Dry-Reagent Strips for Measuring Phenytoin in Serum

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A new reagent strip has been developed for measuring phenytoin in serum or plasma. It utilizes the chemistry of the ApoEnzyme Reactivation Immunoassay System (ARIS) and is designed for use with the Seralyzer® reflectance photometer. The 90-s test relies on comparison of strip reactivities with a two-point calibration line, which can be validly stored in the instrument for two weeks. Within-run CVs for controls at five concentrations ranged from 2.7 to 5.6%, between-run CVs from 2.3 to 6.3%. Phenytoin values obtained for clinical serum samples correlated well ($r > 0.99$) with those obtained by liquid chromatography or substrate-labeled fluorescent immunoassay. The speed and convenience of this assay for monitoring phenytoin in serum make it well suited for decentralized test sites such as emergency rooms, urgent-care centers, and physician's offices.

Phenytoin, an anticonvulsant drug, has a relatively narrow therapeutic range in which it is effective against major generalized seizures (1–3). Because seizure control is directly related to the concentration of phenytoin in plasma (4), and because a given dose of phenytoin can result in various concentrations of the drug in plasma or serum (5), frequent assessment of its concentration is necessary for maintaining therapeutic values while avoiding higher, potentially toxic concentrations.

Numerous methods have been used to assay phenytoin in serum. These include spectrophotometry (6), oxidation (7, 8), gas–liquid chromatography (9–11), liquid chromatography (12, 13), radioimmunoassay (14), enzyme-mediated immunoassay (15), substrate-labeled fluorescent immunoassay (16), and fluorescence polarization immunoassay (17). We report here the development and evaluation of a dry reagent strip assay for phenytoin in serum and plasma. This homogeneous immunoassay method utilizes the ApoEnzyme Reactivation Immunoassay System (ARIS) (18, 19). In this system (Figure 1) a conjugate of phenytoin linked to flavin adenine dinucleotide (FAD) competes with phenytoin in the sample for the limited number of binding sites on the phenytoin-specific antibody. Phenytoin–FAD conjugate bound by antibody is unable to reactivate apoglucose oxidase, the inactive enzyme that remains after FAD is removed from glucose oxidase (EC 1.1.3.4). Conjugate not bound by antibody binds to the apoglucose oxidase and reconstitutes the active enzyme. The reactivated glucose oxidase converts glucose to gluconolactone and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide oxidizes the indicator 3,3',5,5'-tetramethylbenzidine (TMB) to a blue product. This charge transfer complex formed between reduced and oxidized TMB (20) absorbs maximally at 660 nm, but routinely the reflectance of the phenytoin reagent strips is measured at 740 nm, to be within the optimal operating range of the Seralyzer reflectance photometer (Ames Division, Miles Laboratories, Inc., Elkhart, IN 46515).

Materials and Methods

Apparatus. For single-wavelength measurements we used a Seralyzer® reflectance photometer with a 740-nm, three-cavity interference filter (Ditric Optics, Hudson, MA). The data generated by the photometer were collected with and analyzed by a HP-85 computer (Hewlett Packard, Palo Alto, CA 94304). Reflectance spectra measurements were obtained with a rapid scanning reflectometer, an instrument

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previously described (21). We also used MLA™ pipettes (Medical Laboratory Automation, Inc., Mt. Vernon, NY 10550) and Dip-Stat™ pipettes (Godax Laboratories, Inc., New York, NY 10013).

Reagents. Goat antiserum to phenytoin was prepared by immunizing goats with phenytoin conjugated to keyhole limpet hemocyanin, and goat antiserum to glucose oxidase was prepared by immunizing goats with glucose oxidase. Apoglucoase oxidase and phenytoin–FAD conjugate were prepared as previously described (19). Peroxidase (EC 1.11.1.7; Grade VI) was from Sigma Chemical Co., St. Louis, MO 63178. Glucose was from Mallinckrodt, Inc., St. Louis, MO 63147. The Ames TDA™ Phenytoin Kit was from Ames Division.

