

## FLUORESCENCE POLARIZATION IMMUNOASSAY OF GENTAMICIN IN BLOOD SPOTTED ON FILTER PAPER

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We have developed a simple method for monitoring aminoglycoside antibiotic concentrations in dried blood spots on filter paper. Gentamicin was recovered from the blood spots most effectively by incubation for 60 min at 35°C in an ultrafiltration tube containing 500  $\mu$ l of 0.5 M  $\text{Na}_2\text{HPO}_4$  solution. The eluates from the paper were centrifuged and transferred to an Abbott TDX cartridge for measurement by fluorescence polarization immunosay.

Gentamicin in the dried blood spots on filter paper was stable for at least ten days at ambient temperature. The inter-run precision (RSD) was below 8.5%. Analytical recovery of gentamicin from the filter paper exceeded 92%. Although the applicability of the method is limited because the quantitation limit of this method is more than 1  $\mu$ g/ml for whole blood (2  $\mu$ g/ml when the hematocrit value of whole blood was taken into account) this method permits simple blood collection.

For monitoring the effective concentration of gentamicin in serum, particularly for newborns.

**Key words:** Gentamicin, Aminoglycoside antibiotic, Dried blood spots, Drug monitoring, FPIA

### INTRODUCTION

Gentamicin, an aminoglycoside antibiotic, is widely used against infections from Gram-negative bacteria. Monitoring serum levels of this drug has been suggested to optimize therapy, thus limiting related toxicity and therapeutic failure<sup>1</sup>. In current procedures for therapeutic drug monitoring in adults, venous blood is widely used. However, this is often very difficult and sometimes impossible for infants and children. Capillary blood spotted on filter paper has been successfully used for detecting inborn errors of metabolism for phenylalanine<sup>2</sup>, prolactin<sup>3</sup>, or for monitoring concentrations of theophylline<sup>4-6</sup>. Since the collection of blood by finger prick or venipuncture is minimally traumatic and can be performed easily and rapidly, multiple samples can be collected from newborn patients.

We previously reported the use of HPLC for the determination of sisomicin in dried blood spots (DBS) on filter paper<sup>7</sup>. In this study, we applied the fluorescence polarization immunoassay (FPIA) to the determination of gentamicin in DBS of

pediatric patients.

### MATERIALS AND METHODS

#### Reagents

For gentamicin-free whole blood, we used citrated or heparinized whole blood from healthy male volunteers. The blood-sampling paper was filter paper type I (Fig. 1, Toyo-Roshi, Tokyo, Japan). The absorbing area of this paper holds 0.1 ml of blood when the paper is dried with the blood-wetted surface on top. The hemoglobin kit (cyanmethemoglobin method) was from Wako Pure Chemical Ind., Osaka, Japan; the FPIA reagent for the gentamicin assay (TDX) from Dinabot Co. Ltd., Tokyo, Japan. The ultrafiltration tubes ("Minicent"<sup>®</sup>; molecular mass cutoff 30,000 Da) were from To-So Co. Ltd., Tokyo, Japan. Gentamicin sulfate was from Essex Nippon K. K., Osaka, Japan; all other chemicals used were of analytical reagent grade.

#### Procedures

**Preparation of the calibration curve.** We prepared aqueous standard solutions of gentamicin 0~20  $\mu$ g/ml, by adding appropriate quantities of aque-

ous stock solution of gentamicin sulfate (1 mg/ml) to distilled water. We also prepared gentamicin standards in blood, 0~20  $\mu\text{g/ml}$ , by adding appropriate quantities of the aqueous stock solution to drug-free pooled blood (hemoglobin content : 12 g/dl).

**Preparation of DBS.** We spotted 100  $\mu\text{l}$  of the standard aqueous solutions or blood solutions of gentamicin, then dried these at 50°C for 10 min in an air-circulating oven or at ambient temperature for 3 h.

**Preparation of ultrafiltrates from DBS.** Using scissors, we cut the blood-absorbing area of the DBS into five or six pieces and placed all of them in a Minicent tube. We added 500  $\mu\text{l}$  of pre-warmed (35°C) 0.5 M  $\text{Na}_2\text{HPO}_4$  buffer, and incubated in an oven (35°C) for various times up to 90 min. We then centrifuged the extracted solution for 15 min (3,000 Xg) and transferred the clear colorless filtrate to the specimen-well of the TDX cartridge for measurement by FPIA.

