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An On-line Clean-up Procedure for Large Sample Volume Analysis of Serum Aminoglycoside Antibiotics by Reversed-Phase High-Performance Liquid Chromatography

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A reversed-phase (RP) liquid chromatography procedure using column switching was applied to assay one of the aminoglycoside antibiotics, sisomicin. The drug in serum was derivatized with *o*-phthalaldehyde (OPA) and *β*-mercaptopropionic acid (*β*-MP) in 0.05 M borate buffer (pH 9.0), then directly injected onto a RP pre-column (ODS-silica, 50×4 mm i.d.) to remove protein and interfering compounds (confirmed by ultraviolet monitoring at 280 nm). The sisomicin derivatives retained on the pre-column were desorbed by back-flushing onto an analytical column (5 *μ*m ODS-silica 200×4 mm i.d.) with 0.05 M potassium dihydrogen phosphate–0.05 M disodium hydrogen phosphate buffer (pH 7) and were separated by elution with a mixture of methanol, ethylene glycol and counter ion solution (sodium 1-heptanesulfonate/acetic acid/water, 2.5 g/42 ml/208 ml). This system could be utilized for more than 50 samples with a 1 ml injection volume. A good linear response curve was found between the injected sample amounts and the peak heights for the concentration range of 0.5 to 5 *μ*g/ml of sisomicin. The limit of detection for sisomicin in serum was 62.5 ng/ml for 0.1 ml of sample. The mean recovery from serum was 97.5%.

Keywords——HPLC; direct injection; column-switching; venting method; *o*-phthalaldehyde/*β*-mercaptopropionic acid; fluorescence detection; sisomicin

Introduction

Aminoglycoside antibiotics (AGs) are used for the therapy of serious infections caused by aerobic gram-negative bacilli.[1] The pharmacokinetic monitoring of these drugs is necessary for dosing without nephrotoxicity and ototoxicity.[2] Various methods have been reported for the determination of AGs in serum, including microbiological assay, radioenzymic assay, homogeneous enzymic immunoassay and high-performance liquid chromatography (HPLC). Several post-column derivatizations with *o*-phthalaldehyde (OPA) and thiol are currently in use for the determination of AGs by HPLC.[3] However, this method lacks sensitivity.

We have previously reported the pre-column derivatization of sisomicin with OPA/*β*-mercaptopropionic acid (*β*-MP) and its application to the reversed-phase (RP) HPLC analysis of serum samples.[4] We have also reported a sensitive analysis of sisomicin by HPLC with column-switching after pre-column derivatization using OPA and thioglycolic acid (TGA).[5] However, the deproteinization steps in both methods ere very time-consuming and unsuitable for routine laboratory analysis. A pre-column venting method, which was developed by Wahlund *et al.*[6] can remove the protein and other interfering substances in serum by the use of a pre-column. The method appears to be particularly appropriate for direct injection of a large volume of serum sample.

In the present study, we coupled a back-flush procedure with the column venting method

in a HPLC system for direct injection of sisomicin in serum using commercially available instruments.

Experimental

Chemicals——OPA and β-MP were purchased from Nakarai Chemicals Ltd. (Kyoto, Japan). Siseptin sulfate (sisomicin) was obtained from Yamanouchi Pharmaceutical Co. (Tokyo, Japan). The human control serum was from Hyland Diagnostics (Div. of Cooper Biomedical, Inc., Malvern, U.S.A). All other chemicals were of analytical reagent grades. Distilled water filtered through a MILLI-QII was used.

