

Separation Characteristics of a Phosphatidylcholine-Coated ODS Column for Direct Sample Injection Analysis of Biological Fluid Samples

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An octadecylsilyl silica (ODS) column coated with phosphatidylcholine (PC) was prepared by using a dynamic coating method, and its separation characteristics were evaluated for direct sample injection analysis of biological fluids. First, fundamental separation behaviors of various kinds of large and small molecules/ions were examined by HPLC using the PC-coated ODS column with UV absorption detection, for which pure water was used as a mobile phase. In the experiments, proteins (large molecules) were rapidly eluted in the range of 2.07 min (dead time)–3.20 min, while small molecules, such as inorganic anions, amino acids, organic acids, and organic drugs, were eluted after 3.50 min. The fact that proteins and small molecules/ions were separated on the PC-coated ODS column suggested that the present column can be used for direct sample injection analysis of biological fluid samples, such as urine and blood serum. Thus, the PC-coated ODS column was used in the analysis of arsenic species in urine and in the analysis of nicotinamide, theophylline, tryptophan, and caffeine in a model blood serum solution without any sample pretreatment.

Recently, "metallomics" has been proposed as integrated biometal science,[1] in which the importance of chemical speciation of trace elements in biological samples is especially emphasized from analytical points of view.[1,2] In chemical speciation analysis for species identification, high-performance liquid chromatography (HPLC) is generally used for separation of various chemical species, together with the highly sensitive detection methods, such as inductively coupled plasma atomic emission spectrometry (ICP-AES) and inductively coupled plasma mass spectrometry (ICP-MS).[1–3] In biological sample analysis, however, the high content of salts and proteins in the samples often cause severe deterioration of the column efficiency because they are adsorbed onto the separation column, which makes direct sample injection for chemical speciation analysis of urine or blood serum difficult. In order to overcome such experimental difficulties, pretreatment, such as deproteinization and desalting, is usually required. However, some pretreatment increases the risks of contamination from analytical reagents as well as the alteration of unstable species. Thus, considerable efforts have been dedicated to developing direct sample injection analysis without any sample pretreatment. Up to now, various types of restricted access-type stationary phases with a protein-repelling hydrophilic exterior have been exploited, mainly for therapeutic drug analysis and monitoring.[4–14] The use of micellar mobile phases has also been investigated for similar purposes.[15,16] Such efforts also involve a column switching technique that allows preconcentration of trace analytes.[17] Furthermore, it has been reported that a conventional reversed-phase column immobilized with biomolecules, such as proteins, often works as a restricted access-type stationary phase, which provides some additional separation functions, such as ion-exchange separation and enantiomeric separation.[18–20]

In recent years, we have demonstrated that an octadecylsilyl silica (ODS) column coated with CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate), a zwitterionic bile acid derivative, as well as sulfobetein-type zwitterionic surfactants (C12SB and C14SB) makes it possible to separate simultaneously proteins and small analytes, such as inorganic anions and drugs.[21–30] Such separation characteristics of the CHAPS-coated ODS column can be explained by the unique structure of CHAPS as a surfactant with zwitterionic properties,[22,23,26,30] which enables us to perform multi-mode separation in electrostatic ion chromatography with electrostatic interaction, hydrophobic interaction, ion exchange, and so forth. Thus, the CHAPS-coated ODS column has been used for separation analysis of inorganic cations and anions, amino acid or protein-binding metal species, drug compounds, etc. in urine, blood serum and salmon egg cytoplasm by direct sample injection without any pretreatment. However, it has been found that the column coated with CHAPS can be unstable, and so CHAPS has had to be added to the mobile phase to prevent the deterioration of the column.[27]

In the present work, we have attempted to develop a new type of ODS separation column using phosphatidylcholine (PC) as the stationary-phase modifier for multi-functional separation. PC is a phospholipid with double-chained zwitterionic properties having phosphate and quaternary ammonium groups, which means that it can act as a surfactant with unique characteristics to form micelles as well as mono- and bi-layer membrane structures.[37] So far, in order to separate certain inorganic anions[31–35] or proteins,[36,37] PC has been used

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as a modifier of various types of separation columns, such as ODS and electrophoresis capillary. In the present study, the PC-coated ODS column was prepared by dynamic coating, and its separation characteristics in terms of proteins and small molecules/ions was systematically examined using pure water as mobile phase. Since proteins and small molecules/ions could be separated with the PC-coated ODS column, direct sample injection analysis of arsenic species in human urine and some drug compounds in model blood serum was attempted.

