Comparison of Different Tobramycin Assays

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Three commercial available tobramycin assays, the fluorescence immunoassay (AMES), the Diagnostic Products Corp. radioimmunoassay, and the Nuclear Medical System radioimmunoassay, were compared with the microbiological assay and the chemical assay involving high-performance liquid chromatography. The results obtained with each assay were evaluated in terms of usefulness, reproducibility, and cost-effectiveness.

Tobramycin, an aminoglycoside antibiotic, has proved to be a useful agent in the treatment of serious infections due to gram-negative microorganisms (6, 13). Because tobramycin is mainly excreted in the urine, accumulation and consequent toxicity can result in patients with impaired renal function (7). The low therapeutic index and the rather unpredictable serum levels, even in patients with normal renal function, require accurate monitoring of the serum concentration during therapy (4, 14).

Several methods for assays of antibiotics have been developed recently, including a fluorescence immunoassay (FIA), a radioimmunoassay (RIA), an enzyme multiplied immunoassay technique, and chemical assays with gas-liquid chromatography and high-performance liquid chromatography (HPLC) (1–3, 5, 8, 10–12, 15, 16, 22).

Traditionally, assays of antibiotics are performed by a bioassay technique (19, 20). This method has proved to be reliable and simple; no special equipment is required. Attendant with the use of the bioassay is the delay time (i.e., the time between obtaining the serum sample and getting the results available) of at least 4 h and the lack of specificity (the interference of coadministered antibiotics).

The increasing number of different antibiotic assays justified the present study in which the FIA and the RIA (commercially available kits) were compared with the conventional microbiological assay (MA) and the chemical assay involving HPLC as developed in our laboratory.

The results obtained with each assay were analyzed in terms of usefulness, reproducibility, and cost-effectiveness.

MATERIALS AND METHODS

Serum samples collected from patients receiving tobramycin alone (n = 30) or in combination with other antibiotics (n = 18) were stored at −20°C until tested. Standard concentrations were made by a two-fold dilution of the stock solution (32 µg/ml in distilled water in pooled human sera obtained from healthy volunteers). All assays were performed in duplicate. Dilutions were made with adjustable pipettes (Pipetman Gilson). Tobramycin (potency, 95.6%) was a generous gift of Eli Lilly & Co.

MA. The MA was performed by an agar diffusion method (3) with large bioassay plates (Nunc bio assay disk PS SI) and ISO sensitest agar (Oxoid). Klebsiella edwardsii subsp. atlantae (NCTC 10896) was used as the test organism.

Wells of 7-mm diameter were filled with standard and patient sera in duplicate. After incubation for 4 h at 42°C the diameters of zones of growth inhibition were measured (20).

FIA. The FIA (AMES, therapeutic drug assay) uses the principle of the competitive protein binding method to measure the antibiotic concentration in the serum samples. Competitive binding reactions are set up with a constant amount of the fluorogenic tobramycin reagent (nonfluorescent under the conditions of the assay), a limiting amount of antibody against tobramycin, and the serum sample.

The tobramycin in the serum sample competes with the fluorogenic tobramycin reagent for the antibody binding sites. The fluorogenic tobramycin reagent, not bound to antibody, is hydrolyzed by β-galactosidase to produce a fluorescent product. The fluorescence produced is proportional to the tobramycin concentration in the serum samples.

The fluorescence was measured with a Perkin Elmer spectrofluorometer (type 1000 M). Reagent preparations, storage, and specimen handling were as described by the manufacturer's instructions.

RIA. The RIA is based upon the competition between [125I]tobramycin and unlabeled antigen (i.e., tobramycin in the serum samples) for the antibody binding sites. The higher the antibiotic concentration in the serum sample, the fewer antibody binding sites will be occupied by the labeled tobramycin. Bound and unbound [125I]tobramycin were separated by centrifugation after adding a second antibody (goat or sheep anti-rabbit immunoglobulin G). The radioactivity in the pellet (bound tobramycin) was counted, and a standard curve was constructed by plotting the concentration of the standards against the percentage of bound labeled tobramycin.

The Diagnostic Products Corp. RIA (RIA-DPC) and
the Nuclear Medical Systems RIA (RIA-NMS) were tested. The differences in procedure of the assays used were limited to minor differences in volume and dilutions of the serum samples (2.3 ml of serum diluted 1:200 and 0.5 ml of serum diluted 1:100) and shorter incubation time at room temperature (10 min versus 40 min). The procedure followed that described in the manufacturer's manual.

