Improving the Sensitivity of Gentamicin Enzyme Immunoassay

To the Editor:

Monitoring of inter-dose concentrations of gentamicin in plasma presents an additional problem when the EMIT® assay (Syva, Palo Alto, CA 94303) is used. This enzyme immunoassay is designed to measure plasma concentrations of 1.0 to 18.0 mg/L, with precision being best between 2.0 and 10.0 mg/L. The desired minimum concentration of gentamicin is <2.0 mg/L, which means that gentamicin concentrations can be routinely below the limits of the assay. This limitation is most important when the elimination half-life and pharmacokinetic dosing predictions are calculated from assay data. We sought to determine whether the sensitivity of the EMIT assay for gentamicin could be increased by a dilutional modification of the standard procedure and compared the performance of this modified procedure with the unmodified procedure and with radioimmunoassay (RIA; done with a kit from Nuclear Medical Systems, Inc., Newport Beach, CA 92663) at gentamicin concentrations of <1.0 mg/L.

We prepared samples by adding gentamicin for injection (Garamycin, Schering) to pooled drug-free human plasma to give concentrations of 0.13, 0.32, 0.62, 0.81, 0.95, and 1.10 mg/L. These solutions were stored at −20 °C until needed. Each of the three assays was done in quadruplicate on each of the seven sample concentrations. The assays were done during a single work day, and the reagents were in each case those provided by the manufacturers. After reconstitution, the EMIT reagents were not used for 24 h, to assure complete solution.

The RIA gentamicin assay was performed as described in the manufacturer’s literature, except that, because the lower limit of the RIA is 1.0 mg/L, the 4 mg/L standard was added to all of the samples being assayed. This placed the sample concentrations within the normal range of concentrations measured by the RIA. The stated within-assay precision (CV) for this RIA is 2.4%.

The unmodified EMIT assay was performed as described in the manufacturer’s literature, as was the modified assay except that the second sixfold dilution was not made. Thus in the modified procedure we used a sixfold dilution of plasma rather than the prescribed 36-fold dilution, and the subsequent resulting concentration was then divided by six.

We plotted the RIA curve on logit-log graph paper. The standard deviation from the regression line was 1.49. The EMIT data were analyzed with a Syva CP-5000 Clinical Processor. The standard deviation from the regression line was 0.92.

Fig. 1. Predicted vs assayed gentamicin concentration by the RIA (A), the unmodified EMIT (B), and the modified EMIT (C) assays. Each point represents the mean of four determinations ± SD.

RIA and the unmodified EMIT were not significantly different from one another. The sensitivity of the EMIT and RIA assays can be increased to include concentrations <1.0 mg/L. This increase in sensitivity was at the expense of a decrease in precision for the RIA and standard EMIT methods. Both Figure 1 and Table 1 demonstrate the large degree of variation with these two assay techniques in this concentration range. However, the precision of the modified EMIT technique did not change over the range tested. The CV was <10% only at concentrations >0.95 mg/L for the RIA and 0.81 mg/L for the unmodified EMIT, but was <10% over the entire range for the modified EMIT. The accuracy of both EMIT techniques was within 10%.

Using theoretical patient data, we find that dosing-prediction errors could exceed 10% if gentamicin results <1.0 mg/L obtained with the RIA or unmodified EMIT were used in the pharmacokinetic calculations. Therefore, we recommend that all gentamicin concentrations of <1.0 mg/L obtained with the unmodified EMIT technique be repeated with use of the modified EMIT technique when pharmacokinetic dosing profiles will be generated from the assay values obtained.