HPLC——A schematic representation of the HPLC system used in the present study is illutrated in Fig. 1. The prepared sample was injected through a model 7125 sample injector (Rheodyne, Cotati, Ca, U.S.A.) onto the pre-column C1 (50 × 4 mm i.d.). A model KHD-16 pump P1 (Kyowa Seimitsu Co. Ltd., Japan) was used for flushing the injected sample on C1 at the flow rate of 0.5 ml/min. A model ERC-8710 UV detector D1 (Erma Optical Works, Ltd., Tokyo, Japan) was used for monitoring the rinsing buffer. A model ERC-8710 pump P2 (Erma Optical Works. Ltd., Tokyo, Japan) was used for back-flushing from C1 onto the analytical column C2 which was filled with Nucleosil C_{18} (5 μm) at a flow rate of 0.5 ml/min. The monitoring of the derivatives eluted from C2 was carried out with a model RF-500LC fluorescence detector D2 at Ex. 340 nm and Em. 455 nm (Shimadzu, Kyoto, Japan). Chromatograms were recorded using a model C-R2AX Chromatopac (Shimadzu, Kyoto, Japan). A model 7000 six-port rotary valve V1 (Rheodyne, Cotaci, Ca, U.S.A.) was used for conversion of concentration mode and back-flushing mode.

Mobile phase 1 for sample clean-up consisted of 30% ethylene glycol in 0.05 M phosphate buffer (pH 7). Mobile phase 2 for back-flushing consisted of 80% methanol and 10% ethylene glycol in counter ion solution. The counter ion solution was prepared from sodium 1-heptanesulfonate (2.5 g), acetic acid (42 ml) and distilled water (208 ml).

Sample Derivatization Procedure——For pre-column derivatization, we mixed 100 μl of the serum sample containing sisomicin, 500 μl of methanolic OPA solution (2 mg/ml), 500 μl of methanolic β-MP solution (0.1 M) and 0.9 ml of 0.1 M borate buffer (pH 9). The resulting mixture was allowed to react at 20 °C for 1 h. After centrifugation for 10 min at 4500 × *g*, 1 ml of the supernatant was injected onto C1, pre-equilibrated with the mobile 1. Then, C1 was rinsed with the mobile 1 for 20 min in order to remove the serum protein with monitoring at 280 nm by D1. V1 was switched to the back-flushing mode and the mobile 2 was pumped out to C2 by P2 for 5 min. After re-switching of V1, C1 was washed with mobile 1 by P1 for 35 min for re-injection of the sample. Up to 60 samples could be applied to a pre-column without changing it.

Results and Discussion

Effect of Pre-treatment of Serum Sample

When a large volume of serum sample was passed through a membrane filter of 0.22 or 0.45 μm, the sisomicin derivative could not be determined, as shown in Fig. 2, though in the

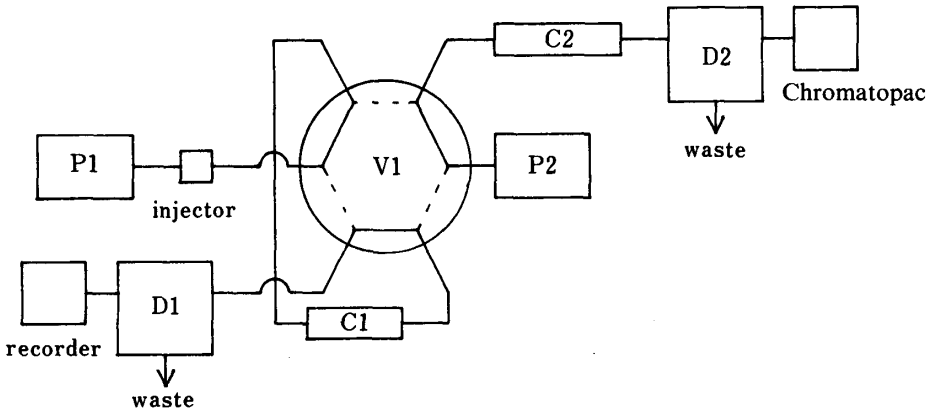

Fig. 1. Schematic Diagram of the Column Switching Assembly

Valve 1, column switching valve; pre-column, Chemcosorb ODS-H, 30 μm (5 × 0.4 cm i.d.); analytical column, Nucleosil C_{18}, 5 μm (20 × 0.4 cm i.d.). Mobile phase: pump 1, 30% ethylene glycol in 0.05 M phosphate buffer (pH 7), flow-rate 0.5 ml/min (clean-up mobile phase); pump 2, 80% methanol and 10% ethylene glycol in counter ion solution, flow-rate 0.5 ml/min.