Experimental

Apparatus. The HPLC system used in the present experiment consisted of an HPLC pump (model PU-980, Jasco, Tokyo, Japan), a sample injector (model 7725, Rheodyne, Cotati, CA, U.S.A.) with a 20 μL sample loop, and a UV absorption detector (model UV-970, Jasco). An ODS column (Mightysil RP-18 GP Aqua packed with 5 μm C_{18}-bonded silica, 4.6 mm i.d. × 250 mm long) was purchased from Kanto Chemicals (Tokyo, Japan). An ICP-MS instrument (model Agilent HP 4500, Yokogawa Analytical Systems, Tokyo, Japan) was used as an element-selective detector for HPLC. The effluent from HPLC was led to the capillary of the nebulizer in the ICP-MS instrument through Teflon tubing after the UV absorption detector.

Reagents. 3-*s*,*n*-PC, derived from egg yolk, was purchased from Wako Pure Chemicals (Osaka, Japan). The fundamental structure of PC used is shown in Fig. 1. The structure of this PC was identified by measuring its molecular weight with MALDI-TOF-MS (model AXIMA CFR plus; Shimadzu, Kyoto, Japan). Proteins and other chemicals (Table 1) used for evaluation of separation characteristics were of analytical reagent grade and were used without further purification. Pure water used throughout the present experiments was prepared using a Milli-Q deionization system (model Element A-10, Nihon Millipore Kogyo, Tokyo, Japan).

Preparation of the PC-Coated ODS Column. The PC-coated ODS column was prepared by using a dynamic coating method. First, the ODS column was washed with pure water at least for 60 min at a flow rate of 0.75 mL min^{-1}. In the second step, methanol–water (50:50, v/v) containing 13 mM PC was passed through an ODS column for 120 min at a flow rate of 0.30 mL min^{-1}. Finally, the column was washed with pure water for ca. 60 min at a flow rate of 0.75 mL min^{-1}.

Results and Discussion

Estimation of the Amounts of PC Coated on the ODS Column. In the present experiment, a PC-coated ODS column was prepared as the stationary phase by using a dynamic coating method. In this procedure, PC, which is a zwitterionic phospholipid molecule, is probably adsorbed on the ODS column surface through hydrophobic interactions, and the amount of PC absorbed on the ODS column was estimated by using the

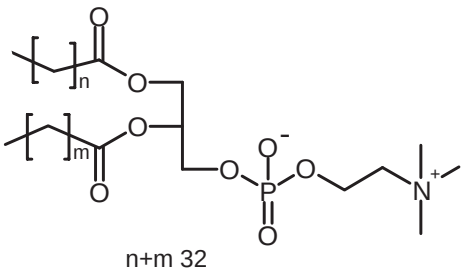

Fig. 1. Chemical structure of phosphatidylchorine.

Table 1. Retention Factors of Various Analytes Obtained by the Proposed HPLC System[a)]

No.	Analyte	MW/Da	pI or pK_a	Retention factor	
				PC[b)]	CHAPS[c)]
1	Blue dextran	2000000	non-ionic	0.000	0.000
2	Alcohol dehydrogenase	150000	pI 5.4	0.502	0.0725
3	Albumin	66000	pI 4.9	0.526	0.0966
4	AsO_3^{3-} (iAs^{III})	126	pK_a 9, 12, 13	0.691	0.870
5	AsO_4^{3-} (iAs^{V})	142	pK_a 2, 7.08, 12	0.691	0.725
6	Cl^-	36		0.778	0.638
7	Br^-	80		0.826	0.516
8	I^-	127		0.947	1.12
9	Monomethylarsonic acid (MMA)	140	pK_a 4.1, 9.1	0.836	0.469
10	Dimethylarsinic acid (DMA)	138	pK_a 6.2	0.836	3.70
11	Arsenobetaine (AB)	178		0.836	3.70
12	Acetic acid	60	pK_a 4.75	1.00	4.15
13	Citric acid	192	pK_a 3.13, 4.76, 6.40	1.24	4.00
14	L-Aspartic acid	133	pK_a 2.10, 3.86, 9.82	0.850	3.40
15	L-Phenylalanine	165	pK_a 2.20, 9.09	1.10	4.33
16	L-Tryptophan	204	pK_a 2.38, 9.39	7.62	10.55
17	Nicotinamide	122	non-ionic	1.73	6.18
18	Theophylline	180	non-ionic	4.68	5.31
19	Caffeine	194	non-ionic	10.33	16.47

