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DETERMINATION OF THE AMINOGLYCOSIDE ANTIBIOTICS SISOMICIN AND NETILMICIN IN DRIED BLOOD SPOTS ON FILTER DISCS, BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH PRE-COLUMN DERIVATIZATION AND FLUORIMETRIC DETECTION

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SUMMARY

A simple method for the determination of the aminoglycoside antibiotic sisomicin or its 1-N-ethyl derivative, netilmicin in whole blood, using dried blood spots (DBSs) on filter-paper punched discs has been developed. Sisomicin or netilmicin in the DBSs were recovered most effectively in 0.5 *M* Na_2HPO_4 using ultrasonication. The eluates from the DBSs were treated by ultrafiltration for deproteinization and subjected to pre-column fluorescent derivatization using *o*-phthalaldehyde and *β*-mercaptopropionic acid in 0.05 *M* KH_2PO_4–borate buffer (pH 9.0), followed by determination by reversed-phase high-performance liquid chromatography. The detection limits of sisomicin and netilmicin in the DBSs on punched discs (10.1 *μl* of whole blood) were 0.053 and 0.50 *μg* per ml of whole blood, respectively (signal-to-noise ratio ≥ 2). The method permits a simple collection of blood at the microlitre level and should prove particularly useful for monitoring sisomicin and netilmicin in blood at therapeutic levels in geriatric and paediatric patients.

INTRODUCTION

Aminoglycoside antibiotics (AGs) are widely used against serious infections with Gram-negative bacilli. However, in treating life-threatening infections in

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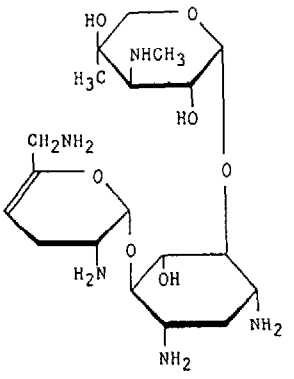
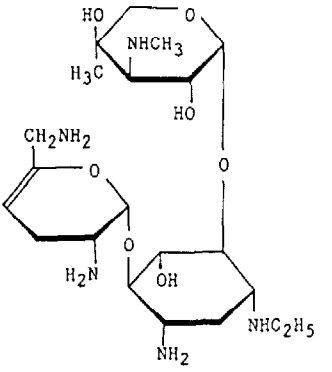


(a) Sisomicin



(b) Netilmicin

Fig 1. Structures of sisomicin and netilmicin.

patients with impaired renal function or when the therapy is of long duration, their potential ototoxicity and nephrotoxicity require the careful monitoring of AG concentrations in blood, because of their narrow therapeutic index [1–3]. From the clinical point of view it is desirable to determine AGs in microlitre samples. For example, 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 [4]. Although the dried blood spot (DBS) produced by finger- or heel-pricking has been used in diagnostic screening programmes for many years, the applicability of the method is limited [5–7] and no reports have been published concerning the determination of AGs in DBS on filter-paper.

We have already developed a sensitive method for the determination of the AG sisomicin in serum by reversed-phase high-performance liquid chromatography (HPLC) using pre-column derivatization with *o*-phthalaldehyde (OPA) and *β*-mercaptopropionic acid (*β*-MP) [8]. In this work, we have applied this HPLC method to the determination of sisomicin or its 1-N-ethyl derivative, netilmicin (Fig. 1), in DBSs on punched discs (5 mm diameter) of filter-paper.

EXPERIMENTAL

Materials

Sisomicin sulphate (Yamanouchi Pharmaceutical, Tokyo, Japan) and netilmicin sulphate (Essex Nippon, Osaka, Japan) were used as the injectable forms with labelled potencies of 75 mg per 1.5 ml and 100 mg per 2 ml, respectively. Heparinized blood was obtained by venepuncture from healthy adults. OPA, *β*-MP and sodium 1-heptanesulphonate were purchased from Nakalai Tesque (Kyoto, Japan). All other chemicals were of analytical-reagent grade.

Distilled water was filtered through a Milli-Q II water purification system (Nippon Millipore, Tokyo, Japan).

Instrumentation

The ultrasonic bath used was a 50-kHz Model C-051 A (Nippon Denshi Kagaku, Kyoto, Japan). The water-bath was a Model UA-100 (Tokyo Rikakikai, Tokyo, Japan). The HPLC system consisted of a Model ERC-8710 liquid chromatograph pump (Erma Optical Works, Tokyo, Japan) equipped with a Model RF-530 fluorescence HPLC monitor (Shimadzu, Kyoto, Japan).

DBS preparation

Whole blood samples, 20 or 100 μl, containing known concentrations of sisomicin or netilmicin were spotted on to blood sampling paper (disc type, Toyo Roshi, Tokyo, Japan). The papers were allowed to dry at 50°C for 10 min and stored in an air-tight glass bottle at 4°C until measured.

