Radioimmunoassay, Enzyme Immunoassay, Spectrophotometry, and Gas–Liquid Chromatography Compared for Determination of Phenobarbital and Diphenylhydantoin

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Sera from epileptic patients were assayed for phenobarbital and diphenylhydantoin by four different analytical procedures. Quantitative results obtained by radioimmunoassay (I) and enzyme immunoassay (II) were compared to each other and to the results obtained on aliquots of the same sample by gas–liquid chromatography (III) and ultraviolet spectrophotometry (IV). For phenobarbital the correlation coefficients were I vs. II, 0.909; I vs. III, 0.947; II vs. III, 0.917; I vs. IV, 0.950; II vs. IV, 0.953. For diphenylhydantoin the correlation coefficients were I vs. II, 0.953; I vs. III, 0.951; II vs. III, 0.957; I vs. IV, 0.862; II vs. IV, 0.898. The immunoassays can be substituted for liquid chromatography or ultraviolet spectrophotometry without changing the resulting clinical interpretations.

Diphenylhydantoin (Dilantin, Parke-Davis), phenobarbital, and primidone (Mysoline, Ayerst), are the drugs most commonly used in the treatment of grand mal, psychomotor epilepsy, and other forms of focal epilepsy. Because the clinical symptoms of epilepsy are not conveniently monitored, clinicians have turned to the measurement of these antiepileptic drugs in the blood to determine the adequacy of dosage and the possibility of toxicity. Concentrations of the drug in serum and therapeutic or toxic effects have been correlated (I). Such monitoring allows the physician (a) to detect non-compliance; (b) to evaluate the patient with inadequate seizure control, or a change in degree of seizure control caused by poor absorption or rapid metabolism of the drug; and (c) to establish baseline concentrations and to confirm suspected intoxication. Moreover, in the case of a patient with symptoms of drug intoxication, who is receiving several drugs, the offending drug may be precisely identified and the dose corrected.

Because of the importance of monitoring antiepileptic drug blood concentrations, new or improved methods have been continually introduced. Our objective was to compare the quantitative results obtained for phenobarbital and diphenylhydantoin by radioimmunoassay (RIA) and enzyme-mediated immunoassay (EMIT) (2–6) with results from spectrophotometric (7–10) and gas–liquid chromatographic (GLC) (11–14) procedures.⁵

Materials and Methods

Samples

We assayed sera from 90 epileptic patients referred by physicians for antiepileptic drug screen and from 50 patients admitted to a hospital emergency room for suspected drug overdose. Samples were assayed by the two immunoassay methods and by either spectrophotometry or chromatography.

Methods

Phenobarbital

EMIT

The reagents for the enzyme immunoassay of phenobarbital were obtained from Syva Corp., Palo Alto, Calif. 94304, and used according to the manufacturer’s

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Received Oct. 16, 1975; accepted Dec. 30, 1975.

⁵ Nonstandard abbreviations used: RIA, radioimmunoassay; EMIT, enzyme-mediated immunoassay; and GLC, gas-liquid chromatography.
instructions. Absorbance was measured at 340 nm on a Model 24/25 double-beam spectrophotometer with a heated cuvette cell and a Model 701 Printer/Calculator (all from Beckman Instruments, Inc., Irvine, Calif. 92664).

The antibody to phenobarbital, obtained from Syva Corp., showed the following cross reactivity: phenobarbital 100.0%, mepobarbital 20.0%, secobarbital 1.0%, amobarbital 0.7%, pentobarbital 0.5%, diphenylhydantoin 0.5%, and primidone 0.5%.

**RIA**

The reagents for radioimmunoassay of barbiturates were obtained from Roche Diagnostics, Hoffmann-La Roche, Inc., Nutley, N.J. 07110 and used according to the manufacturer's protocol for quantitative assay. Phenobarbital solutions of 1000, 500, 250, 125, 63, 32 and 16 μg/liter were used in constructing the standard curve. The supernate was counted on a "Biogamma" gamma counter (Beckman Instruments).

The antibody to barbiturates, obtained from Roche Diagnostics, showed the following cross reactivity: secobarbital 100.0%, pentobarbital 45.0%, butobarbital 45.0%, amobarbital 35.0%, phenobarbital 25.0%, and barbital 10.0%. The manufacturer has found no significant cross reactivity with diphenylhydantoin.

