Homogeneous Nonisotopic Assay for Phenytoin Evaluated

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We evaluated a new homogeneous immunoprecipitation assay for phenytoin in human serum. No sample dilution or pretreatment is required. The new method is based on spectrophotometry of the inhibition by free phenytoin of the precipitating reaction between anti-phenytoin antibody and a phenytoin–human serum albumin conjugate. A serum test sample is simultaneously mixed with the phenytoin–albumin conjugate and rabbit antiserum to phenytoin in a centrifugal analyzer, and the subsequent reaction is monitored at 3 min. Within-run and between-run coefficients of variation were well below 7%. The relation between results for patients' test samples as determined by immunoprecipitation assay (y) and an enzyme immunoassay (x) can be expressed as y = 1.10x + 1.1 (r = 0.966, n = 66).

Additional Keyphrases: centrifugal analyzer • drug assay • immunoprecipitation assay

Several nonisotopic immunoassay methods for determining phenytoin have been reported (1–4) since the inception of the radiolmmunoassay method for this drug (5, 6). A recent nonisotopic, homogeneous immunooassay of phenytoin (7) is based on an immunoprecipitation assay (IPA) and avoids the use of enzyme activity, fluorescent tags, radioactive isotopes, or an expensive laser nephelometer. The IPA for phenytoin relates a two-point measurement of absorbance at 340 nm to the concentration of phenytoin in a test sample. Reaction between anti-phenytoin antibody and phenytoin–human serum albumin conjugate increases the absorbance at that wavelength relative to the absorbance of a test blank; reaction of free phenytoin with anti-phenytoin does not. The presence of free phenytoin in a test sample displaces the phenytoin–albumin conjugate from reaction with anti-phenytoin antibody, thus inhibiting the increase of absorbance as compared with that produced in the absence of free phenytoin.

We compared results obtained by the present method with those obtained by an enzyme immunoassay.

Materials and Methods

We used a centrifugal analyzer that incorporates a spectrophotometer (Gemini; Electro-Nucleonics, Inc., Fairfield, NJ 07006).

Reagents. Anti-phenytoin antibody and phenytoin–albumin conjugate are supplied as lyophilized powders. Accelerator reagent, containing polyethylene glycol (Mr = 6000), is used to reconstitute working antibody reagents. Standard solutions provided contain 0.0, 5.0, 10.0, 15.0, 20.0, and 30.0 mg of phenytoin per liter. All these reagents were provided to us by Electro-Nucleonics Laboratories, Inc., Bethesda, MD 20814.

Procedures. The Gemini centrifugal analyzer is provided with special polypropylene discs; each disc has 20 cuvettes and each cuvette has two wells, a sample well and a reagent well. The pipetting sequence is as follows: pipet 500 µL of distilled water into the reagent well of cuvettes 1 and 2, the reference wells; 10 µL of standards (0.0, 5.0, 10.0, 15.0, 20.0, and 30.0

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mg/L) into sample wells of cuvettes 3 through 8; 10 µL of patients’ samples into sample wells of cuvettes 9 through 20; 10 µL of working conjugate reagent into sample wells of cuvettes 3 through 20; and 500 µL of working antibody reagent into reagent wells of cuvettes 3 through 20. Then warm the disc containing the samples and reagents in the reaction chamber for 5 min at 30 °C. The initial absorbance at 340 nm is measured 5 s after the samples and reagents are mixed together, and the final absorbance is measured 3 min later. The change in absorbance for standards and samples is automatically recorded with use of the appropriate program from Gemeni.

Results and Discussion

Standard curve. Figure 1 shows a typical IPA standard curve for phenytoin as obtained by two-point rate analysis of a 3-min reaction and graphed on linear graph paper. The range of change of absorbance from 0.0 to 30.0 mg/L is > 0.300 A.

Precision. To determine within-run precision, we assayed 24 replicates of each of two phenytoin concentrations in serum. The CV at each concentration was about 5% (Table 1). Between-run studies involved assays of three concentrations of phenytoin on six days during a 14-day period. The CV at each concentration was between 4.0 and 7.0%.

Correlation with enzyme immunoassay. We assayed the same patients’ samples by IPA (y) and homogeneous enzyme immunoassay (EMIT; Syva Co., Palo Alto, CA 94303) (x). Linear regression of the results gives the equation 

\[ y = 1.10x + 1.1 \]

\( r = 0.966, n = 66, S_{yx} = 2.72 \), indicating that the IPA results are as reliable as those of the comparison method.

Because the IPA method obviates the use of enzyme activity and enzyme substrate, it has the advantages of simplicity and long-term reagent stability. Also, the “spread” (magnitude of ∆A) of the standard curve of the IPA method appears to be at least twice as great as that obtained with the enzyme immunoassay, suggesting that greater precision may result with the IPA method. We are currently evaluating the IPA method for use with other anti-epileptic drugs and antibiotics.

References


Table 1. Precision Evaluation

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<th>Control 1</th>
<th>Control 2</th>
<th>Control 3</th>
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<tr>
<td>Mean, mg/L</td>
<td>10.44</td>
<td>14.15</td>
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<td>SD, mg/L</td>
<td>0.55</td>
<td>0.61</td>
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<td>CV, %</td>
<td>5.2</td>
<td>4.3</td>
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Between-run (n = 6 each)

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<td>SD, mg/L</td>
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<td>0.64</td>
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<td>CV, %</td>
<td>6.9</td>
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* From Fisher Diagnostics, Orangeburg, NY 10902.