Substrate-Labeled Fluorescent Immunoassay for Phenytoin in Human Serum

Raphael C. Wong, John F. Burd, Robert J. Carrico, Robert T. Buckler, Jerry Thoma, and Robert C. Boguslaski

A homogeneous substrate-labeled fluorescent immunoassay has been applied to the measurement of phenytoin concentrations in human serum. We coupled a fluorogenic enzyme substrate, galactosyl-umbelliferone, covalently to a derivative of phenytoin. Under assay conditions, this drug–substrate conjugate was nonfluorescent but became fluorescent upon hydrolysis catalyzed by bacterial β-galactosidase. When antibody to phenytoin is bound to the drug–substrate conjugate, it is inactive as an enzyme substrate. Addition of phenytoin to competitive-binding reactions relieves the inactivation, and the resulting fluorescence is proportional to the phenytoin concentration. We validated the fluorescent immunoassay by comparing values for phenytoin obtained with this technique to those obtained by gas chromatography and by enzyme immunoassay (EMIT®). All three methods correlated well. The major metabolite of phenytoin, 5-(p-hydroxyphenyl)-5-phenylhydantoin, and other drugs at concentrations expected in serum had no effect on the assay. The fluorescent immunoassay is rapid and simple to perform and requires only 2 μL of serum sample per test.

Additional Keyphrases: anticonvulsants • competitive protein-binding assay • monitoring drug therapy • fluorescent enzyme immunoassay

Phenytoin (PHT) is a widely used antiepileptic drug with a narrow therapeutic range. Its determination in human serum can assist physicians in prescribing the appropriate dosage (1). The major assay methods for PHT are EMIT (2), gas chromatography (3), “high-pressure” liquid chromatography (4), and radioimmunoassay (5).

We have developed several nonradioisotopic immunoassay procedures (6–8) and have used one of these, substrate-labeled fluorescent immunoassay (SLFIA), to determine therapeutic drugs such as gentamicin (9) and tobramycin (10). We have now extended this assay to the measurement of serum PHT.

The principles of the SLFIA are shown schematically in the following reaction:

Enzymatic Reaction:

\[
\beta G-U-PHT \xrightarrow{\text{enzyme}} G + U-PHT \quad \text{(fluorescent product)}
\]

Antibody-binding Reaction:

\[
\beta G-U-PHT + Ab \xrightarrow{\text{enzyme}} \beta G-U-PHT:Ab \quad \text{no reaction}
\]

Competitive-binding Reaction:

\[
\beta G-U-PHT + Ab + PHT \xrightarrow{\text{enzyme}} \text{fluorescent product}
\]

PHT is labeled with a fluorogenic enzyme substrate, β-galactosyl-umbelliferone (βG-U). Under assay conditions, the βG-U-PHT conjugate is nonfluorescent; but when treated with an enzyme, a fluorescent product is produced. When the βG-U-PHT is bound by a specific antibody (Ab), a complex is formed that prevents interaction of the bound βG-U-PHT with the enzyme. Addition of PHT from the serum samples results in a competition with the βG-U-PHT for binding sites on the antibody, thus reducing the amount of antibody-bound βG-U-PHT. Hence the amount of βG-U-PHT available for reaction with the enzyme is proportional to the concentration of PHT being assayed. This fluorescent immunoassay does not require separation steps because the βG-U-PHT bound to antibody does not react with enzyme. In addition, a relatively small volume of serum is needed and the fluorescent assay is rapid and simple.

Materials and Methods

Instruments

Fluorescence was measured with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, MD 20910) with excitation and emission wavelengths set at 400 and 450 nm, respectively. Polystyrene disposable cuvets (Evergreen Scientific, Los Angeles, CA 90058) were used for assay measurements. All fluorescence determinations were measured at room temperature. Fluorescence excitation and emission spectra were uncorrected.

