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Dibekacin assay in serum by automated fluorescence polarization immunoassay (Abbott Tdx): Comparison with high-performance liquid chromatography, substrate-labelled fluorescent immunoassay and radioimmunoassay

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Abstract: An immunoassay based on fluorescence polarization detection (FPIA) has been recently adapted for dibekacin. This has been compared with a reference method (high-performance liquid chromatography), and two other methods used in clinical laboratories for monitoring this aminoglycoside, namely substrate-labelled fluorescent immunoassay (SLFIA) and radioimmunoassay (RIA). FPIA was fast and more reliable than SLFIA or RIA, and offered therefore superior performance. However, its nominal cost per assay is high.

Keywords: *Dibekacin; fluorescence polarization immunoassay; substrate-labelled fluorescent immunoassay; high-performance liquid chromatography; radioimmunoassay; serum.*

Introduction

Aminoglycosides remain very useful antibiotics for the treatment of moderate and severe infections, especially with gram-negative organisms [1]. The therapeutic levels in the serum are, however, close to the toxic levels [2]. Moreover, the elimination rate of aminoglycosides may considerably vary among patients and is somewhat difficult to predict accurately, even when doses are adjusted for individual's weight and kidney function [3–6]. Trough levels over 2 mg/l for gentamicin or tobramycin may indicate accumulation of the aminoglycoside [8] and have been correlated with nephrotoxicity [9–13]. Elevated peak levels of aminoglycosides have been correlated with an increased risk of ototoxicity which is usually considered as irreversible [14, 15]. On the other hand, it is not infrequent that the recommended dosages result in serum levels that are below the MIC 90 of the sensitive organism, in which case readjustment of the posology is

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essential. Thus, a recent clinical investigation has demonstrated that a too low initial peak level (<5 mg l^{-1} for gentamicin or tobramycin) was associated with a greater mortality in patients with severe gram-negative bacteremia [16]. Thus, the monitoring of the aminoglycoside blood levels may both reduce their toxicity and improve their efficacy in life-threatening and severe infections.

Many assay methods of aminoglycosides have been described, based on either their antibacterial activity [17], their chromatographic behavior [18, 19] or enzyme-catalysed specific reactions [20–22]. These methods are often slow, insensitive or subject to many interferences. The immunological detection of aminoglycosides has therefore gained a wide popularity, and a variety of detection procedures have been proposed [23–27]. Recently, an immunoassay based on fluorescence polarization detection (FPIA) has been adapted for aminoglycosides [29], with a totally automated bench-top analyser (Abbott Tdx [30]). This method has been now adapted to Dibekacin.

Dibekacin (DKB) is an aminoglycoside used in Japan, Korea, several European countries and South America. It is 3′, 4′- dideoxykanamycin B and is thus closely related to tobramycin (which is 3′-deoxykanamycin B [31, 32]). Both drugs display essentially similar antimicrobial properties. Few reports have been published concerning the toxicity of DKB in humans. Animal data on nephrotoxicity are controversial [33–35], although recent evidence shows that DKB is more accumulated by rat kidney than tobramycin [36]. It is probably less ototoxic [37].

An evaluation of the FPIA method, performed according to the manufacturer's recommendation, on serum of patients treated with DKB, is here reported. The results are compared with (i) a reference method allowing a positive identification of the molecule, namely *high-performance liquid chromatography* (HPLC), and (ii) two other methods widely used in clinical laboratories, namely *substrate-labelled fluorescent immunoassay* (SLFIA) and *radioimmunoassay* (RIA). These two assays were run with antitobramycin antibodies which cross-react with DKB.

Materials and Methods

Specimens

Fifty-two serum samples were obtained from patients receiving DKB for severe infections. All patients had been hospitalized for abdominal surgery; all received other antibiotics in association with DKB: ampicillin, metronidazole, clindamycin or minocycline. They also received other non-antibiotic drugs, according to their needs. Samples were obtained at random from 30 min to 10 h after a regular administration of DKB (usually 1–1.5 mg kg^{-1} per dose). The serums were kept frozen until analysis, which was performed independently by two investigators (B.R. for FPIA and HPLC, and P.V.D.A. for SLFIA and RIA). In addition, serums were also assayed for triglycerides, cholesterol, total bilirubin and protein according to standard laboratory procedures. Visible hemolysis was noted in nine samples.