References

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Determination of Thioethers in Urine

To the Editor:

Certain chemically unreactive compounds induce disease because they are converted in the body to reactive derivatives. Covalent binding of these toxic metabolites is counteracted by nucleophilic sulfhydryl compounds such as glutathione (1). The products formed through combination with reduced glutathione are excreted in urine as mercapturic acids or as cysteine derivatives, pre-mercapturic acids, or other thioethers (e.g., thioglycollic acid in the case of vinyl chloride). The presence of these acids in urine may be considered indicative of the formation of reactive metabolites in the organism. Seutt-
Filtered urine at pH 8.0
↓
AG 50-X8 column
↓
NaBH₄ reduction
↓
Affigel 501 column
↓
Hydrolysis
↓
NaBH₄ reduction
↓
Ellman reaction (A)

**Fig. 1. Reaction scheme for thioether determination**

The Ellman reaction was normally carried out under the following conditions: 2 mL of sample (corresponding to 0.1 mL of urine) plus 3 mL of phosphate buffer (250 mmol/L, pH 8.0) plus either (a) 0.1 mL of DTNB solution or (b) 0.1 mL of doubly distilled water. Absorbance (A) is measured at 412 nm. A₀ - Aₘ = absorbance of sulfhydryl groups; A₀ - Aₙ = absorbance of mercapturic acid.

Berlage et al. (2) proposed increased excretion of thioethers in urine as an indication of hazardous environmental chemicals. In agreement with this, Vainio et al. (3) and Van Doorn et al. (4) found greater excretion of thioethers in people exposed to environmental chemicals. Summer et al. (5) observed a high basal thioether excretion in humans.

All the methods described for application to human urine are based on determination by the method of Ellman (6) of the SH groups released by alkaline hydrolysis. The application of this method to urine by Seutter-Berlage et al. (2) was only slightly modified by the introduction of reducing agents such as ascorbic acid into crude urine (3) and of sodium borohydride reduction (4) before hydrolysis to correct for free SH groups and disulfides in the urine. This was criticized by Pentz (7), who found large intra-individual variations in estimated thioether excretion by humans, and found no significant difference between urinary thioether excretion by untreated rats and those treated with many toxic compounds. Pentz (7) therefore doubted the value of Ellman’s reagent as an indicator of exposure to xenobiotics.

Our experience is very much in agreement with Pentz’ result. We found it impossible to estimate thiol compounds added to human urine by direct application of the Ellman reagent. We therefore wanted to develop a method that would be highly specific for the estimation of urinary thioethers. The method, based on the Ellman reagent test after alkaline hydrolysis of thiocysteine and cysteine, differs from the original in that: (a) free SH and SS groups in the urine are removed before hydrolysis by reduction and affinity chromatography, (b) cysteine and yellow urinary pigments are partly removed by treatment with a cation-exchange resin (AG50-X8; Bio-Rad Lab., Milan, Italy); the residual pale yellow color that interferes at 412 nm is estimated and the absorbance subtracted from that obtained with the Ellman reagent.

Mercapturic acids are hydrolyzed by sodium hydroxide to thiols and thioethers, according to the conditions described by Parke and Williams (8). Because during hydrolysis the sulfhydryl groups set free are oxidized and consequently their recovery is very low, we improved the recovery as follows: Hydrolyze 5 mL of urine for 40 min at 105 °C with 5 mL of 5 mol/L sodium hydroxide. Transfer the solution quickly to an ice-water bath, then add 400 mg of solid sodium borohydride, shake well, keep the solution in the ice-water bath, adjust to pH 6.0 with hydrochloric acid (10 mol/L), dilute to 25 mL with sodium phosphate buffer (250 mol/L, pH 8.0) and clarify by centrifugation at 5000 x g for 15 min. We used the Ellman reagent (5,5'-dithiobis-2-nitrobenzoic acid) because it reacts both with thiols and thioethers in the proportion of 2 mol to 1 mol of 5,5'-dithiobis-2-nitrobenzoic acid. The pH optimum for the reaction is between 7 and 8. The molar absorptivity is 13 600 (±100 SEM) L mol⁻¹ cm⁻¹ for cysteine and for thiophenol (mean of 10 determinations, each at five different concentrations of both compounds).

