed the adsorption of proteins, while the internal surface of MC-ODS retained various types of drugs, such as ketoprofen, propranolol, caffeine and atenolol in plasma samples. In addition, MC-ODS allowed direct analysis of drugs in a 1000-μL plasma sample to monitor trace amounts of analytes contained. Reduced efficiency and clogging of the MC-ODS precolumn and/or the analytical column were not observed even after the repetitive injection of plasma sample up to 40 mL. Our results indicated that the MC-ODS precolumn could be used in pharmacodynamic and clinical studies.

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Introduction

High performance liquid chromatography (HPLC) is a powerful tool for analyzing small analytes such as drugs in biological samples. However, analysis of analytes present in matrices such as plasma requires well-designed sample preparation procedures, such as protein precipitation, centrifugation, extraction and filtration, that might introduce experimental errors that may ultimately reduce the precision and accuracy of measurement.

It is well known that direct injection of a biological sample such as plasma into a specially designed column is an attractive alternative to off-line sample preparation techniques. For direct analysis of plasma samples, a number of restricted access media (RAM) columns were designed and are currently available commercially. The internal surface reversed phase (ISRP) invented by Pinkerton and Hagestam[1-3] set a benchmark for performance of RAM columns, and an improved ISRP column bed was later developed by Haginaka.[4] Other types of ISRP columns, several alkyl/diol silica (ADS),[5-8] dual zone[9] and mixed functional phase (MFP) types[10,11] had been developed. As a protein-immobilized stationary phase capable of plasma direct injection, octadecylsilane (ODS) treated with human plasma was developed and plasma direct injection capability was shown by Yoshida *et al*.[12,13] α_1-Acidglycoprotein (α1-AGP)-, ovomucoid- and avidin-coated columns had been developed,[14-16] and are capable of plasma direct injection.[17-20] Furthermore, direct analysis of drugs in serum using zwitterionic bile acid derivative-coated ODS was reported by Umemura *et al*.[21]

Several polymer-modified reversed-phase column materials such as the shielded hydrophobic phase (SHP) and the semi-permeable surface (SPS) had also been reported.[8,22-24] SHP consists of a hydrophilic, polymeric, water-solvated phase, covalently bonded to a silica bed. SPS is a conventional reversed-phase packing adsorbed to polyoxyethylene-based nonionic surfactant as a monolayer and exhibits RAM function.

These RAM columns offer several benefits. First, there is no need to process the off-line sample preparation; thus, one can analyze the sample rapidly and obtain accurate data with high reproducibility. Second, the use of the RAM precolumn in a column switching system allows us to concentrate the analytes of interest and thus results in high sensitivity. Furthermore, this method shows some unique advantages: longevity of the analytical column and ease for adaptation for different separation and detection principles. Third, automation of the procedure using the column switching system should increase the throughput of analysis.

In order to prepare a new RAM material with suitable properties for evaluating small analytes in a protein matrix, we prepared the methylcellulose (MC)-immobilized reversed-phase silica material (MC-ODS) by modification of the external surface of porous silica with MC, followed by the internal surface modification with ODS. Evaluation of the MC-ODS precolumn was carried out by direct analysis of drugs in plasma samples using a column switching HPLC system.

Experimental

Chemicals

Silica gel particles for chromatography (particle size, 40 – 60 μm; pore size, 12 nm) (GL science, Tokyo, Japan) were used for the preparation of the RAM material. Octadecyltrichlorosilane and MC (average M.W. 8×10^4) were purchased from Shinetsu Chemical Industries (Tokyo). Sodium dihydrogenphosphate dihydrate, disodium hydrogenphosphate·12hydrate, ammonium acetate, phosphoric acid and hydrochloric acid were purchased from Wako Pure Chemicals (Tokyo). Triethylamine and hexane were purchased from Tokyo Chemical Industries (Tokyo). Acetonitrile was purchased from Kanto Chemicals

† To whom correspondence should be addressed.

