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Note

Determination of anticonvulsant drugs and methyl xanthine derivatives in serum by liquid chromatography with direct injection: column-switching method using a new internal-surface reversed-phase silica support as a precolumn

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A column-switching technique has been widely used for sample concentration and clean-up in complex matrices in high-performance liquid chromatography (HPLC) [1]. The precolumns used for direct serum injection assays of drugs by column switching can be divided into two types [1]: one type retains small molecules such as drugs but not macromolecules such as serum proteins; with the other type the situation is the reverse. Recently, supports of the former type have been designed for the purpose of assaying drugs by direct serum injection either alone or in combination with a conventional C_{18} column [2–5]. Hagestam and Pinkerton [3] designed a so-called internal-surface reversed-phase (ISRP) silica support, which is prepared by removing the hydrophobic ligands from the external surfaces of the porous silica particulates by a proteolytic enzyme such as carboxypeptidase A [3,6,7] or α-chymotrypsin [8,9]. The commercially available ISRP support, designed by Hagestam and Pinkerton [3], has glycyl-L-phenylalanyl-L-phenylalanine (GFF) and glycine residues directly bonded to glycerylpropyl groups as its internal and external surfaces, respectively. Thus, the negatively charged GFF ISRP support can

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retain positively charged aromatic substances, and the recommended eluent pH range was limited to 6.0–7.5.

Previously we reported [5,10] the preparation of a new ISRP silica support with N-octanoylaminopropyl and N-(2,3-dihydroxypropyl)aminopropyl phases as internal and external surfaces. Also, the neutral ISRP support can be used for assays of hydrophilic or hydrophobic drugs by direct serum injection over an eluent pH range of 3–7. The aim of the present study is to apply this ISRP silica support to direct serum injection assays of anticonvulsant drugs and theophylline, which require monitoring in therapeutic and clinical usage, by a column-switching method.

EXPERIMENTAL

Reagents and materials

(3-Aminopropyl)trimethoxysilane was obtained from Shin-etsu Silicon Chemicals (Tokyo, Japan). Octanoyl chloride, triethylamine, glycidol (2,3-epoxy-1-propanol), caffeine and theobromine were purchased from Nacarai Tesque (Kyoto, Japan). Other reagents and organic solvents of analytical-reagent grade and control human serum (Control Serum I) were obtained from Wako (Osaka, Japan). Primidone, phenobarbital, phenytoin, carbamazepine and theophylline used in this study were kindly donated by Dainippon Pharmaceutical (Osaka, Japan), Sankyo (Tokyo, Japan), Nippon Ciba-Geigy (Takarazuka, Japan) and Eisai (Tokyo, Japan). A porous Spherisorb silica (Phase Separations, Hauppage, NY, U.S.A.) with a particle diameter of ca. 5 μm, a nominal pore diameter of 80 Å and a specific surface area of 220 m^2/g was used for the preparation of an ISRP silica, as reported previously [10].

Water purified with a Nanopure II unit (Barnstead, Boston, MA, U.S.A.) was used to prepare the eluent and the sample solution.

Instrumentation

The chromatographic system was controlled by an 802-SC system controller (Japan Spectroscopic, Tokyo, Japan). It had two Model 880-PU pumps for delivering the eluents for pretreatment and analysis; a Model 875-UV variable-wavelength detector; a Model 892-01 six-port switching valve; a Model 851-AS automatic injector (all from Japan Spectroscopic) or a Model 8125 injector (Rheodyne, Cotati, CA, U.S.A.). The chromatograms were recorded and integrated using a Chromatopac CR-6A (Shimadzu, Kyoto, Japan). Detection was performed at 230 and 275 nm for the assays of anticonvulsant drugs and methyl xanthine derivatives, respectively.

The commercially available TSK gel ODS-80TM column (150 mm × 4.6 mm I.D.) (Tosoh, Tokyo, Japan) and a laboratory-made Nucleosil C_{18} column (5 μm, 150 mm × 4.6 mm I.D.) (Macherey-Nagel, Düren, F.R.G.) were used as analytical columns. The former was used for the assay of methyl xanthine

derivatives and the latter for anticonvulsant drugs. The prepared ISRP silica was packed into a 30 mm×4.6 mm I.D. stainless-steel tube by conventional high-pressure slurry-packing procedures [11] and used as a precolumn.

