Enzymatic Immunoassay vs. Gas/Liquid Chromatography for Determination of Phenobarbital and Diphenylhydantoin in Serum

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The antiepileptic drugs diphenylhydantoin and phenobarbital were measured in serum by a newly commercially available enzyme immunoassay procedure ("EMIT," Syva Corp.). The procedure requires <5 min and no more than 50 μl of serum per determination. It is simple; only four steps (pipetting and diluting with an automatic pipettor-dilutor) are required before spectrophotometry. Twenty replicate analyses of a serum containing phenobarbital and diphenylhydantoin gave results with a CV of 6.8% and 9.1%, respectively. Results attained in a large series of patients were compared with results by a gas/liquid chromatographic procedure. For phenobarbital \( r = 0.97 \), and for diphenylhydantoin \( r = 0.98 \). No false negatives or false positives were encountered.

The clinical value of determining the concentration of antiepileptic drugs in serum is well established and has recently been reviewed (1). Such results are most effective if the clinician knows them at the time the patient is in the clinic. Gas/liquid chromatography, widely used for this purpose, is complicated and time consuming. Relatively large samples are needed, even though the amount of serum that can be obtained from the patient is limited, particularly in the case of children. Immunoassay techniques that are simple, quick, and require small amounts of serum would offer some advantages.

Recently an enzymatic immunoassay ("EMIT"); Syva Corp., Palo Alto, Calif. 94309) has become commercially available for such measurements of two of the most frequently used antiepileptic drugs, diphenylhydantoin and phenobarbital. The enzyme, glucose-6-phosphate dehydrogenase (EC 1.1.1.49), has been coupled to the drug to be measured, and this drug/enzyme complex retains its enzymatic activity. The antibody binds with both native drug and drug/enzyme complex. Drug/enzyme complex bound to antibody is inactive. In the reaction of enzyme with substrate (glucose-6-phosphate), NAD\(^+\) is converted to NADH. Change in concentration of NAD\(^+\) is measured spectrophotometrically.

In the actual procedure, controlled amounts of antibody are added to the specimen. If the specimen contains the test drug, it and the antibody bind to one another. As an excess of antibody is added, the concentration of unbound antibody is directly related to the original concentration of the test drug. When the drug/enzyme complex is then added, the degree of inactivation of the enzyme is directly related to the concentration of unbound antibody (2).

In addition to the accuracy and reliability of any new procedure, the clinical validity of the results must be established. The clinical validity of determining the concentration of antiepileptic drugs in serum has predominantly been based on gas chromatographic methods. The purpose of the present study was to compare results obtained by the EMIT assay method with results obtained by a gas chromatographic method that we have used for several years.

**Materials and Methods**

**Gas/Liquid Chromatography**

(by a modification of 3)

**Apparatus:** We used an F & M Model 402 gas chromatograph (Hewlett-Packard, Avondale, Pa. 19311) equipped with dual hydrogen-flame detectors. Columns were of glass, U-shaped, 150 × 2 mm (i.d.). They were packed with 3% OV-17 on 80/100 mesh high-performance Chromosorb W (Hewlett-Packard). Carrier gas flow rate was 50 ml/min; hydrogen, 40 ml/min; and air, 280 ml/min. The column was maintained at 260 °C. The flash heater and detector were maintained at 290 °C.

**Procedure:** Add 0.5 ml of an aqueous solution of 5-(p-methylphenyl)-5-phenylhydantoin to 1 ml of each standard and unknown for the internal standard. Acidify the serum with 0.5 ml of HCl (0.5 mol/liter). Extract the drugs with CHCl\(_3\). Evaporate the CHCl\(_3\) and dissolve the residue in CS\(_2\). Inject an aliquot of the resulting solution onto the gas chromatograph column, and measure the resulting peaks.

The retention time for phenobarbital is 1.1 min, for primidone 6.0 min, for diphenylhydantoin 7.1 min, and for 5-(p-methylphenyl)-5-phenyl hydantoin 9.3 min.