**HPLC.** HPLC involves a sample preparation by a combined extraction-deproteinization procedure, followed by postcolumn derivatization, separation by reversed phase chromatography, and measurement of the fluorescent product.

The sample preparation was as follows: 400 μl of phenol solution and 700 μl of trichloroacetic acid (0.25 mol) were added to 400 μl of serum. After blending in a Vortex mixer, the mixture was centrifuged for 6 min at 10,000 rpm, and the supernatant was injected by using a Valco valve with a 2-μl injection loop.

An SP 3500 B pump (Spectra Physics) was used to deliver the mobile phase. A Schoeffel model 970 fluorometer (Schoeffel Instrument Corp., Westwood, N.J.) was used to detect fluorescent products, formed by continuous flow, postcolumn derivatization with ortho-phthalaldehyde. Fluorescence excitation was at 340 nm, a KV 415 filter was used for emission, and the photomultiplier voltage was 420 V.

Ortho-phthalaldehyde was supplied by a tube Minipulse-2 pump (Meyvis) with a flow rate of about 0.5 ml/min by using isoswavelne tubes (these tubes are specially made for use with aggressive reagents). Outlets from the HPLC column and tube pump were connected with a low dead-column T-piece (Chrompack).

A reaction coil consisting of a stainless steel coil (1.0 m by 0.25-mm inner diameter) was used between the T-piece and the detector. Analysis was performed by using a lichrosorb 5 RP 8 15-cm column (Chrompack). The detector signal was recorded by a Vitatron recordor.

**Derivatizing reagent (ortho-phthalaldehyde).** Boric acid (24.7 g) was dissolved in 950 ml of water, adjusted to pH 10.4 with 8 N sodium hydroxide solution (320 g/liter), and diluted to 1,000 ml. To 250 ml of this solution ortho-phthalaldehyde reagent (150 mg dissolved in 5 ml methanol) and 0.5 ml of 2-mercaptoethanol were added. After filtration the reagent was stored at 4°C and used within 1 week.

**Phenol solution.** Phenol (100 g) was dissolved in 6 ml of water. After the addition of 25 ml of dichromomethane the mixture was blended in a Vortex mixer and stored in a dark place at room temperature.

**Chromatographic eluent.** The solvent used was water–methanol–acetic acid (99:7:0.2:0.1 mol%), containing 28.4 g (0.2 mol) of sodium sulfate and 3.84 g (0.02 mol) of sodium pentane sulfate. Column flow rate was 1 ml/min at 130 atm (13.169 kPa).

Sodium pentane sulfonate was obtained from Eastman Kodak Co. All other chemicals were of reagent grade, obtained from Merck & Co., Inc. Distilled water was used throughout.

### RESULTS

**Measurements of standards.** The standard curves obtained using the different methods are shown in Fig. 1a (MA), 1b (FIA), 1c (RIA-DPC), and 1d (RIA-NMS). The data are expressed as the mean values of duplicate measurements. The standard curve for the HPLC was constructed by plotting the standard concentration against the peak heights (Fig. 2). A straight line was obtained with the MA and the HPLC. The data with the RIA assays yielded a sigmoid shaped curve, but on logit-logit paper the curve was converted into a straight line.

**Intra-assay variations.** The reproducibility of each assay has been assessed by calculating the coefficient of variation and the standard deviation of the duplicate measurements of the patient sera (Table 1). The lowest coefficient of variation and standard deviation were obtained with the HPLC (coefficient of variation, 9.0%; standard deviation, 0.22 μg/ml) followed in increasing order by the FIA, RIA-DPC, MA, and RIA-NMS.

**Inter-assay variations.** A comparison between the different assays has been made by calculating the coefficient of correlation, with the MA and the HPLC as the reference methods (Table 1). When sera of patients receiving tobramycin alone (n = 30) were included in the calculation, correlation coefficients from 0.74 (RIA-DPC and MA) to 0.98 (HPLC and MA) were obtained. The presence of other antibiotics resulted in a decrease of the r values to 0.65, at least when the MA was used as the reference method. The regression analysis of the different assays is given in Fig. 3 (MA as reference) and 4 (HPLC as reference). The parameters of the equations of the regression lines in form of \( y = ax + b \) are given in Tables 2 and 3.

**DISCUSSION**

Recently several methods have been developed; each method has problems attendant with its use. To be practically useful for a clinical routine laboratory an assay method should (i) be accurate and specific, (ii) require a minimum of patient serum, (iii) yield the results before the next dose will be given, (iv) require a minimum of skilled technician time, and (v) preferably make use of equipment already available (17).

