Radioimmunoassay Results for Phenytoin in Serum Increased by Abnormally High Concentrations of Free Fatty Acids in External Quality-Control Samples

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Performance of a radioimmunoassay kit for phenytoin in a quality-control scheme for antiepileptic drugs was affected by deterioration, during mailing, of the liquid control sera. Formation of nonesterified (free) fatty acids appears to have caused bias in assay results.

Additional Keyphrases: drug assay • variation, source of • sample handling

Amersham International's kit for radioimmunoassay of phenytoin in serum or plasma has been extensively tested with lyophilized human control sera, and comparison with gas-liquid chromatography in a clinical trial gave excellent agreement between the two methods (1). However, unsatisfactory results were obtained by this laboratory during participation in an American quality-control (QC) scheme for antiepileptic drugs. Here, I present the results of our internal investigation into this problem.

Materials and Methods

I used the following special materials: Phenytoin RIA Kit (IMS90; Amersham International plc, Amersham, Bucks., U.K.), arachidonic acid (Sigma Chemical Co. Ltd., Poole, Dorset, U.K.), and phenytoin, >99% pure ("Gold Label"; Aldrich Chemical Co., Gillingham, Dorset, U.K.).

Phenytoin radioimmunoassay kit. The Amersham kit measures phenytoin in serum or plasma by competing with an iodinated derivative of phenytoin for the limited number of binding sites on an antibody raised against a phenytoin conjugate. This phenytoin-specific antibody has been combined with a second antibody to give an insoluble phenytoin-binding reagent, which is separated from unbound phenytoin by centrifugation. To avoid a sample-dilution stage, the sensitivity of the phenytoin kit was reduced by use of a labeled antigen that is more closely related to the immunogen than phenytoin (2).

Quality-control-scheme samples. Three samples of normal human serum containing precise gravimetrically determined amounts of five antiepileptic drugs were received by mail from Dr. C. E. Pippenger, Department of Neurology, College of Physicians and Surgeons, Columbia University, New York, NY 10032, once a month by each participating laboratory. The samples were liquid and contained bacteriostats.

Studies of serum stability. To freshly prepared serum from two men and three women volunteers was added phenytoin to give a concentration of approximately 19 mg/L. Samples were stored for various times at -20, 20, and 37 °C. Serum from another man, which did not contain added phenytoin, was used as a control. In a separate study, phenytoin was added to serum from three men and two women, to the same final concentration as before and stored for various times at -20 and 4 °C. A portion of serum from one of the men, without added phenytoin, was used as a control. Phenytoin in all the samples was measured with the Amersham kit.

Measurement of nonesterified fatty acids. Nonesterified fatty acids (NEFA) in serum were determined by the method of Trout et al. (3).

Effect of arachidonic acid on the kit assay. Arachidonic acid, a nonesterified fatty acid, was added to pooled serum from treated epileptic patients (NEFA content, 0.6 mmol/L) to concentrations of 0.1, 1.0, and 10 mmol/L. These and a sample of the same serum without any addition were assayed with the Amersham kit.

Results and Discussion

Performance in the quality-control scheme. The Amersham International laboratory in the U.K. consistently overestimated the phenytoin concentration in the distributed QC-scheme samples. Between December 1976 and March 1978 the average monthly bias ranged from -1% to +28%, averaging +16%. For most samples, the Amersham Corp. laboratory in Chicago was also positively biased from the target gravimetric concentration, but less so than the U.K. laboratory. This led us to speculate that the bias may have been caused by assay sample instability, because the mailing time to the U.K. was about two to three weeks longer than to Chicago. Recovery experiments performed with pure phenytoin added to fresh serum showed an average recovery of 102% (range, 100–109%).

Serum stability. The results of the serum stability experiments are shown in Figure 1. At 4 °C there was little change even after 35 days (2–12% increase, Figure 1a). Storage at 20 °C (Figure 1b) brought about a 15–23% increase in apparent phenytoin concentration after 12 days, similar in magnitude to the bias experienced with QC-scheme samples assayed after three to four weeks at ambient temperature during mailing. At 37 °C (Figure 1c) there was a much greater increase, especially in the sera from the three youngest volunteers. This temperature dependence supported the notion that enzyme action was involved.

The sera with no phenytoin added did not change significantly from zero at any of the storage temperatures. This indicates that a factor in the serum was interacting with phenytoin in the assay system to increase the apparent phenytoin concentration.

