Heparin Interferes with the Radioenzymatic and Homogeneous Enzyme Immunoassays for Aminoglycosides

Donald J. Krogstad, George G. Granich, Patrick R. Murray, Michael A. Pfaller, and Roland Valdes

Heparin interferes with measurement of aminoglycosides in serum by biological, radioenzymatic, and homogeneous enzyme immunoassay techniques, but not with radioimmunoassay. At concentrations ≥10^5 and ≥3 × 10^6 USP units/L, respectively, it interferes with the radioenzymatic assay by inhibiting the gentamicin 3-acetyltransferase (EC 2.3.1.60) and kanamycin 6'-acetyltransferase (EC 2.3.1.55) enzymes used in the assay. It interferes with the homogeneous enzyme immunoassays for gentamicin and tobramycin (at concentrations ≥10^5 and ≥10^4 USP units/L, respectively), but not with the commercially available homogeneous enzyme immunoassays for other drugs. Heparin interference with the homogeneous enzyme immunoassays for aminoglycosides requires both the heparin polymer and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) bound to a cationic aminoglycoside. This interference can be reproduced with dextran sulfate (but not dextran), and does not occur with free enzyme (glucose-6-phosphate dehydrogenase) alone. We describe how heparin interferes with these two assays and demonstrate interference at heparin concentrations that may be present in intravenous infusions or in seriously underfilled blood-collection tubes.

Additional Keyphrases: analytical error • drug assays • gentamicin • tobramycin • amikacin • radioimmunoassay • side effects of anticoagulants

Aminoglycoside antibiotics are widely used. Their measurement in serum is clinically important because they have narrow toxic/therapeutic ratios, and because optimal concentrations in serum are difficult to achieve by use of nomograms alone (1). In addition to bioassays, which are slow and nonspecific, at least six other techniques are available for routine measurement of serum aminoglycoside concentrations (2), their major advantages over bioassay being their speed (minutes vs 1–2 h), sensitivity (<1 mg/L), and specificity (no false-positive results from other antibiotics the patient is receiving).

We find that high concentrations of heparin interfere with the available commercial radioenzymatic (REA)1 and homogeneous enzyme immunoassays for aminoglycosides, and we have examined the mechanisms of these interferences. The possibility of heparin interference was first suggested to us when we found (by REA) inordinately low aminoglycoside concentrations in the serum of a child who was receiving a heparin infusion.

Materials and Methods

Chemicals and antibiotics. Compounds tested for their ability to interfere with the measurement of aminoglycosides in serum included sodium heparin (Organon, Inc., West Orange, NJ 07092), trisodium citrate, ammonium and potassium oxalate, EDTA, dextran, and dextan sulfate (all from Sigma Chemical Co., St. Louis, MO 63178).

Aminoglycosides used were gentamicin complex (Scherer-Plough Corp., Bloomfield, NJ 07003), tobramycin (Eli Lilly Co., Indianapolis, IN 46206), and amikacin (Bristol Laboratories, Syracuse, NY 13201). Radiolabeled [14C]gentamicin was kindly provided by Dr. J. Allan Waitz (Scherer-Plough Corp.).

Aminoglycoside bioassays. Bioassays were performed in trypsinase soy agar (Difco Laboratories, Detroit, MI 48232), using the agar well diffusion method and Bacillus globigii strain described by Winters et al. (3).

