Assay of Phenytoin: Adaptation of "EMIT" to the Centrifugal Analyzer

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We describe adaptation to the centrifugal analyzer of the homogeneous enzyme immunoassay ("EMIT") for phenytoin (diphenylhydantoin). The assay was modified to give greater range and sensitivity, and less reagent is needed. Precision and accuracy are excellent, and results correlate well with those by gas-liquid chromatography; the slight systematic bias is of no clinical significance.

Measurement of phenytoin (diphenylhydantoin) in serum is used as an aid in managing treatment of patients who are subject to generalized epileptic seizures (1), to increase the efficacy and safety of therapy. Such monitoring facilitates individualization of dosage regimens, reveals irregular drug intake, and identifies the responsible agent in drug-intoxicated patients who are being administered several drugs simultaneously (2).

Frequent monitoring of the concentration of the drug in serum is necessary to achieve satisfactory seizure control in ambulant patients with grand mal epilepsy. Therapeutic concentrations may vary among individuals, and it is therefore important to measure them in each individual patient over periods of time. The estimation of phenytoin has been done for the most part by using gas-liquid chromatography or ultraviolet spectrophotometry. Rubenstein et al. (3) introduced the "enzyme multiplied immunoassay technique" (EMIT). The Syva Research Institute, Palo Alto, Calif. 94304, has developed this particular assay so that at the present time one can use the system for the measurement, in urine, of opiates as a class, amphetamine and methamphetamine, and barbiturates as a class including pentobarbital, phenobarbital, butobarbital, secobarbital, and amobarbital. Methods have also been developed for the measurement in urine of cocaine and cocaine metabolites as well as methadone. Broughton and Ross (4) have recently adapted the EMIT to the centrifugal analyzer in the measurement of the above classes of drugs in the urine.

It is now possible to use this system to measure phenytoin and phenobarbital in serum. Other antibodies are being prepared to measure other anti-epileptic drugs, including ethosuximide, primidone, and carbamazepine.

In EMIT assays, enzyme labels are used instead of radioactive labels. An antibody specific to phenytoin is used. An enzyme (from Leuconostoc mesenteroides), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), which is not present in human serum, is attached to the phenytoin molecule in such a way that the enzyme cannot act on its substrate, glucose 6-phosphate, when the molecule is captured by the antibody. When the sample material, which in this case is serum, is mixed with the antibody and enzyme-labeled molecule, any free molecules of the compound of interest in the sample will compete with the enzyme-labeled molecules for the limited number of antibody binding sites. The more free molecules available in the sample, the more enzyme will remain unbound and therefore active in the mixture. NAD+ is also added to the substrate mixture. The enzyme converts NAD+ to NADH, resulting in an absorbance change that is measured at 340 nm. The assay, therefore, is essentially a competitive binding type of assay. Schneider et al. (5) have given a detailed discussion of the homogeneous enzyme immunoassay.
The manufacturer recommends that the EMIT system be run with very careful automatic timing. The instrument suggested for use in analysis of urine and serum samples is the Model 300N spectrophotometer (Gilford Instrument Co., Oberlin, Ohio 44074). A timer-printer and a small automatic diluter (Syva Corp.) for the purpose of semi-automating the system is mandatory. Certain predilution steps are required, and assay time is exactly 80 s. The immunoassay of phenytoin has been shown to give a very close correlation with gas-liquid chromatography (6, 7).

A recent report (8) discusses measurement of phenytoin with a centrifugal analyzer and reports the adaptation of the EMIT system to the centrifugal analyzer. The reagents were prediluted; pipetting into the transfer disc was manual. The reported coefficient of variation was between 5 and 10%, depending on thermal conditions. A pre-incubation time of 3 min was used. There was no mention of correlation with results by ultraviolet or gas/liquid chromatographic methods, nor was the sensitivity of the assay mentioned.

We wished to adapt the EMIT assay of phenytoin to a centrifugal analyzer and to avoid the use of manual pipetting into the transfer discs. We also wished to expand the range of the assay substantially more than the present 2.5 to 30 mg/liter, because we not only must measure phenytoin as a drug of abuse in patients, but also must measure phenytoin half-lives.