——, sample loading; ----, back-flush elution to the analytical column. Details of the operation are described under·in the experimental section.

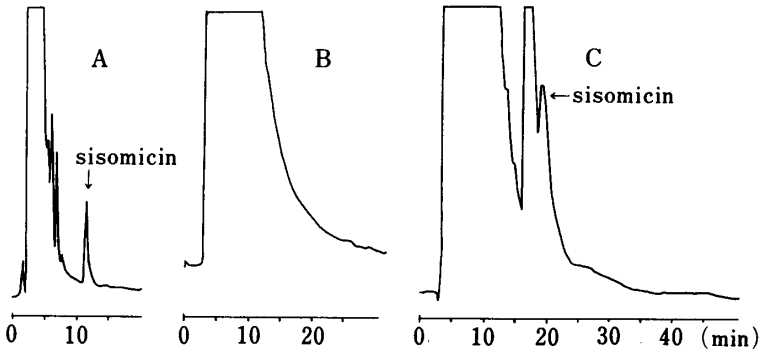

Fig. 2. Injection of Serum Sample Spiked with 1 μg/ml of Sisomicin directly into the Analytical Column

The injection volume was 1 ml. Analytical column, Nucleosil C_{18}, 5 μm (20 × 0.4 cm i.d.); eluent, 75% methanol in counter ion solution; flow-rate, 0.7 ml/min.
A) Anhalt's extraction method with CM-sephadex 0.2 M sodium sulfate was used for elimination of the interfering substances and 0.01 M sodium hydroxide in 0.2 M sodium sulfate for elution of the sisomicin derivatives. B) Membrane filtration (0.45 μm). C) Membrane filtration (0.22 μm). Injected samples are described in the text.

TABLE I. Influences of the Support in the Pre-column on the Stability of the Analytical Column[a)]

	VR (ml)[b)]	$W_{1/2}$ (mm)[c)]	*N*[d)]	*L* (cm)[e)]	*H*[f)]	*dp* (μm)[g)]	*h*[h)]	*Ch* (%)[i)]	P.H. (μV)[j)]	R.S.D. (%)[k)]
Starting value										
Nucleosil $5C_{18}$	4.54	17.4	1.09	5	46	5	9174	—	—	—
Chemcosorb 7-ODS-H	3.12	11.3	1.22	5	41	7	5857	—	—	—
Chemcosorb 30-ODS-H	5.14	39.9	0.27	5	188	30	6267	—	—	—
After 10 times										
Nucleosil $5C_{18}$	3.0	13.3	0.81	5	61	5	12248	25.1	401	6.96
Chemcosorb 7-ODS-H	3.4	12.7	1.14	5	43	7	6211	5.7	343	6.53
Chemcosorb 30-ODS-H	6.2	49.2	0.25	5	196	30	6521	3.9	441	4.93

a) Injection volume was 1 ml. *b*) Elution volume. *c*) Half width. *d*) Plate number. *e*) Column length. *f*) The theoretical plate height. *g*) Particle diameter. *h*) Reduced plate height. *i*) The change width of h after 10 injections. *j*) The mean of peak heights for 10 samples. *k*) The relative standard deviation.

case of a small volume of the sample, the procedure was effective. Anhalt's method[7)] using carboxymethyl (CM)-Sephadex can also eliminate most of the protein in serum, but this method gave poor reproducibility and is more tedious.