As stated earlier by Philips et al. (17), a 20% level of inaccuracy should be acceptable, at least for use in a clinical laboratory. The lines indicating these 20% limits of error (i.e., ±20% of the true value) are given in Fig. 3 and 4. However, for research purposes and especially for non-microbiological methods a lower level of inaccuracy should be more acceptable.

The RIA-DPC showed the lowest accuracy, with correlation coefficients of 0.74 and 0.79, respectively, with the MA and the HPLC as reference methods. In a similar study comparing the RIA-DPC with the MA, Lantz et al. (9) found nearly the same results (r = 0.798).
FIG. 1. Standard curves, expressed as the mean value of duplicate measurements, with the MA (a), the FIA (b), the RIA-DPC (c) and the RIA-NMS (d).

Also our findings with the FIA and the RIA-NMS are generally in agreement with the results of similar studies in which $r$ values of 0.81 to 0.95 were found, at least when clinical samples were tested (9, 18, 23). However, in some studies better results were obtained. White et al. (25) found, comparing the FIA with the RIA and the MA, $r$ values of 0.95 and 0.98, respectively. Differences in experience and probably differences in manufacturer (Technia Diagnostics Ltd. versus the AMES therapeutic drug assay) might explain the high $r$ values in their study. Although differences in procedure of both RIA assays tested were limited to minor differences in incubation time and in volume and dilutions of the serum samples, in our study better results were obtained with the RIA-NMS. This could be explained by the rather flat slope of the standard curve of the RIA-DPC. Small differences in the percentage of bound tobramycin could therefore result in distinct changes in tobramycin concentrations in the serum samples. This problem was not observed with the RIA-NMS.

Aminoglycosides are often given in combination with other antibiotics. Interference in the assay results by the presence of other antibiotics was only observed with the MA (Fig. 3).

The amount of patient serum required varied from 40 µl for the RIA-DPC to 400 µl for the HPLC. These volumes are acceptable, even in pediatric patients.

Measurement of the antibiotic concentration is of the greatest value when the results are available before the next dose is given. To make dosage adjustment possible, the delay time (i.e., the time between obtaining the serum sample and getting the results available) should preferably be less than 4 h. This time in the assays tested varied from 51 min for the FIA to 240 min for the MA. Even if a regimen every 6 h is to be followed, the delay time for the MA will still make accurate monitoring possible. However, when it is necessary to have the results available...
more quickly another method such as FIA, RIA, or HPLC must be used. The inaccuracy of the RIA-DPC argued, at least for the RIA assays tested, in favor of the RIA-NMS.

The set-up time (i.e., the time needed to perform the assay) for one sample, using the FIA and the RIA-NMS, was 50 and 90 min, respectively (Table 4). Up to 20 samples could be tested within the same time. Using the HPLC the set-up time of 30 min included 10 min for the sample preparation (i.e., the extraction-deproteinization procedure) and 20 min of retention time. Several samples could be prepared at the same time, but for each additional sample tested, the 20 min more of retention time was required. Despite the simplification of the HPLC method we developed in terms of the combined extraction-deproteinization procedure, the retention time of 20 min was the limiting factor for the number of sera which could be tested daily. Only 25 sera could be tested each day: 20 patient sera and five standards.

In making a cost analysis of the different methods one has to take into account the price of the commercially available kits and the set-up of the laboratory, as workload, staff experience, labor cost and equipment available.

The workload within the shelf life of the solutions necessary for the different methods as well as the daily workload are important. The shelf life of the solutions of the FIA, RIA-DPC, and RIA-NMS, each of them suitable for 100 assays, was 8, 6, and 4 weeks, respectively.

Therefore, for an optimal use of the commercially available kits at the lowest cost, this number of assays should be performed within the shelf life of each kit. For the MA and the HPLC the shelf life of the solutions is of minor importance. For the MA no specific solution is necessary. The derivatizing reagent used for the HPLC has to be made by the technician himself. The cost of performing one tobramycin assay as a single determination, varied from $2.00 for the MA to $20.00 for the RIA-NMS (The price includes labor cost and cost of material for the serum sample and the standard curve).

If more than one sample can be tested at the same time, the reduction in cost for one sample using the FIA, RIA-DPC, RIA-NMS, and MA is obvious (Table 4). This reduction is due to the use of one standard curve when several samples

![CHROMATOGRAM](http://jcm.asm.org/)

FIG. 2. Standard curve, expressed as the mean value of duplicate measurements, with the HPLC and constructed by plotting the peak height against the concentration.