Materials and Methods

Apparatus

We performed the immunoassays with an “Aminco Roto-Chem II” centrifugal analyzer (American Instrument Co., Silver Spring, Md. 20920).

Table 1. Phenytoin—Precision of GLC

<table>
<thead>
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<th>Concentration</th>
<th>Within-day</th>
<th>Between-day</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Low</td>
<td>4.3</td>
<td>0.22</td>
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<tr>
<td>Therapeutic</td>
<td>13.2</td>
<td>0.71</td>
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<tr>
<td>High</td>
<td>52.6</td>
<td>3.11</td>
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Table 2. Phenytoin—Precision of EMIT

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Within-day</th>
<th>Between-day</th>
</tr>
</thead>
<tbody>
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<td>Mean</td>
<td>SD</td>
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<tr>
<td>Low</td>
<td>4.6</td>
<td>0.25</td>
</tr>
<tr>
<td>Therapeutic</td>
<td>13.3</td>
<td>0.80</td>
</tr>
<tr>
<td>High</td>
<td>55.5</td>
<td>2.91</td>
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Reagents

All reagents used for the immunoassay procedure were those commercially available from the Syva Corp. Reagents A and B are provided as a matched set for each of the assays to be performed and are specific for phenytoin. The buffer solution and the calibrators are also supplied with the kit. Reagent A is the antibody/substrate reagent; it contains NAD+, the specific antibody to phenytoin, and the substrate, glucose-6-phosphate. Reagent B is the drug/enzyme complex, phenytoin coupled to glucose-6-phosphate dehydrogenase. The two reagents are in lyophilized form. Reconstituted with distilled water, they are stable for four weeks if kept refrigerated.

Buffer solution. Tris(hydroxymethyl)aminomethane hydrochloride buffer (55 mmol/liter, pH 7.8) with added surfactant is prepared from a buffer concentrate supplied with each reagent kit; the solution is stable at room temperature.

Calibrators. A series of serum standards containing phenytoin in concentrations ranging from 0–30 mg/liter are provided as a lyophilized serum-based preparation.

We prepared another set of standards from our own stock diphenylhydantoin solution. The standards were made up using a stock solution added into a commercial control serum (“Chemvarion”; Clinton Laboratories, Santa Monica, Calif. 90404).

The gas-liquid chromatography method of Barrett (9) was used, with methylation and programmed temperature.

Procedure

Standard EMIT procedure. The procedure recommended by the manufacturer is as follows: Place 50 μl of standard or unknown serum plus 250 μl of buffer in a 1-ml disposable beaker. This step and the succeeding dilution steps may be done with a pipettor-dilutor. Before proceeding, at least 1 min should elapse. Deliver 50 μl of diluted serum from the first beaker plus 250 μl of buffer into a second beaker. Then dispense into the second beaker 50 μl of reagent A plus 250 μl of buffer. Next add 50 μl of reagent B plus 250 μl of buffer to the second beaker. Immediately upon addition of Reagent B, aspirate the contents of the second beaker into the spectrophotometer (Model 300N, Gilford Instrument Co.) flow cell. This automatically activates the printer/calculator to time and
record the measurement. Make two absorbance readings at 340 nm, the first at 15 s, the second at 95 s. The difference (ΔA) over the 80-s measurement period is used to calculate the results. Prepare a standard curve by plotting ΔA - ΔA₀ (ΔA₀ is the absorbance of the zero standard at 80 s) for each standard on EMIT graph paper (a modified logit function paper). A linear relation should be obtained.

Modification for centrifugal analyzer. We modified the EMIT procedure as follows:
1. The buffer is diluted twofold with water.
2. Reagent A is diluted 10-fold (rather than fivefold) with buffer.
3. Reagent B is diluted 10-fold (rather than fivefold) with buffer.
4. After dilution of Reagent A and Reagent B, these are allowed to set at room temperature for at least 1 h. The dilutions may be used for as long as five days if stored at 4 °C.
5. Load the sample cups as follows: cup 1, negative serum blank; cups 2–14, standards and sera. Set the Rotofil Automatic Samplet/Dilutor (American Instrument Co.) as follows: Serum/standard pump, 8 μl; buffer pump, 200 μl; Reagent A, 200 μl; Reagent B, 200 μl. Deliver sample, buffer and Reagent A to the inner well, and Reagent B to the middle well. Then place the transfer disc on the centrifugal analyzer. The assay may be begun immediately. The analyzer is controlled with Kinetic Rate III tape, a program supplied by the American Instrument Co. It allows the user to take nine absorbance readings at equal intervals chosen by the operator. There is a slight nonlinearity between 15 and 45 s; the curve then becomes linear. The 3-min reading time ensured an adequate absorption change, for reasonable sensitivity.