Measurement of NEFA. A pool of the QC-scheme sera from July and August 1977 contained 2.6 mmol of NEFA per liter (normal range for human serum, 0.45–0.90 mmol/L). The phenytoin concentrations in these samples were biased by an average of +10.5% from the weighed-in concentration when assayed with the Amersham kit.

Effect of arachidonic acid on the kit assay. Arachidonic acid is known to have a strong influence on competitive protein-binding assays for thyroxin (4). The results of phenytoin assays after arachidonic acid was added to samples of pooled serum from epileptic patients are shown in Table 1. The increase in the measured value of phenytoin correlated with the concentration of added arachidonic acid. Total NEFA of 1.6

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Received Aug. 24, 1981; accepted June 30, 1982.

2292 CLINICAL CHEMISTRY, Vol. 28, No. 11, 1982
mmol/L caused a bias of +8.4%. The magnitude of the bias was comparable to that found in the QC-scheme results and to the NEFA concentration of the pool of QC-scheme sera.

Possible mechanism of bias in phenytoin kit assay. The release of NEFA in serum and plasma at ambient temperature is well known (5, 6) and is thought to result from the action of lecithinase A (6, 7). Unusual peaks noted by some QC-scheme participants who used gas–liquid chromatography were possibly caused by NEFA.

NEFA released in serum samples have caused increased thyroxin values in competitive protein-binding assays (4, 7), but similar effects have not been observed with radioimmunoassays for thyroid hormones. Phenytoin and thyroxin have similar chemical properties: both are insoluble in water and both bind to thyroxin-binding globulin. The binding between phenytoin and antibody is comparatively weak in the phenytoin kit, the structure of the drug being less like that of the immunogen than is the tracer (as mentioned before, this technique is commonly used to reduce assay sensitivity).

Phenytoin and NEFA effectively displace more tracer from the antibody than does phenytoin alone, although NEFA cannot displace the tracer in the absence of phenytoin. Moreover, NEFA released by nerve stimulation of blood-perfused subcutaneous adipose tissue decreased plasma binding of phenytoin in dogs (8). In vivo increases of NEFA by physical exercise or epinephrine injection double the unbound fraction of phenytoin in plasma in rats (9).

The mechanism of NEFA interference in the phenytoin assay therefore appears to be as follows. Most of the phenytoin is bound to nonspecific lipophilic binding sites on serum proteins; some may even bind to the plastic assay tube. The unlabeled phenytoin binds less strongly to the antibody than the tracer does, so the nonspecific binding sites compete with the antibody for the phenytoin. This reduces the proportion of unlabeled phenytoin bound to the antibody but does not affect assay quality, because standards and unknowns are similarly affected. NEFA in the serum sample at abnormally high concentrations release phenytoin from nonspecific binding sites, as occurs in vivo (8, 9). More of the phenytoin may now bind to the antibody, unhindered by nonspecific binding, and hence decreases tracer binding. This results in a higher apparent concentration as read from the standard curve. It is not clear how NEFA release phenytoin from the nonspecific binding sites.

This mechanism can apply to any ligand-binding assay found to be affected by NEFA. If the tracer binds less strongly than the unlabeled ligand, the roles are reversed and NEFA may cause a decrease in apparent concentration. Preliminary results, not presented here, indicate that such is the case with a steroid radioimmunoassay of this type.

The effect on phenytoin values only becomes apparent after sample storage for relatively long periods at room temperature or after incubation at 37 °C for at least 24 h. Under these conditions NEFA interference may not be limited to phenytoin RIA and thyroxin competitive protein-binding assays but may occur in many other ligand-binding assays in which the ligand is relatively insoluble in water.

Other sources of NEFA should not be ignored. Serum obtained commercially may contain increased NEFA, which would cause problems with standards and control sera. NEFA also find their way into reagents from serum or as impurities, e.g., in albumin.