Elution of AGs from the DBS

The spotted area (20 μl) as cut with scissors into three or four pieces. Discs of 5.0 mm diameter were punched out from blood-spotted paper (100 μl), dipped in glass tubes containing 500 μl of solvent and placed for various times in the ultrasonic or water bath (37 and 50°C).

Derivatization

We used an ultrafiltration tube (Ultrafree®, Type C3-TK, molecular mass cut-off 30 000 Da; Nippon Millipore) with centrifugation (2000 *g*, 10 min, ambient temperature) for deproteinization of the eluates from the DBS.

Procedure A: derivatization of sisomicin. A 40-μl aliquot of the eluate from the Ultrafree was mixed with 50 μl of methanolic β-MP solution (0.1 *M*), 50 μl of methanolic OPA solution (2 mg/ml), 400 μl of methanol and 460 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 [8].

Procedure B: derivatization of netilmicin. A 40-μl aliquot of the eluate was mixed with 50 μl of methanolic β-MP solution, 50 μl of methanolic OPA solution, 300 μl of methanol and 560 μl of 0.05 *M* KH_2PO_4–borate buffer (pH 9.0) containing 1% (w/v) sodium 1-heptanesulphonate. The mixture was allowed to react at 20°C for 1 h. The fluorescent derivative of netilmicin was stable for at least 6 h under the conditions used (Fig. 2).

Chromatographic conditions

The separations were achieved using a column (200 mm × 4 mm I.D.) packed with Nucleosil C_{18} (particle size 5 μm) (Macherey & Nagel, Düren, F.R.G.) fitted with a C_{18} (particle size 5 μm) guard column (10 mm × 4 mm I.D.). The mobile phase for the sisomicin derivative was a mixture of 800 ml of methanol

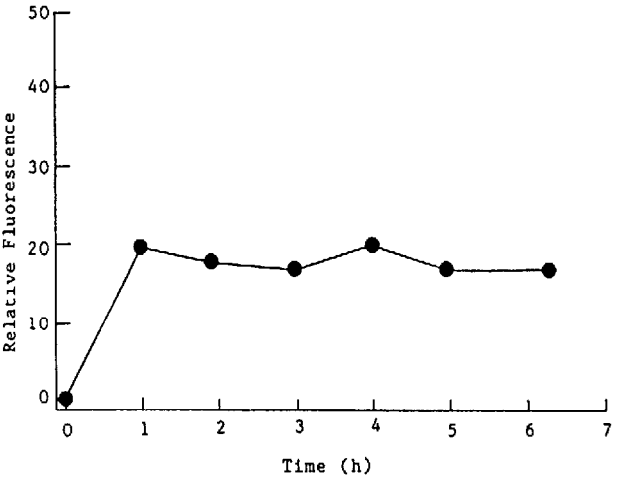


Fig. 2. Stability of OPA-β-MP derivative of a standard solution of netilmicin (7.4 μg/ml). Concentration of OPA, 0.1 g/l; concentration of β-MP, $5 \cdot 10^{-3}$ mol/l; 50 mmol/l KH_2PO_4-borate buffer (pH 9.0) containing 1% (w/v) sodium 1-heptanesulphonate; temperature, 0°C. Other conditions as described under Experimental.

and 200 ml of a counter ion solution consisting of sodium 1-heptanesulphonate (2.5 g) and acetic acid (42 ml) in 208 ml of distilled water, and deaerated ultrasonically. This ion-paired mobile phase gave a single peak for the sisomicin derivative with a shorter retention time for analysis than that in our previous work [8]. The mobile phase for the netilmicin derivative was a mixture of 750 ml of methanol and 250 ml of the counter ion solution as described above. The flow-rate was 0.9 ml/min. The fluorescence intensity of the column eluent was monitored at 450 nm with excitation at 340 nm. The volume of the derivatized sample injected was 400 μl.

RESULTS

Elution of sisomicin and netilmicin from the DBSs on the punched disc

The elution of sisomicin or netilmicin from the DBSs was dependent on several factors, including the nature of the solvent and the duration and the method of elution. Distilled water, physiological saline and 60% (v/v) ethanol had no effect on the elution of sisomicin or netilmicin; 0.5 *M* Na_2HPO_4 solution (pH 8.7) was more effective than 0.05 *M* KH_2PO_4–borate buffer (pH 9.0) for the elution of sisomicin by ultrasonication (Table I), although the haemoglobin was also released from the DBSs.