**GLC**

The gas–liquid chromatographic analysis was performed according to the method of Perchalski et al. (14). Methylene chloride was substituted for ethyl ether as the extraction solvent. "Concentratubes" (Laboratory Research, Los Angeles, Calif. 90036) were used for the final extraction. Trimethylanilinium hydroxide in methanol, 25 ml/100 ml, from Southwest Analytical Chemicals, Austin, Tex., was used to methylate the drugs on the column as described elsewhere (10, 12–14). 5-Methylphenylhydantoin (Aldrich Chemical Co., Milwaukee, Wis. 53233) was used as an internal standard. A Model 5710A gas chromatograph was used for this assay with 3% OV-17 on Gas Chrom Q in a 1.82 m × 2 mm (i. d.) column (Hewlett-Packard, Avondale, Pa. 19311).

**Spectrophotometry**

Spectrophotometry was performed according to the method of Goldbaum (7) as modified by Bogan and Smith (8). Absorbance of the unknown at 260 nm was compared with that of a butobarbital standard.

**Diphenylhydantoin**

**EMIT**

The reagents for the enzyme mediated immunoassay of diphenylhydantoin (Syva Corp.) were used according to the manufacturer's instructions. Absorbance was read as described previously.

The antibody to diphenylhydantoin obtained from Syva Corp. showed the following cross reactivities: diphenylhydantoin 100.0%, 5-hydroxyphenyl-5-phenylhydantoin 0.5%, phenobarbital 0.5%, primidone 0.5%, methsuximide 0.5%, ethosuximide 0.5%, and mepobarbital 0.5%.

**RIA**

The reagents for radioimmunoassay of diphenylhydantoin (Wien Laboratories, Inc., Succasunna, N. J. 07876) were used according to the manufacturer's instructions. The supernate was counted on a Model 350 liquid scintillation counter (Beckman Instruments).

The antibody to diphenylhydantoin, obtained from Wien Laboratories, showed the following cross reactivities: diphenylhydantoin 100.0%, 5-hydroxyphenyl-5-phenyldantoin 76.5%, 5-ethyl-5-phenylhydantoin 1.0%, phenobarbital 0.1%, mepobarbital 0.1%, and primidone 0.1%.

**GLC**

The method of Perchalski et al. (14), modified as described in the above GLC section, was used for the gas–liquid chromatographic analysis.

**Spectrophotometry**

The procedure of Wallace (9) as modified by Dill et al. (10) was used for the ultraviolet spectrophotometric analysis of diphenylhydantoin. The absorption of the oxidation product, benzophenone, in isooctane was measured at 247 nm vs. a reagent blank.

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**Table 1. Statistical Comparison of Immunoassay, Spectrophotometry and Gas-Chromatography for Determining Phenobarbital and Diphenylhydantoin**

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<thead>
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<th>Phenobarbital</th>
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<th>a. RIA vs. EMIT</th>
<th>b. RIA vs. GLC</th>
<th>c. EMIT vs. GLC</th>
<th>d. RIA vs. spectr.</th>
<th>e. EMIT vs. spectr.</th>
<th>f. RIA vs. EMIT</th>
<th>g. RIA vs. GLC</th>
<th>h. EMIT vs. GLC</th>
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- **m** = slope of the least-squares regression line
- **b** = intercept of the least-squares regression line
- **S_y** = standard error of the estimate
- **r** = correlation coefficient
- **n** = number of cases
- **t** = t-statistics testing linear regression
Statistical Analysis

Concentrations of phenobarbital found by radioimmunoassay and enzyme immunoassay were compared to each other and to concentrations of phenobarbital found by gas–liquid chromatography or ultraviolet spectrophotometry on aliquots of the same sample. Errors were estimated from the least-squares parameters (slope of the least-squares line, y intercept, standard error of estimates $S_{yx}$) as recommended by Westgard and Hunt (15).

Systematic errors may be constant (a systematic error in concentration units) or proportional (a systematic error in percentage units). Using a simulated test set of data, Westgard and Hunt (15) demonstrated that constant error is reflected exactly in the intercept of the least-squares line, as well as in the bias. The slope, standard error, correlation coefficient, and standard deviation are not sensitive to constant error. The exact magnitude of proportional error is quantitated by changes in the slope, m.

Westgard and Hunt (15) pointed out that both the standard error and the standard deviation reflect the magnitude of the random error. The correlation coefficient decreases as random error increases, but the changes are small.

Cases showing large discrepancies ($\pm 3$ SD) were not included in the linear regression and statistical calculations (16).