**Assay of hemoglobin in extracts.** We reserved 20  $\mu\text{l}$  of each extract after incubation and mixed this with 5 ml of cyanmethemoglobin reagent. After 5 min we measured the absorbance at 540 nm and compared it with that of a standard solution containing 3.58 mg of cyanmethemoglobin per 5 ml.

**Assay of gentamicin.** The gentamicin assay was performed with the TDX according to the instruction manual of FPIA method from Dinabot Co. Ltd..

## RESULTS

### Assay of hemoglobin.

To obtain the hemoglobin contents in the DBS, 100  $\mu\text{l}$  of the whole blood was spotted on the DBS paper, which was extracted with 500  $\mu\text{l}$  of 0.5 M  $\text{Na}_2\text{HPO}_4$  for 1 h in a water-bath (35°C). The absorbance of eluates was measured by spectrophotometry and the content of hemoglobin was calculated from the standard calibration curve ( $y=0.273 X+0.007$ ,  $r=0.999$ ) of the hemoglobin assay kit. The eluate from the DBS spotted with 100  $\mu\text{l}$  of the whole blood contained about 2.4 g/dl of the hemoglobin.

### Removal of hemoglobin.

It has been shown that more than 0.86 g/dl of the hemoglobin caused interference on the FPIA

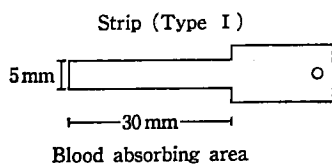


Fig. 1. Blood sampling paper

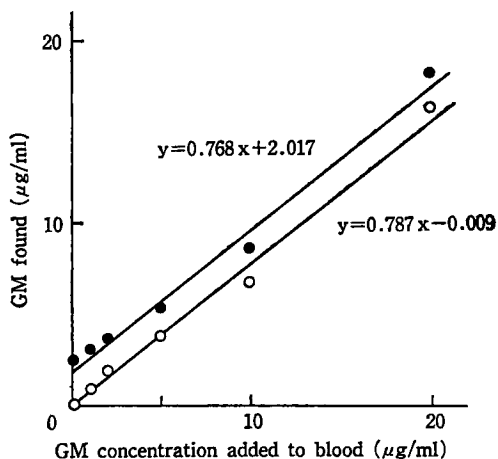
gentamicin assay<sup>8)</sup>. In the present study the eluate from the gentamicin-free DBS paper gave about 2.0  $\mu\text{g/ml}$  as gentamicin value, as shown in Fig. 2 (closed circle symbol). So, in order to remove the hemoglobin (66,000 Da) the eluates from the DBS papers were transferred into the ultrafiltration apparatus (Minicent, the molecular mass cut off was 30,000 Da) and were centrifuged at 3,000 g for 15 min. For the clear and colorless filtrates the present FPIA assay gave less than 0.2  $\mu\text{g/ml}$  as measurement value of gentamicin, which was equal to the detection limit of the used FPIA instrument. As shown in Fig. 2 (open circle symbol), therefore, the hemoglobin in the eluates was almost removed by the present ultrafiltration procedure.

### Effect of elution time.

At each elution period, the eluate was transferred to the Minicent for assay. Since the elution of gentamicin from the DBS paper reached a maximum after 60 to 90 min under these extraction conditions, an elution time of 60 min was adopted for this study (Fig. 3).

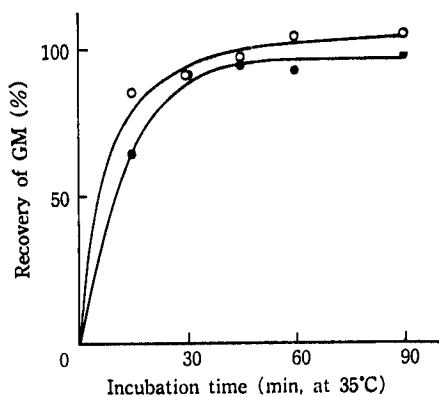
### Analytical recovery and reproducibility.