Effect of Pre-column Using Venting Method on Stability of the Analytical Column

The reduced plate height (*h*) was calculated from the theoretical plate height (*H*) and the particle diameter (*dp*) as follows:

$$h = H/dp$$

where *H* was calculated from the column length (*L*) and the plate number (*N*) as

$$H = L/N$$

where *N* was calculated from the retention time and the base width. *Ch* values define the *h* value changes at the start to 10 measurements, that is, they indicate the stability of the pre-column. When the *Ch* value is lower, the support is more suitable for the pre-column.

Table I shows the average of peak heights and the peak efficiency of the analytical column when using the pre-column filled with various supports with different particle sizes

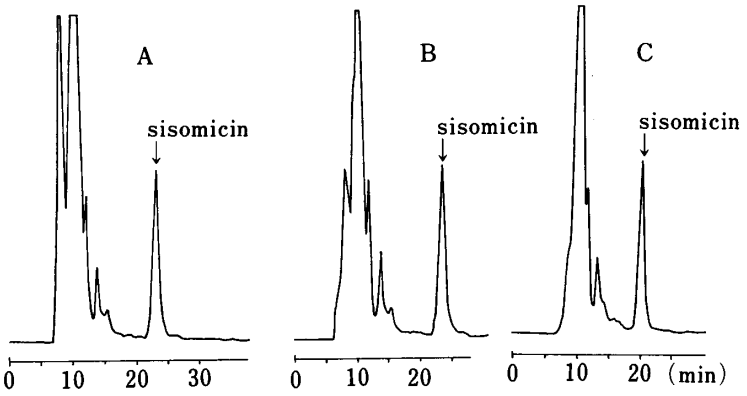

Fig. 3. Analysis by the Venting Method with Different Rinsing Buffers

Serum spiked with sisomicin, concentration 0.4 μg/ml. Pre-column, Nucleosil C_{18}, 5 μm (5 × 0.4 cm i.d.); analytical column, Nucleosil C_{18}, 5 μm (20 × 0.4 cm i.d.). Mobile phase for analysis, 80% methanol and 10% ethylene glycol in counter ion solution; flow rate 0.5 ml/min; flow-rate of the rinsing buffer 0.5 ml/min.

A) 0.05 M phosphate buffer (pH 7). B) 20% ethylene glycol in 0.05 M phosphate buffer (pH 7). C) 40% ethylene glycol in 0.05 M phosphate buffer (pH 7). Injected samples are described in the text.

and different numbers of residual silanol groups. When a support with large particle size was placed in the pre-column, the analytical column gave greater peak heights than in the case of small particle size. In general, the interparticle channels are wider so that denatured proteins tend to adhere to the particle surfaces to a lesser extent, so the stability is greater for pre-columns with large particles. Advidsson *et al.* pointed out that this beneficial effect of the stability of large particles in the pre-column can only be utilized for less demanding separations.[8]

However, then the pre-column was used for the pre-treatment of serum samples, residual silanol groups should be into account as well as particle size in evalnating the stability of the pre-column. To investigate the effect of the residual silanol groups we examined two kinds of supports, Nucleosil C_{18} (5-μm) and Chemcosorb ODS-H (7-μm). Chemcosorb ODS-H has a small amount of residual silanol groups, compared with Nucleosil C_{18}. As shown in Table I, the efficiency of the analytical column was enormously stabilized by the use of the pre-column filled with Chemcosorb ODS-H. Nucleosil C_{18} was less stable and acquired a yellowish color, probably caused by strongly retained lipid material on the support (this was not observed with Chemcosorb ODS-H). Consequently, the stability of the support may be highly dependent on the amount of residual silanol groups.

Therefore, two points have to be considered in adopting a pre-column for the pre-treatment of biological fluids: (1) particle diameter should be chosen so as to exclude interfering substances as early as possible from the pre-column and (2) packing materials should be chosen to give stable efficiency of the analytical column.