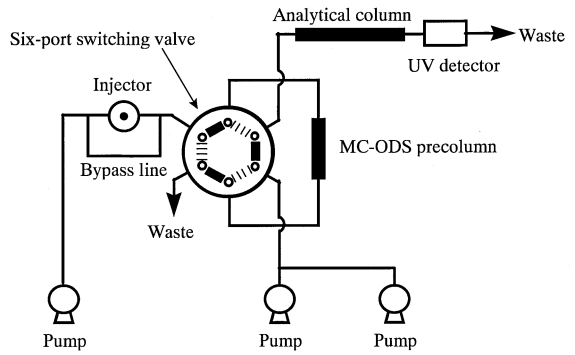

Fig. 1 Schematic diagram of the column switching HPLC apparatus.

Table 1 Recovery of plasma protein from the MC-ODS precolumn under various mobile phase conditions

pH	Mobile phase[a]	Recovery, %[b]
2	PB	99.2
	PB–ACN (9:1, v/v)	99.2
4	PB	97.4
	PB–ACN (9:1, v/v)	98.6
7	PB	100.8
	PB–ACN (9:1, v/v)	101.1
	AmAc	100.4
	AmAc–ACN (9:1, v/v)	100.0

a. PB, 20 mmol/L phosphate buffer; ACN, acetonitrile; AmAc, 20 mmol/L ammonium acetate buffer.
b. $n = 2$.

(Tokyo). Trifluoroacetic acid was purchased from Pierce (Rockford, IL). All reagents were used as obtained without further purification. Water used in all experiments was deionized and purified by a Milli-Q purification system from Millipore (Bedford, MA).

Equipment

A schematic diagram of the instrument setup is shown in Fig. 1. A Reodyne 7125 six-port injection valve (Berkeley, CA) equipped with a stainless-steel sample loop (200 μL) was used as the injector. An LC-5A pump (Shimadzu, Kyoto, Japan) was used to deliver the flow though the MC-ODS precolumn (4.0 mm i.d. × 10 mm). This system was used as an extraction system.

A Reodyne 7000 six-port switching valve was connected to an LC-10Avp analytical system (Shimadzu) with an extraction system. An LC-10ADvp pump delivered the gradient flow to elute the analytes from the MC-ODS precolumn and to perform the separation on an Inertsil ODS-2 analytical column (4.6 mm i.d. × 150 mm) (GL Science).

In the extraction procedure, a sample was injected into the MC-ODS precolumn at 2 mL/min with dilution using a bypass line,[25,26] and eight-fold dilution was prepared from every sample. The MC-ODS precolumn was directed to waste during this time. Plasma proteins and other matrix components were removed while the analytes were retained on MC-ODS to be enriched. This loading and washing step was completed after 2 min when the switching valve was switched to the other position. In the elution procedure, the analytes on MC-ODS were eluted by the LC-10ADvp pump at 1 mL/min to the analytical column. Following the completion of the elution procedure, the six-port valve was switched to the extraction position to re-equilibrate the system for 2 min prior to the next injection.

Preparation of MC-ODS

Silica particles were treated with hydrochloric acid to activate the silanol group and to remove impurities such as metals on the surface, followed by careful washing with water in a Buchner funnel with a fritted disk with a maximum pore size of 20 – 30 μm. The cleansed silica particles were dried at 120˚C in a convection oven (Sanyo, Tokyo) for several hours. In order to modify the silica particles using hydrophilic MC, 30 mL of 0.2% (w/v) MC solution was applied to the silica particles. The mixture was stirred and ultrasonicated for 30 min under ambient temperature, followed by washing and drying as described above.

The MC solution-treated silica particles (MC-silica) were added to 30 mL of hexane. One milliliter of octadecyltrichlorosilane and 0.5 mL of triethylamine were added during stirring at 50 – 60˚C in a water bath. The MC-ODS was carefully washed with ethanol and water and dried at 120˚C as described above. MC-ODS was poured into a column cartridge (4.0 mm i.d. × 10 mm) with 2- and 10-μm end frits (GL Science) by the tapping method and compressed by a suitable tip moistened with an adequate amount of ethanol.

Sample preparation

Human plasma from a healthy male and rat plasma were used as blank plasma. Plasma samples spiked with ketoprofen, propranolol, and a mixture of caffeine and atenolol (10 – 1000 ng/mL) were used as the test plasma samples. Ketoprofen and propranolol in 50% acetonitrile solution and a mixture of caffeine and atenolol aqueous solution (10 – 1000 ng/mL) were used as the calibration standard solutions.

