concentrations from 1 to 10 mg/L, were re-analyzed after cold storage for seven days in Teflon-lined screw-capped culture tubes; benzene averaged 92% of the originally measured concentrations. The slightly greater loss from this set of samples than from the holding-time-study samples was probably due to the greater headspace in the storage tubes. Dilute standards of benzene in toluene stored at room temperature are stable for up to a week, but solutions of benzene in water are unstable and must be prepared freshly each working day.

We thank Dr. Thomas Fritz (Biological and Medical Research Division, Argonne National Laboratory) for supporting this work and supplying the sample materials. This work was performed under the auspices of the United States Department of Energy.

Results by Fluorescent Immunoassay for Phenytoin Compared with those by Enzyme Immunoassay, Liquid Chromatography, and Discrete Analysis (Dupont aca)

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We describe the Ames Fluorescent Enzyme Immunoassay procedure for the detection of phenytoin in serum, and compare the results with it with those by enzyme immunoassay (EMIT), "high-pressure" liquid chromatography, and the Dupont aca. Sera from 48 patients who were receiving phenytoin were promptly analyzed in duplicate by EMIT, frozen, then assayed within four weeks by the other three methods. Correlation was good between results by the fluorescent immunoassay and the other methods. The fluorescent immunoassay procedure is sensitive and requires a small sample volume (100 μL). Sample reading time can be shortened from 30 to 15 s as the technician becomes more proficient, with no sacrifice of accuracy. The procedure is fast, easy to perform, specific, sensitive, and inexpensive; involves no radioactivity; and requires no temperature control. It is the current method of choice for detection of phenytoin in serum, in terms of precision and accuracy.

Additional Keyphrases: drug assay - anticonvulsant drugs

The fluorescent immunoassay for phenytoin, from Ames Co, Elkhart, IN 46514, is based on the principle of competitive protein binding. Phenytoin is labeled with a derivative of the fluorogenic enzyme substrate umbelliferyl-β-D-galactoside that is not fluorescent under the conditions of the assay. On hydrolysis catalyzed by β-galactosidase (EC 3.2.1.23), the product is fluorescent. When antibody to phenytoin reacts with this substrate, the product is virtually inactive as a substrate for β-galactosidase. Competitive binding reactions are set up with a constant amount of antibody to phenytoin. The substrate and phenytoin in the serum sample compete for antibody-binding sites, and any unbound substrate is hydrolyzed with β-galactosidase to produce the fluorescent product. The intensity of this fluorescence is related to the phenytoin concentration by means of a standard curve.

We therefore analyzed sera from 48 patients being treated with phenytoin by using this assay, enzyme immunoassay (1), "high-pressure" liquid chromatography (2), and the Dupont aca discrete analyzer. The data correlated well statistically (3), not only with this fluorescent assay but also with one another, for all except the chromatographic method, which gave lower values, especially at higher concentrations of phenytoin.

Phenytoin is an effective drug in the therapy of grand mal epilepsy. Its therapeutic concentration in serum ranges from 10 to 20 mg/L. Although concentrations above this range benefit some patients, toxic symptoms such as ataxia, nystagmus, and lethargy have been reported when concentrations exceed 30 mg/L (4). Because the difference between therapeutic and toxic concentrations is narrow, and effective dosage may vary substantially for different patients (5), an accurate, precise, and specific method for the emergency measurement and monitoring of phenytoin is important.

Materials and Methods

Samples

Sera from 48 patients were sampled from specimens submitted for emergency testing from hospitalized or outpatients.
Some of these patients were known epileptics who were receiving phenytoin as anticonvulsant therapy; others were receiving the drug for the treatment of other convulsions. The specimens selected were representative of subtherapeutic, therapeutic, and toxic concentrations of the drug. Severely hemolytic, lipemic, or icteric samples were not included in our assays, nor was plasma. The drug was quantitated by the enzyme immunoassay (EMIT) and other aliquots were stored frozen. Within four weeks all samples were assayed by the other three methods.