a) Organic compounds (proteins, organic acids, amino acids, and drug compounds) were detected by UV absorption detection at 210 nm. Inorganic ions (Cl^-, Br^-, and I^-) and arsenic species were detected by ICP-MS at m/z 51 (as $^{35}Cl^{16}O$), 79, 127, and 75, respectively. b) PC-coated ODS column, pure water was used as the mobile phase. c) CHAPS-coated ODS column, 0.1 M Tris-HNO_3 buffer solution containing 0.2 mM CHAPS (pH 7.4) was used as the mobile phase.

breakthrough method. Figure 2 shows the breakthrough curve for the UV absorption monitored at 210 nm, during which time 13 mM PC in methanol–water (50:50, v/v) was passed through the ODS column for 150 min at a flow rate of 0.30 mL min^{-1}. In this experiment, 45 mL of the PC solution was supplied into the ODS column. Under these conditions, a breakthrough point was observed at ca. 67.5 min, which corresponded to a solution volume of 20.3 mL, as is shown in Fig. 2. Thus, the net adsorbed amount of PC was estimated to be 0.38 mmol per column. This adsorbed amount of PC on the ODS column was similar to that of CHAPS (0.40 mmol per column).[22] The surface coverage of PC on the column was estimated to be approximately 20% from the binding amounts of ODS, which were calculated from the commercially reported basic physicochemical properties of the ODS phase, in which the amount of packing materials and total carbon content were 2.8 g and 15%, respectively. In other words, the total amount of ODS was 1.9 mmol per column.

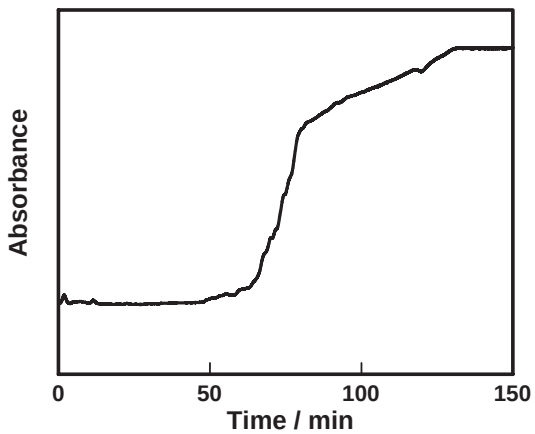

Fig. 2. The breakthrough curves for coating phosphatidylcholine on the ODS column by a dynamic coating method. Coating solution, 13 mM phosphatidylcholine in methanol–water (50:50 v/v); flow rate, 0.30 mL min^{-1}; detection wavelength, 210 nm.

A schematic structure model of the PC-coated ODS column is shown in Fig. 3, in which PC might be adsorbed on the ODS column through the hydrophobic interactions between the hydrocarbon groups of PC and the octadecyl groups on ODS. Therefore, the ionic groups of PC may be oriented to the mobile phase, which results in forming the protein-repelling hydrophilic exterior. As a result, the PC-coated ODS column act as a restricted access-type stationary phase, in which large molecules, such as proteins, may be eluted rapidly without retention. On the other hand, small molecules or ions should be retained through hydrophobic interactions as well as electrostatic and ion-exchange interactions by penetrating into the inner area of the PC-coated ODS column. As a result, the PC-coated ODS column should have multi-functional separation capability for various kinds of compounds even in pure water as the mobile phase. Such separation characteristics were examined in the following experiments.

Separation Characteristics of PC-Coated ODS Column. In electrostatic ion chromatography, the ODS columns coated with CHAPS, C12SB, and C14SB (zwitterionic substances) have been used as the stationary phase and the fundamental separation characteristics of various compounds have been investigated using pure water as the mobile phase.[22–25,30] In the present experiments, the fundamental separation characteristics of a PC-coated ODS column as the stationary phase were examined in terms of proteins and small molecules/ions, such as inorganic ions, amino acids, organic acids, and some drug compounds, where pure water was also used as the mobile phase.