The recovery of gentamicin was assessed by multiple analyses ( $n=5$ ) of the DBS samples, using pooled whole blood supplemented with known amounts of gentamicin ranging from 1~10.0  $\mu\text{g/ml}$ . The recoveries of gentamicin ranged from 92 to 134% (Table 1), which indicates that the pre-assay treatment presented in this study is also adequate for clinical samples. The recovery of 134% for 1  $\mu\text{g/ml}$  of gentamicin was considered to be due to the error in the concentration of gentamicin in



Recovery of gentamicin in extracts of aqueous standard solutions (O), and in dried blood spot standards (●) which contained hemoglobin 2.4 g/dl.

Fig. 2. Influence of hemoglobin



\* GM concentration: 20  $\mu\text{g/ml}$   
 ○ : dried blood spots (aqueous solution, control)  
 ● : dried blood spots (blood)

Fig. 3. Effect of incubation time on the recovery of gentamicin\*

whole blood. The intra-assay precision (relative standard deviation: RSD,  $n=5$ ) was 8.5, 5.6 and 6.3% for gentamicin at 1, 5 and 10  $\mu\text{g/ml}$ , respectively. The inter-assay variability (RSD,  $n=6$ ) was 6.9% for a gentamicin concentration of 5  $\mu\text{g/ml}$ . When the quantitation limit was settled within 10% of RSD, the assay limit of gentamicin was less than 1.0  $\mu\text{g/ml}$  (RSD=10%) for the DBS sample spotted with 100  $\mu\text{l}$  of whole blood. A typical standard curve is illustrated in Fig. 4.

#### Estimation of serum or plasma concentration.

If the hematocrit value is obtained, the concentration of gentamicin in serum or plasma can be estimated from the data by the present method. In this study, these concentrations ( $C$   $\mu\text{g/ml}$ ) were calculated as follows:

gentamicin ( $\mu\text{g/ml}$ ) in DBS/100-hematocrit value (%)  $\times 100 = C$   $\mu\text{g/ml}$

#### Stability of DBS.

The DBS paper, containing 5  $\mu\text{g/ml}$  of gentamicin in whole blood, was stored at ambient temperature

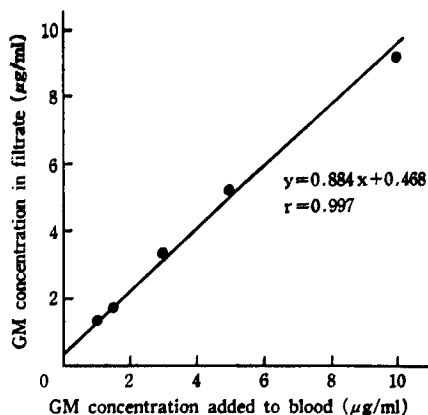


Fig. 4. Calibration curve of gentamicin from dried blood spots by ultrafiltration

or 35°C, which was assayed four times during ten days. As shown in Table 2, no significant loss of gentamicin was observed, indicating that gentamicin is stable in the DBS paper matrix for at least ten days.

#### DISCUSSION

The monitoring of aminoglycoside antibiotics (AGs) in serum or plasma is important for safe and effective therapy because of their narrow therapeutic range. AGs in biological fluids have been determined by a variety of methods, e.g., bioassay, gas chromatography, high-performance liquid chromatography, enzyme immunoassay and others. With the development of monoclonal antibodies and fully-automated assay equipment, the enzyme immunoassay has become a rapid, precise and easy means of assaying AGs. For the patient, especially children, dried blood spots (DBS) collected on filter paper mean an atraumatic and safe form of sampling at the bed side. However, DBS have not been used because of difficulties in extracting AGs from DBS on filter paper. In a previous study, we first reported the use of HPLC for determining sisomicin in DBS on filter paper. The procedure described here combines DBS on filter