REA for aminoglycosides. We used Gentamicin REA and Tobram REA kits (P-L Biochemicals, Milwaukee, WI 53205) with two different enzymes: gentamicin 3-acetyltransferase (GAT; EC 2.3.1.60) for gentamicin, and kanamycin 6' -acetyltransferase (KAT; EC 2.3.1.55) for tobramycin and amikacin. Each reaction mixture contained 10 μL of serum, 25 μL of buffer (0.1 mol/L Tris, pH 7.5), 2.5 μL of [1-14C]acetyl Co-A (10 mCi/L, with specific activity of 67 Ci/mmol; New England Nuclear, Boston, MA 02118), and 2.5 μL of enzyme (GAT or KAT, 20–25 U/L) in a total volume of 40 μL. After incubation for 20 min at 37 °C, 25–μL samples of the reaction mixture were pipetted onto phosphocellulose filter paper (Whatman P-81 paper; Whatman, Inc., Clifton, NJ 07014), washed twice in glass-distilled water to remove any [14C]acetyl Co-A not bound to the aminoglycosides, and dried briefly. Radioactivity was counted with a liquid scintillation counter (LS-8000; Beckman Instruments, Inc., Fullerton, CA 92634) after addition of 0.5 mL of NH₄OH (1.4 mol/L) and 8 mL of Bray’s solution (New England Nuclear). The negatively charged filter paper adsorbed the positively charged aminoglycosides and the [14C]acetyl could be bound to them. Thus, the number of counts per minute (cpm) was directly proportional to the amount of aminoglycoside in the serum specimen (4, 5).

Mechanism of heparin interference with the REA. We examined the effect of heparin on the adsorption of gentamicin by the phosphocellulose filter paper, using [14C]gentamicin. In these experiments we mixed 20 mg/L of [14C]gentamicin in assay buffer with an equal volume of serum, with and without 20 × 10⁶ USP units of heparin per liter. Thus, each specimen contained, per liter, 10 mg of [14C]gentamicin and either zero or 10 × 10⁶ USP units of heparin. We pipetted 25–μL aliquots of this mixture onto phosphocellulose filter paper, washed and dried it, and counted the radioactivity.

To define the effect of heparin on enzymatic acetylation, we diluted acetyltransferase enzymes in Tris assay buffer (0.1 mol/L, pH 7.5) to slow the rate of the reaction (50-fold for GAT, 10-fold for KAT). Two controls were used in each ex-

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1 Nonstandard abbreviations: REA, radioenzymatic assay; GAT, gentamicin 3-acetyltransferase (EC 2.3.1.60); KAT, kanamycin 6'-acetyltransferase (EC 2.3.1.55); G6PD, glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

Received March 11, 1982; accepted April 13, 1982.
experiment: (a) one kept in an ice-water bath (the reaction does not proceed at 0 °C) and (b) another to which buffer (instead of heparin) was added at 37 °C to control for the effect of dilution.

Homogeneous enzyme immunoassays. These assays were performed with “EMIT-AMD” kits provided by Syva Co., Palo Alto, CA 94303. Serum specimens were twice diluted sixfold in Tris buffer (55 mmol/L, pH 8.0) to produce a 36-fold working dilution. We mixed 300 μL of this working dilution in sequence with 50 μL of reagent A (containing glucose 6-phosphate, NAD+, and sheep antibody to the aminoglycoside), an additional 250 μL of Tris buffer, 50 μL of reagent B (G6PD bound to an aminoglycoside), and another 250 μL of Tris buffer. An aliquot (~500 μL) of this reaction mixture was then aspirated into the flow cell of a spectrophotometer maintained at 30 °C (Stasar II; Gilford Instrument Laboratories, Inc., Oberlin, OH 44074). The change in absorbance at 340 nm during 30 s of incubation was related to aminoglycoside content through a probit transformation performed with the Syva CP-5000 EMIT Clinical Processor, with use of 0, 1, 2, 4, 8, and 16 mg/L aminoglycoside standards (6, 7).

Mechanism of heparin interference with the homogeneous enzyme immunoassay for aminoglycosides. To determine whether heparin interfered with this assay by interacting with the aminoglycosides bound to G6PD, we compared the effect of heparin on G6PD bound to gentamicin or tobramycin with the effect of heparin on the activity of free G6PD. In these experiments, we used a modified reagent A without aminoglycoside antibody (i.e., containing only glucose 6-phosphate and NAD+), and measured changes in absorbance at 340 nm with the two-point mode on the EMIT Clinical Processor. The other homogeneous enzyme immunoassays examined for the effect of heparin were tested similarly, with reagents provided by the manufacturer.