A Model 2100 gas chromatograph equipped with a flame-ionization detector (Varian Instrument Co., Palo Alto, CA 94303) was used to determine PHT in serum. The chromatography was run on a 0.2 × 91 cm glass column packed with a mixed phase of 2% SP2110 and 1% SP2510-DA on 100/120 Supelcoport (Supelco Inc., Bellefonte, PA 16823) under the following conditions: column temperature, 230°C isothermal;
injection temperature, 300 °C; detector temperature, 275 °C; nitrogen carrier gas at 40 mL/min.

For correlation studies with EMIT, absorbances were measured at 340 nm with a Model 26 double-beam spectrophotometer, a heated sipper cell, and a Model 701 printer/calculator (all from Beckman Instruments, Inc., Irvine, CA 92713). Other spectrophotometric measurements were made with a Cary Model 16 spectrophotometer (Varian Instrument Co.).

Reagents

**Enzyme.** β-Galactosidase (EC 3.2.1.23) (from *Escherichia coli*, grade IV; Sigma Chemical Co., St. Louis, MO 63178) was assayed at 25 °C in 50 mmol/L Bicine buffer (see below), pH 8.2, containing, per liter, 15.4 mmol sodium azide and 3 mmol o-nitrophenyl-β-D-galactoside. Under these conditions, the millimolar extinction coefficient for the product of this reaction, o-nitrophenol, is 4.27 at 415 nm. One unit of enzyme activity hydrolyzes 1.0 μmol of substrate per min under these conditions.

**Chemicals.** Bicine buffer, N,N-bis(2-hydroxyethyl)glycine (Grade A, Calbiochem, LaJolla, CA 92037), 50 mmol/L, was used at pH 8.2 (25 °C). Sodium azide was purchased from Fisher Scientific Co., Fair Lawn, NJ 07410.

2-Hydroxybenzophenone, N-(4-bromobutyl)phthalimide, pyridine-2-propanol, ethyl chloroformate, 4-dimethylamino-2-pyrindine, ethyl-5-bromovalerate, and o-nitrophenyl-β-D-galactoside were from Aldrich Chemical Co., Milwaukee, WI 53223. Silica gel 60 was from E. Merck Lab. Inc., Elmsford, NY 10523. Tri-N-butylamine and Tween-20 were from J. T. Baker Chemical Co., Phillipsburg, NJ 08865. Bovine serum albumin was from Research Products, Miles Laboratories, Inc., Elkhart, IN 46515.

**Drugs.** PHT and the gas-chromatographic internal standard, 4-methylpridine, were purchased from Aldrich Chemical Co. Other drugs used in the cross-reactivity study were obtained from Applied Science Lab, Inc., State College, PA 16801; United States Pharmacopoeia-NF Reference Standards, Rockville, MD 20852; Sigma Chemical Co.; or the manufacturers.

**PHT serum samples and PHT standards.** Sera from patients receiving PHT were provided by South Bend Medical Foundation, South Bend, IN 46601. The PHT standards for SLFIA were prepared in serum according to a published procedure (17). Both the calibrator and the control sera for the EMIT determinations were obtained from Syva Corp. The serum control used in gas chromatography was obtained from Ortho Diagnostics Inc., Raritan, NJ 08869, and the standards were made up in the laboratory from drug-free human serum (12).

**Procedures**

**SLFIA for the measurement of serum PHT.** To a series of cuvets add 3.0-mL aliquots of an antibody-enzyme reagent containing per liter, 50 mmol of Bicine buffer (pH 8.2), 15.4 mmol of sodium azide, 18.0 units of β-galactosidase, and an amount of antiserum sufficient to decrease the fluorescence production to 10% of that observed in the absence of antibody. Dilute the standard and unknown sera 50-fold in a 5 g/L solution of Tween-20, and add 100 μL of each diluted sample to the corresponding cuvet. Finally, add 100 μL of an aqueous solution of 1.05 μmol of β-G-U-PHT per liter of Tween-20 (1 g/L) to each cuvet at 30-s intervals and mix immediately. After incubating the first reaction at room temperature for 20 min, record the fluorescence and then measure the fluorescence intensities of the subsequent reactions at 30-s intervals. The unknown PHT concentrations are determined from a plot of fluorescence vs. standard PHT concentration.