To eliminate urinary substances that interfere with the determination of sulfhydryl groups with the Ellman reagent, we treated specimens as follows: 10 mL of urine was adjusted to pH 8.0, filtered, and passed through a column (Pasteur pipet) containing 1 g of AG50-X8 in acid form (200–400 mesh), which had been equilibrated in water. The eluate was collected, then 10 mL of 25 g/L ammonia solution was passed through the column and the eluate was combined with the first one. Ten milliliters of the combined eluates was treated in ice with sodium borohydride, ethanol, and HCl, and then diluted to 20 mL with the sodium phosphate buffer. After centrifugation (6000 x g, 15 min), two 5-mL samples of this solution were passed through two columns (Pasteur pipets) containing 0.5 g of “Affigel 501” (Bio-Rad, Milan, Italy), which had been equilibrated in the sodium phosphate buffer. One eluate was used for the Ellman reaction, the other for the hydrolysis.

The scheme of the full procedure is outlined in Figure 1. We evaluated analytical recovery of the hydrolysis procedure by using L-naphthylmercapturic acid at 30–90 mg/L; it ranged from 78 to 82%. The mean recovery from the AG50-X8 elution was 99% (n = 5); from Affigel 501 it was 90% (n = 5). The mean yield for the total procedure, tested with a solution of 50 mg of naphthylmercapturic acid per liter of the sodium phosphate buffer, was 80% (n = 5); for human urine the mean yield was 88% (n = 5).

In the daily urine of 15 apparently healthy subjects the mean thioether content was 0.45 (SEM 0.05) mmol/L in terms of SH groups. This group contained both smokers and non-smokers. In further experiments, five smokers were found to excrete a statistically significantly (Student’s t-test) higher amount of thioethers than five non-smokers: the SH groups were 0.42 (SEM 0.05) mmol/L vs 0.23 (SEM 0.03) mmol/L.

This method, although quite laborious, gives a reasonable yield and accuracy, and is not difficult to execute. It reveals the presence of mercapturic acids or other thioethers in human urine in apparently significant amounts. The high variation of thioether excretion in human urine was probably due to the fact that the diet and living conditions of the subjects were not controlled.

**References**

4. Van Doorn, R., Boe, P. R. Lajidekkers, C. M., et al., Thioether concentration and mutagenicity of urine free from cigarette smok-
Table 1. Effect of Preservatives on Recovery of 5-HIAA from a 50 μmol/L Aqueous Solution

<table>
<thead>
<tr>
<th>Preservatives added</th>
<th>Normal assay procedure</th>
<th>Omitting extraction step a</th>
<th>Total extracted into ether b</th>
<th>Residue in ether after reextraction c</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>40.1</td>
<td>13.8</td>
<td>39.0</td>
<td>40.1</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>50.0</td>
<td>48.7</td>
<td>50.4</td>
<td>47.9</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>43.2</td>
<td>42.2</td>
<td>39.8</td>
<td>43.6</td>
</tr>
<tr>
<td>Boric acid</td>
<td>3.1</td>
<td>28.9</td>
<td>0.4</td>
<td>3.5</td>
</tr>
</tbody>
</table>

a Solid 5-HIAA and the appropriate preservative were dissolved in phosphate buffer (0.1 mol/L) and 2 ml aliquots used for the nitrosanaphthol color reaction. b After the initial extraction, 20 ml of the ether layer was evaporated, the residue was dissolved in 4 ml of phosphate buffer (0.1 mol/L), and 2 ml aliquots were used for the color reaction. c Same as b but 20 ml of the ether was taken after reextraction with phosphate buffer (0.1 mol/L).

Interference by Acetic Acid In Urinary 5-Hydroxyindoleacetic Acid Determination

To the Editor:

Slaunwhite et al. (1) have reported that the use of acetic acid as a preservative for urine collections results in an apparent interference in Goldenberg's method (2) for 5-hydroxyindoleacetic acid (5-HIAA) estimation. As they presented no data, we investigated the mechanism of this interference.

