Determination of Sisomicin in Eluate from Dried Blood Spot on Filter Paper Disc for Monitoring of Blood Level in Rat, by Reversed-Phase High-Performance Liquid Chromatography after Pre-column Fluorimetric Derivatization

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About 20 μl of whole blood obtained by venipuncture from rat tail vein was spotted onto a filter paper and the blood spot was punched out (5 mm diameter). Sisomicin (SISO) in the dried blood spot (DBS) was extracted effectively into 0.5 M Na_2HPO_4 solution by ultrasonication and determined by reversed-phase high-performance liquid chromatography with pre-column derivatization using *o*-phthalaldehyde and β-mercaptopropionic acid. This method could be used for the pre-clinical study of SISO blood levels of a number of mice or rats without killing. The results were identical with those for SISO in serum, if corrected for hematocrit values, and were used for the calculation of pharmacokinetic parameters for individual rats. The detection limit of SISO in DBS (10.1 μl of whole blood) was 1.0 μg per ml of whole blood.

Keywords sisomicin; dried blood spot; punched disc; pharmacokinetic; pre-column derivatization; HPLC; rat

Sisomicin (SISO) is an aminoglycoside antibiotic (AGs) that is active *in vivo* against a wide range of bacteria. Although AGs are often chosen to treat many gram-negative infections, their usage has been hampered by their toxicity. Monitoring the serum concentration of AGs has been considered to be helpful in achieving suitable therapeutic ranges and avoiding undesirable side-effects. Multiple sample collection by finger pricking is advantageous for pediatric patients, where venipuncture is often difficult or impossible to perform on multiple occasions. The dried blood spot (DBS) method has been used in diagnostic screening,[1–3] and it has an advantage in terms of multiple sample collection from pediatric patients[4,5] and small animals, *e.g.*, mouse or rat. However, the applicability of the method is limited, and no report has been published concerning determination of AGs in dried whole blood spotted on filter paper. Recent work in our laboratories has dealt with this method[6,7] of sample collection for therapeutic drug monitoring and pharmacokinetic studies.

In the present study, we investigated the determination of SISO in blood collected from the tail vein of individual rats using the DBS method by means of pre-column derivatization high-performance liquid chromatography (HPLC),[8] and its application to pharmacokinetic studies of SISO after single intramuscular administration in individual rats.

Experimental

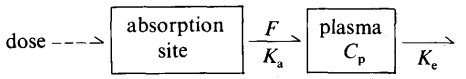
Animals Male CD rats weighing about 150 g were used (Shimidzu Animal Labs., Kyoto). The rats were acclimatized in a temperature (23 °C) and humidity (60%) controlled room before treatment.

Chemicals and Apparatus The chemicals and HPLC equipment used were as described previously,[6] unless otherwise mentioned.

Dosing and Blood Sampling Two experiments were performed with groups of three rats. The rats were fixed on a rat-holder (KN-325 A, Natsume Co., Tokyo). One group received the test solution equivalent to 10 mg/kg of SISO (Yamanouchi Pharmaceutical Co., Ltd., Tokyo) i.m., and the other group received the test solution equivalent to 30 mg/kg of SISO i.m. The blood was collected by from the tail vein of each rat by venipuncture with a lancet at the following times: predose, 15, 30 and 45 min and 1, 2, 4 and 7 h after the injection. It was spotted on paper (blood sampling paper, strip type, Toyo Roshi Co., Tokyo). The papers were allowed to dry at ambient temperature, and stored in an air-tight bottle at −20 °C until assay.

Sample Preparation To obtain a calibration curve of SISO in the DBS, the dried blood spot on a filter paper spotted with blood (50 μl) containing a known concentration of SISO was punched out with an office punch (5 mm diameter) and placed in a test tube. Then 500 μl of 0.5 M Na_2HPO_4 solution was added, and the tube was ultrasonicated (28 kHz) for 30 min. The extract from the paper disc, 200 μl, was transferred to an Ultrafree® tube (CT3K, Nippon Millipore Co., Ltd., Tokyo) and centrifuged at 2000 *g* for 30 min. For monitoring of SISO in a DBS sample

