Fluorescence Polarization Immunoassay of Gentamicin or Netilmicin in Blood Spotted on Filter Paper

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In this improved simple method for determination of aminoglycoside antibiotics in dried-blood spots on filter paper, gentamicin or netilmicin is recovered from the blood spot most effectively by incubation for 60 min in an ultrafiltration tube containing 500 μL of 0.5 mol/L NaHPO4 buffer. The eluates from the paper are centrifuged, then transferred to an Abbott TDx cartridge for measurement of gentamicin or netilmicin by fluorescence polarization immunoassay. The dried sample on paper is stable for about eight days at ambient temperature. Intra-assay CVs for gentamicin and netilmicin are <8.5% and <6.1%, respectively. Analytical recovery of gentamicin and netilmicin from the paper exceeded 90%. This method permits simple blood collection and monitoring of the therapeutic concentration of gentamicin or netilmicin in serum, particularly that of newborn infants and small children.

Materials and Methods

Reagents

For antibiotic-free whole blood, we used citrated or heparinized specimens from male volunteers. The blood-sampling paper was filter paper type I (Toyobo, Tokyo, Japan). This paper can hold 0.1 mL of blood when the paper is dried with the blood-wetted surface on top. The hemoglobin assay kit (cyanmethemoglobin method) was from Wako Pure Chemical Indus., Osaka, Japan. The FP1A reagent for gentamicin and netilmicin assay (TDx) was from Dinabot Co. Ltd., Tokyo, Japan. The ultrafiltration tubes (“Mini-cent,” molecular mass cutoff 30 000 Da) were from Toyo Co. Ltd., Tokyo, Japan. Gentamicin sulfate and netilmicin sulfate were from Essex Nippon K.K. (a subsidiary of Schering-Plough, Osaka, Japan). All other chemicals were used of analytical reagent grade.

Procedures

Preparation of the calibration curve. We prepared aqueous standard solutions of gentamicin and netilmicin, 0 to 20 μg/mL, by adding appropriate quantities of aqueous stock solution of gentamicin sulfate (1 mg/mL, expressed as the base) or netilmicin sulfate (1 mg/mL, expressed as the base) to distilled water. We prepared gentamicin standards and netilmicin standards in blood, 0 to 20 μg/mL, by adding appropriate quantities of the aqueous stock solution to antibiotic-free pooled blood.

Preparation of dried blood spots. Onto the filter papers we spotted 100 μL of the standard solutions or gentamicin in blood, then dried these either at 50 °C for 10 min in an air-circulating oven or at room temperature for 5 h. We prepared the spots containing netilmicin by the same procedures. For netilmicin, we also examined the correlation between the conventional method with the plasma and the present method with the spot samples for pediatric patients.

Preparation of ultrafiltrates from dried blood spots. Using scissors, we cut the blood-containing area of the dried blood spots into five or six pieces and placed all of them into one Minicent tube. We then added 500 μL of warmed (35 °C) 0.5 mol/L NaHPO4 buffer, and incubated the tube in an oven (35 °C) for 60 min. We then centrifuged the sample for 15 min (3000 × g) and transferred the clear, colorless filtrate to the specimen well of the TDx cartridge for measurement by FP1A.

Assay of hemoglobin in extracts. We reserved 20 μL of each extract and mixed this with 5 mL of cyanmethemoglo-

Additional Keyphrases: drug monitoring · newborns · ultrafiltration · pediatric chemistry

Gentamicin and netilmicin, aminoglycoside antibiotics, are widely used to treat infections from Gram-negative bacteria. Monitoring the concentration of these drugs in serum has been suggested to optimize therapy and thus limit the incidence of both toxicity and therapeutic failure (1). Ordinarily, the specimen used for adults in therapeutic drug monitoring is venous blood. However, this is often very difficult, and sometimes impossible, for infants and children, and capillary blood spotted on filter paper has been used successfully for (e.g.) detecting inborn errors of metabolism or monitoring concentrations of phenylalanine (2), prolactin (3), and theophylline (4–6). Collection of blood on filter paper by finger prick or venepuncture is minimally traumatic and can be performed easily and rapidly, so serial samples can be collected from young patients.