Portions of a pool of fresh urine containing (a) no preservative, (b) HCl (50 mmol/L), (c) boric acid (3 mmol/L), or (d) acetic acid (0.33 mol/L) were assayed immediately for 5-HIAA by Goldenberg's method and the following results obtained: 76, 76, 81, and 28 μmol/L, respectively. After storage for eight weeks at 4 °C, the 5-HIAA concentrations in the same specimens were, respectively, 78, 75, 82, and 32 μmol/L. This suggests that the lower results observed in the presence of acetic acid are due to direct interference in the assay and are not a consequence of reduced stability of 5-HIAA in the specimens.

In Goldenberg's modification (2) of Udenfriend's original method (3), 5-HIAA is extracted from acidified urine saturated with NaCl into diethylether, then back-extracted into a phosphate buffer (0.1 mol/L, pH 7.0) before the nitrosanaphthol color reaction is carried out. To investigate the mechanism of this interference, we measured the recovery of 5-HIAA from an aqueous standard solution at the various stages of the assay (Table 1). Omission of the extraction procedure by directly adding 5-HIAA and preservative to the phosphate buffer showed that acetic acid does not interfere with the color reaction. Measurement of the 5-HIAA in the initial ether extract showed that there was no adverse effect on the extraction of 5-HIAA from the urine; however, after the second extraction stage, sufficient 5-HIAA remained in the ether layer to account for the observed interference by acetic acid in the complete assay system.

The pH of the phosphate buffer (initially 7.0) after extraction of the ether fraction was reduced to 5.2 when acetic acid was added to the original sample, but unchanged when HCl or boric acid were added. Goldenberg (2) has shown that re-extraction of 5-HIAA is strongly pH dependent, the efficiency at pH 5.2 being only 30% of that at pH 7.0. Therefore, the interference by acetic acid most likely is caused by its extraction from the sample into the ether layer; its back-extraction into the phosphate buffer and the consequent reduction of the buffer pH from 7.0 to 5.2 decreases the efficiency of the extraction of 5-HIAA from the ether fraction.

Figure 1 shows that increasing the phosphate concentration considerably improves the poor recovery of 5-HIAA observed in the presence of acetic acid. However, this is at the expense of decreasing sensitivity because, as the phosphate concentration is increased, the pH (2.0 at 0.1 mol/L) and so affects the nitrosanaphthol color reaction, which is also pH sensitive. Figure 1 also shows that at the phosphate concentration first used by Udenfriend (0.5 mol/L) the interference by acetic acid is hardly significant although the sensitivity is low.

We can find no reference to the use of acetic acid in Udenfriend's paper (3); its use was apparently first introduced by Macfarlane et al. (4) and did not interfere with their method because no back-extraction was involved. As far as we are aware, no actual stability data on 5-HIAA have been published, although there are reports that 5-HIAA is easily oxidizable in alkaline solution (4-6) and so it is usually recommended that urine collected for this measurement should be maintained at an acid pH. Both HCl (3, 6) and boric acid (2) have been used, but Dalgleish (7) recommends the use of acetic acid to avoid the degradation of 5-HIAA by mineral acids. Because our stability data do not demonstrate any advantage in using acetic acid, we recommend that if Goldenberg's method is to be used for 5-HIAA estimation, then urines should be preserved by the addition of either HCl or boric acid.

References


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Figure 1. Effect of phosphate concentration in the neutral phosphate buffer on the re-extraction of 5-HIAA from the ether extract.

Absorbance readings obtained in the unmodified analysis of a 5-HIAA standard solution (50 μmol/L) containing no preservative (C) and acetic acid (B). Δ, absorbance reading obtained with a 50 μmol/L 5-HIAA standard solution containing acetic acid, when the ether layer after re-extraction with phosphate buffer was evaporated, dissolved in phosphate buffer (0.1 mol/L, pH 7.0), and treated for color reaction in the usual manner.