Fluorescence polarization immunoassay (FPIA)

Specific reagents for dibekacin were made available by Dr Schwenzer from Abbott Laboratories, Diagnostic Division (Irving, Texas) and were used on a standard Abbott Tdx automated fluorescence polarization analyser, using the specific DKB program (assay No. 19) already available on the machine. In this method, unlabelled DKB present in the patient's serum competes with fluorescein-labelled DKB (tracer) for

a limited number of antibody sites; the binding of the tracer to specific antibody results in an increase of its apparent molecular weight. When a mixture of free and bound tracer is illuminated with a polarized exciting light (at 484 nm), the emitted light (530 nm) is depolarized in proportion to the amount of free tracer present. This is because the rotational relaxation time of the free tracer (i.e. its thermal mobility) is much higher than that of the bound tracer. Thus, the loss of polarization indirectly measures the amount of drug in the patient's serum.

During the study period, two different batches of reagents were used. Before each series of assays, the instrument was first calibrated with calibrators provided by the manufacturer (DKB solutions at 0, 0.5, 1.5, 6.0, 10.0 mg l^{-1}); during each series of assay control sera (1.0, 4.0 and 8.0 mg l^{-1}) were included in the samples. Some of these control sera were provided by Abbott Diagnostics; others were prepared by the authors (see below).

In addition to the assays with the specific dibekacin reagents, the sera were also assayed with tobramycin reagents. Thus, standards of DKB (drug-free serum spiked with known amounts of DKB to concentrations of 2–10 mg l^{-1}) were assayed with the tobramycin reagents and the readings of the instrument were converted into their equivalents of DKB concentrations. The correction factor was approximately 1.4 in the 0–6 mg l^{-1} range and increased thereafter to reach approximately 1.8 at 10 mg l^{-1}. A calibration curve was therefore constructed and used for the subsequent assays of DKB-containing clinical samples. Samples above 10 mg l^{-1} were diluted 1:2 in drug-free serum.

High-performance liquid chromatography (HPLC)

The method is derived from that described for gentamicin [18] and we used netilmicin as internal standard. Serum samples were first prepurified by deproteinization with trichloroacetic acid (5% final concentration) and ion-exchange on DOWEX 50 W-X 8 (100–200 mesh) microcolumns. These columns were washed with 10 vol. 0.1 M acetate–0.1 M Na_2SO_4 (pH 7.4); the aminoglycosides were eluted with 0.1 N NaOH and quickly neutralized with *p*-toluene sulphonic acid (40 μM) and acetic acid (40 μM). Samples (50 μl) of eluates were applied to HIBAR Lichrosorb RP 18–10 μm column (25 cm) equipped with a RP 18 precolumn and chromatography was performed with 0.02 M *p*-toluene sulphonic acid–0.02 M acetate–0.2 M Na_2SO_4–0.04 M acetic acid–5% acetonitrile at a flow rate of 1.0 ml min^{-1}. Detection was made by post-column derivatization with *o*-phtalaldehyde/mercaptoethanol reagents [18] and fluorescence measurement (340 nm exciting light; 440 nm emitted light). Figure 1 shows typical chromatograms of drug-free and of DKB-containing serums. A linear response was observed in the range 0–10 mg dibekacin per litre of serum, with a correlation coefficient of at least 0.995. Each assay was done at least in duplicate.

Substrate-labelled fluorescence immunoassay (SLFIA)

Fluorescence was measured with an AMES Fluorostat (Miles, Brussels, Belgium). The procedure was performed according to the instructions of the manufacturer. In this method, antibodies to the aminoglycoside are added to the patient's serum together with β-galactosidase. A second addition of a conjugate between the same aminoglycoside methylumbelliferyl-β-galactoside (protracer) is made. In the absence of aminoglycoside in the patient's serums, most of the protracer reacts with the antibodies and becomes therefore unaccessible to β-galactosidase. Conversely, if aminoglycoside is present, part