**Gas chromatography.** Place a 0.5-mL aliquot of serum, 0.1 mL of internal standard (4-methylpridine, 50 mg/L) and 0.5 mL of ammonium sulfate (1.5 mol/L) into a 16 × 100 mm screw-cap culture tube. After agitation the contents for about 5 s on a vortex-type mixer, add three drops of 3 mol/L HCl to the tube and agitate again for 5 s. Add 5 mL of methylene chloride to the tube and cap with a Teflon-lined cap. Mix the contents with a horizontal shaker at 280 oscillations per min for 3 min. After centrifuging at 200 × g for 3 min, remove and discard the aqueous upper layer. Evaporate the lower methylene chloride layer at 50 °C with a stream of nitrogen. Reconstitute the residue with 30 μL of methylene chloride, then inject 1 μL of this into the gas chromatograph.

**EMIT.** Use the “EMIT-aed” phenytoin kit (Syva) according to the manufacturer's instructions.

**Synthesis of the drug-labeled substrate.** The synthetic scheme for preparation of the PHT-labeled fluorescent substrate, β-G-U-PHT, is presented in Figure 1. The first step was the synthesis of 2-[4-(N-phthalimido)butoxy]benzophenone (I), which was carried out by reacting 34.4 g of 2-hydroxybenzophenone and 8.64 g of sodium hydride with 52 g of N-(4-bromobutyl)phthalimide in dimethylformamide for 18 h at room temperature. Dilution of the reaction mixture with 200 mL of water yielded 49 g of (I), which was recrystallized from ethanol to give white needles with mp 121–122 °C. Analysis—calculated for C_{25}H_{21}N_{2}O_{5}; C, 75.17; H, 5.30; N, 6.35; found: C, 74.78; H, 5.26; N, 5.79. NMR spectrum (CDCl₃): δ 1.5 (m, 4H), 3.5 (m, 2H), 3.9 (m, 2H).

The second step, the synthesis of 3-[2-(4-N-formylamino-4-butoxy)phenyl]-5-phenylhydantoin (II), was carried out by heating a mixture of 22.4 g of (I), 4.15 g of potassium cyanide, 17.3 g of ammonium carbonate, 24 mL of water, and 200 mL of dimethylformamide in a steel autoclave for four days at 110 °C. After cooling, the reaction mixture was adsorbed onto 100 g of silica gel 60 and chromatographed on a 700 g column of
solvent. Twenty-milliliter fractions were collected, and fractions 276 to 803 were combined. The solvent was removed by evaporation to yield 4.65 g of solid. Recrystallization from ethanol gave a white solid with mp 201–203 °C. Analysis—calculated for C_{18}H_{21}N_{3}O_{4}; C, 65.38; H, 5.76; N, 11.44; found: C, 65.23; H, 5.79; N, 11.47. NMR spectrum (NaOD–D_{2}O): δ 1.2 (m, 4H), 3.0 (m, 2H), 3.2 to 4.0 (m, 2H).

For synthesis of 5-[2-(4-aminobutoxyphenyl)]-5-phenylhydantoin (III), 3.5 g of (II) in 100 mL of 1 mol/L sodium hydroxide was heated on a steam bath for 24 h. The aminohydantoin (III) precipitated when carbon dioxide was added to the cooled reaction mixture. Recrystallization of the precipitate from pyridine-2-propanol and then from methanol yielded 1.5 g of fine white crystals, mp 225 °C (d). Analysis—calculated for C_{18}H_{21}N_{3}O_{4}; C, 62.24; H, 6.24; N, 12.38; found: C, 67.56; H, 6.23; N, 12.56. NMR spectrum (NaOD–D_{2}O): δ 0.09 (m, 4H), 2.2 (m, 2H), 3.2 (m, 2H).