Protein exclusion ability of MC-ODS precolumn

A 100 μl portion of blank plasma was injected into the MC-ODS precolumn with 20 mmol/L phosphate buffer solution (pH 2.0, 4.0 and 7.0), or 20 mmol/L ammonium acetate solution with or without 10% acetonitrile at 2 mL/min using the HPLC system shown in Fig. 1. Under each mobile phase condition, the initial 4.0-mL fraction of the extraction mobile phase was collected in duplicate. A portion of the collected fraction was taken into the solution-holding glass cell (path length 1 cm), then the glass cell was placed in a UV-2400PC spectrophotometer (Shimadzu). Quantitative absorbance measurement was carried out at a wavelength of 280 nm using the test samples and standard solutions. The protein exclusion ability of the MC-ODS precolumn was assessed by the recovery of protein in the collected fractions.

HPLC analysis of ketoprofen and propranolol

Linearities of the standard solutions and sample solutions and recovery from human plasma samples were assessed at ketoprofen and propranolol concentrations of 10, 30, 100, 300 and 1000 ng/mL. Reproducibility of the recovery was obtained for 30-ng/mL plasma samples by triplicate injections. Reproducibility of the retention time of analyte was also obtained for standard solution and plasma sample ($n = 3$).

HPLC analysis of mixture of atenolol and caffeine

Linearity in standard solutions of 10, 100 and 1000 ng/mL of atenolol and caffeine, and recovery from a plasma sample containing 100 ng/mL of atenolol and caffeine was assessed for

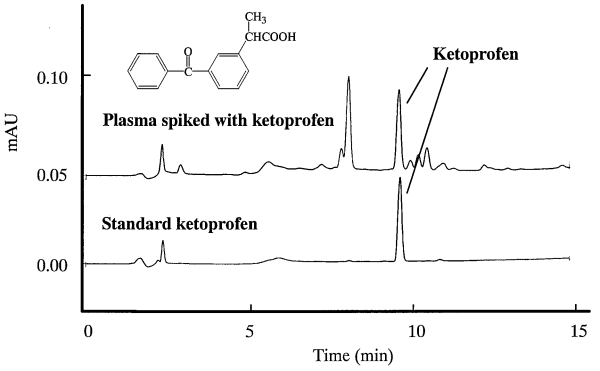

Fig. 2 HPLC-UV chromatograms of ketoprofen. LC conditions: extraction mobile phase, 20 mmol/L phosphate buffer (pH 2)–ACN (9:1, v/v); flow rate, 2 mL/min; extraction time, 2 min; elution mobile phase, gradient of 34 – 75% acetonitrile in 0.1% TFA; flow rate, 1 mL/min; detection wavelength, 254 nm; injection, 1000 ng/mL ketoprofen, 100 μL.

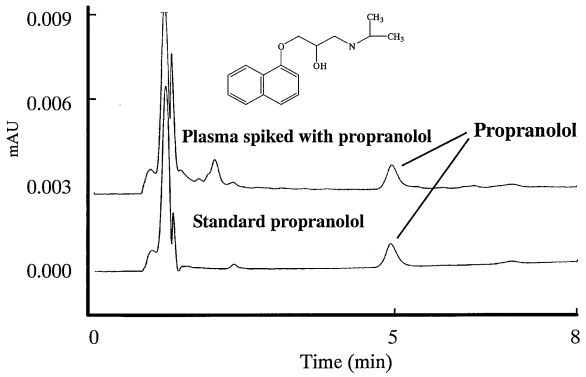

Fig. 3 HPLC-UV chromatograms of propranolol. LC conditions: extraction mobile phase, 20 mmol/L phosphate buffer (pH 7)–ACN (9:1, v/v); flow rate, 2 mL/min; extraction time, 2 min; elution mobile phase, gradient of 30 – 38% acetonitrile in 0.1% TFA; flow rate, 1 mL/min; detection wavelength, 280 nm; injection, 10 ng/mL propranolol, 100 μL.

each compound.

Direct injection of 1000-μL plasma sample

In experiments of direct injection of 1000 μL of plasma sample, a Reodyne 7125 six-port injection valve equipped with a 1000-μL stainless-steel sample loop was used, and the extraction time was 10 min.