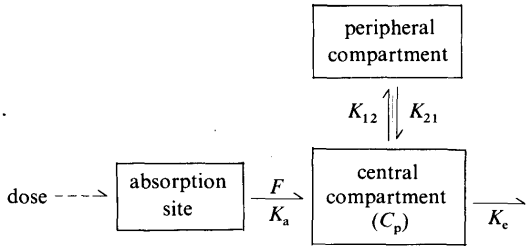
one-compartment model with first-order absorption



$$C_p = \frac{K_a \cdot F \cdot \text{dose}}{V_d \cdot (K_a - K_e)} [\exp^{(-K_e \cdot t)} - \exp^{(-K_a \cdot t)}]$$

C_p: plasma concentration at time t
K_a: first-order absorption rate constant
K_e: first-order elimination rate constant
V_d: apparent volume of distribution
F: fraction of dose absorbed

two-compartment model with first-order absorption



$$C_p = \frac{D}{V_c(\alpha - \beta)} [(\alpha - K_{21})e^{-\alpha t} + (K_{21} - \beta)e^{-\beta t}]$$

K_{12}, K_{21}: first-order distribution rate constants
K_e: first-order elimination rate constant
V_c: volume of central compartment
K_a: first-order absorption rate constant
F: fraction of dose absorbed

$$\alpha = \frac{1}{2}[(K_{12} + K_{21} + K_e) + \sqrt{(K_{12} + K_{21} + K_e)^2 - 4K_{21}K_e}]$$

$$\beta = \frac{1}{2}[(K_{12} + K_{21} + K_e) - \sqrt{(K_{12} + K_{21} + K_e)^2 - 4K_{21}K_e}]$$

Chart 1

the filter paper was treated as described above.

Pre-column Derivatization A 40 μl aliquot of the filtrate from the Ultrafree® tube was mixed with 50 μl of methanolic β-mercaptopropionic acid solution (0.1 M), 50 μl of methanolic *o*-phthalaldehyde solution (2 mg/ml), 460 μl of 0.05 M KH_2PO_4–borate buffer (pH 9.0) and 400 μl of methanol. 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., FRG) 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 0.8 ml/min. The fluorescence intensity of the column effluent was monitored at 450 nm with excitation at 340 nm. The volume of the derivatized sample solution injected was 200 or 400 μl.

Pharmacokinetics The SISO concentration in blood *versus* time data for each rat were simultaneously fitted to an open one-compartment pharmacokinetic model by using a non-linear curve fitting program or a two-compartment pharmacokinetic model by using the same procedure.

Results

Extraction Conditions The optimum elution method was investigated for extraction of SISO from the DBS. In the present study, 0.5 M Na_2HPO_4 solution gave the most effective elution of SISO from the DBS paper disc under the conditions used, although hemoglobin was also released from the DBS.

The extraction time of SISO was examined with 500 μl of 0.5 M Na_2HPO_4 solution, with gentle shaking in a water-bath (35 or 50 °C) or ultrasonication. Figure 1 shows that extraction under ultrasonication over 30 min ensures good recovery of SISO; this was adopted as the standard elution technique.

Blood Volume To obtain information on the blood volume in the punched-out discs and the reproducibility, six filter papers were punched in duplicate. The resulting twelve discs were extracted with 0.5 M Na_2HPO_4 solution as described above and absorbance of the eluates was measured by spectrophotometry at 575 nm to determine hemoglobin. The blood volumes in these filter paper discs were found to average 10.1 μl with a standard deviation of 0.33, based on the standard calibration curve of the whole blood. The coefficient of variation for the blood volume of the twelve discs was thus 5.2% ($y = 0.13x - 4.02$, $r^2 = 0.996$).

Quantitation of SISO in Paper Disc A typical chromatogram is shown in Fig. 2, demonstrating a well-resolved peak of SISO derivative, free from apparent interference. The relationship between the peak height and the injected volume of samples was linear over 200—1000 μl for 5.0 μg/ml of SISO in the DBS. The calibration curve for SISO in whole blood was linear over the concentration range of 1.0—50.0 μg/ml ($y = 4.30x - 5.68$, $r^2 = 1.000$). The limit of detection was 1.0 μg per ml of whole blood by the DBS method under the conditions used. The limit may possibly be further improved by modification of the sample preparation procedure for HPLC assay.