Column-switching procedure

Assay of anticonvulsant drugs. The precolumn was equilibrated with 14 m*M* sodium dihydrogenphosphate plus 6 m*M* disodium hydrogenphosphate solution (eluent A), and a 20-μl aliquot of a serum sample was loaded. The precolumn was washed for 1.5 min with the eluent A at a flow-rate of 0.8 ml/min to remove proteinaceous components and hydrophilic substances. Then the six-port switching valve was actuated, and the drugs retained on the precolumn were swept to the analytical column in the back-flush mode by 14 m*M* sodium dihydrogenphosphate plus 6 m*M* disodium hydrogenphosphate–acetonitrile–methanol (6.5:1.5:2, v/v) (eluent B) at a flow-rate of 0.8 ml/min. The precolumn was switched back after 1 min and equilibrated with eluent A. The drugs were separated on the analytical column with eluent B.

Assay of methyl xanthine derivatives. The assay was performed by similar procedures except that (i) eluents A and B were 20 m*M* sodium dihydrogenphosphate solution and 100 m*M* sodium dihydrogenphosphate–acetonitrile (9:1, v/v), respectively, (ii) eluent B was delivered at a flow-rate of 1.0 ml/min and (iii) the washing time of the precolumn to remove proteinaceous components and hydrophilic substances was 1.0 min.

Preparation of human serum samples

Anticonvulsant drugs and methyl xanthine derivatives were dissolved in human serum at a known concentration, and an appropriate volume of serum sample was applied to the precolumn after filtration through a 0.22-μm membrane filter.

RESULTS AND DISCUSSION

Pretreatment and analytical conditions

In previous studies [5,10], we reported the preparation of a new, neutral ISRP support with N-octanoylaminopropyl and N-(2,3-dihydroxypropyl)aminopropyl phases on the internal and external surfaces, by using a porous silica as the base silica material. In this study, the neutral ISRP silica column was placed in-line with an analytical C_{18} column, and direct serum injection determinations of anticonvulsant drugs and methyl xanthine derivatives were investigated by a column-switching method. The hydrophobicity of the bonded-phase of a precolumn is important for the column-switching method: a low hydrophobicity causes the elution of drugs in washing away proteinaceous components and hydrophilic compounds [12]; and a high hydrophobicity needs a purge wash of the precolumn after an injection to remove

hydrophobic compounds in serum and reequilibration before the next injection [13,14].

First, the assay of hydrophobic anticonvulsant drugs was examined with the forflush mode. The disadvantages with the forflush mode were as follows: a long time is required for elution of drugs of interest from the precolumn; peak-broadening occurs; and elution of background components of serum left by the preceding injection interferes with the assay of drugs. This last effect was also observed when the precolumn was connected to the analytical column for 3 min in backflush mode. Thus, the precolumn was backflushed for 1 min to transfer drugs of interest to the analytical column. Fig. 1 shows a chromatogram of anticonvulsant drugs (primidone, phenobarbital, phenytoin and carbamazepine) in serum under the optimum pretreatment and analytical con-

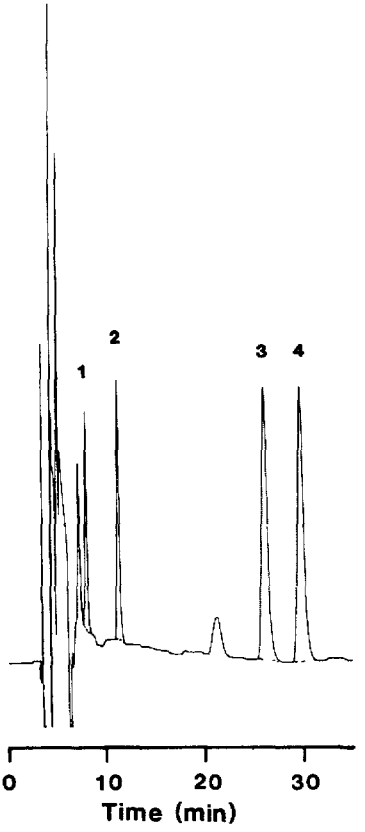

Fig. 1. Chromatogram of (1) primidone (10 μg/ml), (2) phenobarbital (10 μg/ml), (3) phenytoin (20 μg/ml) and (4) carbamazepine (5 μg/ml) after pretreatment of serum with the new ISRP precolumn. Injection volume, 20 μl; detection, 230 nm. Dotted lines indicate serum blank. Other conditions as in Experimental.