Instrumentation

**Enzyme immunoassay (EMIT).** For the EMIT procedure we used a Stasar III spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH 44074) equipped with a temperature-controlled cuvette (thermocuvette) and a Model 1000 computer-printer (Syva Co.). To dilute samples and reagents, we used a Model 1500 automatic pipetter-diluter (Syva Co.). With the instrument in the absorbance mode, de-ionized water was aspirated and the display was set at 1000 with the zero-control knob. The mode control was switched to "concentration," and the display was set to 2.667 with the concentration calibration knob. The display was adjusted to zero with the "zero-control" knob.

**Liquid chromatography.** We used a Model 65T "high-pressure" liquid chromatograph (Perkin-Elmer Corp., Norwalk, CT 06856) that included an ODS-Sil-X-1 high-capacity reversed-phase column, 0.25 X 25 cm, together with a Model 56 LC-65T variable-wavelength UV-VIS detector or spectrophotometer and a Model 56 chart recorder (all from Perkin-Elmer). The instrument was equipped with a Rheodyne Model 7105 Injection System (a syringe-loading sample-injector). Special labware included 1-mL disposable polypropylene microcentrifuge tubes with caps, which we used in a Model 152 high-speed microcentrifuge (Spinco Div., Beckman Instruments, Irvine, CA 92713).

**Fluorescent immunoassay.** For the fluorescent immunoassay we used a fluorocolorimeter (Aminco, Silver Spring, MD 20910) equipped with a 405-nm narrow-bandpass interference (excitation) filter and no. 5-56 (blue), no. 3-73 (yellow) glass (emission) filters.

**Dupont (aca).** We also analyzed for phenytoin with the aca, equipped with their Computer II (Dupont Instruments, Wilmington, DE 19898).

**Reagents**

**Enzyme immunoassay (EMIT).** Reagent A—which contains the antibody against phenytoin, the enzyme substrate glucose 6-phosphate, and NAD+—was reconstituted with 6 mL of de-ionized water. Reagent B, which contains phenytoin labeled with the enzyme glucose-6-phosphate dehydrogenase (EC 1.1.1.49), was reconstituted with 6 mL of de-ionized water.

After reconstitution, both reagents were stored at room temperature for at least 8 h before use. Buffer solution containing a surfactant was prepared by diluting the 10-mL vial of buffer concentrate to 150 mL with de-ionized water.

All samples, calibrator standards, and controls were diluted fivefold with the buffer solution with a diluter (Syva Co.) before assay.

**Fluorescent immunoassay.** The reagents were obtained from the Ames Co. Concentrated N,N-bis(2-hydroxyethyl)-glycine (Bicine) buffer (pH 8.5) was diluted 20-fold with distilled water. The concentrated antibody–enzyme reagent, containing β-galactosidase and antiserum to phenytoin, was diluted 30-fold with the Bicine buffer solution. The "fluorogenic phenytoin reagent" containing β-galactosyl-umbeliferone–phenytoin conjugate in formate buffer was used undiluted. The "range adjustment solution," which contains 7-hydroxycoumarin-3-[N-(2 hydroxyethyl)]carboxamide in formate buffer, was not diluted. Five standards containing 0, 5, 10, 20, and 30 μg of phenytoin per liter of human serum were used.

The procedure for the phenytoin fluorescent immunoassay is as follows: Into a 13 X 100-mm glass test tube, place 5.0 mL of the dilute buffer solution and 100 μL of standard, control, or serum sample. Cover the tubes with Parafilm and mix well; run tests in duplicate. To two separate cuvettes, each containing 3.0 μL of the diluted antibody/enzyme reagent, add 100-μL aliquots of the above dilutions and mix well. While starting a timer, add 100 μL of the fluorogenic phenytoin reagent to the cuvette and mix immediately. Repeat this step at 30-s intervals for the remaining standards and serum samples.

Incubate the tubes at room temperature for exactly 20 min, then read in sequence at 30-s intervals, so that each cuvette is incubated with the fluorogenic phenytoin reagent for exactly the same interval during each run. Make a standard curve by plotting the average fluorescence intensity of each pair of standard solutions (0, 5, 10, 20, 30 μg of phenytoin per liter) vs concentration.