**Immunoassay**

**Apparatus:** We used a Model 300 N microsample spectrophotometer equipped with a thermally regulated flowcell (Gilford Instrument Laboratories Inc., Oberlin, Ohio 44074) to measure enzymatic activity. The spectrophotometer is integrally connected to an EMIT printer-calculator. We made the sample and reagent volume measurements with an EMIT pipettor-dilutor.
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 either diphenylhydantoin or phenobarbital. The buffer solution and the calibrators are common to both determinations. Reagent A is the antibody/substrate reagent, and Reagent B is the drug/enzyme complex. These preparations are in lyophilized form and are reconstituted with distilled water. They are stable for three weeks if refrigerated when not in use.

Buffer solution: Tris(hydroxymethyl)aminomethane hydrochloride buffer (55 mmol/liter, pH 7.9), with added surfactant, is prepared from a buffer concentrate supplied with each reagent kit. The buffer solution is stable at room temperature.

Calibrators: A series of serum calibrators containing phenobarbital and diphenylhydantoin in concentration ranges 0–80 and 0–30 mg/liter, respectively, are provided as a lyophilized serum base preparation.

Procedure

The procedure has been standardized so that in each step 50 μl of active substance is diluted with 250 μl of the buffer. Use of an automatic pipettor/diluter, pre-set to these volumes, greatly facilitates and simplifies the procedure.

With the pipettor/diluter, transfer 50 μl of standard or unknown serum plus 250 μl of buffer to a 1-ml disposable beaker. This diluted sample may be used for as many as five assays. More consistent results are obtained by waiting 1 min after this initial dilution before proceeding. Transfer 50 μl of the diluted serum plus 250 μl of buffer to a second beaker. Add 50 μl of reagent A plus 250 μl of buffer. Next, add 50 μl of reagent B plus 250 μl of buffer. Immediately upon addition of reagent B, aspirate the contents of the beaker into the spectrophotometer flowcell. This automatically activates the printer/calculator to time and record the measurement. Two absorbance readings are made at 340 nm, the first after 15 s and the second after 95 s. The difference, ΔA, over the 80-s measurement period is used to calculate results.

When preparing the standard curve, determine ΔA of the negative calibrator (ΔA₀). Program the printer/calculator to automatically subtract ΔA₀ from subsequent standards and unknowns. Prepare a standard curve by plotting ΔA − ΔA₀ (EMIT units recorded by the printer/calculator) for each calibrator vs. concentration of drug. With EMIT graph paper (a modified logit function paper), a linear curve will be obtained. Convert the EMIT units of unknown samples to concentration by use of the standard curve.

Results

Precision: Within-day reproducibility of the gas chromatography was determined by the repeated analysis (20 times) of a serum containing phenobarbital (25 mg/liter) and diphenylhydantoin (12.5 mg/liter). The average phenobarbital concentration was 24.99 mg/liter, with a SD of 0.98 and a CV of 3.9%. The average diphenylhydantoin concentration was 12.52 mg/liter, with a SD of 0.28 and a CV of 2.2%.

Within-day reproducibility of the EMIT system was determined by 20 replicate analyses of a serum containing phenobarbital (20 mg/liter) and diphenylhydantoin (10 mg/liter). For phenobarbital the average concentration was 19.8 mg/liter, with a SD of 1.34 and a CV of 6.8%. For diphenylhydantoin the average concentration was 9.95 mg/liter, with a SD of 0.91 and a CV of 9.1%. Day to day variation was determined by analyzing on 25 days a calibration serum containing phenobarbital (30 mg/liter) and diphenylhydantoin (15 mg/liter). The average phenobarbital concentration was 31.1 mg/liter, with a SD of 2.27 and a CV of 7.3%. The average diphenylhydantoin
concentration was 15.3 mg/liter, with a SD of 0.87 and a CV of 5.76.