Elsewhere (7), we have reported the use of HPLC for determination of sisomicin or netilmicin in dried blood spots on filter paper. Here we have applied fluorescence polarization immunoassay (FP1A) to the determination of gentamicin or netilmicin in blood of pediatric patients.

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bin reagent. After 5 min, we measured the absorbance at 540 nm and compared it with that of a standard solution containing 3.58 of cyanmethemoglobin per 5 mL.

Results and Discussion

Removal of hemoglobin. Hemoglobin below 8.6 g/L did not affect the gentamicin assay by FPIA. The extracts, before ultrafiltration from blood-spotted paper free of gentamicin, contained 24 g of hemoglobin per liter as determined by the cyanmethemoglobin method; these gave a value of about 2 μg/mL for apparent gentamicin. To avoid any interference by hemoglobin, we transferred the extracts into the Minicent tube, centrifuged at 3000 × g for 15 min, and then assayed. The clear, colorless filtrates for gentamicin-free spots gave values for gentamicin corresponding to less than 0.2 μg/mL, showing that hemoglobin in the filtrates was almost removed by the ultrafiltration procedures (Figure 1).

Effect of elution time. A dried blood-spot paper containing gentamicin was cut and placed in a test tube containing 500 μL of 0.5 mol/L Na₂HPO₄. The tube was sealed and stored in a water bath or circulating-air oven at 35 °C for up to 90 min. At each elution period, the eluate was transferred to the Minicent tube for assay. Elution of gentamicin from the spotted paper was maximum after 60 to 90 min under our extraction conditions, so we adopted an elution time of 60 min for gentamicin, and subsequently also for netilmicin.

Analytical recovery. We assessed recovery of gentamicin by multiple analyses (n = 5) of the dried blood-spot samples of pooled whole blood supplemented with gentamicin to give concentrations ranging from 1.5 to 20 μg/mL. Recovery of gentamicin was from 92% to 115%, indicating that the pre-assay treatment was satisfactory.

The lower limit of detection of gentamicin or netilmicin with the FPIA reagent kits was 1 μg/mL each for the sample spotted with 100 μL of whole blood. However, the recovery for 1 μg/mL of gentamicin was 134%, owing to the small concentration of gentamicin in whole blood.

The calibration curve for gentamicin or netilmicin in dried blood spots was linear over the concentration range of 1.0 to 20 μg/mL (y = 0.873x + 0.568, r = 0.996, Sₓₓ = 0.170) or 1 to 20 μg/mL (y = 0.965x - 0.037, r = 0.999, Sₓₓ = 0.262), respectively. The intra-assay CV (n = 5) was 5.8% and 6.3% for gentamicin concentrations of 5 and 10 μg/mL, 4.6% and 1.6% for netilmicin concentrations of 10 and 20 μg/mL. The interassay CV was 4.5% (n = 4) for a gentamicin concentration of 5 μg/mL and 9.7% (n = 13) for a netilmicin concentration of 10 μg/mL.

We applied the present procedure in a preliminary way to clinical samples from some pediatric patients who had been treated with netilmicin. We observed an excellent linear correlation between these antibiotics concentration in plasma from pediatric patients, when corrected for hematocrit, confirming the validity of this simple and accurate procedure (Figure 2).

Stability of dried blood spots. The stability of gentamicin or netilmicin in paper spotted and stored at ambient temperature or 35 °C was evaluated by using 5 μg of gentamicin or 10 μg of netilmicin per milliliter in whole blood and checking during 10 days. In all the specimens analyzed (Table 1) we observed no significant loss in gentamicin and netilmicin contents, indicating that gentamicin and netilmicin are stable in the paper matrix for at least eight days.

In conclusion: we have described a method for the determination of gentamicin or netilmicin in dried-blood spots by FPIA. The applicability of the method is limited, because the quantification limit exceeds the effective range of the antibiotics. We propose this method for monitoring these antibiotics in serum and in pharmacokinetic studies in pediatrics. Results of our clinical studies in pediatrics will be reported elsewhere.