Procedures. The reagent paper was impregnated in two steps. In the first step, filter paper was dipped in an aqueous solution containing goat antibody to phenytoin, apoglucoase oxidase complexed to goat antibody to glucose oxidase, peroxidase, glucose, and buffer, and then dried in a stream of warm air. The antibody to glucose oxidase allows the apoglucoase oxidase to be reactivated by phenytoin–FAD at 37 °C (22). In the second step, the paper was dipped in an organic solvent containing phenytoin–FAD and TMB, then dried in warm air. The treated paper was then cut into 0.5 x 1 cm sections and mounted with double-sided adhesive on 0.5 x 8.3 cm polystrene supports. The phenytoin reagent strips were stored in capped glass bottles with silica gel and molecular sieves desiccant materials.

Clinical samples, calibrators, and controls were diluted by dispensing 30 μL of sample, with a MLA pipette, into 800 μL of distilled water dispensed from a Dip-Stat. The 30-μL MLA pipette was again used to apply diluted sample to the reagent pad to begin the reaction. Reflectance was measured every 5 s with the reflectance photometer and the results were converted into the corresponding Kubelka–Munk ratio (23), K/S (K/S = (1 – R)^2/R, where K is the absorption coefficient, S is the scattering coefficient, and R is the reflectance). To determine the phenytoin concentration we used the rate of change in K/S from 70 to 90 s.

For comparison, we measured phenytoin by the substrate-labeled fluorescent immunoassay as described in the Ames TDA product insert, and by liquid chromatography as previously reported (24).

Results

Strip response. Figure 2 shows the kinetic responses of a series of reagent strips prepared with various antibody/conjugate ratios when samples containing no phenytoin were applied. With increasing ratios of antibody to phenytoin relative to phenytoin–FAD the reactivation of apoglucoase oxidase by phenytoin–FAD is inhibited. At high antibody/conjugate ratios, this inhibition exceeds 95%. Figure 3 shows the plot of the K/S at 90 s vs the ratio of anti-phenytoin to phenytoin–FAD conjugate for samples with and without phenytoin. The region between the two curves corresponds to the valid working range for an immunoassay for phenytoin. The best linearity for the dose–response curve was obtained with the strip formulation containing 590 μL of antisera per nanomole of phenytoin–FAD in conjunction with a rate measurement of K/S from 70 to 90 s. Figure 4 shows that the response of the strip varied linearly with phenytoin concentration up to 40 mg/L, which is well beyond the therapeutic range for this drug. This allowed the use of a two-point calibration at 5 and 25 mg of phenytoin per liter.

Precision and accuracy. Precision of the assay were determined by calibrating the Seralyser Reflectance Photometer and assaying serum controls in the range of 5 to 30 mg/L, in triplicate. This was repeated 20 times. Table 1 summarizes the within-run and between-run precision of the assay. The bias is <0.5 mg of phenytoin per liter for all concentrations tested.

Analytical recovery. For these studies we assayed samples prepared by mixing equal volumes of clinical samples and phenytoin standards. For concentrations ranging from 6.1 to 34.2 mg/L, the mean recovery was 98% (range 87–107%).

Comparison with other methods. Clinical serum specimens were assayed for phenytoin by the present method and by liquid chromatography (Figure 5) and substrate-labeled fluorescent immunoassay. For the comparison with Ames

Fig. 2. Kinetic responses of reagent strips (prepared with phenytoin–FAD, 250 nmol/L, and various amounts of goat antiserum to phenytoin) to the application of 30 μL of diluted serum not containing phenytoin. The antibody/conjugate ratio tested were (top to bottom): 0, 150, 290, 440, 590, 730, 890, 1050, and 1170 μmol

Fig. 3. Titration the anti-phenytoin antiserum on reagent strips via the K/S response at 90 s of the strips prepared as stated in Fig. 2. The samples were dilutions of serum containing either 0 or 40 mg of phenytoin per liter

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System calibration. The validity of using a stored calibration line was tested by assaying eight clinical serum specimens at two- to four-day intervals during two weeks, after calibrating the Seralyzer on the first day (day 0). Clinical samples were subdivided and stored frozen; freshly thawed aliquots were used for testing at each time point. The data in Table 2 show that phenytoin concentrations can be determined from a calibration line for two weeks after it is established.