Results and Discussion

Preparation of MC-ODS

The MC-ODS packing materials were prepared from large porous silica particles for short-time extraction and prevention of column clogging.[27,28] In this study, we tried to modify the ODS silica particles with a hydrophilic polymer such as MC, hexadimethrine, polyvinylalcohol, polyvinylpyrrolidone, certain dextran derivatives and alginic acid in various solvents (mixture of water and organic solvents, and aqueous solutions at various pHs). Unfortunately, the prepared material did not show satisfactory RAM function. However, our attempt was successful in modifying the external surface of porous silica with these hydrophilic polymers such as MC, followed by drying at 120˚C and modification of the internal surface with ODS. The increase in percent carbon relative to unmodified control confirmed the surface modification with MC and ODS, respectively. Actually, 0.00, 0.14 and 8.12% carbon were found in unmodified control, MC-silica and MC-ODS, respectively.

The surface modification with MC is based on the covalent-bonding originating in the physisorption of MC on the silica surface. Physisorption studies of triethylcarbinol on the silica surface showed that the interaction is based on hydrogen bonding with the surface hydroxyl group, indicating that free hydroxyl groups mainly take part in the physisorption,[29-31] and that at higher temperatures chemisorption occurs through the formation of the covalent bond between silanol group and hydroxyl group.[29] Furthermore, in the field of thin-layer chromatography, a polymeric compound is considered to adsorb on the silica surface by hydrogen bonding interaction and so the adsorbed polymer can not be mobilized by any mobilizing solution. Adsorbed hydrophilic polymers give the surface a hydrophilic feature. Therefore, the coverage of MC on the silica surface was presumed to suppress the adsorption of proteins.

Protein exclusion ability of MC-ODS column

The protein exclusion ability of RAM materials depends on the mobile-phase pH. We evaluated the mobile phase pH-dependent recovery of plasma protein from the MC-ODS precolumn. Acidic and neutral conditions (pH 2.0 and 7.0) were employed for the mobile phase. Considering the pI point for albumin, we used a mobile phase at pH 4.0 in these studies. The recovery of plasma proteins from the precolumn is shown in Table 1. The precolumn contained 40-μm MC-ODS particles and 10-μm end frit, which allowed large protein molecules to pass easily through the column without plugging. Therefore, no pressure build-up was observed during the analysis. Most of the plasma protein was recovered at pH 2.0 – 7.0 even under 100% aqueous mobile phase conditions (97.4 – 101.1%). These results indicated that the MC-ODS material suppresses the adsorption of plasma protein and that the MC-ODS precolumn can be applied to the HPLC analysis of drugs in plasma using a mobile phase at pH 2.0 – 7.0.

Direct analysis of drugs in plasma

First, we show the result of acidic ketoprofen with plasma. The HPLC chromatogram is shown in Fig. 2. For plasma samples spiked with ketoprofen, sufficient separation of the ketoprofen was observed. On the other hand, the peak shape and retention time of ketoprofen were similar to those of the standard solution. The RSD(%) value of retention time of ketoprofen was 0.098% for standard solution and 0.095% for plasma sample. We evaluated the linearity of ketoprofen in the standard solutions and plasma samples. A linear relationship was observed for both the standard solutions and plasma samples at concentrations ranging from 10 to 1000 ng/mL (y = 405.78x + 305.81, r = 1.000 for standard solution, y = 403.96x + 980.85, r = 1.000 for plasma sample). These results indicated a satisfactory recovery of ketoprofen from plasma samples (99.6 – 110.2%). The RSD(%) value of the recovery for triplicate injection was 1.169%, which is permissible.