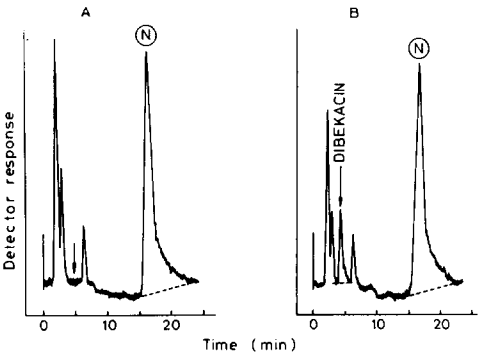

Figure 1
Typical recording after HPLC analysis of serum samples (after extraction).
(A) Serum from a control patient. (B) Serum from a patient treated with dibekacin. The fast-moving material eluting before and after the dibekacin peak represents aminated contaminants that were not removed by the extraction procedure. The retention time of dibekacin was fairly constant at 4 min 25 sec. Netilmicin, used as internal standard, elutes at 16 min 50 sec.

of the protracer will remain free and will be cleaved into aminoglycoside-β-galactose and free methylumbelliferone (tracer). The latter is highly fluorescent and its amount of fluorescence is therefore directly related to the amount of aminoglycoside present in the patient's serum. Commercially available kits for the detection of tobramycin were used, since the antibodies cross-reacted with DKB. The readings of the instrument were corrected by means of a calibration curve constructed as described for the FPIA assay. The correction factor was approximately 1.2 up to 10 mg DKB l^{-1}. Samples with a DKB concentration >10 mg l^{-1} were diluted 1:2 in drug free serum.

Radioimmunoassay (RIA)

The reagents (tobramycin kit) were obtained from Travenol-Diagnostics (Belgium). The procedure was performed according to the instructions of the manufacturer. As for SLFIA and FPIA, a calibration was made with standards of DKB. The correction factor was close to 1 up to 7 mg l^{-1}, but increased sharply above this concentration. Thus, a detailed calibration was made between 7 and 10 mg l^{-1}. Samples with a concentration of DKB ≥10 mg l^{-1} were diluted 1:2 in drug-free serum.

Antibiotics

Patients received DKB as DIKACINE (dibekacin sulphate solution), manufactured and supplied by Belphar, s.a., Brussels, Belgium under licence from Meiji Seika Kaisha Ltd, (Tokyo, Japan). Standards for assays were either from Continental Pharma (Brussels, Belgium) or Meiji Seika Kaisha Ltd and supplied as dried sulphate powder. After further drying *in vacuo* at 110°C for 2 h, samples were weighed and stock solutions were prepared, taking into account the potency indicated by the supplier (usually approx. 700 mg g^{-1} dried powder). Standards of netilmicin and tobramycin were obtained from Schering Corporation (Kenilworth, NJ) or E. Lilly (Indianapolis, Ind), respectively, and treated as dibekacin samples.

Results and Discussion

Figure 2 shows the linearity of the readings of the Abbott Tdx instrument after analysis of drug-free serum spiked with increasing amounts of dibekacin. In some circumstances, a non-linear response was observed above a concentration of approximately 7 mg l^{-1}, irrespective of the reagent batch used (Fig. 2A). A correction curve was therefore constructed. During the test period, however, the linearity of the assay was improved by

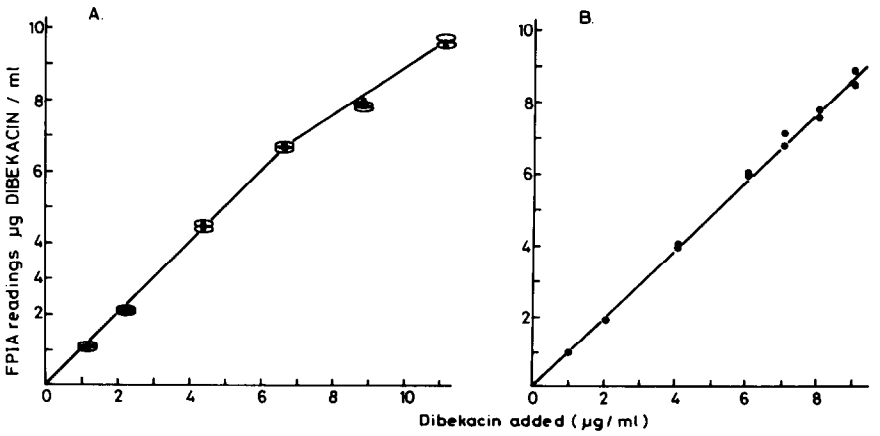

Figure 2
Observed readings on Abbott Tdx fluorescence polarization analyzer with spiked serums. Known amounts of dibekacin (Meiji) were added to blank sera. Panel A: assays were done with two reagent batches (○ 9246–18; ●: 12278–203) at the beginning of the study period; panel B: a new calibration was obtained after a change in the instrument software at the end of the study period.

a change of the instrument software carried out by the manufacturer (Fig. 2B). Variations among successive assays of the samples remained below 5%.