The final step involved the reaction of (III) with 7-β-galactosylcoumarin-3-carboxylic acid (IV), which had been prepared as described by Burd et al. (9). We combined 216 mg of ethyl chloroformate with 308 mg of (IV) in 20 mL of dimethylformamide at 0 °C. After stirring for 1 h at this temperature to allow the formation of the mixed anhydride of (IV), we added 638 mg of (III), 244 mg of 4-dimethylaminopyridine, and 5 mL of dry pyridine. The reaction was allowed to proceed overnight at 0 °C. Then the solvent was removed by evaporation and the residue was added to 7 g of silica gel 60 and chromatographed on 200 g of silica gel 60. The chromatogram was developed with a linear gradient generated with 2 L of ethyl acetate and 2 L of a solution of equal volumes of ethyl acetate and ethanol. Ten-milliliter fractions were collected. The solvent was evaporated from fractions 143 to 160 to yield 200 mg of solid, which we rechromatographed on a 3.2 × 45 cm column of Sephadex LH-20 equilibrated with methanol. Seven-milliliter fractions were collected, and fractions 30 to 40 were combined and evaporated. One hundred milligrams of (V) in the form of a white, glassy solid was obtained. Analysis—calculated for C_{36}H_{38}N_{4}O_{12}H_{2}O: C, 59.40; H, 5.27; N, 5.94; found: C, 59.51; H, 5.04; N, 6.14. Optical rotation [α]_{D}^{20} = −39.04 (10 g/L methanol).

Synthesis of the immunogen. Antibodies against PHT were produced by immunization of rabbits with PHT—bovine serum albumin immunogen that had been synthesized as follows. 2-Hydroxybenzophenone (19.8 g) was added to a solution of 2.52 g of sodium in 400 mL of ethanol. After 45 min, 20.9 g of ethyl-5-bromomalonate was added, and the reaction was refluxed for 44 h. The reaction mixture was cooled and diluted with 250 mL of sodium hydroxide (1 mol/L) and then extracted with 250 mL of ether. The aqueous layer was saturated with sodium chloride and re-extracted with four 200-mL portions of ether. The combined ether extracts were dried over anhydrous magnesium sulfate and filtered, and the solvent was removed by evaporation to give 40 g of a yellow oil that was then heated to reflux for 15 h in a mixture of 500 mL of dioxane and 60 mL of concentrated hydrochloric acid. A brown, oily residue was left after evaporation of the solvent. The residue was partitioned between 200 mL of ether and 200 mL of saturated sodium bicarbonate. After re-extraction of the ether phase with another 200 mL of sodium bicarbonate, the ether was removed by evaporation to give 23 g of a brown oil; this oil was chromatographed on 200 g of silica gel 60 eluted with carbon tetrachloride/acetone, 19/1 (by vol). Twenty-milliliter fractions were collected, and fractions 1271 to 1670 were combined and evaporated to give 11.1 g of 2-(4-carboxybutoxy)benzophenone. Recrystallization from methylene chloride gave white crystals, mp 80–81 °C. Analysis—calculated for C_{18}H_{13}O_{2}: C, 72.48; H, 0.69; found: C, 72.23; H, 6.07.

A mixture of 17.8 g of 2-(4-carboxybutoxy)benzophenone, 4.15 g of potassium cyanide, 17.3 g of ammonium carbonate, 24 mL of H_{2}O, and 200 mL of dimethylformamide was placed in a steel autoclave and heated to 110 °C for five days. The cooled reaction mixture was then dissolved in 800 mL of 100 g of sodium hydroxide per liter of water, washed with ether, and acidified to pH 4.5 with 6 mol/L hydrochloric acid to give a precipitate of 5-[2-(4-carboxybutoxy)phenyl]-5-phenylhydantoin, which when dried weighed 24.6 g. Recrystallization from aqueous methanol gave a white solid, mp 231–232 °C. Analysis—calculated for C_{36}H_{29}N_{4}O_{5}; C, 65.20; H, 5.48; N, 7.61; found: C, 64.92; H, 5.62; N, 7.90. NMR spectrum (C_{2}D_{3}N): δ 1.2 (m, 2H), 1.6 to 2.0 (m, 4H), 3.2 (m, 2H).