Next, we carried out HPLC analysis of basic propranolol in plasma. A typical chromatogram for plasma spiked with propranolol is shown in Fig. 3. Propranolol was separated from the background peaks. The RSD(%) value of the retention time of propranolol was 0.861% for standard solution and 0.581%

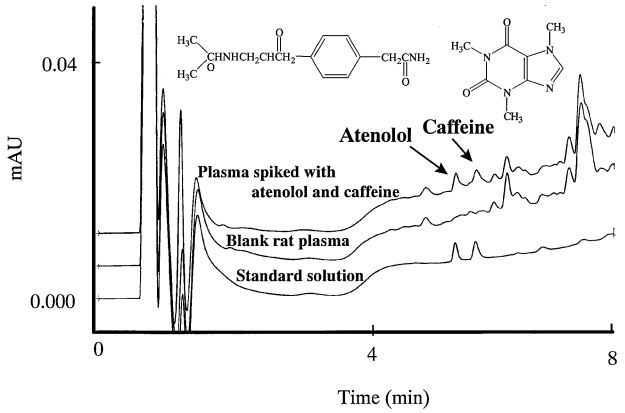

Fig. 4 HPLC-UV chromatograms of a mixture of atenolol and caffeine. LC conditions: extraction mobile phase, 20 mmol/L ammonium acetate; flow rate, 2 mL/min; extraction time, 2 min; elution mobile phase, gradient of 0 – 38% acetonitrile in 0.1% TFA; flow rate, 1 mL/min; detection wavelength, 230 nm; injection, 100 ng/mL atenolol and caffeine, 100 μL.

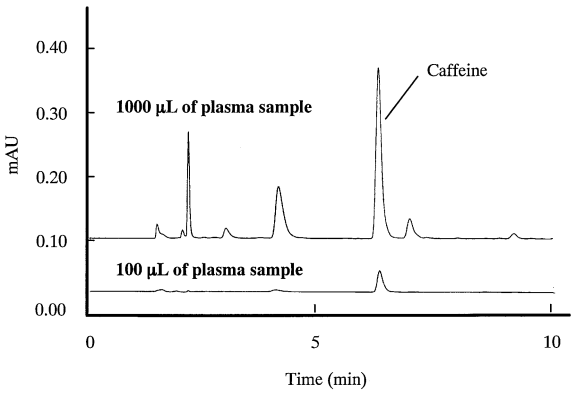

Fig. 5 HPLC-UV chromatograms of human plasma containing caffeine. LC conditions: extraction mobile phase, 20 mmol/L ammonium acetate; flow rate, 2 mL/min; extraction time, 10 min; elution mobile phase, gradient of 0 – 38% acetonitrile in 0.1% TFA; flow rate, 1 mL/min; detection wavelength, 230 nm.

for plasma sample. We also evaluated the correlation between propranolol in the standard solutions and plasma samples, and that recovered from the plasma samples. There was a good correlation between both standard solutions and plasma samples in the concentration range of 10 to 1000 ng/mL ($y = 1137.4x - 3901.6$, $r = 0.999$ for standard solution, $y = 1155.8x - 4427.9$, $r = 0.999$ for plasma sample). These data indicated a sufficient recovery of propranolol from the plasma samples. The recovery of propranolol from the plasma samples ranged from 95.6 to 104.2%. The RSD(%) value of the recovery for triplicate injection was also satisfactory (0.460%).

In general, column-switching HPLC analysis for hydrophilic compounds is difficult because of its weak retention on reversed-phases of precolumns. Therefore, we evaluated the performance of the MC-ODS precolumn for the polar compounds of atenolol and caffeine. Typical HPLC chromatograms for blank rat plasma, a mixture of atenolol and caffeine standard solution and rat plasma spiked with atenolol and caffeine are shown in Fig. 4. These drugs were separated in HPLC chromatograms of the standard solution and plasma sample. There was a good correlation with both atanolol and caffeine in the standard solution (10, 100 and 1000 ng/mL) ($y = 141.8827x + 169.8617$, $r = 1.000$ for atenolol, $y = 146.7275x - 11.9526$, $r = 1.000$ for caffeine). The amounts of recovered atenolol and caffeine at 100 ng/mL were 110.3% and 102.6%, respectively. The RSD(%) value of retention time were 0.103% for atenolol and 0.059% for caffeine in the standard solution.

These results indicated that external surface of the MC-ODS removed the plasma proteins from the precolumn sufficiently. On the other hand, the internal surface of MC-ODS retained acidic, basic and neutral compounds in plasma by reversed-phase mode. This explanation is supported by the fact that all the drugs tested were separated from proteins and fully recovered with satisfactory reproducibility by the MC-ODS precolumn. As a result, it was concluded that the MC-ODS precolumn could be used for column-switching HPLC analysis of various types of compounds that can be retained on the reversed-phase material.