Cross reactivity. We tested for cross reactivity drugs that might be prescribed with phenytoin, compounds with chemical structures similar to phenytoin, and phenytoin metabolites. At a concentration of 1 g/L in serum the following compounds increased the apparent value for a 15 mg/L phenytoin control by <20%: acetaminophen, acetylsalicylic acid, amobarbital, barbital, butabarbital, caffeine, carbamazepine, ethosuximide, fluphenazine·2HCl, hexobarbital, p-hydroxyphenobarbital, indomethacin, mepobarbital, meprobamate, methaqualone, methyuximide, methyprylon, phenylbutazone, phensuximide, primidone, and secobarbital. Compounds which exhibited greater than 20% cross reactivity were retested at lower concentrations. Table 3 shows the concentrations at which they were 20% cross reactive. The metabolite HPPH shows 20% cross reactivity at 7 mg/L; however the HPPH concentration in serum is seldom >0.5 mg/L and that of its glucuronide is usually <5 mg/L (25).

Interferences. We examined some endogenous compounds for potential interference with the present assay. We added phenytoin to dilutions of clinical serum specimens containing above-normal amounts of the substance being tested for interference. None interfered at the highest concentrations tested, which were: total bilirubin (100 mg/L), uric acid (160 mg/L), cholesterol (5 g/L), and triglycerides (7 g/L).

Interference from hemoglobin was studied by adding hemolysate to diluted phenytoin-containing control sera. Hemoglobin did not interfere at 1 g/L but decreased the apparent phenytoin concentration by 2 mg/L for each gram per liter increase above this value; therefore, hemolysed samples must not be used.

Interference by other compounds was also tested by supplementing the diluted samples of phenytoin controls with the compound of interest. Ascorbic acid reduces the apparent phenytoin value by approximately 17 µg/L for each milligram of ascorbic acid per liter. No substantial decrease in the assayed phenytoin values should result from the ascorbic acid values usually found in serum, 5–15 mg/L. Sodium fluoride was found to decrease the apparent phenytoin value by up to 0.4 mg/L for each gram of sodium fluoride per liter. Thus samples should contain no fluoride. Anticoagulant compounds, at the indicated concentrations did not interfere: disodium EDTA (10 g/L), heparin (100 000 USP units/L), sodium oxalate (10 g/L), and trisodium citrate (20 g/L). However, when plasma was sampled from patients taking phenytoin, only heparinized plasma gave plasma phenytoin values that were consistently the same as concurrently drawn serum samples. Thus heparin is the only anticoagulant that should be used with samples for this assay.

Discussion

A dry-reagent immunoassay for the monitoring of theophylline in serum has previously been described (26). We describe here a colorimetric dry-reagent immunoassay for the quantitative determination of phenytoin in serum. The
Table 2. Two-Week Calibration Hold Study

<table>
<thead>
<tr>
<th>Assayed phenytoin concon, mg/L</th>
<th>Sample mean</th>
<th>SD</th>
<th>CV, %</th>
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<td>14.5</td>
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<td>11</td>
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<td>9</td>
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<td>13.1</td>
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<td>25.6</td>
<td>25.9</td>
<td>26.0</td>
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</table>

Daily mean

14.8

Table 3. Concentration of Compounds Yielding a 20% Increase in the Apparent Concentration of Phenytoin

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration, mg/L</th>
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<tr>
<td>5-Ethyl-5-phenylhydantoin</td>
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<tr>
<td>Mephenytoin</td>
<td>200</td>
</tr>
<tr>
<td>5-Methyl-5-phenylhydantoin</td>
<td>200</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>400</td>
</tr>
<tr>
<td>Diazepam</td>
<td>500</td>
</tr>
<tr>
<td>Glutethimide</td>
<td>100</td>
</tr>
<tr>
<td>HPPH (5-(p-hydroxyphenyl)-5-phenylhydantoin)</td>
<td>7</td>
</tr>
<tr>
<td>HPPH glucuronide</td>
<td>400</td>
</tr>
</tbody>
</table>

*15 mg/L in control serum

We appreciate helpful discussions with Dr. Stephan Thompson and Patricia Rupchock and the excellent technical assistance of Charlotte Acrey, Denise Clay, Joan Clayborn, Marie Clements, Cathy Halter, Oseola Skinner, and Lisa VanHouten.