<table>
<thead>
<tr>
<th>Method</th>
<th>Intra-assay variation</th>
<th>Inter-assay variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CV (%)</td>
<td>SD (μg/ml)</td>
</tr>
<tr>
<td>MA</td>
<td>15.2</td>
<td>0.59</td>
</tr>
<tr>
<td>RIA-DPC</td>
<td>10.8</td>
<td>0.24</td>
</tr>
<tr>
<td>RIA-NMS</td>
<td>20.8</td>
<td>0.47</td>
</tr>
<tr>
<td>FIA</td>
<td>10.8</td>
<td>0.24</td>
</tr>
<tr>
<td>HPLC</td>
<td>9.0</td>
<td>0.22</td>
</tr>
</tbody>
</table>

^n CV, coefficient of variation.
TOBRAMYCIN ASSAYS

FIG. 3. Regression line analysis with the MA as the reference method. Interrupted lines indicate 20% limits of error. Each point represents one serum sample. Samples containing other antibiotics are indicated with ×.

were tested at the same time as well as to a decrease in set-up time for one assay when tested among a batch of several samples.

With the HPLC the effect is less remarkable. Although also for the HPLC one standard curve can be used when more samples are tested at the same time, each additional sample requires a 20-min retention time.

The experience necessary to perform the different assays is given in Table 4. The several steps necessary in the RIA assays make the procedure complicated and prone to error. Especially, decanting of the tubes and blotting of the rim have to be done carefully. The time required to perform each assay depends on the experience of the technicians. In consequence, in our bacteriological laboratory the MA was the easiest method to perform and therefore the least time consuming. The FIA, RIA, and the HPLC followed the MA in special training and experience of the technicians to perform the assays.

The essential apparatus necessary to perform the different assays was varied: a zone reader (or a ruler), a fluorometer, a gamma counter, and a liquid chromatograph for the MA, FIA, RIA, and HPLC, respectively. Depending on the set-up of the laboratory, the above-mentioned equipment could be already available and used for other purposes (21, 24).

In this regard it is important to note that with a daily workload of 20 patient samples the HPLC cannot be used for other purposes.

In making a decision on which method a laboratory should choose, not only are the characteristics of each method important, but also (even more) the number of sera tested daily, the set-up of the laboratory, and the use of the
equipment already available are important factors. For a routine bacteriological laboratory the MA should be a suitable method. For laboratories familiar with fluorometry or with RIA the FIA or the RIA is preferable. The accuracy of the HPLC argues in favor of the use of the HPLC for research purposes, especially for pharmacokinetic studies.

Considering the advantages and disadvantages of the different methods we conclude that the MA is the easiest method to perform. The assay does not require specialized equipment or highly qualified technicians. One of the disadvantages of this method, interference with other antibiotics, can be eliminated in some cases by using a suitable test microorganism or by adding an enzyme that destroys the second antibiotic. The method is also very suitable for emergency
TABLE 4. Cost-effectiveness of different tobramycin assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Set-up time (min)</th>
<th>Delay time (min)</th>
<th>Costs ($)</th>
<th>Detection limit (g/ml)</th>
<th>Experience required</th>
<th>Necessary equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st</td>
<td>2nd</td>
<td></td>
<td>Kit (100 assays)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>60</td>
<td>60</td>
<td>2</td>
<td>0.70</td>
<td>0.8</td>
<td>Zone reader</td>
</tr>
<tr>
<td>FIA</td>
<td>50</td>
<td>50</td>
<td>0.70</td>
<td></td>
<td>++</td>
<td>Fluorimeter</td>
</tr>
<tr>
<td>RIA-DPC</td>
<td>60</td>
<td>60</td>
<td>1.0</td>
<td>2.50</td>
<td>++</td>
<td>Gamma counter</td>
</tr>
<tr>
<td>RIA-NMS</td>
<td>90</td>
<td>90</td>
<td>2.50</td>
<td></td>
<td>++</td>
<td>Gamma counter</td>
</tr>
<tr>
<td>HPLC</td>
<td>30</td>
<td>450</td>
<td>12</td>
<td>4.10</td>
<td>++</td>
<td>Liquid chromatograph</td>
</tr>
</tbody>
</table>

* Number of samples.
*b Price includes labor cost at $10.00/h.
*c If one sample would be tested in a batch of 20.

use as the agar plate and the standards can be stored in the refrigerator. At the time of obtaining the sample only the plate has to be inoculated; wells have to be made and filled with the standards and the patient sera. The FIA, RIA, and HPLC require more time and expertise in setting up. However, when on occasion the delay time of the MA causes difficulties, the FIA should be a reasonable alternative among the assays we tested.

LITERATURE CITED