RIA for aminoglycosides. The RIA kits we used were “[125I] Gentamicin and Tobramycin RIA” kits manufactured by Diagnostic Products, Los Angeles, CA 90064. For these assays, specimens were first diluted 200-fold in Tris buffer (50 mmol/L, pH 7.2). Aliquots (100 μL) of these diluted specimens were mixed with equal volumes of goat anti-rabbit gammaglobulin, and [125I]-labeled rabbit antibody to gentamicin or tobramycin, incubated for 5 min at 25 °C, and 2 mL of cold 60 g/L polyethylene glycol was added to the reaction mixture, which was then centrifuged (1200 × g, 4 °C, 15–20 min). The radioactivity of the precipitate was counted for 1 min with a gamma counter. The aminoglycoside content of the specimens tested was determined from a standard curve that was calculated by weighted logit-log analysis of 0, 1, 2, 4, 8, and 16 mg/L gentamicin and tobramycin standards (6, 9).

Statistical testing. Differences between groups of data were tested by the Student’s t-test (10); p values > 0.05 were considered not significant.

Results and Discussion
Effect of heparin on the bioassay. As noted previously (11, 12), heparin concentrations ≥10^6 USP units/L interfered with measurement of gentamicin, amikacin, and tobramycin by the agar well-diffusion bioassay (Figure 1).

Effect of heparin on the RIA. Heparin interfered with measurement of gentamicin by use of GAT and KAT (at concentrations ≥10^6 USP units/L), and with measurement of both tobramycin and amikacin by KAT (at concentrations ≥3 × 10^6 USP units/L) (Figure 2). This effect increased with increasing concentrations of heparin (up to 10^7 USP units/L). From the results for gentamicin (which is acetylated by both enzymes), we conclude that heparin had a greater effect on aminoglycoside determinations performed with GAT.

Effects of other anticoagulants on the RIA. Testing of
other anions used as anticoagulants for their effect on the REA was limited by their solubility in serum (≤10 g/L for ammonium oxalate, ≤100 g/L for potassium oxalate and citrate). At the maximal concentrations attainable in serum, neither citrate nor oxalate interfered with the REA. EDTA, which is more soluble in serum, interfered with the REA at concentrations ≥30 g/L (Figure 3). However, such high EDTA concentrations are unlikely unless the blood-collection tube is seriously underfilled (Table 1). Thus, EDTA can interfere with the REA, but this effect should not cause difficulty in the clinical laboratory.

Mechanism of heparin interference with the REA. Because measurement of aminoglycosides by REA depends on their adsorption to a negatively-charged phosphocellulose filter paper (4, 5), heparin could interfere with the REA by decreasing the adsorption of aminoglycosides to the filter paper. However, using [14C]gentamicin, we found that heparin (106 USP units/L), had no effect at concentrations that interfered with the REA (1175 ± 50 cpm with heparin, 1132 ± 42 cpm without).

In contrast, addition of heparin to the reaction mixture at different times during incubation at 37 °C stopped acetylation as effectively as transferring the tubes to the ice-water bath (Figure 4). The addition of buffer alone (in a volume equal to that of the heparin solution) had no effect on the REA (data not shown). We conclude that heparin interferes with the REA by inhibiting the acetylation of aminoglycosides by GAT and KAT.

Effect of heparin on the homogeneous enzyme immunoassay. With this technique, heparin concentrations ≥106 USP units/L interfered with measurement of gentamicin, and concentrations ≥104 USP units/L interfered with the measurement of tobramycin (Figure 5), as previously suggested by Nilsson et al. (13).

Heparin at a concentration of 106 USP units/L did not interfere with the other homogeneous enzyme immunoassays tested. Kits examined included those for quinidine, procainamide, lidocaine, phenytoin, phenobarbital, theophylline, carbamazepine, primidone, and ethosuximide (data not

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**Table 1. Effects of Anticoagulants in Blood-Collection Tubes on Aminoglycoside Assay Results**

<table>
<thead>
<tr>
<th>Anticoagulant</th>
<th>Concentration (g/L)</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>4-4.2</td>
<td>Does not interfere, even at limits of solubility (100 g/L)</td>
</tr>
<tr>
<td>EDTA</td>
<td>≥1.5</td>
<td>≥30 g/L (REA)</td>
</tr>
<tr>
<td>Heparin</td>
<td>1.4-2.8 × 10^4 usp units/L</td>
<td>≥10^6 USP units/L (bioassay, gentamicin REA, tobramycin and gentamicin homogeneous enzyme immunoassays); ≥3 × 10^4 usp units/L (tobramycin and amikacin REA)</td>
</tr>
<tr>
<td>Oxalate</td>
<td>1.4-2.9</td>
<td>Does not interfere, even at limits of solubility (10 g/L for ammonium oxalate, 100 g/L for potassium oxalate)</td>
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**Fig. 3.** Effect of EDTA on apparent concentrations of gentamicin (■) and tobramycin (□) as measured by the REA.