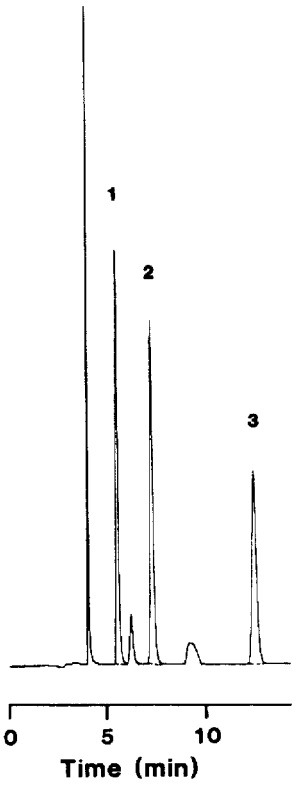

Fig. 2. Chromatogram of (1) theobromine, (2) theophylline and (3) caffeine after pretreatment of serum with the new ISRP precolumn. Injection volume, 20 μl; detection, 275 nm; concentration of each compound, 20 μg/ml. Dotted lines indicate serum blank. Other conditions as in Experimental.

ditions obtained. All drugs were completely recovered and repeated injections could be used without a purge wash of the precolumn. Fig. 2 shows a chromatogram of hydrophilic methyl xanthine derivatives in serum. The transfer of methyl xanthine derivatives to the analytical column was performed in the backflush mode for 1 min. In this system, drugs of interest were not eluted at the stage of removing proteinaceous components and repeated injections could also be performed without a purge wash of the precolumn. The results reveal that the hydrophobicity of the neutral ISRP support was suitable for direct serum injection assays of hydrophobic and hydrophilic drugs by a column-switching method.

Reproducibility and recovery

Tables I and II illustrate the within- and between-day assay precisions and recovery of anticonvulsant drugs and methyl xanthine derivatives, respec-

TABLE I

REPRODUCIBILITY AND RECOVERY OF DRUGS FROM HUMAN SERUM

The concentrations were 20 μg/ml for phenytoin, 10 μg/ml for primidone and phenobarbital, and 5 μg/ml for carbamazepine.

Drug	C.V. (%)		Recovery (%)
	Within-day ($n=10$)	Between-day ($n=3$)	
Primidone	0.6	2.3	99
Phenobarbital	1.3	1.7	100
Phenytoin	1.7	2.3	101
Carbamazepine	0.4	1.7	101

TABLE II

REPRODUCIBILITY AND RECOVERY OF DRUGS FROM HUMAN SERUM

The concentration of each drug was 20 μg/ml.

Drug	C.V. (%)		Recovery (%)
	Within-day ($n=10$)	Between-day ($n=3$)	
Theophylline	0.5	1.0	100
Theobromine	0.4	0.7	100
Caffeine	0.3	0.8	100

tively: good reproducibility [coefficient of variation (C.V.) of 0.3–2.3%] was obtained. The recovery of drugs from serum was determined by comparing peak areas with those obtained for a standard solution containing the same concentrations of drugs, which was directly injected onto analytical column. These data reveal that the drugs were completely recovered (100–101%) in this system.

Linearity and sensitivity

The calibration graphs of peak area versus concentration over the range 1–100 μg/ml were linear and passed through the origin. Table III shows therapeutic and potentially toxic serum concentration ranges and limits of detection (LOD) for drugs examined in this study. The present method will be useful for therapeutic monitoring of these drugs.

Durability of the precolumn

Both the precolumn and analytical column maintained efficiency after ca. 300 injections of 20-μl serum samples. When an increase in the back-pressure

TABLE III

THERAPEUTIC AND POTENTIALLY TOXIC SERUM CONCENTRATION RANGES AND LIMITS OF DETECTION (LOD) FOR DRUGS USED IN THIS STUDY

Drug	Concentration (μg/ml)		
	Therapeutic range[a]	Potentially toxic range[a]	Relative LOD by the present method[b]
Phenobarbital	15–40	>50	0 2
Phenytoin	10–20	>30	1
Carbamazepine	8–12	>15	0.1
Theophylline	10–20	>20	0.05

[a]Ref. 15.
[b]Signal-to-noise ratio of 3.

was observed, the stainless-steel frits or the precolumn were replaced. Further injections were performed without an increase in the back-pressure or loss of column efficiency.

In conclusion, this neutral ISRP support can be used for direct serum injection assays of hydrophobic and hydrophilic drugs in combination with conventional reversed-phase packings, such as C_{18}.

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