Table 1. Antiepileptic Drug Concentrations in a Drug-Supplemented Plasma Sample, Measured Six Months Apart

<table>
<thead>
<tr>
<th>Antiepileptic drug</th>
<th>Measured concn. mean (SE) mg/liter</th>
<th>5/7/77 (n = 6)</th>
<th>p*a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mephenytoin</td>
<td>11.0 (0.3)</td>
<td>11.4 (0.1)</td>
<td>.194</td>
</tr>
<tr>
<td>5-Ethyl-5-phenylhydantoin</td>
<td>21.1 (0.3)</td>
<td>21.9 (0.3)</td>
<td>.087</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>18.8 (0.1)</td>
<td>19.7 (0.5)</td>
<td>.094</td>
</tr>
<tr>
<td>Primidone</td>
<td>10.3 (0.3)</td>
<td>9.9 (1.0)</td>
<td>&gt;0.3</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>21.0 (0.3)</td>
<td>20.9 (0.1)</td>
<td>&gt;0.3</td>
</tr>
</tbody>
</table>

*a One way analysis of variance, null hypothesis of equal means.

°C for 48 h, and then filtered through glass wool to remove precipitated fi- brin.

Drugs were added to the control sample by evaporating a solution of the drugs in absolute ethanol in a 50-ml volumetric flask, then adding 50 ml of drug-free plasma to the residue and stirring for 4 h. Sixteen-milliliter aliquots of this sample were stored in 50-ml scintillation vials.

The control sample was assayed for antiepileptic drugs along with patients' sera from the Epilepsy Center Clinic on six occasions in October 1976; all determinations were made from the same 16-ml aliquot, which was stored at 4 °C between runs. A second 16-ml aliquot was stored at room temperature (20–25 °C), without exposure to light, for six months. In May 1977 this aliquot was returned to 4 °C, and then run along with patients' sera on six occasions during this month. Before each assay, the sample was stirred on a vortex-type mixer.

All measurements were done by the same technologist, using a gas–liquid chromatographic method (3). The following internal standards were used: 5-methyl-5-phenylhydantoin for mephenytoin; 5-ethyl-5-phenylhydantoin (the principal serum metabolite of mephenytoin) and 5-allyl-5-phenyl barbituric acid for phenobarbital and primidone; and 5-(4-methylphenyl)-5-phenylhydantoin for phenytoin.

Results before and after storage for each of the five antiepileptic drugs were compared by one-way analysis of variance on the CLINFO Prototype Data Management and Analysis System (4). Because only two means were compared in each test, use of one-way analysis of variance is equivalent to use of an unpaired Student's t-test.

After storage at room temperature for six months, a film of green bacterial or fungal growth was present on the surface of the control sample. Care was taken to mix the sample well before each assay.

Table 1 summarizes the results of this experiment. For each drug, mean concentrations before and after storage were not significantly different at the .05 level.

After storage, one major peak appeared in the chromatograms that had not been observed before storage. The peak has a retention time of 1.05 relative to the phenytoin internal standard, 5-(4-methylphenyl)-5-phenylhydantoin, and is well resolved from this internal standard (which is usually the final peak to elute in a given run). The identity of the compound represented by this peak is unknown.

Our results suggest that disagreements over the stability of these compounds in serum may be a result of the use of different assay methods. It was simply fortuitous that the major peak that arose during storage did not interfere with drug or internal standard peaks in our system. In other systems this peak could merge with the phenytoin internal standard peak, resulting in a lower estimate of phenytoin concentration. If, as seems likely, the unknown peak is a result of bacterial or fungal overgrowth, it would increase with time and thus phenytoin concentration would appear to decrease with time.

Two other variables that might affect drug stability in serum are the bacteria themselves and the pH. Pippenger et al. (5) compared the concentrations of phenytoin, phenobarbital, primidone, and ethosuximide in samples before and after mailing or storage at room temperature for up to 26 working days. After mailing or storage there was significant bacterial growth, but this did not result in significant changes in drug concentrations. Our results agree with these findings. The pH of the samples was not measured during this experiment. In future studies, we plan to examine the effect of sample pH on drug stability, and to investigate the reported instability of carbamazepine in aqueous solution (6).