Results

Phenobarbital (Table 1)

Radioimmunoassay vs. enzyme immunoassay (Table 1, line a). The slope of the line is 0.867, which indicates a proportional error of 13.3%. Constant error is estimated at 1.19 mg/liter from the intercept. The random error is estimated at 5.15 mg/liter from the standard error of the estimate, $S_{yx}$. The correlation coefficient is 0.909. For example, a radioimmunoassay value of 30 mg of phenobarbital per liter would correspond to an average value of 27.4 mg/liter $\pm 5.2$ (SD) by enzyme immunoassay.

Immunoassays vs. gas chromatography (Table 1, lines b and c). The slope is 0.795 for radioimmunoassay and 0.901 for enzyme immunoassay, which indicates a proportional error of 20% and 10%, respectively. Constant error is estimated as 1.45 mg/liter (RIA) and 0.79 mg/liter (EMIT). Random error estimated from the standard error is 3.84 mg/liter (RIA) and 4.65 mg/liter (EMIT). The correlation coefficients were 0.947 and 0.917, respectively. For example, a phenobarbital value of 30 mg/liter by gas–liquid chromatography would correspond to an average value by RIA of 34.9 $\pm 3.84$ mg/liter and an average value by EMIT of 32.4 $\pm 4.65$ mg/liter.

Immunoassays vs. spectrophotometry (Table 1, lines d and e). From the least-squares parameters, proportional error between the immunoassays and Goldbaum’s method (7) was much less than that found when comparing immunoassays to gas–liquid chromatography. Proportional error was estimated at 0.5% (RIA) and 10.0% (EMIT), constant error as $-2.36$ mg/liter (RIA) and $-1.47$ mg/liter (EMIT), and random error as 3.5 mg/liter (RIA) and 3.0 mg/liter (EMIT). The correlation coefficients were 0.950 and 0.953, respectively. For example, a phenobarbital value of 30 mg/liter obtained by spectrophotometry would correspond to an average value by radioimmunoassay of $32.5 \pm 3.5$ mg/liter and an average value by enzyme immunoassay of $35.0 \pm 3.0$ mg/liter.

Diphenylhydantoin (Table 1)

Concentrations of diphenylhydantoin found by radiommunoassay and enzyme immunoassay were compared to each other and to concentrations of diphenylhydantoin found by gas–liquid chromatography and by spectrophotometry according to Wallace (9) as modified by Dill et al. (10).

Radioimmunoassay vs. enzyme immunoassay (Table 1, line f). Error estimates based on the least-squares parameters as described by Westgard and Hunt (15) indicate a proportional error of 10% and a constant error of 0.12 mg/liter. Random error is estimated as 2.78 mg/liter. The correlation coefficient is 0.953. A diphenylhydantoin concentration of 10 mg/liter by radioimmunoassay would correspond to an average value of $9.14 \pm 2.78$ mg/liter by enzyme immunoassay.

Immunoassay vs. gas–liquid chromatography (Table 1, lines g and h). The slopes were 0.661 and 0.786, which suggest 34% and 22% proportional error for RIA and EMIT when gas chromatography is taken as the reference method. The constant error is small, 1.61 mg/liter and 0.976 mg/liter, and the random error was 2.19 and 2.04 mg/liter, respectively. The correlation coefficients were 0.951 and 0.957.

A diphenylhydantoin value of 10 mg/liter by gas chromatography would correspond to an average value.

Fig. 1. Immunoassay vs. gas–liquid chromatography compared for diphenylhydantoin determination
of 12.7 ± 2.2 mg/liter (RIA) or 11.5 ± 1.0 mg/liter (EMIT).

Immunoassays vs. spectrophotometry (Table 1, lines j and i). Estimated from the linear regression parameters, the proportional error was about 30%, the constant error was 1.8 mg/liter, and the random error range was 1.6 to 1.9 mg/liter for both immunoassays. The correlation coefficients were 0.862 and 0.898, respectively.

In our hands, concentration of 10 mg/liter as determined by the method of Wallace (9) as modified by Dill et al. (10) would correspond to an average value of 11.4 ± 1.9 mg/liter (RIA) or 11.3 ± 1.6 mg/liter (EMIT).

Interferences

A scattergram of the immunoassay results for diphenylhydantoin plotted against the results obtained by gas–liquid chromatography is shown in Figure 1. When the case histories were investigated some of the outlying points seen on this scattergram (and on the corresponding graphs for the other comparisons) could be explained in some cases by the different cross-reactivities of the immunoassays and by interference from other drugs taken by the patient.