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![Graph](image)

**Fig. 1.** Analytical recovery of gentamicin in extracts of aqueous standard solutions (O) and in dried blood-spot standards (●)

![Graph](image)

**Fig. 2.** Correlation of netilmicin concentrations as measured in serum and calculated from results for dried blood spots

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Days stored</th>
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<tr>
<td>Storage at ambient temp.</td>
<td>0</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>100</td>
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<tr>
<td>Netilmicin</td>
<td>100</td>
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<tr>
<td>Storage at 35 °C</td>
<td>100</td>
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<tr>
<td>Gentamicin</td>
<td>100</td>
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<td>Netilmicin</td>
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*Gentamicin or netilmicin measured as a percentage of original gentamicin or netilmicin concentration (5 μg/mL whole blood, 100 μL spotted); average of two determinations each.
Concentrations of Tumor-Associated Trypsin Inhibitor and C-Reactive Protein in Serum in Acute Pelvic Inflammatory Disease

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We measured tumor-associated trypsin inhibitor (TATI) and C-reactive protein (CRP) in serum of 29 patients with proven pelvic inflammatory disease (PID). TATI values were increased in seven (24%), paralleling increases in CRP. TATI was increased by about 3.5-fold in seven of eight patients with CRP concentrations >90 mg/L, but in none of 21 patients with CRP concentrations <90 mg/L. TATI concentration and severity of PID as determined by laparoscopy or endometrial biopsy were not correlated. These results suggest that, in severe infections, regulation of TATI synthesis resembles that of acute-phase proteins.

Microbial invasion, tissue injury, and acute inflammation turn on the acute-phase response in the host. During the response, the liver increases the synthesis of certain acute-phase proteins. Of these, C-reactive protein (CRP) is most widely measured in clinical practice. Acute pelvic inflammatory disease (PID) is a polymicrobial infection with a wide range of symptoms and signs. Determination of CRP in serum has hastened the diagnosis of PID (1).

In a previous study (2) we demonstrated a high prevalence of above-normal concentrations of ovarian cancer antigen CA 125, but not of other tumor markers (CEA, APP, CA 15-3, β2-microglobulin), in patients with proven PID. Values for CA 125 were correlated with severity of PID, but not with CRP in serum. We have now studied the correlation between CRP and another marker for ovarian cancer, tumor-associated trypsin inhibitor (TATI) (3). TATI, a 6-kDa peptide, is immunologically identical to the pancreatic secretory trypsin inhibitor (4, 5). Its concentrations are high in serum in all cases of mucinous ovarian tumors and in some serous tumors (6). However, its concentrations may also be increased in bronchitis (3) and hepatobiliary obstruction (7). In patients with various nonmalignant gynecological disorders—including mild infections—TATI concentrations are not increased (8). Patients with severe gynecological infections have not yet been studied. We now report a relatively high prevalence of high values for TATI in serum of patients with proven PID, and a strong nonlinear correlation between TATI and CRP.

Materials and Methods

Study population. The study population consisted of 29 women with acute PID seen in the Department of Obstetrics & Gynecology, University Central Hospital, Tampere, between June 1983 and June 1984. All were hospitalized and underwent laparoscopy and endometrial sampling as detailed elsewhere (9).

Definition of endometritis and salpingitis. The histopathological diagnosis of endometritis was based on the identification of plasma cells (9). The severity of plasma-cell endometritis was graded into mild, moderate, or severe on the basis of histopathological criteria (10). The presence of salpingitis was assessed by laparoscopic criteria and classified as mild, moderate, or severe (11). The minimum criteria for salpingitis were the presence of erythema and edema of the fallopian tubes, with mucopurulent exudate expressed from the fimbrial end of the tubes.

Collection of specimens. Collection of culture specimens for isolation of specific microorganisms from the vagina, cervix, and the upper tract was done as detailed elsewhere (9). Endometrial biopsy specimens were fixed in formalin and processed by routine histological methods. Sera were sampled at the time of admission and again at the time of discharge.

Other procedures. Procedures for isolation of specific microorganisms have been previously described in detail (9). TATI in serum was measured by RIA (3). The mean concentration of TATI in serum of healthy men and women is 11 μg/L; the normal reference interval is 3 to 21 μg/L (3). Thus, we used a cutoff value of 22 μg/L. Serum CRP was

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5 Nonstandard abbreviations: CRP, C-reactive protein; PID, pelvic inflammatory disease; TATI, tumor-associated trypsin inhibitor; IL, interleukin; and TNF, tumor necrosis factor.
6 Received December 5, 1988; accepted February 14, 1989.

References