The data for retention factors of the various compounds are summarized in Table 1, together with their molecular weights, isoelectoric point (pI) and/or dissociation constant (pK_a). In Table 1, the retention factors of the same analytes that were obtained by using the CHAPS-coated ODS column are also shown for comparison. It is noted here that, in the case of the CHAPS-coated ODS column, proteins were eluted with 0.1 M Tris-HNO_3 buffer solution containing 0.2 mM CHAPS (pH 7.4) as the mobile phase, because they could not be eluted with pure water. In order to gain insight into the separation

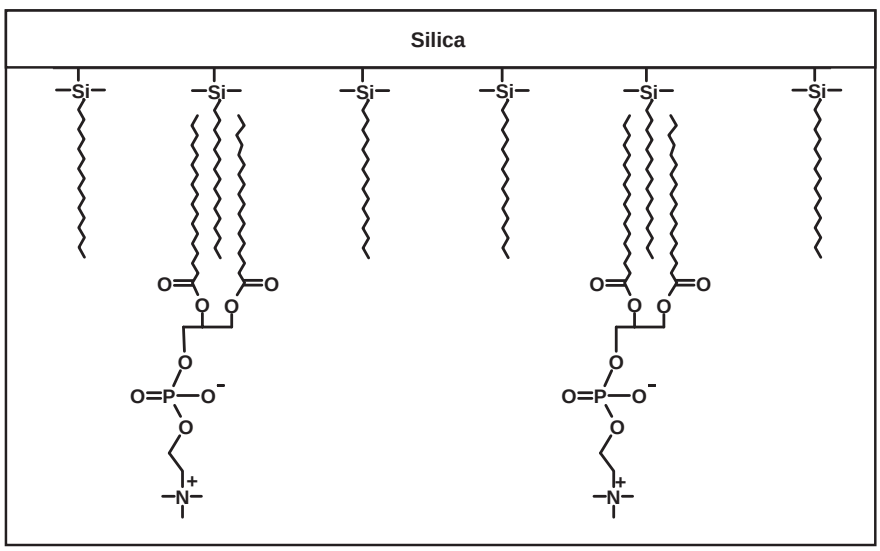

Fig. 3. A schematic structure model of the PC-coated ODS column.

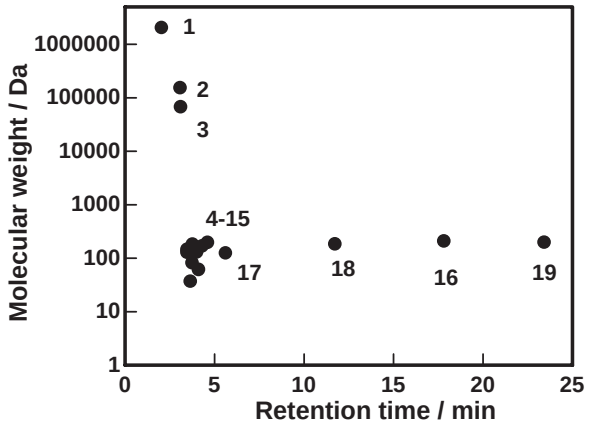

Fig. 4. Relationship between molecular weights and retention times of analytes.

characteristics of the PC-coated ODS column, a plot of the retention time vs molecular weight was prepared, as is shown in Fig. 4, where the numbers correspond to the analytes listed in Table 1. It is seen from Fig. 4 that large molecules, such as blue dextran (MW 2000 kDa) and proteins (alcohol dehydrogenase and albumin), have retention times between 2.07 min (dead time) and 3.2 min, and showed only slightly different retention times with each other. In other words, the PC-coated ODS column has a weak size-exclusion property. The recovery of albumin from the PC-coated ODS column with pure water as the mobile phase was about 95%.

It can also be seen in Fig. 4 that all of the small molecules, such as inorganic anions, organic acids, and amino acids, were separately eluted at retention times longer than 3.5 min. Inorganic anions (Cl^-, Br^-, and I^-) were eluted together in the retention time range from 3.5 to 4.0 min even in the water mobile phase. This might be caused by the electrostatic attraction force of the quaternary ammonium groups of PC on the zwitterionic stationary phases, similar to the CHAPS-coated ODS column.[22,23] Aromatic compounds (Nos. 16–19), except for L-phenylalanine, had the retention times longer than 5.0 min, which might be caused by hydrophobic interactions with the octadecyl groups on ODS or hydrocarbon groups of PC. The elution order of the aromatic compounds was similar to that using a conventional ODS column. However, the retention times for the aromatic compounds using the PC-coated ODS column were shorter than those with the conventional ODS column, maybe, because of the weaker interactions with PC.