Table 1. Recovery of gentamicin from dried blood spots

Gentamicin concentration added ( $\mu\text{g/ml}$ )	n	Gentamicin concentration found ( $\mu\text{g/ml}$ )	Analytical recovery (%)	RSD (%)
1.0	5	1.34 $\pm$ 0.114*	134	8.5
1.5	5	1.72 $\pm$ 0.110	115	6.4
3.0	5	3.32 $\pm$ 0.110	111	3.3
5.0	5	5.20 $\pm$ 0.291	104	5.6
10.0	5	9.16 $\pm$ 0.577	92	6.3

\* : mean  $\pm$  SD

Table 2. Stability of gentamicin in dried blood spots on filter paper

Storage temp.	Days stored			
	0	3	6	10
Ambient	100*	104	104	101
35°C	100	97	99	102

\* Gentamicin measured, expressed as a percentage of the original gentamicin concentration (5  $\mu\text{g}/\text{ml}$  blood, 100  $\mu\text{l}$  spotted), average of two determinations each.

paper with a specific, rapid and accurate method, "FPIA", for monitoring the therapeutic range of gentamicin.

The instruction manual for the FPIA assay points that the hemoglobin levels below 0.86 g/dl gave no effect in the assay. The eluate from the DBS paper contained 2.4 g/dl hemoglobin. In this study, in order to avoid any interference with hemoglobin, the eluates from the DBS paper were transferred into the ultrafiltration apparatus. After centrifugation, the clear and colorless filtrates caused no interference in the present FPIA assay.

Regarding the detection limit of filtrates, the present data supported that the detection limit of a gentamicin assay is 0.2  $\mu\text{g}/\text{ml}$  as described in the FPIA instruction manual. JOLLEY, et al<sup>9</sup>, also reported that the 0.5  $\mu\text{g}/\text{ml}$  gentamicin was recovered completely by the FPIA method. As the 100  $\mu\text{l}$  of whole blood in DBS was diluted to 500  $\mu\text{l}$  during the extraction procedure, the detection limit of gentamicin was increased from 0.2 to 1.0  $\mu\text{g}/\text{ml}$ . In this study, however, the lower limit of quantitation was less than 10% RSD for 1  $\mu\text{g}/\text{ml}$  of gentamicin in whole blood and the recovery from the DBS was considered to be more than 92%.

When the hematocrit value was 50%, the present method gave about 2  $\mu\text{g}/\text{ml}$  as the minimum amount of gentamicin in serum or plasma.

RIFF and JACKSON reported<sup>9</sup> that the drug was estimated at a higher concentration in adult patients with a low hematocrit. On the other hand, MC-CRACKEN et al. reported<sup>10</sup> that no correlation was observed between peak levels of gentamicin in serum and hemoglobin or hematocrit values in infants at two weeks of age.

This discrepancy should be determined by further clinical studies. In conclusion, we have described a method for determining gentamicin in serum using DBS and FPIA. This method gave excellent correlation between the gentamicin concentrations in plasma and in DBS, if corrected for hematocrit value. However, the applicability of the method is limited, because the quantitation limit is within the effective range of gentamicin.

Finally, we propose this method for monitoring

of the therapeutic range of gentamicin in serum and pharmacokinetic studies in pediatrics. The results of our preliminary clinical study in pediatrics will be reported<sup>11</sup> elsewhere.

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### FPIA 法によるろ紙中のゲンタマイシン測定

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乾燥ろ紙血を用いた、アミノ配糖体系抗生物質の簡便な測定法を開発した。限外ろ過用のチューブ内で、ろ紙に 500  $\mu$ l の 0.5 M  $\text{Na}_2\text{HPO}_4$  を加え、35°C、60 min のインキュベーションを行なうことにより、最も効率良くゲンタマイシンを回収できた。TDX<sup>®</sup>全自動測定装置を用い、この抽出ろ液の測定を行なった。ろ紙血中のゲンタマイシンは、室温で少なくとも 10 日間は安定であった。日内変動 (CV) は 8.5% 以下で、ろ紙からの回収率は 92% 以上であった。本法は、全血中 1  $\mu$ g/ml 以上、また、ヘマトクリット値を用いて換算すれば血清中約 2  $\mu$ g/ml 以上のゲンタマイシンの測定に適した方法である。この簡便な採血方法によって、特に新生児における有効治療域のゲンタマイシン濃度のモニタリングが可能になった。

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