Direct analysis of drugs in plasma using the MC-ODS precolumn contributed the throughput of analysis. For a batch of 20 samples, the sample preparation time for direct injection method was only about 50% of the time required for protein precipitation method. The decrease in sample preparation time improved over all throughput of analysis.

Direct injection of a large volume plasma sample

In order to investigate the capacity of the MC-ODS precolumn, direct injection of 1000 μL of plasma sample was carried out. The caffeine peak was observed in the chromatogram of the plasma sample (Fig. 5). The area of the caffeine peak was proportionate to the sample volume in the range of 100 – 1000 μL. It was found that the MC-ODS precolumn allows a direct injection of 1000 μL of plasma sample and concentration of the hydrophilic compounds. To our knowledge, such a direct injection of 1000-μL plasma sample had not been reported previously. These results indicated that the MC-ODS precolumn has a good sensitivity for hydrophilic compounds and can effectively exclude plasma proteins. The MC-ODS precolumn can contribute to the trace analysis of analyte in plasma in various fields.

Durability of MC-ODS precolumn

The HPLC chromatograms of plasma samples spiked with ketoprofen obtained at baseline and after repetitive injection of plasma sample up to 40 mL are shown in Fig. 6. When the column was operated under the conditions employed in this study, there was little change in column performance and back pressure for either the precolumn or the analytical column even after direct injection of 40 mL of plasma sample. The performance of analytical column was evaluated by the resolution (Rs) between ketoprofen peak and the background peak eluted just behind the ketoprofen peak and the tailing factor (Tf) of ketoprofen peak.

The performance of analytical column at baseline was Rs = 4.70 and Tf = 1.06 with a retention time of 9.697 min, while it was Rs = 4.67 and Tf = 1.18 with a retention time of 9.636 min after repetitive injection of plasma sample up to 40-mL that involved a 1000-μL and more than 200 times injection of 100-μL plasma samples. Reduced efficiency of the MC-ODS precolumn for drug recovery from plasma sample, protein exclusion and clogging of the MC-ODS precolumn and the analytical column were not observed. These results indicated that the MC-ODS precolumn prevents the plasma protein from entering to the analytical column and evidently lengthens the

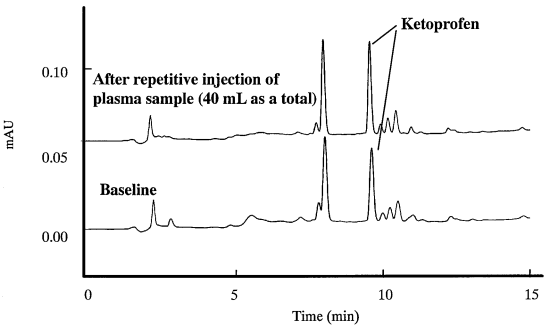

Fig. 6 HPLC-UV chromatograms of ketoprofen for durability evaluation. LC conditions: extraction mobile phase, 20 mmol/L phosphate buffer (pH 2)–ACN (9:1, v/v); flow rate, 2 mL/min; extraction time, 2 min; elution mobile phase, gradient of 34 – 75% acetonitrile in 0.1% TFA; flow rate, 1 mL/min; detection wavelength, 254 nm; injection, 1000 ng/mL ketoprofen, 100 μL.

lifetime of analytical column, and that direct analysis of drugs in plasma using MC-ODS precolumn offer reproducible data.

Hence, we conclude that the MC-ODS precolumn meets the requirements for routine analysis.

Conclusions

We evaluated the utility of the MC-ODS precolumn for direct analysis of drugs in plasma using a column switching LC system. MC-ODS has sufficient stability and durability. Reduced performance of the MC-ODS precolumn was not observed even after the repetitive injection of plasma sample up to 40-mL, including direct injection of a 1000-μL and more than 200 times injection of 100-μL plasma samples. Thus, the performance of the MC-ODS precolumn for various types of drugs (acidic, basic and neutral compounds) indicates its applicability for routine analysis in the field of pharmacodynamic and clinical studies. Further studies on the preparation of the MC-ODS analytical column, off-line solid phase extraction cartridge, a 96-well plate and MC-immobilized materials with ion-exchangeable functions are in progress in our laboratory.

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