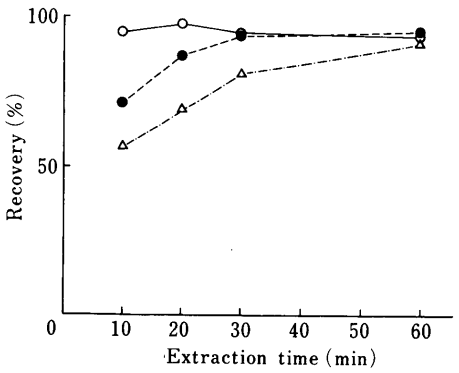


Fig. 1. Efficiency of Extraction

Recovery of sisomicin (7.4 μg/ml) from the DBS after shaking in a water-bath at 37 °C (△) or 50 °C (○) and ultrasonication (●) for various times. The extraction was carried out in a glass tube containing 500 μl of 0.5 M Na_2HPO_4 (pH 8.7).

Recovery and Reproducibility The intra-assay relative standard deviation for 0.74 μg/ml in the DBS sample ($n = 8$) was 7.5% and the mean recovery, which was calculated from the standard curve using the DBS samples, was 93.6% based on the calibration curve for SISO in plasma prepared from the same whole blood samples containing known concentrations of SISO. There was no significant difference between recoveries of SISO in the DBS and in plasma samples (Table I) when the hematocrit value of whole blood was taken into account.

Blood Concentration of SISO Figures 3 and 4 show the individual concentrations of SISO measured in whole blood after i.m. administration. SISO was rapidly absorbed from the injection site, reaching peak concentration at about 30 min (30 mg/kg) or 45 min (10 mg/kg).

Pharmacokinetic Parameters The blood concentration of SISO *versus* time data obtained after single-dose administration in rats (Figs. 3 and 4) were employed to estimate one-compartment pharmacokinetic parameters as summa-

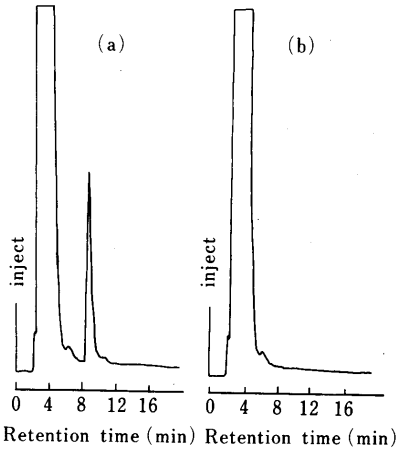


Fig. 2. Reversed-Phase HPLC Separation of Fluorescent Isoindole Derivative of Sisomicin in Dried Blood Spot Using *o*-Phthalaldehyde/β-Mercaptopropionic Acid

HPLC conditions, see Materials and Methods. Samples were (a) sisomicin (20 μg/ml) in a dried blood spot (10.1 μl of whole blood) and (b) blank. Injection volume was 200 μl.

TABLE I. Recoveries of Sisomicin from Dried Blood Spots, Calculated from the Calibration Curve for Sisomicin in Plasma

Concentration (μg/ml)	Concentration, found (μg/ml)[a,b]	Mean recovery (%)	Corrected mean recovery (%)[c]	C.V. ($n = 3$) (%)
5.0	2.28 ± 0.12	45.6	103.5	5.3
10.0	4.59 ± 0.10	45.9	104.2	2.2
20.0	8.96 ± 0.52	44.8	101.7	5.8

a) The concentration was calculated from the calibration curve for sisomicin in plasma ($y = -5.11 + 13.08x$, $r^2 = 1.000$). *b*) Each value is the mean ± S.D. *c*) The hematocrit value was 44.1%. C.V., coefficient of variation.

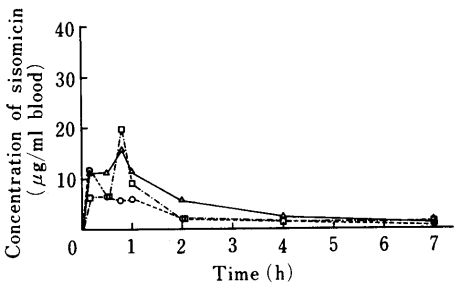


Fig. 3. Time Profiles of Sisomicin after Single-Dose Administration in Rats

Dose, 10 mg/kg; ○, rat No. 1; △, rat No. 2; □, rat No. 3.