References

approach has general utility in the application to therapeutic drug monitoring (27).

To incorporate all of the reagents necessary for the ARIS assay into reagent paper it is necessary to use two solvents as described earlier (19, 26, 28, 29). This keeps the phenytoin–FAD from reacting prematurely with either the antibody to phenytoin or the apoglucone oxidase.

The reagent strip has low cross reactivity to drugs that may be prescribed along with phenytoin and to compounds with chemical structures similar to that of phenytoin. The reagent strip does cross react with the phenytoin metabolite HPPH (Table 3), but this is not of concern because of the usually low concentrations of HPPH in serum (25). However, HPPH may accumulate in the serum of uremic patients (30), so phenytoin values obtained for samples from uremic patients should be interpreted with extreme caution.

No interference was observed with samples containing abnormally high concentrations of uric acid, bilirubin, lipids, or <1 g of hemoglobin per liter. Heperinized plasma performs well with the assay; other anticoagulants should be avoided.

With the optimized formulation, response is linear over the entire test range. The system requires only a two-point calibration, which can validly be stored for as long as two weeks, to minimize reagent use. Incorporation into a dry reagent format eliminates all reagent manipulations, and no separation steps are required. A single dilution of a 30-μL sample is all that is ordinarily required to perform the assay. The assay is rapid, accurate, precise, and exceptionally easy to perform.

The format for the phenytoin immunoassay is particularly appropriate for those facilities where rapid testing is desirable, such as the emergency room, urgent-care centers, or in the physician's office (31).
The Lability of Estrogen Receptor: Correlation of Estrogen Binding and Immunoreactivity

Frederic Clayton and James Wu

We compared the Abbott enzyme immunoassay method for estrogen receptor with a standard dextran-coated charcoal method, and studied the effects of treatments causing denaturation. Estrogen receptor values were slightly higher by the Abbott method, but the methods agreed with regard to receptors being present or absent in 94–98% of 50 cases. Heat lability of immunoreactivity by the Abbott enzyme immunoassay is comparable to the lability of estrogen binding by the estrogen receptor. Estrogen and molybdate substantially stabilize estrogen receptor during the assay, but improper handling of tissue before the assay may cause similar, substantial decreases in estrogen receptor by either method. The Abbott method is easier to use than the dextran-coated charcoal method, requires less tissue, and measures receptor with or without endogenously bound estrogen, but reagent cost is high.

Additional Keyphrases: enzyme immunoassay • dextran-coated charcoal procedures compared • variation, sources of • sample handling • cancer

The marked lability of estrogen receptor is a serious problem, which undoubtedly is responsible for falsely negative results for estrogen receptor. Substantial decreases in estrogen receptor activity have been documented after a delay in cooling the tissue (1), after mastectomy as opposed to biopsy (1, 2), after electrocautery (3), and after prolonged storage except at -70 °C (4). Furthermore, a falsely negative result is clearly the most clinically deleterious error that could occur. One may hope that this problem could be averted by studying immunoreactivity, which in other proteins may be preserved even when biological activity is lost. We have studied the heat inactivation of estrogen receptor by the Abbott enzyme immunoassay (ER-EIA) method in parallel with a conventional dextran-coated charcoal (DCC) technique.

Materials and Methods

Breast carcinoma tissue, promptly frozen and stored at -70 °C until analysis, was homogenized and diluted to approximately 2 mg of protein per milliliter, by use of the method and reagents of the NEN estrogen receptor [125I] RIANEN assay system (New England Nuclear, North Billerica, MA 01821; cat. no. NEA-089). This six-point dextran-coated charcoal procedure measures estrogen receptor binding capacity in tissue cytosol (5). The homogenized and diluted breast tissue extract was then assayed by that method or by the Abbott monoclonal enzyme immunoassay (ER-EIA) for the quantitative measurement of human estrogen receptor in tissue cytosol (Abbott Laboratories, Diagnostics Division, North Chicago, IL 60064) (6) after treatment as described below.

Results

Comparison with the dextran-coated charcoal (DCC) method. Estrogen receptor analysis was performed simulta-