**Fig. 4.** Effect of transfer to an ice-water bath at 0 °C and addition of heparin (10^6 USP units/L) on acetylation of gentamicin by GAT (●, ○, left) and tobramycin by KAT (■, □, right).
Mechanism of heparin interference with the homogeneous enzyme immunoassay for aminoglycosides. Heparin reduced the activity of G6PD bound to gentamicin or tobramycin (at heparin concentrations ≥10^6 and ≥10^4 USP units/L, respectively), but had no effect on the activity of free G6PD (Figure 6). These results indicate that heparin interferes with this assay by inhibiting the activity of G6PD bound to aminoglycosides.

To define the mechanism of this effect, we performed additional experiments, which revealed that dextran sulfate also inhibited the activity of G6PD bound to tobramycin or gentamicin (Table 2). Because dextran (an otherwise similar, but neutral, molecule) had no effect, this effect may be due to interaction between negatively charged polyanions (heparin, dextran sulfate) and the positively charged aminoglycosides bound to G6PD, an interpretation supported by the observation that neither heparin nor dextran sulfate decreased the activity of free G6PD.

**Table 2. Effect of Heparin, Dextran Sulfate, and Dextran on the Activity of G6PD Bound to Aminoglycosides**

<table>
<thead>
<tr>
<th></th>
<th>G6PD–gentamicin</th>
<th>G6PD–tobramycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>660</td>
<td>776</td>
</tr>
<tr>
<td>Heparin, 10^6 USP units/L</td>
<td>514</td>
<td>483</td>
</tr>
<tr>
<td>Dextran sulfate, 1 g/L</td>
<td>501</td>
<td>391</td>
</tr>
<tr>
<td>Dextran, 1 g/L</td>
<td>658</td>
<td>809</td>
</tr>
</tbody>
</table>

*Values are expressed as the average rate of change in absorbance (ΔA × 10^5) at 340 nm during a 30-s incubation at 30°C with 10 mg of either gentamicin or tobramycin per liter in serum (± heparin, dextran sulfate, or dextran).*

**Effect of heparin on the RIA.** At concentrations as great as 10^7 USP units/L, heparin had no effect on results of RIA for gentamicin or tobramycin, in accord with other investigators (14). Results for gentamicin and tobramycin samples with and without heparin were 10.8 vs 10.4 and 11.1 vs 11.4 mg/L, respectively.

**Clinical implications.** From these data, we conclude that the various types of aminoglycoside assay examined all give valid results when the concentrations of heparin do not exceed those usually present in serum with anticoagulation treatment (1 to 3 × 10^4 USP units/L). However, if the specimen to be examined for aminoglycosides is drawn through an intravenous line containing heparin or is placed in an underfilled heparin tube (Table 1), the resulting heparin concentration in the serum sample (≥10^6 USP units/L) may interfere with measurement of all three aminoglycosides by the bioassay, with homogeneous enzyme immunoassay measurements of tobramycin and gentamicin, or with RIA measurements of gentamicin (by GAT). Heparin concentrations great enough to interfere with RIA measurements of tobramycin or amikacin (≥3 × 10^6 USP units/L) can be produced only with high-dose heparin infusions, which are rarely used (even in children) because small changes in the infusion rate can produce excessive anticoagulation and potentially life-threatening hemorrhage. As noted above, heparin has no effect on the RIA for aminoglycosides.

Our studies of nine additional homogeneous enzyme immunoassay assay kits indicate that it is unlikely that clinically achievable heparin concentrations will interfere with the homogeneous enzyme immunoassays in current use for drugs other than aminoglycosides. However, heparin will probably interfere with the measurement of amikacin by this technique (when that assay becomes available).