**Liquid chromatography.** We prepared the samples by the method of Soldin and Hill (6). The extracting reagent, acetonitrile, contained an internal standard (cybeptamide; Pierce Chemical Co., Rockford, IL 61105), 80 mg/L. Standards were prepared in drug-free sera to which we added phenytoin (Sigma Chemical Co., St. Louis, MO 63178) in methanol. We placed 200 μL of the sample, control, or standard in a Microtainer Capillary Blood Serum Separator (Becton Dickinson Co., Rutherford, NJ 07070) that had the inert barrier material removed, then added 200 μL of the extracting reagent containing internal standard. The tubes were capped to avoid evaporation and their contents vortex-mixed to extract the drug and to precipitate the proteins. The tubes were then centrifuged (9000 X g) for 1 min. We then injected 10 μL of the samples or standard into the liquid chromatograph, which had a mobile phase consisting of acetonitrile and phosphate buffer, 0.19 mol/L, pH 4.4 (20/80 by vol). Absorbance at 195 nm was measured. Concentrations were calculated from peak-height ratios of phenytoin to internal standard.

**Dupont aca.** The aca phenytoin method is an adaptation of Syva's homogeneous EMIT procedure. Samples giving results for phenytoin >30 mg/L were diluted with a solution of serum albumin (Armour Pharmaceutical Co., Tarrytown, NY 10591).

Results

Between-lot reproducibility was determined by assaying a group of 48 serum samples with two different lot numbers of reagents. The results were essentially the same, giving data that after linear regression resulted in a slope of 0.9944, a y-intercept of -0.0110, and a correlation coefficient of 0.9957.

Within-run precision was estimated from results for duplicate analyses on serum samples having low, therapeutic, and toxic concentrations of the drug. The comparison between fluorescent immunoassay and EMIT gave a slope of 0.9862, a y-intercept of -0.2383, and a correlation coefficient of 0.9954 (n = 45). The correlation with the Dupont aca method gave a slope of 0.9436, a y-intercept of -0.2846, and a correlation coefficient of 0.9752 (n = 48). Of the three methods, the chromatographic results were in general lower than by fluorescent immunoassay; for two samples, the difference was 20%. The comparison of the latter two procedures gave a slope of

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1.025, a y-intercept of 0.3886, and a correlation coefficient of 0.9894 (n = 48).

A standard curve was prepared for each run of 12 patients each. The fluorescence values were plotted against the standards, which were subjected to the same procedure as the patients' samples and a control.

Day-to-day precision was estimated by daily analysis over a four-week period of three controls with low, therapeutic, and toxic concentrations of the drug. The mean, SD, and CV for the respective controls were 7.1 ± 0.365 μg/L (CV 5.1%), 14.4 ± 0.390 μg/L (CV 2.7%), and 26 ± 0.129 μg/L (CV 0.5%).

Within-run precision was determined from results for 24 runs of a control of known concentration of the drug. This gave a mean value of 14.06 (SD 0.187) μg/L and a CV of 1.33%. Although the actual fluorescence intensity of the reading for the standard shifted from day to day, the actual concentration of the control and patients did not change significantly. For the Ames procedure, the standards were first diluted 50-fold with a buffer.

In the final procedure we mixed 100-μL aliquot of the diluted sample with 3.0 mL of antibody/enzyme reagent. The time of addition of 100 μL of the fluorogenic reagent was considered time zero; this step was repeated every 30 s with each subsequent sample. After the 20-min incubation we read the fluorescent intensity of each sample at 30-s intervals. We found that thorough mixing of the fluorogenic reagent is critical. Precision (reproducibility) improved if samples were remixed during the incubation.

Discussion

Many techniques have been developed for the detection and measurement of levels of antiepileptic drugs in serum. Some of these procedures, such as gas chromatography, have the disadvantage of lengthy extractions. Other techniques require large sample volume, making them particularly unwieldy for pediatric patients.

The fluorescent immunoassay offers several advantages. The use of nonradioactive reagents precludes precautions with radioimmunoassay procedures, and the reagent shelf-life of approximately two years makes the assay more economical.

Linear regression and cumulative data indicate a good correlation between the fluorescent immunoassay and the other three methods. The values obtained for the patients' samples and controls, with two different lots of reagents, were essentially the same. The methodology is sensitive and requires a small sample size (100 μL). After the initial incubation of 20 min, samples are read every 30 s; as technicians become more proficient in the procedure, this could be reduced to 15 s without sacrificing accuracy.

The instrumentation is inexpensive, and no temperature control is required. At present, we consider this the method of choice for precision and accuracy.

References