From Table 1, the PC-coated ODS column generally has separation characteristics for large and small molecules/ions similar to those of the CHAPS-coated ODS column. However, longer retention times were generally obtained for organic molecules, such as proteins, amino acids, organic acids, and drugs, with the present PC-coated ODS column than those with the CHAPS-coated ODS column, although the retention times of inorganic arsenic species, such as AsO_3^{3-} and AsO_4^{3-}, were significantly shorter in the former. It should be noted here that the retention factors obtained using the PC-coated ODS column system had no significant relationship to the values of pI and/or pK_a.

The stability of the PC-coated ODS column was estimated from the repeated measurements of various analytes. The run-to-run ($n = 5$) and day-to-day ($n = 5$) repeatabilities in terms of retention times were 0.20 and 1.1%, (the relative standard deviation; RSD), respectively. These data were more satisfactory than those reported in the literature.[36,37]

Speciation of Arsenic Species in Human Urine. As reported previously,[39] arsenic species, such as AsO_4^{3-} (iAs^V), AsO_3^{3-} (iAs^{III}), MMA (monomethylarsonic acid), DMA (dimethylarsinic acid), and AB (arsenobetaine), have been found to be excreted in urine after ingestion of *hijiki* (a type of seaweed) by using an ordinary ODS column as the stationary phase in reversed-phase ion-pair chromatography. Since human urine contains a lot of salts, column efficiency often decreases after the repeated experiments. Therefore, a more efficient and ragged separation column is necessary even for urine analysis.

Thus, the present PC-coated ODS column was used to analyze the speciation of arsenic species in human urine samples with high salt content. As is seen in Table 1, when pure water was used as the mobile phase, inorganic arsenic species, iAs^V and iAs^{III}, and organic arsenic species could not be separated on the PC-coated ODS column. As mentioned earlier, the PC-coated ODS column has reversed-phase separation abilities. In the present experiment, the PC-coated ODS column was used in ion-pair formation mode for separation of arsenic species, in which 5 mM citric acid containing 5 mM sodium 1-dodecanesulfonate and 5 mM tetramethylammonium hydroxide (pH 4.0) was used as the mobile phase. A urine sample was collected from a healthy student volunteer 7 h after ingestion of *hijiki*, and it was directly injected to the PC-coated ODS column, using the same instrumental setup as shown in Fig. 2.

The chromatograms for arsenic species in urine are shown in Fig. 5, together with those for standard arsenic compounds, where arsenic was detected by ICP-MS at m/z 75 (^{75}As). In Fig. 5, the chromatograms observed for the 1st and 50th injections are shown to evaluate the ruggedness of the PC-coated ODS column in the repeated experiment. It is seen in Fig. 5 that arsenic species, such as iAs^V, iAs^{III}, MMA, DMA, and AB, were clearly found in urine after ingestion of *hijiki* even after 50 injections. These results indicate that the present PC-coated ODS column can be used to analyze the species in urine samples via reversed-phase ion-pair chromatography, similar to a simple ODS column. In addition, the long term stability of the PC-coated ODS column was significantly better than that of the CHAPS-coated ODS column.

Direct Injection Analysis of Drugs in Serum Sample. As has been described so far, the PC-coated ODS column afforded shorter retention times for proteins and longer retention times for small molecules and ions. These results indicate that the PC-coated ODS column can also be used as a restricted access-type stationary phase with a protein-repelling hydrophilic exterior, which allows direct sample injection analysis of biological fluids. Thus, in order to evaluate the separation capability of the PC-coated ODS column as the restricted access-type stationary phase, direct sample injection analysis of drug compounds in a model serum sample was attempted, because conventional and rapid analysis of drugs in blood serum is important in clinical analysis. A solution containing protein (10000 mg L^{-1} of albumin) and a few drugs (10 mg L^{-1} of nicotin-

