Comparative Determination of Phenytoin by Spectrophotometry, Gas Chromatography, Liquid Chromatography, Enzyme Immunoassay, and Radioimmunoassay

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Sera from patients being treated with phenytoin were analyzed for the drug by spectrophotometry, gas chromatography, radioimmunoassay, enzyme immunoassay, and liquid chromatography. The assay values obtained were intercompared statistically. Enzyme immunoassay and liquid chromatography appear to be attractive alternatives to the more traditional methods of spectrophotometry and gas chromatography. Our radioimmunoassay data correlated poorly with results by the four other methods.

Additional Keyphrases: intermethod comparison - drug assay

Phenytoin is a most effective drug for use in the therapy of grand mal epilepsy. Its concentrations in plasma of persons being treated with it range between 2.5 and 30 mg/liter; seizures are usually effectively controlled when concentrations are 10 to 20 mg/liter. Most adult patients can tolerate doses of 300 to 400 mg daily without experiencing toxic side effects, but when such effects occur they can be severe. Thus, accurate measurement of phenytoin in the blood is important.

The proliferation of methods for measuring phenytoin in blood evokes the need for comparative evaluation of their accuracy and precision. Results by rapid methods for quantitation in emergency situations should correlate significantly with those by methods routinely used for monitoring or screening. A recent study in Europe of laboratories engaged in determining antiepileptic drug concentrations in blood has demonstrated great variability (1). More recently, Pippenger et al. (2) showed a great interlaboratory variation in reported antiepileptic drug concentrations, attributable in part to the use of different methods and lack of familiarity with the assay pitfalls.

We compare herein results for phenytoin in serum as measured by chromatography, liquid chromatography, enzyme immunoassay, and radioimmunoassay.

Materials and Methods

Samples

Sera from 50 blood specimens submitted routinely from hospitalized epileptic patients for monitoring of phenytoin were quantitated spectrophotometrically and frozen. Within four weeks all samples were assayed by the four other methods.

Spectrophotometry

For this we used the method of Wallace, as modified (3), in which phenytoin is extracted from 2 mg of the specimen with chloroform and oxidized with permanganate. The resulting heptane solutions of benzophenone were scanned from 340 to 200 nm with a Model DK2 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif. 92834).

Radioimmunoassay

Reagents for radioimmunoassay of phenytoin were obtained from Wein Laboratories, Succasunna, N.J. 07876.³ The supplier’s procedure was followed, except that we used as scintillator a mixture of 100 g of naphthalene, 7 g of 2,5-diphenyloxazole, and 300 mg of 2,2’-p-phenylenebis(4-methyl-5-phenyloxazole) in 1 liter of 1,4-dioxane.

Radioactivity in the supernatant scintillation cocktail mixture was counted in a Searle Isocap/300, 6872 Liquid Scintillation Counter.

Enzyme Immunoassay (EMIT)

We used a Model KA-150 kinetic analyzer (Perkin-Elmer Corp.) to perform the EMIT (Syva Corp., Palo Alto, Calif. 94304) assay for phenytoin.⁴ The supplier’s reagents were reconstituted as follows: Buffer concentrate was diluted to 58 ml with de-ionized water; antibody–substrate reagent A, used as reagent 1, was reconstituted with 24.8 ml of de-ionized water; and enzyme reagent B, used as reagent 2, was reconstituted with 24.8 ml of buffer. All samples, calibrator standards, and controls were diluted fivefold with de-ionized water by use of a diluter (Micromedic Systems, Inc., Philadelphia, Pa. 19105) before they were placed in the KA 150.