The quality of control sera should be such that they are indistinguishable from patients' samples in the assay system. Use of lyophilized controls from good-quality human serum

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**Table 1. Effect of Arachidonlic Acid on Phenytoin Measurements**

<table>
<thead>
<tr>
<th>Pooled serum from epileptic patients</th>
<th>Measured phenytoin, mg/L</th>
<th>Bias, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ arachidonate, 0.1 mmol/L</td>
<td>8.3</td>
<td>—</td>
</tr>
<tr>
<td>+ arachidonate, 1.0 mmol/L</td>
<td>8.5</td>
<td>+2.4</td>
</tr>
<tr>
<td>+ arachidonate, 10 mmol/L</td>
<td>9.0</td>
<td>+8.4</td>
</tr>
<tr>
<td>+ arachidonate, 10 mmol/L</td>
<td>11.7</td>
<td>+41</td>
</tr>
</tbody>
</table>
is highly desirable, if not essential, for interlaboratory QC schemes, where shipped samples may be exposed for a substantial period to ambient temperature. I acknowledge the technical assistance from Quality Control staff at Amersham International plc. I am indebted to Drs. E. H. D. Cameron and P. G. Malan for detailed and helpful criticism of the manuscript, and I am grateful to Dr. J. S. Burgess, Managing Director of Amersham International plc, for permission to publish this work.

References

CLIN. CHEM. 28/11, 2294–2296 (1982)

Urine Contains an Inhibitor for Turbidimetric Determinations of Protein

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Our examination of urine components separated by gel filtration revealed the presence of an inhibitor that decreases the analytical recovery of protein in a turbidimetric assay involving sulfosalicylic acid as reagent (Proc. Soc. Exp. Biol. Med. 92: 748, 1956). The apparent relative molecular mass of this inhibitor was in the range 160 000–240 000. A study with purified proteins showed a similar inhibition by gamma-globulin, glycoprotein, and beta-lipoprotein in the assay of albumin by the same turbidimetric method. In contrast, measurement of protein by a dye-binding method was not affected by these materials. The low values for apparent urinary protein given by the turbidimetric method as compared with those by the dye-binding method are at least partly ascribable to the inhibitor.

Additional Keyphrases: gel filtration · albumin assay

Quantitative determination of protein in urine is important in clinical diagnosis and screening of patients. Techniques for doing so include turbidimetry, colorimetry, and dye-binding (1–5). A recent dye-binding technique (5) for the determination of protein in urine and cerebrospinal fluid has several advantages for routine assays. Although it gives higher values for urinary protein than those obtained by the turbidimetric method, no detailed study to assess the reason for this discrepancy has been performed. Therefore, we compared results by the two methods for urine samples that we had submitted to gel filtration.

Materials and Methods

As known proteins we used human alpha-globulin, Cohn Fraction IV (ICN NBC Laboratories Inc., Cleveland, OH 44128); human gamma-globulin, Cohn Fraction II, and human glycoprotein, Cohn Fraction VI (Miles Laboratories Inc. Elkhart, IN 46515); myoglobin, equine muscle Type I (Sigma Chemical Co., St. Louis, MO 63178), and beta-lipoprotein, bovine Cohn Fraction III-0 (United States Biochemical Corp., Cleveland, OH 44122). These were dissolved in isotonic saline (NaCl, 8.5 g/L). Human albumin, Cohn Fraction V (Sigma), was used as the routine standard for the protein assay.

SSA method for protein. A urine sample, 0.2 mL, was mixed with 3 mL of sulfosalicylic acid (SSA) solution, 30 g/L. After the mixture had stood for 10 min, the absorbance at 660 nm was measured with a spectrophotometer (Model 100-50; Hitachi, Japan).

CBB method for protein. The reagent, obtained from Ohtsuka Assay Laboratories, Ohsaka, Japan, contained per liter, 0.2 g of Coomasie Brilliant Blue G-250 (CBB) (Sigma), 0.5 g of methyl cellulose, 0.3 mol of hydrochloric acid, and 0.3 mol of sodium acetate. For 20 μL of urine we used 3 mL of the reagent, and measured the absorbance at 590 nm.

Sugar content was determined by the phenol–H2SO4 method, with glucose as a standard (6).

For gel filtration we used a 2 × 60 cm column of Sephadex G-200 that had been equilibrated with a buffer solution (7 mmol of sodium phosphate, pH 6.5, and 0.15 mol of NaCl per liter). We collected 3.9-mL fractions, and assayed them for protein and sugar. The column was calibrated with catalase, albumin, and chymotrypsinogen A (respective Mf’s of 232 000, 67 000, and 25 000). The void volume of the column was determined by the elution of Blue Dextran 2000 (Pharmacia).

Results and Discussion

The correlation coefficient for the relation between protein concentrations as measured by the CBB (y) and SSA (x) methods was 0.967 for 91 urine samples. The calculated regression equation was y = 1.24x + 0.692. The ratio of protein