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Fluorometric Determination of Sisomicin, an Aminoglycoside Antibiotic, in Dried Blood Spots on Filter Paper by Reversed-Phase High-Performance Liquid Chromatography with Pre-column Derivatization

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A simple method for the determination of sisomicin (SISO) in dried blood spots (DBS) on filter paper has developed. SISO in DBS was recovered most effectively by ultrasonication in 0.05 M KH_2PO_4–borate buffer (pH 9.0). The eluates from the DBS were treated with a carboxymethyl-Sephadex column, followed by determination by reversed-phase high-performance liquid chromatography after pre-column fluorescence derivatization using *o*-phthalaldehyde and β-mercaptopropionic acid in 0.05 M KH_2PO_4–borate buffer (pH 9.0). The detection limit of SISO was below 0.05 μg per ml of whole blood (100 μl). The method permits a simple blood collection method for monitoring of aminoglycoside antibiotics at therapeutic levels.

Keywords——sisomicin; dried blood spot; filter paper; HPLC; pre-column derivatization; *o*-phthalaldehyde/β-mercaptopropionic acid; aminoglycoside antibiotic

Aminoglycoside antibiotics (AGs) are widely used against serious infections with gram-negative bacilli. However, their ototoxicity and nephrotoxicity require careful monitoring of blood levels especially in treating life-threatening infections in patients with impaired renal function or when therapy is of long duration, because of their narrow therapeutic range.[1-5]

From the clinical point of view it is potentially useful to determine the AGs in microliter samples. Sample collection by finger pricking instead of venepuncture is advantageous in geriatric and pediatric patients where venepuncture is often difficult or on some occasions impossible to perform. The dried blood spot (DBS) method has been used in diagnostic screening programmes for many years.[6-10] Although the applicability of the DBS method is limited, it has an advantage in terms of sample collection. However, no reports have been published concerning determination of AGs in dried whole blood spotted on filter paper.

In the present study, we have developed a method for determination of sisomicin (SISO) in DBS on filter paper by reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection by using pre-column derivatization.

Experimental

Materials——SISO sulfate as an injectable form (labelled potency 75 mg/1.5 ml) was obtained from Yamanouchi Pharmaceutical Co. Heparinized blood was obtained by venepuncture from healthy adults. *o*-Phthalaldehyde (OPA), β-mercaptopropionic acid (β-MP), Brij 35 and sodium 1-heptanesulfonate were purchased from Nakarai Chemicals Co. All other chemicals used were of reagent grade. Distilled water used was filtered through a Milli-Q II water purification system (Nippon Millipore Ltd.).

Apparatus——The ultrasonic bath used was a Medel C-051 A (50 kHz, Nippon Denshi Kagaku Co., Ltd.). The HPLC equipment was described previously.[11]

Blood Spot Preparation——Whole blood samples, 100 μl, with known concentration of SISO were spotted onto

blood sampling paper (strip type, Toyo Roshi Kaisha Ltd.). The paper was allowed to dry at 50 °C for 10 min and stored in an air-tight glass bottle at 4 °C until measured. The spotted area was cut with scissors into 3—4 pieces and dipped in a glass the containing 500 μl of solvent.

Sample Preparation——We used Anhalt's method with a modification for deproteinization.[12] A carboxymethyl (CM)-Sephadex (C-25, Pharmacia) column was prepared with a bed volume of 0.5 ml in a disposable polypropylene tube (50 × 5 mm i.d., Muromachi Kagaku Kogyo Kaisha Ltd.). The extract from the DBS, 300 μl, was applied to the column, which was washed with 1 ml of 0.2 M Na_2SO_4 twice in succession, and eluted with 350 μl of a buffer containing 10 mM NaOH in 0.2 M Na_2SO_4 (elution buffer). After the column had drained completely, 200 μl of the elution buffer was added and the eluate was collected in an Eppendorf tube.

Pre-column Derivatization[11]——A 200 μl aliquot of the eluate from the CM-Sephadex column was mixed with 50 μl of methanolic β-MP solution (0.1 M), 50 μl of methanolic OPA solution (2 mg/ml), 350 μl of methanol and 350 μl of 0.05 M KH_2PO_4–borate buffer (pH 9.0). The resulting mixture was allowed to react at 20 °C for 1 h.

HPLC Conditions——The separations were achieved using a column (200 × 4 mm i.d.) packed with Nucleosil C_{18} (5-μm, Macherey, Nagel & Co.) fitted with a C_{18} (5-μm) guard column (10 × 4 mm i.d.). The mobile phase was prepared by mixing 800 ml of methanol (HPLC grade) with 200 ml of a solution containing sodium 1-heptanesulfonate (2.5 g) and acetic acid (42 ml) in 208 ml of distilled water, and deaerated by ultrasonication. The flow rate was 0.9 ml/min. The column effluent was monitored at 450 nm with excitation at 340 nm. The volume of the derivatized sample injected was 800 μl.

Results and Discussion

The extraction efficiency of SISO from the DBS was dependent on the solvent and the elution method. Distilled water, physiological saline, 60% ethanol and buffers were investigated. All, except 60% ethanol, released hemoglobin from the DBS, and 0.05 M KH_2PO_4–borate buffer (pH 9.0) gave the most effective elution of SISO from the DBS under the conditions used (Table I). The addition of Brij 35 detergent had no effect of the recovery of SISO.