Precision. Within-day and day-to-day precision for phenytoin measured on both the gas-liquid chromatograph (Table 1) and the Amino Roto-Chem II (Table 2) are shown; the patients’ samples include concentrations below, within, and above the therapeutic range. The within-day samples were analyzed 20 times on the same day by both procedures; the between-day analyses were done on 25 successive days. The precision of the EMIT system compares favorably with that of the comparison method.

Accuracy. The analytical recovery for the method is shown in Table 3. Two experiments were done. Phenytoin was added to the negative control serum (Chemvarion) to give concentrations of 4.0 and 15.0 mg/liter, respectively. The samples were analyzed on each of 20 separate days. The resulting coefficient of variation was satisfactory and the analytical recovery adequate.

Table 4 shows a linearity study of a serum with a high phenytoin concentration, diluted with the negative control serum (Chemvarion). Linearity is evidently satisfactory down to low concentration of the drug.

Thirty-two sera that contained both phenobarbital and phenytoin were obtained from patients undergoing maintenance therapy for convulsive disorders. Results for these particular samples compared well with measurements by gas–liquid chromatography; phenobarbital does not interfere in the EMIT method. (These sera are included in the comparison study between the EMIT system and gas/liquid chromatography.)

A linear regression analysis of the comparison study is shown in Table 5. The coefficient of correlation is acceptable. The standard error of the estimate shows very modest scatter about the line. The intercept is close to 0, and the standard error of the intercept has a t-score of −7.71, essentially no significant difference from an 0 intercept. The slope, however, shows a t-score of 4.919, which is slightly significant. EMIT values evidently have a tendency to be slightly higher than the values obtained by gas/liquid chromatographic analysis. These data are very similar to the study of Booker and Darcey (7), in which the authors discuss the correlation between the en-
zymatic immunoassay as done by the recommended technique on the Gilford instrumentation vs. gas/liquid chromatography done in a standard manner. The t-value of 3.81 shows a definite but small difference in the two means.

Interferences. Bilirubin concentrations up to 150 mg/liter did not affect results of the assay. Extremely lipemic sera also had no effect. There was, however, a significant suppression of absorbance by hemoglobin. The following interferences were noted: at a hemoglobin concentration of 5000 mg/liter, 70%; at 2500 mg/liter, 55%; at 1250 mg/liter, 10%; and at 1000 mg/liter or less, no interference.

Discussion

The measurement of phenytoin is of great value clinically. Measurement by gas/liquid chromatography is slow and may be difficult technically. It also requires at least 1.0 ml of serum, whereas the modification of the EMIT system proposed uses only 8 microliters in the assay. The EMIT technique is simple, rapid, and many samples may be processed at one time. The accuracy is quite satisfactory. Our reproducibility is somewhat better than that reported by Booker and Darcey (7).

Our modification increases the detectable range over previous immunoassay techniques and is reliable from 1–70 mg/liter. There is a slight systematic proportional bias when comparing EMIT assay to gas/liquid chromatographic assay. The slight bias is not significant clinically. The greater dilution of the reagents than recommended by the Syva Corp. diminishes the expense of the test. The use of Chemvarion has proved very effective as the base serum for our own standards. The standards prepared in our laboratory compare extremely well with those supplied by the company. Up to 10 sera may be analyzed in 5 min; reagent cost per sample is about 40¢, as compared to $1 for the standard EMIT procedure.

When we performed the assay in a kinetic mode, we observed nonlinearity for the first 45 s. To ensure complete linearity, we therefore read the absorbance change from 45 to 225 s, a total elapsed time of 3 min.

The specificity of the antibody has been shown in this assay. This is particularly important because patients taking phenytoin are often taking other drugs as well. The only interference we have observed is that of hemolysis, and this appears to be a very substantial problem.

References