Determination of Diphenylhydantoin in Human Serum by Spin Immunoassay


A spin immunoassay for diphenylhydantoin is reported, which appears to give an accurate and precise estimate of serum diphenylhydantoin concentrations, as judged by the disappearance of [14C]diphenylhydantoin from the serum of a rabbit. The assay also appears to be a reliable technique for routine diphenylhydantoin determinations, as judged from our experience with 28 patients. Serum diphenylhydantoin concentrations in the range of 1.0–50.0 mg/liter are easily determined on a 50-μl sample. Except for primidone, no significant cross reactivity was observed with eight drugs that are commonly used in conjunction with diphenylhydantoin therapy. This fast, simple, and precise method therefore appears to be readily applicable to routine determination of diphenylhydantoin.

Additional Keyphrases: gas chromatography • radioimmunoassay • electron spin resonance

Radioimmunoassay represents a major step forward in the development of rapid, sensitive, and economical assays for determination of serum drug concentrations. Yet there remain two areas in which this assay could be improved.

The first is that frequently not only the parent compound but also its metabolites and congeners can bind to the antibody, so that the method may lack specificity. This is a problem with all immunoassays, which can only be resolved by a thorough study of a wide variety of antigens in a wide range of animals with the hope of developing more specific antibodies.

The second problem is that the radioimmunoassay requires that the free and the antibody-bound label somehow be physically separated. Although some elegant and reliable methods of doing this have been devised, they require an added two to three steps that may introduce error, entail added labor and cost, and are difficult to fully automate. These problems prompted us to examine an alternative detection system for the immunoassay, the spin-labeled immunoassay (1–4).

In this assay the rapidly rotating free spin-labeled drug gives three sharp peaks in the ESR1 spectrometer. In contrast, when the drug becomes bound to the antibody, the peaks broaden out so that the maximum peak-height drops by a factor of 104. Hence, when the drug binds to the antibody, the ESR spectrum essentially disappears. On the other hand, when unlabeled drug competitively displaces the spin-labeled drug from the antibody, the ESR spectrum reappears. The height of the observed peaks are, therefore, directly proportional to the unlabeled drug concentration. Since the ESR spectrometer only detects the free drug, this assay does not require that the free and bound drug be physically separated.

This method was originally used to detect morphine congeners in urine (2–4) and, more recently, in serum (1). It has been found to be quick (requiring only 2 min of technician's time), simple, and sufficiently sensitive for many of the drugs routinely determined in the serum [minimum sensitivity, about 20–30 nmol (1)].

In this work we have examined a new spin immunoassay for the determination of serum DPH and compared it to a standard gas-chromatographic method. Our results suggest that the spin immunoassay is sufficiently precise, accurate, and convenient to warrant its application in the routine clinical analysis of this drug in serum.

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1 Nonstandard abbreviations used: ESR, electron spin resonance; and DPH, diphenylhydantoin.
Materials and Methods

**ESR instrumentation and instrument settings.** All ESR determinations were performed on a Varian E-4 spectrometer utilizing glass capillary tubes graduated to a volume of 50 μl (“FRAT” Disposable Microcapillaries; Syva Corp., Palo Alto, Calif. 94304). The ESR settings for serum DPH determinations were: scan rate 2.5 Gs (gauss)/min; field set 3376 Gs (uncalibrated); time constant 3 s; modulation amplitude 3.2 Gs; modulation frequency 100 kHz; receiver gain 3.2 × 10^4; microwave power 12 mW; microwave frequency 9.538 GHz. Dry nitrogen was constantly blown through the magnetic cavity to maintain temperature stability.

**Antibodies and spin-labeled diphenylhydantoin. Preparation of 3-N-(cyanomethyl)-diphenylhydantoin.** Diphenylhydantoin sodium (2 g, 7.3 mmol) was dried at 55 °C and 6.65 Pa for 3 h, dissolved in 50 ml of dimethyl sulfoxide, and chloroacetonitrile (550 mg, 7.3 mmol) was added. The resulting mixture was stirred under nitrogen for one hour, the dimethyl sulfoxide removed by vacuum distillation (50 °C oil bath) and the residue taken up in 40 ml of hot absolute ethanol, filtered, and cooled to yield 1.2 g of white crystals (mp 193–190 °C). An additional 250 mg (mp 190–194 °C) could be obtained from the mother liquor after concentration under reduced pressure.