The optimum elution of sisomicin was checked with 500 μl of 0.5 *M* Na_2HPO_4 solution by monitoring the recovery of sisomicin as a function of time, by gently shaking the tubes in a water bath (37 and 50°C) or by ultrasonication. Fig. 3

TABLE I

INFLUENCE OF SOLVENTS ON THE EXTRACTION OF SISOMICIN FROM THE DBS ON THE PUNCHED DISC

The concentration of sisomicin in the DBS was 7.4 μg/ml. Samples were treated in 500 μl of the solvent by ultrasonication for 30 min.

Solvent	*n*	Recovery (mean ± S.D.) (%)
0.05 *M* KH_2PO_4-borate buffer (pH 9.0)	3	68.9
0.5 *M* Na_2HPO_4 (pH 8.7)	6	93.6±8.5

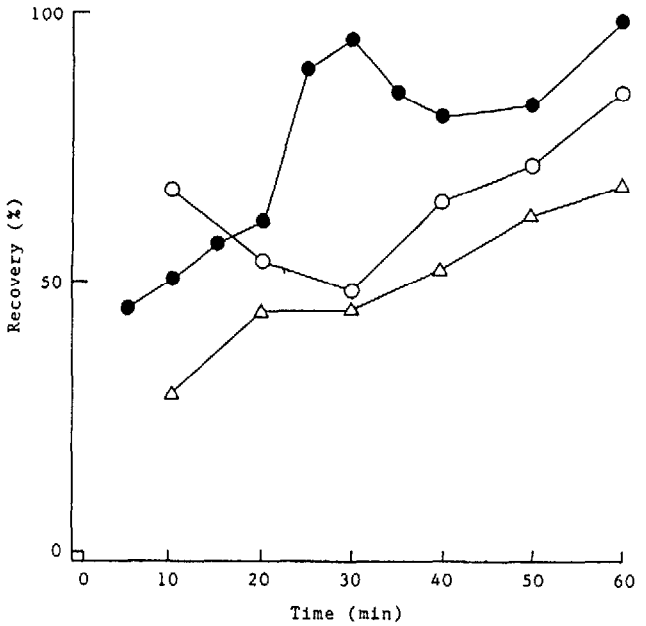


Fig. 3. Efficiency of extraction methods: effect of shaking in a water bath at 37°C (△) or 50°C (○) and ultrasonication (●) for various times on the recovery of sisomicin (7.4 μg/ml) in a DBS punched disc (5 mm diameter). The extraction was carried out in a glass tube containing 500 μl of 0.5 *M* Na_2HPO_4 solution (pH 8.7).

shows that 30 min of ultrasonication ensured good recoveries of sisomicin, and also that ultrasonication for more than 40 min denatured the protein in the eluates; this was subsequently adopted as a standard elution technique for netilmicin.

Blood volume on disc

Using a paper punch as a means of quantification of the volume of blood, it is necessary to analyse the blood in the punched disc (5 mm diameter) of filter-

paper. To obtain information on the volume of blood 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 the eluates were measured by spectrophotometry based on the absorption of the haemoglobin at 575 nm. The blood volume in these filter-paper discs was found to average 10.1 μl with a standard deviation of 0.33 μl ($n = 6$), based on the calibration graph for the whole blood. The intra-assay coefficient of variation for the blood volume in twelve discs was thus 3.3%.

Quantitation of sisomicin and netilmicin in DBSs

Fig. 4 shows chromatograms obtained from DBSs spiked with sisomicin or netilmicin and from a blank DBS, which demonstrates the well resolved peaks of sisomicin and netilmicin derivative, free from apparent interferences in blood. The relationship between the peak heights and the volume of sample injected was linear over the range 200–1000 μl for 5.0 μg/ml sisomicin in the DBSs. The calibration graphs for sisomicin and netilmicin in the DBSs were linear over the concentration ranges 0.1–7.4 μg/ml ($y = 0.049+0.334x$, $r^2 = 0.999$) and 1.0–10.0 μg/ml ($y = -0.552+0.306x$, $r^2 = 0.998$), respectively. The limits of detection of the assay were 0.053 μg/ml for sisomicin in whole blood on the DBSs and 0.5 μg/ml for netilmicin with a signal-to-noise ratio $\geqslant 2$. Both detection limits are sufficient for monitoring these drugs in serum, considering