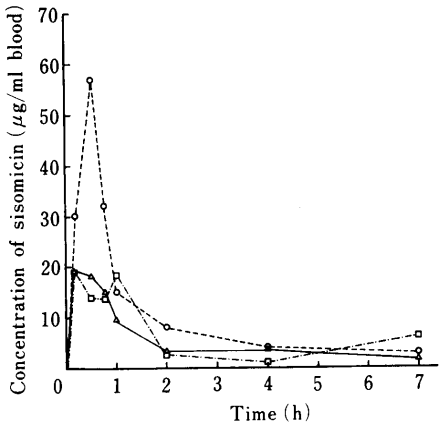


Fig. 4. Time Profiles of Sisomicin after Single-Dose Administration in Rats

Dose, 30 mg/kg; ○, rat No. 4; △, rat No. 5; □, rat No. 6.

TABLE II. One-Compartment Pharmacokinetic Parameters of Sisomicin after Single-Dose Administration in Rats

Dose mg/kg	Rat No.	AIC[a]	SS[b]	K_a (h^{-1})	K_e (h^{-1})	V_d (l/kg)
10	1	5.93	0.98	11.334	1.057	0.645
10	2	8.44	1.50	6.615	0.495	0.556
10	3	21.63	9.32	2.288	1.163	0.532
30	4	21.85	14.04	5.252	1.254	0.455
30	5	12.52	2.96	9.885	0.985	1.163
30	6	15.73	5.06	10.487	0.986	1.220

a) An information criterion. *b*) Sum of squares.

TABLE III. Two-Compartment Pharmacokinetic Parameters of Sisomicin after Single-Dose Administration in Rats

Dose mg/kg	Rat No.	AIC[a]	SS[b]	K_{12} (h^{-1})	K_{21} (h^{-1})	K_a (h^{-1})	K_e (h^{-1})	V_c (l)
10	1	9.93	0.98	0.187	7.106	10.788	1.092	0.624
10	2	−3.15	0.11	0.662	0.281	3.541	0.548	0.355
10	3	24.47	7.90	−0.285	−0.145	2.017	1.737	0.451
30	4	22.96	8.67	1.708	0.214	3.340	0.936	0.287
30	5	−1.14	0.16	−0.205	−0.387	6.077	1.767	0.890
30	6	19.73	5.06	1.214	0.965	10.872	0.977	1.227

a) An information criterion. *b*) Sum of squares.

rized in Table II. The calculation of two-compartment pharmacokinetic parameters was also examined using the above concentrations of SISO. The disposition curves of SISO in blood showed a biexponential decline at dose of 10 and 30 mg/kg. There were small but significant differences between individual rats (Table III).

Discussion

Mice or rats have been utilized for toxicity studies, safety studies, absorption, distribution, metabolism or excretion studies in the initial stages of development of various drugs. For the determination of drug levels in serum, blood samples are usually collected from the heart or retro-orbit sinus of mice or rats under adequate anesthesia and assayed. It has been believed for many years that blood can not be collected more than twice from a mouse or a rat without serious damage to the animal.

We have developed[6] the DBS sampling method and applied it to the HPLC determination of SISO in whole blood. The concept of monitoring the concentration in blood using the DBS technique arises from the practical problems of obtaining samples that are suitable for micro assay in small animals, so as to avoid the need to kill a number of mice or rats just for pre-clinical or toxicity studies of blood levels. By applying DBS, definitive time course assay for pharmacokinetic studies of AGs in individual rats is feasible.

The data summarized in Tables II and III suggest that it will be possible to obtain pharmacokinetic parameters for various AGs in rat plasma, although individual differences in pharmacokinetic parameters were found. If more rats were used, mean pharmacokinetic parameters of SISO could be established more precisely. The principal problems with the punched disc are to ensure constant blood volume for different samples and consistent elution of blood constituents from the punched disc. However, these factors can be corrected for by measuring of the hemoglobin values.

In a clinical study in pediatric patients,[7] this method gave an excellent linear correlation with the concentrations of AGs in plasma. Therefore, we consider that this sampling method is also suitable for use in pre-clinical studies of AGs with rats.

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