Choice of Rinsing Buffer in the Venting Method

Although monohydric alcohols are utilized to regulate the retention of the desired peak, they tend to denature protein. On the other hand, polyhydric alcohols can be used to prevent the denaturation of proteins in serum. In the present study, we adopted ethylene glycol as an organic modifier in the rinsing buffer. To examine clean-up of serum samples, the contents of ethylene glycol in the rinsing buffer were varied. As shown in Fig. 3, the void peaks were reduced with increasing amount of ethylene glycol up to 40%, but addition of over 60% caused elution of the sisomicin derivatives from the pre-column and also increased the column back-pressure owing to the high viscosity. We employed mobile phases for rinsing and elution of 30% ethylene glycol in 0.05 M phosphate buffer (pH 7) and 10% ethylene glycol in counter ion solution containing 80% methanol, respectively. Addition of ethylene glycol to the rinsing

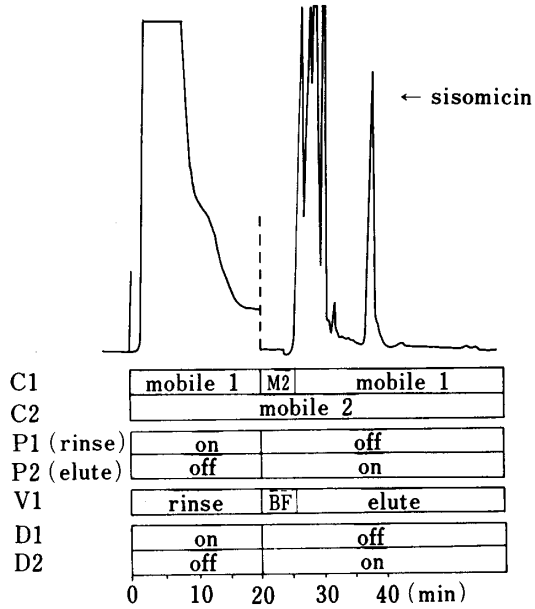

Fig. 4. Typical Chromatogram of a Serum Sample Containing 0.25 μg/ml Sisomicin with the Sample Pre-treatment System

Pre-column, Chemcosorb ODS-H, 30 μm (5 × 0.4 cm i.d.), analytical column, Nucleosil C_{18}, 5 μm (20 × 0.4 cm i.d.). Mobile phase for clean-up, 30% ethylene glycol in 0.05 M phosphate buffer (pH 7); flow-rate, 0.5 ml/min. Mobile phase for analysis, 80% methanol and 10% ethylene glycol in counter ion solution flow-rate 0.5 ml/min.

M2: Mobile phase 2. BF: Back-flush mode. Details of the Chromatographic conditions are described in the text.

TABLE II. Analytical Data for Serum Samples with the Pre-treatment System

Concentration of sisomicin	5 μg/ml	0.25 μg/ml
P.H.	899 V	44.5 V
S.D.	39.92	2.07
R.S.D.	4.44%	4.65%
Limit of detection	62.5 ng/ml	
Mean recovery	97.5%	

The injection volume was 1 ml.

buffer improved the durability of this system and more than 50 samples could be analyzed. A typical chromatogram obtained by this system is shown in Fig. 4.

Recovery and Precision

The mean recovery of sisomicin (5 μg/ml) from serum using the present method was 97.5% ($n=10$) and the standard deviation was ±2.07. A small amount might have been adsorbed by protein.[9] When amounts of 0.5 to 5 μg/ml of sisomicin spiked into serum were injected, the calibration curve for the peak height (y) *versus* the concentration of sisomicin (x) was $y=-0.694+16.753\ x$ ($r=0.9996$; $n=8$). The relative standard deviations (R.S.D.) of 5 and 0.25 μg/ml were 4.44% and 4.65%, respectively. The variation at higher concentrations may be due to the concentration procedure on the column rather than errors in the injected volume. On the other hand, the variation in peak height at lower concentrations may be dependent on the base-line noise of the detector. The detection limit of sisomicin in serum was 62.5 ng/ml with an injection volume of 1 ml (Table II).

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