In preparation of the PHT–bovine serum albumin immunogen, 17.5 μL of tri-N-butylamine was added to a suspension of 27.6 mg of the 5-[2-(4-carboxybutoxy)phenyl]-5-phenylhydantoin in 1.5 mL of dioxane at about 10 °C. Then 6 μL of ethyl chloroformate was added. After 15 min, the mixture was added to 125 mg of bovine serum albumin in 3.4 mL of sodium hydroxide (0.04 mol/L) and 3.25 mL of dioxane. The reaction mixture was stirred for 1.75 h at 5 °C, then chromatographed on a 2.8 × 42 cm column of G-25 Sephadex with saline (8.5 g/L) as the eluant. The PHT–bovine serum albumin immunogen eluted in a void volume. Analysis of the ultraviolet absorbance spectrum of the immunogen indicated 8.1 mol of 5-[2-(4-carboxybutoxy)phenyl]-5-phenylhydantoin coupled with each mole of bovine serum albumin.

Immunization schedule and procedure. For immunization,
1.3 mg of the immunogen in 1 mL of 8.5 g/L saline was emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously into New Zealand white rabbits. Booster injections were administered intravenously with 1.1 mg of the immunogen at 21 and 23 days after the initial injection. The animals were bled 10 days after the last injection. Subsequently, the booster was repeated every 30 days and blood was collected 10 days after each booster.

**Results**

**Absorbance and Fluorescence Spectra of βG-U-PHT**

Analysis of the absorbance spectrum of βG-U-PHT in Bicine buffer, 50 mmol/L, pH 8.2, showed a peak at 343 nm. After hydrolysis, catalyzed by bacterial β-galactosidase, a new maximum appeared at 403 nm while the one at 343 nm decreased. The absorbance maximum of the hydrolyzed βG-U-PHT (i.e., U-PHT) was 1.6 times that of the untreated conjugate.

Hydrolysis of βG-U-PHT by β-galactosidase shifted the fluorescence-excitation maximum from 355 to 410 nm, and the emission maximum was changed from 405 nm to 453 nm. Coincidentally, a 12.4-fold increase in fluorescence was observed after enzymatic hydrolysis. All additional fluorescence measurements were conducted with excitation and emission wavelengths of 400 and 450 nm, respectively. Thus, the unhydrolyzed βG-U-PHT contributes negligible fluorescence under the assay conditions.

**Enzymatic Hydrolysis of βG-U-PHT**

Kuby and Lardy (13) found that the maximum rate of o-nitrophenyl-β-D-galactoside hydrolysis by E. coli β-galactosidase is in the pH range of 7.2 to 7.4. Under the conditions of our experiments, in which βG-U-PHT is the substrate, the enzyme has an optimum activity between pH 6 and 8. Because the fluorescent intensity of hydrolyzed βG-U-PHT increases up to pH 8.0 and remains nearly constant to at least pH 10, we conducted further investigations at pH 8.2 to maximize fluorescence intensity and to obtain adequate β-galactosidase activity. The enzyme activity at pH 8.2 is 0.67 times the activity at pH 7.

Various amounts of βG-U-PHT were added to a series of cuvets containing 3.1 mL of Bicine buffer (50 mmol/L, pH 8.2), 15.4 mmol/L of sodium azide, and 0.04 unit of β-galactosidase. The reaction mixtures were allowed to stand at room temperature for 20 min, after which the fluorescence intensities were recorded. The correlation between fluorescence and βG-U-PHT concentration was linear over the range 0 to 65.5 nmol/L. The amount of βG-U-PHT typically used in the SLFIA, 35 nmol/L, was within the linear range.

**Antibody-Binding Reactions**

The ability of βG-U-PHT to serve as an enzymatic substrate in the presence of antiserum to PHT was examined. Various volumes of antiserum or normal rabbit serum were added to 3.0 mL of buffer containing, per liter, 50 mmol of Bicine, 15.4 mmol of sodium azide, and 40 units of β-galactosidase. βG-U-PHT was added to give a final concentration of 33 nmol/L, and the reactions were allowed to proceed for 20 min at room temperature. Fluorescence was measured at the end of the incubation period. The fluorescence decreased in cuvets containing antiserum; it was unaffected in cuvets containing normal rabbit serum (Figure 2).