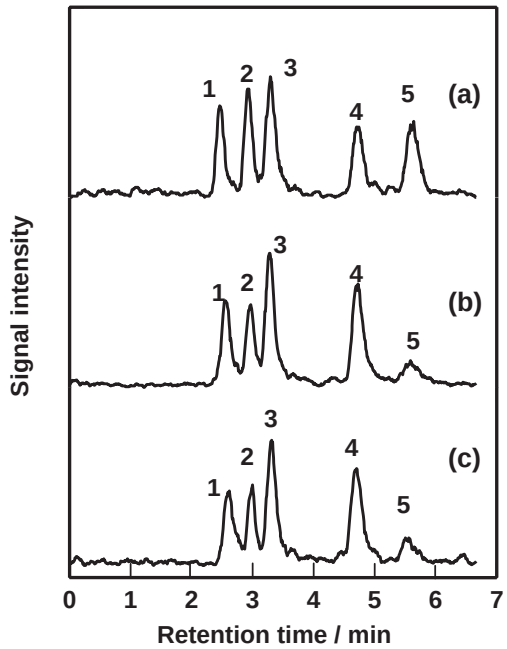

Fig. 5. Chromatograms (a) arsenic standards in pure water, and (b, c) arsenic metabolites in human urine collected 7 h after ingestion of *hijiki*, with ICP-MS detection at m/z 75, where (b) and (c) were obtained from the 1st and 50th measurements, respectively. Peaks: 1. iAs^{V}, 2. MMA, 3. iAs^{III}, 4. DMA, 5. AB. Mobile phase, 5 mM citric acid containing 5 mM sodium 1-dodecanesulfonate and 5 mM tetramethylammonium hydroxide (pH 4.0); flow rate, 0.75 mL min^{-1}; sample injection volume, 20 μL; concentrations of standard arsenic compounds: 10 ng of As g^{-1} each.

amide, theophylline, tryptophan, and caffeine) was prepared as a model serum sample and subjected to direct injection analysis via HPLC using the PC-coated ODS column. The chromatogram measured with UV absorption detection at 210 nm is shown in Fig. 6. Figure 6 shows that the drug compounds (small molecules) are well separated from albumin on the PC-coated ODS column with satisfactory recovery. These results indicate that the PC-coated ODS column can also be used as a restricted access-type stationary phase for biological fluid analysis.

Conclusion

It was demonstrated that proteins are eluted at the retention times shorter than 3.5 min, and inorganic anions, amino acids, and organic acids are eluted significantly later than proteins, when the PC-coated ODS column was used for separation with pure water elution. In addition, direct sample injection analysis of arsenic species in urine with high salt contents as well as of drugs in a model blood serum sample with high protein contents were possible using the PC-coated ODS column. These results suggest that the PC-coated ODS column may be used to analyze the speciation of trace metals bound and unbound by proteins in biological fluids, similar to analysis with the CHAPS-coated ODS column.[25–29] Such work is now in progress, and the results will be published in the near future.

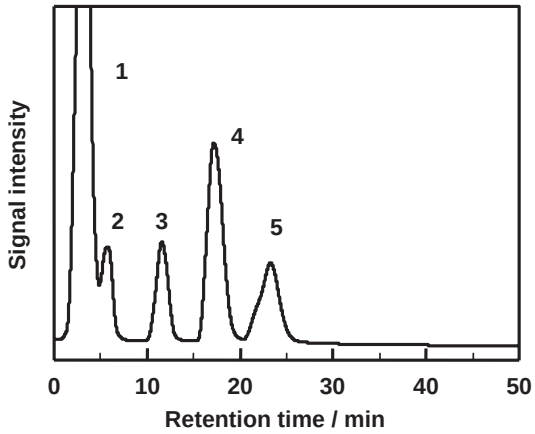

Fig. 6. Chromatogram for drugs in a model blood serum sample obtained by HPLC with detection by UV absorption, using a PC-coated ODS column. Peaks: (1) albumin, (2) nicotinamide, (3) theophylline, (4) tryptophan, (5) caffeine. Model sample, aqueous solution containing 10000 mg L^{-1} of albumin and 10 mg L^{-1} of nicotinamide, theophylline, tryptophan, and caffeine; mobile phase, pure water; flow rate, 0.75 mL min^{-1}; injection volume, 20 μL; detection, UV absorption at 210 nm.

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