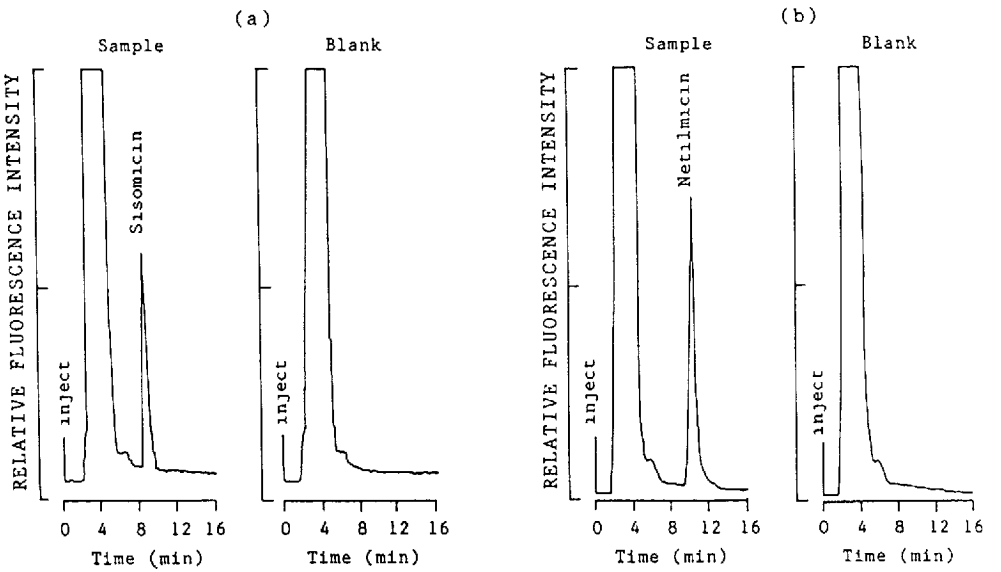


Fig. 4. HPLC of the OPA-β-MP derivatives of (a) sisomicin in the DBS and the blank and (b) netilmicin in the DBS and the blank. Concentrations of sisomicin and netilmicin, 20 and 50 μg per ml of whole blood, respectively. Separation conditions as described under Experimental.

TABLE II

ANALYTICAL RECOVERIES OF SISOMICIN AND NETILMICIN FROM THE DBS ON THE PUNCHED DISC

AG	Added (A) (μg/ml)	n	Recovered (R) (μg/ml)	Accuracy[a] (%)	Coefficient of variation (%)	
					Intra-assay	Inter-assay
Sisomicin	0.74	6	0.69 ± 0.052	−6.8	7.5	
	2.0	6	2.0 ± 0.18	0	9.0	
		5	2.1 ± 0.22	+5.0		10.5
Netilmicin	5.0	8	5.2 ± 0.72	+4.0	13.7	
	10.0	5	10.2 ± 0.89	+2.0	8.7	
		6	10.3 ± 1.69	+3.0		16.4
	25.0	8	24.7 ± 2.10	−1.2	8.3	
	50.0	6	48.3 ± 2.30	−3.4	4.8	
	75.0	5	79.7 ± 2.75	+6.3	3.5	

[a] $[(R-A)/A]\cdot 100$.

that the physiological levels of AGs in patients are in the range 4–30 μg/ml. The higher detection limit for netilmicin may possibly be due to the lower fluorescence intensity or lower stability of the OPA derivative of netilmicin than that of sisomicin under the conditions used [9].

Spiked DBS samples, each containing different concentrations of sisomicin or netilmicin, were analysed by the overall assay technique using the punched discs. The results are summarized in Table II, where the mean recoveries were calculated from the calibration graph using the DBS samples. There were also no significant differences between the recoveries of sisomicin in DBS and plasma samples prepared from blood containing sisomicin concentrations from 2.0 to 20.0 μg/ml, assuming that the ratio of the slopes of the calibration graphs obtained for the DBS and the plasma samples was 2.3 (DBS samples, $y = -0.409 + 0.384x$, $r^2 = 1.000$; plasma samples, $y = -0.340 + 0.872x$, $r^2 = 1.000$) and that the haematocrit value in whole blood is about 50% (v/v) [9].

None of the other commonly used AGs were found to interfere in the determination of sisomicin, but astromicin and dibekacin produced peaks that interfered with netilmicin.

DISCUSSION

Capillary sampling has been used interchangeably with venous sampling for the determination of theophylline, phenytoin and tobramycin and various biochemical and haematological measurements. The micro-volume techniques make it possible to assay small-volume samples, collected, by a finger lancet puncture. The methodology presented here combines the advantage of micro-

sampling, inherent in blood collection on filter-paper, with an accurate, precise and selective HPLC method for the determination of sisomicin in serum and of netilmicin in the absence of astromicin and dibekacin, which interfere. This is the first time that the DBS method has been applied to the determination of sisomicin and netilmicin, although further improvements might be made for use in clinical laboratories, such as the use of an internal standard. Simpler methods, e.g., fluorescence polarization immunoassay, may also be applied to the determination of AGs in filter-paper, although the sensitivity of detection is lower than that of the present HPLC method, so the blood volume in the DBSs would need to be increased. The present micro-method is particularly useful in paediatrics and for patients with renal failure, where sample size is of major concern. Also, as sisomicin and netilmicin on filter-paper are stable for up to ten days (at ambient temperature or 35°C) [10], the DBSs can be easily handled and stored in the laboratory.

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