Patients receiving mephobarbital gave different apparent phenobarbital concentrations by the different methods. For example, in one of the cases in which the patient received both mephobarbital and phenobarbital, the apparent phenobarbital concentrations (mg/liter) were: 54 (GLC), 50 (EMIT), 42 (RIA), and 32 (spectrophotometric).

The gas–liquid chromatographic method involving methylation does not distinguish between phenobarbital and mephobarbital (12). The two drugs cannot be separately measured by this procedure. The extraction efficiency for mephobarbital by this method is not known. However, since the apparent phenobarbital concentrations by GLC in these cases were the highest values, it may be that GLC results represent the sum of the two drugs present.

According to the manufacturer (Syva Corp.), the enzyme immunoassay for phenobarbital cross reacts 20% with mephobarbital. For a mixture of equal amounts of mephobarbital and phenobarbital the phenobarbital result by EMIT would be 20% too high. This appeared to be true in several cases encountered in this study.

In our study, the radioimmunoassay results were slightly less than those obtained by enzyme immunoassay.

The spectrophotometric procedure of Goldbaum gives the sum of 5,5-substituted barbiturates. However, the 1,5,5-substituted barbiturates (hexobarbital, mephobarbital, metharbital, etc.) have characteristically different absorption spectra and do not show a shift in absorption between pH 14.0 and 9.0 (17). For this reason the spectrophotometric method probably suffered the least interference and gave the most nearly accurate value for phenobarbital in these cases.

No other systematic interference from other drugs given concurrently was noted in the phenobarbital or diphenylhydantoin assay. The drugs prescribed concurrently included amitriptyline, primidone (Mysoline, Ayerst), ethosuximide (Zarontin, Parke-Davis) and thioridazine (Mellaril, Sandoz).

Discussion

The immunoassays fill the requirements for an acceptable assay method for antiepileptic drugs. They require very little sample; the Wien radioimmunoassay for diphenylhydantoin requires only 10 μl of serum, the EMIT procedures require 50 μl, and the Roche Diagnostics procedure, 100 μl. The immunoassays can be performed more quickly than the methods now in use, because no extraction or concentration is involved. The fastest procedure is EMIT, which requires less than 2 min per tube; thus, it allows a standard curve and several samples to be run in less than 15 min from receipt of serum. The radioimmunoassays each require 1 h to obtain quantitative results. Because the immunoassays are sensitive in the nanogram range, it was necessary to dilute patients’ serum.

By contrast, gas–liquid chromatography permits simultaneous analysis for several drugs (primidone, phenobarbital, and diphenylhydantoin) which, with batching of specimens, lends itself well to nonemergency analysis. We found the method we used (14) to be convenient and reliable for routine laboratory use.

The immunoassay results for phenobarbital correlate well with each other and with the values found by gas chromatography and spectrophotometry. Plaa and Hine (18) reported that the average phenobarbital concentration in the blood of 30 adult subjects receiving 200 mg of phenobarbital per day for control of seizures was 32 ± 18 mg/liter (range, 7 to 56 mg/liter). As calculated from the least-squares regression parameters, the immunoassays would give average values of 34.9 mg/liter (RIA) and 32.4 mg/liter (EMIT) for a gas-chromatographic value of 30 mg/liter or 32.5 mg/liter (RIA) and 35.0 mg/liter (EMIT) for a spectrophotometric value of 30 mg/liter. Therefore, if the faster, more convenient immunoassays were used in place of the other methods, this substitution would have little effect on the clinical interpretation of blood drug concentrations.

The immunoassay results for diphenylhydantoin correlate well with each other and with the gas–liquid chromatographic method, and somewhat less well with the spectrophotometric procedure of Dill et al. (10). Kutt et al. (19) showed that symptoms of intoxication (nystagmus) caused by diphenylhydantoin begin to appear when its concentration in the blood approaches 20 mg/liter (range 15–25 mg/liter). As calculated from the least-squares regression parameters described above, use of the immunoassays for diphenylhydantoin determination would give average values of 27.8 mg/liter (RIA) and 24.2 mg/liter (EMIT) for a gas chromatographic value of 20 mg/liter and (or) average values of 25.2 mg/liter (RIA) and 25.4 mg/liter (EMIT) for a spectrophotometric value of 20 mg/liter. Therefore, here again, the use of the immunoassay results would not change the clinical interpretation.
References