The βG-U-PHT was used to titer antiserum from several bleedings of one rabbit. The results in Figure 2 show that the titer reached a maximum by the third bleeding and was sustained through the seventh bleeding. A Scatchard plot for the fifth bleeding gave a binding constant of 1.34 L/nmol and a binding-site concentration of 30 μmol/L in the undiluted antiserum. Four rabbits were immunized and all produced antiserum to PHT.

**Competitive-Binding Reactions**

The effect of incubation time with β-galactosidase on the standard curve obtained from competitive-binding reactions was examined. Competitive-binding reactions were conducted by adding various levels of PHT to 3.1-mL reaction mixtures that contained 0.04 unit of β-galactosidase and 5 μL of the antiserum. (This amount of antiserum inhibited the production of fluorescence to 10% of that observed in the absence of antibody.) One hundred-microliter aliquots of βG-U-PHT (1.05 μmol/L) were added sequentially to each mixture at 30-s intervals and mixed. The reaction mixtures were allowed to stand at room temperature for various lengths of time. The fluorescence intensities were measured at 30-s intervals in the same sequence as the βG-U-PHT was added.

As the amount of PHT increased, the fluorescence increased because less βG-U-PHT was bound to antibody (Figure 3).

![Fig. 3. Standard curves for PHT observed after various periods of reaction with β-galactosidase](image)

**Table 1. Comparison between SLFIA and Gas-Chromatographic (GC) Values on Clinical Samples with PHT Concentrations Greater than 30 mg/L**

<table>
<thead>
<tr>
<th>SLFIA values (mg/L)</th>
<th>GC values</th>
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<tr>
<td>39.4</td>
<td>40.5</td>
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<tr>
<td>41.4</td>
<td>40.7</td>
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<tr>
<td>36.8</td>
<td>34.5</td>
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<tr>
<td>48.0</td>
<td>49.6</td>
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<tr>
<td>30.8</td>
<td>32.3</td>
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Correlation coefficient = 0.966.
Longer incubation periods with the enzyme tended to decrease the slope of the curve at high PHT concentrations (>20 mg/L). However, an acceptable standard curve was obtained anytime between 5 min and 7 h after the addition of βG-U-PHT. Incubation of the standards with the antibody-enzyme reagent for 1 to 90 min before addition of βG-U-PHT did not alter the standard curve.

Because it is generally accepted that the therapeutic amount for PHT in blood is 10 to 20 mg/L (1), the range of the SLFIA standard curve adequately covers the amounts most commonly encountered. The concentrations of PHT greater than 30 mg/L in clinical sera are determined with SLFIA by simply reassaying a further twofold dilution of the original 50-fold dilution of these sera. The resulting value for PHT is then multiplied by two. Five samples with greater than 30 mg of PHT per liter were assayed in this fashion, and the results were compared with those found by means of gas chromatography (Table 1). The correlation coefficient between the two assays was 0.966.

We examined the precision of SLFIA measurements by

<table>
<thead>
<tr>
<th>Table 3. Analytical Recovery of PHT in SLFIA</th>
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<tr>
<td><strong>Concentration of added PHT, mg/L</strong></td>
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<tr>
<td><strong>Serum 1</strong></td>
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<tr>
<td>Theoretical, mg/L</td>
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<td>Observed, mg/L</td>
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<td>Recovery, %</td>
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<td><strong>Serum 2</strong></td>
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<td>Theoretical, mg/L</td>
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<td>Observed, mg/L</td>
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<tr>
<td>Recovery, %</td>
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<tr>
<td><strong>Serum 3</strong></td>
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<tr>
<td>Theoretical, mg/L</td>
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<td>Observed, mg/L</td>
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<tr>
<td>Recovery, %</td>
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<td><strong>Serum 4</strong></td>
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<td>Theoretical, mg/L</td>
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<td>Observed, mg/L</td>
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<td>Recovery, %</td>
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<tr>
<td><strong>Serum 5</strong></td>
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<td>Theoretical, mg/L</td>
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<td>Observed, mg/L</td>
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<td>Recovery, %</td>
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<tr>
<td>Mean recovery, %</td>
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<td>SD, %</td>
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</table>

* An equal amount of the original clinical serum sample was mixed with PHT standards containing 5, 10, or 20 mg of PHT per liter of serum. The "theoretical" PHT value was compared to the value determined by SLFIA.