Figure 3 shows the correlations between the results obtained by FPIA, SLFIA and RIA vs those obtained by HPLC. Complementary data are presented in Table 1. The best correlation was obtained with FPIA, using either the specific DKB reagents or the tobramycin kit. SLFIA showed a significantly worse correlation coefficient, but the slope was similar to that of FPIA. Conversely, the RIA showed a better coefficient of correlation but the slope was very different from 1, which resulted in an important underestimation of the values above 5–6 mg l^{-1}. All samples with a DKB content above 9 mg l^{-1} were re-assayed after dilution 1:2 in drug-free serums, and this gave some improvement of the slope.

When the data obtained by FPIA with the specific DKB reagents were compared with those obtained with the reagents designed for tobramycin assay, no major difference was observed. The improvement of the slope and of the intercept values results from the use of a calibration curve for correcting the readings obtained with the tobramycin reagents. The scatter of the points is, however, largely similar. Thus, both reagents can be used interchangeably, as suggested earlier [38].

The serum samples showed variations in their content in triglycerides (37–201 mg dl^{-1}), cholesterol (70–225 mg dl^{-1}), total bilirubin (0.1–2 mg dl^{-1}) and protein (4.1–7.1 g dl^{-1}). None of these constituents affected the results significantly. This was assessed by plotting the difference between the dibekacin concentration measured by each method and that measured by HPLC versus the corresponding value of each constituent, and by computing the linear coefficient of correlation. This coefficient was always lower than 0.2. Similarly, visible hemolysis did not produce systematic differences between the data provided by any of the assay methods.

Although the influence of the various components of the serum on the reliability and accuracy of the assay has not been examined systematically by the present authors, it would nevertheless appear that the conditions set by the manufacturer are satisfactory.

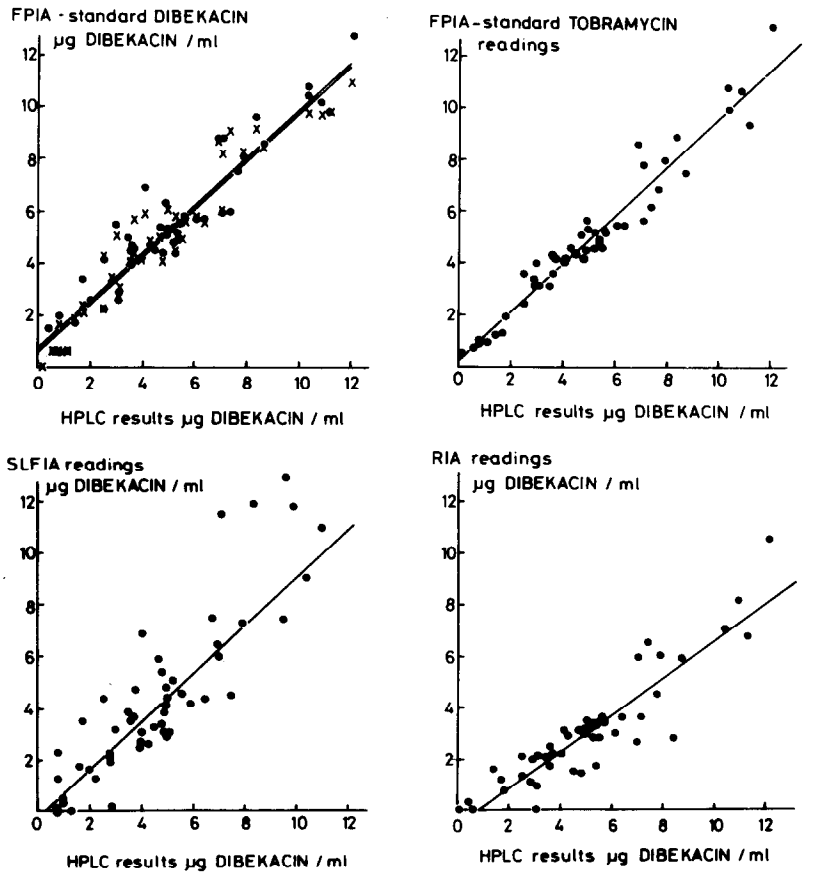

Figure 3
Correlations between the assay of sera by HPLC and the other methods. FPIA–DKB: fluorescence polarization immunoassay with specific DKB reagents (● 9246–18; X: 2278–203). FPIA-Tobra: fluorescence polarization immunoassay with tobramycin-specific reagents. SLFIA: substrate-labelled fluorescent immunoassay with tobramycin-specific reagents. RIA: radioimmunoassay with tobramycin-specific reagents. For FPIA-Tobra, SLFIA and RIA, the readings were corrected by use of a calibration curve. Each value represents the mean of duplicate measurements.