Gas Chromatography

For this we used an alkaline extraction method (4) in which the initial dichloroethane extraction solvent contained 5-(p-methylphenyl)-5-phenylhydantoin as an internal standard. Phenytoin was methylated on the column with trimethylsilylum hydroxide and quantitated by peak-height ratio with 5-(p-methylphenyl)-5-phenylhydantoin (5). The Model 900

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³ DPH Test Set, Package Insert, Wein Laboratories, Inc., P.O. Box 227, Succasunna, N.J. 07876.
Table 1. Statistical Results for Phenytoin Comparison Studies

<table>
<thead>
<tr>
<th>Method</th>
<th>y Method</th>
<th>n</th>
<th>y-intercept</th>
<th>slope</th>
<th>$S_y - x$</th>
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<tbody>
<tr>
<td>Spectrophotometry</td>
<td>Gas chromatography</td>
<td>48</td>
<td>0.396</td>
<td>0.996</td>
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<td>0.200</td>
<td>0.931</td>
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<td>4.7200</td>
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</table>

![Fig. 1. Phenytoin spectrophotometric assay in triplicate was plotted vs. gas chromatographic values](image1)

The y-intercept was 0.4, the slope was 0.996, and the standard error of estimate was 2.42

![Fig. 2. Forty-nine samples were analyzed for phenytoin by spectrophotometric methods and plotted vs. results obtained by the EMIT technique, in triplicate](image2)

The y-intercept was 0.48, the slope was 0.934, and the standard error or estimate was 2.67

![Fig. 3. Sera containing phenytoin were analyzed in triplicate by radioimmunoassay and plotted vs. spectrophotometric values](image3)

The y-intercept was −0.945, the slope was 1.30, and the standard error of estimate was 3.31

Liquid Chromatography

We used an activated charcoal extraction technique (6) and a Model 601 liquid chromatograph (Perkin-Elmer Corp.) equipped with a reversed-phase column. A variable-wavelength spectrophotometer was used to monitor column effluent. The data were processed with a PEP II data processor.

Statistical Analysis and Results

We applied the methods of Westgard and Hunt (7), and present in Table 1 the y-intercept, slope, and standard error of estimate $S_y - x$ to demonstrate the constant, proportional, and random error, respectively. Correlation coefficients are not given in the statistical analysis of the data because they are dependent on range and have a rather doubtful usefulness as a significant barometer of random error.

Discussion

Linear regression data and the cumulative data indicate that, of the five methods compared, there is sufficient correlation among four to make their differences clinically insig-
significant. Only radioimmunoassay appears to have a poor correlation, giving relatively high concentration values consistently. This apparent discrepancy is being reported in light of much other scientific data recorded as giving excellent correlation between radioimmunoassay and the above-mentioned techniques. At this point we do not know the reason for such a difference, whether caused by random metabolite concentrations of uremic patients, inspecificity of antibody, or a possible combination of both (8, 9).

The fundamental differences in the five methods lie in the method of extraction of the phenytoin. In the ultraviolet and gas-chromatographic methods, organic solvents are used to extract the drug from binding proteins. The liquid-chromatographic system uses charcoal while both the EMIT and radioimmunoassay methods rely upon the avidity of an antibody to partition the phenytoin. To some extent we may be seeing variation in extraction efficiencies, and in the case of the two immunoassays, cross reactivity. Both assays are based on antigen–antibody reactions and specificity is germane to results. Recommendations to improve variability will rest not only on the availability of antiepileptic drugs standards in biological matrix, but in a greater control of antibody specificities and cross reactivities produced internally by each laboratory or by commercial companies. Our long experience in the development of radioimmunoassay of drugs has indicated the importance of the specificity and avidity of antibodies (10–15).

The availability of comparison sera for liquid chromatography was rather limited. The cumulative data table, however, shows reasonably good correlation with three other methods. Extensive verification is available through Adams and Vandenmark (6). Total extraction analysis times of 20 min, coupled with simultaneous determinations, make liquid chromatog-
raphy superior to traditional gas-chromatographic methodologies.\textsuperscript{5}

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References


\textsuperscript{5} The raw data are available upon request from the authors or from the Editorial Office of Clinical Chemistry.