For recovery experiments, serum standards containing 5, 10, or 20 mg of PHT per liter were added to equal volumes of five clinical samples with low PHT concentrations (2.2–3.1 mg/L). The average percentage of PHT recovered at each concentration is shown in Table 3. When we assayed 100, 75, 50, and 25 μL of a 100-fold dilution of five clinical serum samples with high PHT concentrations, multiplying the observed values by the appropriate dilution factors gave virtually equivalent results (Table 4). Both of these experiments indicate that there are no interferents in serum that adversely affect the fluorescent immunoassay.

To establish the validity of the SLFIA, we determined PHT amounts in clinical sera by SLFIA, gas chromatography, and EMIT methods and compared the results. The correlation coefficient between the SLFIA and gas-chromatographic methods (Figure 4) was 0.976. The SLFIA and EMIT gave a correlation coefficient of 0.970 (Figure 5), and EMIT and gas chromatography gave a correlation coefficient of 0.971 (SE = 1.79; y = 1.09x + 0.45; n = 102). Thus, the results obtained with the three methods are similar.

To study the specificity of SLFIA for PHT, we examined 23 substances for cross reactivity: 5-(p-hydroxyphenyl)-5-phenylhydantoin, mephenytoin, phenobarbital, secobarbital,
mephobarbital, amobarbital, primidone, phenylethylmalonamide, carbamazepine, ethosuximide, phenytoin, methyamphetamine, methaqualone, methyprylon, diazepam, phenylbutazone, indomethacin, caffeine, theophylline, ethanol, and acetylsalicylic acid. From 10 nmol to 10 μmol of each drug was added to the immunoassay. With the exception of 5-[(p-hydroxyphenyl)-5-phénylhydantoin, none of these substances competed with the βG-U-PHT for PHT antibody. When varying amounts of 5-[(p-hydroxyphenyl)-5-phénylhydantoin were added to the 15 mg/L PHT standard, we found that greater than 35 mg/L of 5-[(p-hydroxyphenyl)-5-phénylhydantoin was required to give an error of 10% with this standard. This is more than 15 times the amount (2 mg/L) normally found in patients' serum (15).

Discussion

In the SLFIA for PHT, we use a single-point reading of fluorescence, which differs from the rate assay we used in the fluorescent immunoassay for gentamicin (9). This modification simplifies the assay procedure and data analysis. In the present assay there is a 20-min incubation period between adding the βG-U-PHT and reading the fluorescence. If the βG-U-PHT is added to each cuvet at 30-s intervals, a maximum of 40 assays can be performed within the 20-min period. However, as the results in Figure 3 indicate, an acceptable standard curve can be obtained anytime between 5 min and 7 h after the addition of βG-U-PHT. Hence, the operator has the flexibility of varying the incubation time according to the number of samples being assayed, as long as all the cuvets are incubated for the same period.

The range of SLFIA is between 0 and 30 mg/L, which should cover the PHT concentrations most commonly encountered in clinical serum specimens. Sera with PHT concentrations greater than 30 mg/L are diluted 100-fold instead of 50-fold and assayed by the usual procedure. The concentration values obtained for these 100-fold dilution are then multiplied by 2 to give the PHT values for these sera (Table 1).

When various amounts of PHT were added to serum, the recoveries measured with SLFIA were generally greater than 100% (Table 3). This positive bias appears to be due to experimental error, because no bias was evident in the dilution studies (Table 4) or the correlation studies (Figures 4 and 5).

PHT amounts determined with SLFIA correlate well with results from both EMIT and gas chromatographic methods. A small serum sample volume, 100 μL of 50-fold diluted serum or 2 μL of undiluted serum, is used for SLFIA, and no extraction procedures are required. Because the antibody-bound and the free βG-U-PHT nead not be separated, the assay is rapid and simple to perform.

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References