Specificity: To determine the specificity of the phenobarbital and diphenylhydantoin procedures, we examined a series of samples from patients receiving other antiepileptic drugs. Thirty-five samples from patients not taking phenobarbital or any drug that is metabolized to phenobarbital but receiving diphenylhydantoin and/or other drugs and 45 samples from patients not receiving diphenylhydantoin but receiving phenobarbital and/or other drugs were analyzed for diphenylhydantoin and phenobarbital by both gas chromatography and EMIT. No false positives or false negatives were encountered.

Comparison. To compare the results of the immunoassay procedure with those of gas chromatographic analysis, we analyzed 202 specimens containing phenobarbital and 197 specimens containing diphenylhydantoin by both methods. The specimens were obtained from patients attending the seizure clinic. EMIT analysis was done in duplicate on these specimens. For phenobarbital, EMIT 1 vs. EMIT 2: r = 0.97, R² = 3.8 mg/liter, intercept = 0.41, and slope = 0.98. For diphenylhydantoin EMIT 1 vs. EMIT 2: r = 0.96, R² = 2.52 mg/liter, intercept = 1.02, and slope = 0.92. (R² is standard error of the estimate.)

For phenobarbital (Figure 1), r = 0.97, R² = 3.8 mg/liter, intercept = 2.15 mg/liter, and slope = 0.79. For diphenylhydantoin (Figure 2), r = 0.98, R² = 1.80 mg/liter, intercept = -0.90 mg/liter, and slope = 1.09.

Discussion

Determination of the concentrations of antiepileptic drugs in serum has become routine in treatment of seizure disorders. While various methods are available, most laboratories currently use gas chromatography. The new EMIT enzyme immunoassay procedure was assessed in this study.

Gas chromatography is time consuming, and results are often not available until the next day. Fortunately, the clinician should know the results of the drug analysis at the time of the patient’s clinic visit. In addition, most such methods require 1 or more milliliters of serum, and, particularly in children, the volume of blood that can be obtained is limited.

The EMIT system offers a potential solution to these problems. Individual analyses can be done in <5 min and as many as five determinations can be performed on 50 µl of serum. The standardized commercially available reagents make precise weighings unnecessary in the individual laboratory. The reagents are nonradioactive, precluding the precautions necessary with radioimmunoassay procedures. The procedure is simple, requiring only four pipetting-diluting steps before results are measured spectrophotometrically. The volumes to be measured are standardized, so that a single setting of an automatic pipettor/dilutor is used. The printer/calculator automatically times the enzyme reaction and prints the results. The EMIT system gives reproducible results, and we have encountered no false positives or false negatives. The procedure is precise, and results obtained correlate well (r = 0.97 for phenobarbital, 0.98 for diphenylhydantoin) with those obtained by a gas chromatographic procedure used in this clinic for several years. Pippenger et al. report similar results. In this study the EMIT analyses were done in duplicate. Correlation between the results was very high (r = 0.96 for phenobarbital, 0.97 for diphenylhydantoin). No significant difference was seen when the gas-chromatographic results were correlated with either the first or second EMIT results or the average of the two. Thus in routine practice a single EMIT analysis should be satisfactory.

Sensitivity for extremely low or high concentrations was not determined in the present study. However, the system was specifically designed to operate in the ranges of concentrations ordinarily present in most patients taking these drugs, including the generally accepted therapeutic range, the low or inadequate range, and the high or toxic range.

The CV with the immunoassay was larger than that for gas chromatography. However, one of us (H.E.B.) was the clinician in charge of the seizure clinic from which these specimens were obtained. Based upon a review of the results, decisions regarding bioavailability and dosage adjustments would have been the same, whichever set of results had been used as a guide.

One current disadvantage of the EMIT system is that it is limited to the analysis of diphenylhydantoin and phenobarbital. It is anticipated that reagents for the determination of primidone, ethosuximide, and carbamazepine concentrations will soon be available.

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References


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