**Note added in proof:** Heparin concentrations ≥3 × 10^6 USP units/L interfere with the measurement of amikacin by the homogeneous enzyme immunoassay.

We thank Jerry L. Shenepe for originally raising the question of heparin interference with the RIA, J. Allen Waltz for kindly providing the [14C]gentamicin, the Syva Co. and P-L Biochemicals, Inc., for kits to carry out these studies, and Gregory S. King and Jack H. Ledson for their helpful suggestions and comments. This work was supported in part by training grant 1-T32-AI 07172 from the National Institute of Allergy and Infectious Diseases, NIH.

**References**


CLIN. CHEM 28/7, 1521–1524 (1982)

Single-Stage Automated Assay for Heparin

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We have developed a single-stage assay for heparin, using reagents modified from the two-stage Dade Protopath heparin synthetic substrate assay. The single-stage assay involves simultaneous mixing of a plasma sample, an antithrombin III source, α-thrombin, and the α-thrombin fluorogenic substrate. The synthetic substrate, antithrombin III, and heparin–antithrombin III complex compete for the α-thrombin active site. The α-thrombin is inactivated by the heparin–antithrombin complex while substrate is being hydrolyzed, so that total product formation decreases with heparin concentration. Day-to-day CV was 9.3% at a heparin concentration of 246 USP units/L. Comparison of results from the single-stage heparin assay with those from a two-stage esterolytic assay yielded the linear regression equation: esterolytic = 0.834 (single-stage) − 7 USP units/L (r = 0.94, n = 47). Bilirubin interfered with the single-stage assay, resulting in an apparent increase in sample heparin concentration. The single-stage heparin assay can be automated for centrifugal analyzers capable of double-reagent addition and fluorometric detection, substantially decreasing reagent requirements and therefore costs.

Additional Keyphrases: centrifugal analysis - fluorometry

Commercially available heparin is a heterogeneous sulfated mucopolysaccharide anticoagulant extracted from bovine lung or porcine intestinal mucosa. This heterogeneity means that heparin is not accurately assayable by immunochemical methods, but several assays are based on heparin's acceleration of the inactivation of α-thrombin by antithrombin III (1–5). In two-stage heparin assays, plasma, an antithrombin III source, and α-thrombin are mixed in the first stage, and then briefly incubated; the resulting inactivation of α-thrombin by the antithrombin III is in direct proportion to heparin concentration. In the second stage, the residual α-thrombin activity is measured with an appropriate substrate. Two-stage assays can be automated for use with some discrete analyzers (5), but are not conveniently adapted to current centrifugal analyzers.

Here we report a single-stage heparin assay in which all reagents are mixed simultaneously.

Materials and Methods

Apparatus. The assays were performed with the fluorescence-equipped Multistat III fluorometer purchased from Instrumentation Laboratory, Lexington, MA 02173.

Chemicals and materials. Sodium chloride, Tris HCl, tetrasodium EDTA, and sodium citrate were obtained from Fisher Scientific, Itasca, IL 60143. Heparin (1000 USP units/ml) was a product of Abbott Laboratories, North Chicago, IL 60064. The Dade Protopath heparin synthetic substrate assay was purchased from Scientific Products, McGaw Park, IL 60085. 4-Methylumbelliferylone was obtained from Instrumentation Laboratory. Bilirubin was obtained from Sigma Chemical Co., St. Louis, MO 63178. Intralipid, a 10% intravenous fat emulsion, was obtained from Cutter Laboratories, Berkeley, CA 94710. Human α-thrombin (specific activity = 2500 United States units/mg; absorptivity = 19.5) (6) was purified as described by Owen (7). The antithrombin III solution was platelet-poor citrated human plasma diluted 10-fold with distilled water. The platelet-poor plasma was prepared by collecting nine volumes of whole blood into one volume of a 38 g/L sodium citrate solution, centrifuging (15 min, 1200 × g), then recentrifuging the plasma supernate (30 min, 1200 × g). Calibrators were prepared in pooled, citrated

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