We conclude that mephenytoin, 5-ethyl-5-phenylhydantoin, phenobarbital, primidone, and phenytoin in serum are stable when stored at room temperature (without exposure to light) for at least six months. For these compounds, shipment of plasma or serum samples for analysis to centrally located laboratories is feasible. The time delays inherent in such shipment evidently will not affect results because of drug breakdown, but problems may arise because of interferences secondary to bacterial or fungal growth in the samples.

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References


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γ-Glutamyltransferase Activity and Bilirubin Concentration

To the Editor:

Combes et al. (1) provided evidence that γ-glutamyltransferase (γGT, EC 2.3.2.2) activity in human serum decreases as bilirubin concentration increases. This seemed to be confirmed by the addition of bilirubin to nonicteric serum. In our laboratory we have observed jaundiced patients with unexpectedly low γGT activities in their serum. For example, in a patient with secondary carcinoma and very high γGT activity, the γGT activity declined dramatically as the patient's condition deteriorated and the bilirubin concentration became markedly elevated (Table 1). We decided, therefore, to investigate the effect of bilirubin on γGT activity in both sera and urines.

The method used to estimate γGT activity, both in serum and urine, was that of Beck and Chaudhuri (2), in which γ-glutamyl-p-nitroanilide is the substrate. The reactions were done at 37 °C, with an LKB 8600 Reaction Rate Analyser, the increase in absorbance...
Table 1. Bilirubin Concentration and γGT Activity in a Patient with Secondary Carcinoma

<table>
<thead>
<tr>
<th>Date</th>
<th>Bilirubin, μmol/l</th>
<th>γGT, U/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.8.76</td>
<td>8</td>
<td>564</td>
</tr>
<tr>
<td>1.17.77</td>
<td>8</td>
<td>564</td>
</tr>
<tr>
<td>3.3.77</td>
<td>10</td>
<td>1786</td>
</tr>
<tr>
<td>5.27.77</td>
<td>32</td>
<td>2800</td>
</tr>
<tr>
<td>8.4.77</td>
<td>120</td>
<td>1245</td>
</tr>
</tbody>
</table>

being measured at 410 nm. The bilirubin was from three sources: human bile, crystalline bilirubin (BDH Chemicals), and the standard used in our laboratory for bilirubin assay (bovine serum with added crystalline bilirubin). Bilirubin concentrations were assayed with a standard Technicon AutoAnalyzer II system. Crystalline bilirubin was initially dissolved in 0.1 mol/liter potassium hydroxide. The concentrations used were in the range 25–250 μmol of bilirubin per liter, dilutions being made in 0.23 mol/liter Tris buffer, pH 8.1. γGT activities of serum/bilirubin mixtures were corrected for γGT present in the bile and standard. Equal volumes of serum or urine and bilirubin were mixed and left at room temperature for 30 min before assay.

The results (Table 2) show that bilirubin does not inhibit γGT. With either bile or the standard as sources of bilirubin, γGT activity increased with increasing bilirubin concentration. A peculiar decrease in γGT activity was observed in all samples with bile at 50 μmol/liter bilirubin concentration. The reason for this is unknown.

Combes et al. incubated sera and bilirubin for 5 min before assay. During our investigations sera were incubated with the various concentrations of bilirubin for 0–30 min, no change in γGT activity being observed with time.

We considered whether the inhibition that Combes et al. found was related to the high pH (10.6) of the bilirubin dissolved in KOH. However, when the pH of the whole reaction mixture was measured with various concentrations of bilirubin no deviation from the expected pH (8.1) was found.

The discrepancies between the two sets of results are difficult to explain. There are differences in methodology, instrumentation, and temperature, but it seems unlikely that these would explain the anomalous results. It is worth noting that Combes et al. found a proportion of zero γGT activities in their series that is somewhat unusual.