Further examination of matrix interferences for the determination of drugs by the FPIA methods has been reported by the manufacturer [29–30] and will be the subject of a forthcoming paper dealing with gentamicin (Van der Auwera *et al.*, in preparation).

Table 2 shows a comparison of the respective merits of the methods studied. The less applicable method for the standard laboratory routine is probably HPLC, due to the very high cost of the apparatus and the need for a well-trained technologist. The time required for setting up the instrument makes it non-cost-effective for a small series of samples. Large series will require considerable time, since each analysis takes about 20 min. These difficulties represent major drawbacks for clinical laboratories, even though they could be partly alleviated by automation.

Among the three immunological methods, RIA shows the lowest cost per assay. It requires the construction of a standard curve and the inclusion of control points for each series of determinations, therefore proportionally increasing the overall cost per sample.

Table 1
Regression parameters of the correlations between assay of Dibekacin by HPLC (reference method) and fluorescence polarization immunoassay (FPIA), substrate-labelled fluorescence immunoassay (SLFIA) and radioimmunoassay (RIA)

Assay method	Reagent	Regression parameters*		
		Intercept	Slope	Coefficient of correlation
FPIA	Dibekacin reagent lot 12278–203	0.748	0.915	0.947
	Dibekacin reagent lot 9246–18	0.677	0.908	0.960
	Tobramycin	0.204	0.944	0.979
SLFIA	Tobramycin	−0.206	0.927	0.847
RIA	Tobramycin	−0.579	0.715	0.907

*Calculated from the data of Fig.2, using linear regression analysis (least square method).

Table 2
Comparative merits of the methods used for the assay of serum dibekacin

	HPLC	FPIA	SLFIA	RIA
Cost of equipment (× BF 10^6)	1.5§	3§	0.3§	1.5§
Cost of 100 tests (× BF 10^3)	1§	22–31§	16§	10–14§
Stability of reagents	months	months	months	weeks
Assay time	2–3 h*	10 min	20 min	2 h
Automation	possible	yes	possible	no
Standard curve stability	internal std	>3 weeks†	10 days†	1 run
Optimal precision in mg l^{-1} of serum	0.5–10	0.2–10‡	0.5–10‡	1–10‡
CV % inter-assay (%)	5	3.5	5	7
Interference with various subs	possible	individual blank allowing correction	possible	possible

*Including extraction procedure.
†Calibration curves are kept in the instrument memory.
‡Because the slope is different from 1, values above 7 mg l^{-1} tend to be underestimated to a level achieving clinical significance especially for RIA.
‡Approximate prices (1984).

Thus, the simultaneous determination of at least 30 samples is required to achieve cost-effectiveness. This number may not be reached easily in medium-sized hospitals, especially if considering that the results of each assay should be made available to the clinician before the next dose, i.e. within a maximum of 5–7 h, to be of more than retrospective interest.

FPIA appears the most effective method, with the lowest inter-assay coefficient of variation, a low delay between the arrival of the sample in the laboratory and the availability of the results, and a complete automation of the procedure. Moreover, the standard curves are kept in the memory of the instrument, so that individual samples can be analysed at any time by themselves without requiring re-calibration, i.e. as soon as

they arrive in the laboratory. An apparent drawback is the high nominal cost per assay, which however, is partly compensated by the savings in laboratory technician work and maintenance. The excellent results obtained with tobramycin reagents should be considered as an advantage since this method does not tie up the laboratory to the individual choice of the prescribing doctors for one or the other of these two aminoglycosides.

SLFIA has a lower cost than FPIA and is almost as easy to perform. The lack of correlation for some of our samples, however, may indicate that interferences are likely to occur. Thus, the reliability of the assay will have to be carefully examined.

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