The extraction time of SISO was examined with 500 μl of 0.05 M KH_2PO_4–borate buffer, with gentle shaking or ultrasonication. Figure 1 shows that extraction under ultrasonication over 30 min ensures good recoveries of SISO; this was subsequently adopted as a standard elution technique, though the recoveries were not complete as shown in Fig. 2. A main reason for this low recovery of SISO from the DBS as compared with the concentration in whole blood is most likely coelution from the CM-Sephadex column with 0.2 M Na_2SO_4 used as the washing buffer, although it was reported that protein binding of AGs can not be neglected at

TABLE I. Influence of Solvents on the Extraction of SISO from DBS[a]

Solvent[b]	Recovery (%)[c]
60% ethanol	<0.2
0.05 M KH_2PO_4–borate buffer (pH 9.0)	82.9±0.1
0.5 M Na_2HPO_4 (pH 8.7)	61.0±6.5

a) The concentration of SISO in DBS was 2.5 μg/ml. *b*) Samples were treated in 500 μl of the solvent with ultrasonication for 30 min. *c*) Values are the mean±S.D. (n=6).

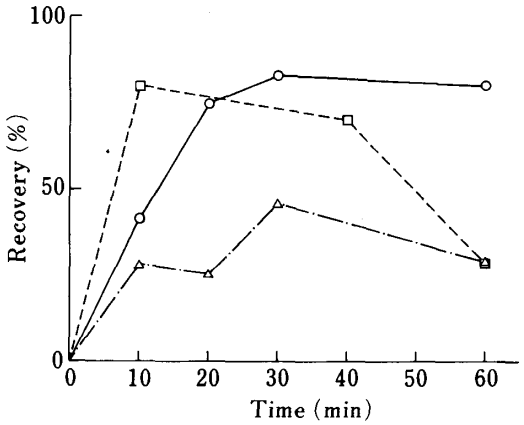

Fig. 1. Influence of Extraction Time on the Recovery of SISO in DBS

The concentration of SISO in the DBS was 5 μg/ml; △, shaking in a water-bath at 37 °C; ○, ultrasonication; □, shaking in a water-bath at 50 °C. Each recovery was calculated from the calibration curve obtained with SISO samples in whole blood (100 μl) (see Fig. 2).

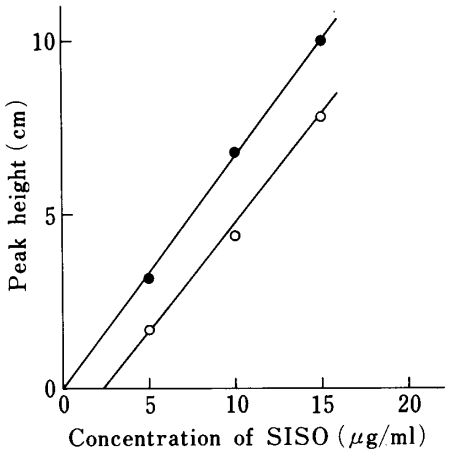

Fig. 2. Comparison of the Recoveries of SISO from Whole Blood Samples and from DBS Samples

●, whole blood samples ($y=-0.014+0.669x$, $r^2=0.980$); ○, DBS samples ($y=-1.400+0.600x$, $r^2=0.995$); HPLC conditions, see Fig. 3.

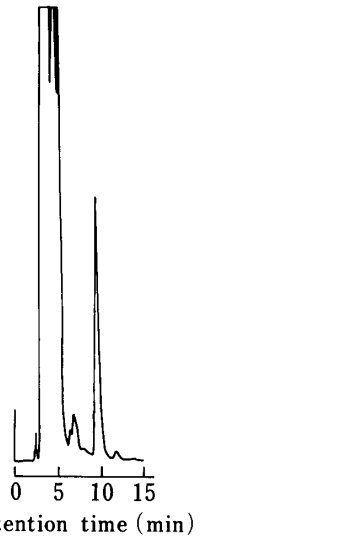

Fig. 3. Chromatogram of Fluorescent Isoindole Derivative of SISO using OPA/β-MP

Mobile phase, 80% methanol in the counter ion solution (see Experimental); detection 340 nm (excitation)/450 nm (emission); flow rate, 0.9 ml/min; 800 μl injection. The concentration of SISO in the DBS (100 μl spotted) was 2 μg/ml.

low concentrations.[13]

A typical chromatogram is shown in Fig. 3, demonstrating a well-resolved peak of SISO derivative, free from apparent interference. The calibration curve for SISO in whole blood was linear over the concentration range of 0.05–5.0 μg/ml ($y=1.37+48.62x$, $r^2=1.000$). The limit of detection was below 0.05 μg/ml for 100 μl of whole blood. The limit may possibly be further improved by modification of the sample preparation procedure for HPLC assay. The intra-assay relative standard deviation for 0.25 μg/ml DBS samples ($n=5$) was 7.9% and the mean recovery, which was calculated from the standard curve using the DBS samples, was 91.6%.

In conclusion, the present method combines the advantage of micro-sampling, inherent in blood collection on a filter paper, with an accurate, precise and specific HPLC method. This is the first time that the DBS method has been tried for AGs analysis, though further improvements should be made for use in clinical laboratories. A simpler method for blood collection on filter paper disks is being developed. This micromethod is particularly useful in pediatrics and for patients with renal failure, where sample size is of major concern. Also, since AGs on filter paper are stable for up to 10 d at room temperature,[14] the DBS can be easily handled and stored in the laboratory and demands on venesection materials and staff are reduced.

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