In conclusion, the unexpectedly low γGT activities sometimes found in patients with very high serum bilirubin concentrations cannot be attributed to bilirubin itself; other, unidentified factors evidently are involved in this phenomenon.

References

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Urinary Enzymes and Renal Transplantation: The Early Warning Controversy

To the Editor:

We read with great interest the Letter from Horpacey et al. (1) and the reply from Keyser et al. (2).

Keyser et al. (2) ask if those who advocate measurement of urinary enzymes are prepared to treat transplant recipients with large doses of steroids when the results of such measurements are unsupported by clinical or other laboratory evidence. Our answer is, in certain circumstances, yes. We stated in our previous Letter [Clin. Chem. 23, 301 (1977)], but we would like to clarify the issue by giving a specific example. Our assay method and criteria for diagnosis have been previously stated (3).

Figure 1 depicts the postoperative course of a patient who received a kidney from his sister. The kidney initially functioned well but then rejection supervened. Ultimately, transplant nephrectomy was performed because of rejection, and histological examination confirmed the diagnosis of rejection. The patient was not infected at any stage. Activity of N-acetyl-β-D-glucosaminidase (EC 3.2.1.30; NAG) in his urine before transplantation was 108 μmol/h per millimole of creatinine. NAG activity in the urine produced 5 hours after transplantation was 131 μmol/h per millimole of creatinine but by the following day the activity had declined to 20 μmol/h per millimole of creatinine.

The transplanted kidney was functioning well and presumably the low activity of NAG in urine from the graft diluted the high activity of NAG in urine produced by the patient's own kidneys. The increase in activity from 20 to 50 μmol/h per millimole of creatinine on day 2 was disregarded because sharp fluctuations are common in the first two or three days. On days 3, 4, and 5 the graft appeared to be reaching its baseline of enzyme excretion. Meanwhile the concentration of creatinine in the serum was steadily decreasing. On day 5, 1 g of methyl prednisolone was given according to our standard practice. On day 6 there was a 50% increase in NAG activity and the serum creatinine increased from 300 to 330 μmol/liter. On days 7 and 8 the serum creatinine concentration again decreased while the patient's clinical state and urine output remained satisfactory. In contrast, NAG activity in the urine increased to 39 μmol/h per millimole of creatinine, a 77% increase since day 5. An increase of 50% or more is taken as indicating possible rejection; accordingly, 0.5 g of methyl prednisolone was given on day 7, when there was no other evidence of rejection. By day 9 there was abundant evidence of rejection: fever, malaise, declining urine output, and increasing serum creatinine. A fivefold increase in NAG from day 5 to day 10 provided further confirmation of rejection. Further doses of methyl prednisolone and radiotherapy were given, but to no avail, and the graft was removed. The decrease in NAG activity on day 9 (18%) might represent an initial response to steroid therapy but it is within the 95% limits of our laboratory error (the day-to-day coefficient of variation was 11%).

We do not claim there is always an early warning, but we do see this sign often enough to justify treatment in appropriate circumstances. We have measured NAG in the urine of more than 200 patients with renal allografts; Horpacey et al. (1) studied 255 grafts and also provided evidence that enzyme excretion could indicate early warning of rejection. Keyser et al. (2), on the other hand, do not believe there is an early warning, but their experience apparently is limited to eight rejection episodes for NAG and 11 episodes for γ-glutamyltransferase (EC 2.3.2.2; γGT). We stress that NAG activities are measured and reported every day, and this is essential if an early warning is to be detected. Decisions about anti-rejection treatment are based on clinical observations and conventional labora-

Table 2. Effect on γGT Activity of Bilirubin from Various Sources and in Various Concentrations

<table>
<thead>
<tr>
<th>Source</th>
<th>Bilirubin concn, μmol/l</th>
<th>Bilirubin activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>Cystalline</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Standard</td>
<td>19</td>
<td>14</td>
</tr>
</tbody>
</table>

* Results are expressed as percentage change in activity as compared to samples without added bilirubin. Positive values indicate apparent activation, negative values inhibition. The results are means for eight samples, analyzed in duplicate.