Calculated for C_{17}H_{13}N_{3}O_{2}: C 70.09%, H 4.50%, N 14.42%. Found: C 70.25%, H 4.64%, N 14.66%. On thin-layer chromatography, the R_f was 0.5 in the system ethyl ether:chloroform (1:1, by vol) on “silica gel GF” (Analtech, Inc., Newark, Del. 19711).

**Preparation of 3-N-(methoxycarbimido-methyl)-diphenylhydantoin.** 3-N-(Cyanomethyl)-diphenylhydantoin (875 mg, 3 mmol, dried at 53 °C and 6.65 Pa for 3 h) was added to a solution of 4 mg (0.09 mmol) of sodium hydride (560 g/liter) in 20 ml of anhydrous methanol, and the mixture was stirred under nitrogen at room temperature for 15 h. Excess solid carbon dioxide was carefully added to quench the reaction and the mixture was evaporated under reduced pressure and maintained at 6.65 Pa for 2 h. The resulting solid was dissolved in 50 ml of dry benzene (stored over sodium), filtered, and the volume decreased under pressure to 10 ml. Petroleum ether was added dropwise to the hot benzene solution until it became cloudy, and the mixture was allowed to cool. Three hundred milligrams of colorless crystals was collected (mp 149–150 °C) and another 150 mg (mp 146–149 °C) could be isolated from the mother liquor by evaporation of the solution and recrystallization from dichloromethane:petroleum ether. Calculated for C_{18}H_{17}N_{3}O_{3}: C 66.86%, H 5.30%, N 13.00%. Found: C 66.67%, H 5.34%, N 13.07%.

**Diphenylhydantoin spin label. 3-N-(Methoxycarbimidomethyl)-diphenylhydantoin (90 mg, 0.28 mmol) and 3-amino-2,2,5,5-tetramethyl-pyrrolidinyl-1-oxyl (44 mg, 0.28 mmol) in 2 ml of chloroform were added to 40 mg (0.30 mmol) of triethylamine hydrochloride and the resulting solution was stirred overnight. The reaction mixture was chromatographed on preparative chromatographic plates (silica gel GF), with concentrated ammonia:ethylacetate (1:50 by vol) as solvent. The yellow band (R_f = 0.7) was scraped from the plate and the drug was eluted from the silica gel with the solvent used above (100 ml). The solvent was evaporated and the residue was dissolved in diethylether. After filtration, the solvent was removed under reduced pressure and the residue dried at 6.65 Pa for 1 h. A yellow glassy residue was obtained (75 mg) (mol wt 448.5; mass spectrum M^+ = 448).

**Preparation of the conjugate with bovine serum albumin.** A solution of 300 mg of 3-N-(methoxycarbimidomethyl)-diphenylhydantoin in 3 ml of dioxane (freshly opened bottle) was added, with constant stirring, to a solution of 300 mg of bovine serum albumin in 10 ml of water at 0 °C and pH 0.5. The pH was maintained with normal sodium hydroxide by use of the “pH-stat” mode of a Radiometer Auto-Titrator. After 16 h the mixture was dialyzed against distilled water. A precipitate appeared. It was removed by centrifugation (12 000 × g for 20 min). The supernatant was desalted on a Pharmacia K-26 column packed with G-25 (medium) Sephadex (Pharmacia Laboratories Inc., Piscataway, N.J. 08854) and lyophilized to yield 200 mg of conjugate with a hapten number of 53. The hapten number was determined by reference to a standard ultraviolet calibration curve, which was obtained by measuring the absorbances of known bovine serum albumin/DPH-derivative (N-3-carbamidomethyl-DPH) mixtures at 280 nm (A_{280}) and 257 nm (A_{257}) and plotting the ratio A_{280}/A_{257} vs. the bovine serum albumin/DPH-derivative ratios.

**Preparation of anti-DPH-γ-globulin.** The conjugate was injected intramuscularly into a sheep at four-week intervals. For the initial injection, 10 mg of the conjugate was dissolved in 1 ml of saline (9 g of NaCl per liter) and emulsified in 3 ml of complete Freund’s adjuvant. The second injection was prepared identically in incomplete Freund’s adjuvant. For the subsequent monthly injections, 2 mg of the conjugate was dissolved in 1 ml of saline and emulsified in 3 ml of incomplete Freund’s adjuvant.

The antiserum was precipitated at 0 °C by addition of an equal volume of saturated ammonium sulfate. The pellet obtained after centrifugation (12 000 g, 20 min) was redissolved in borate buffer (0.4 mol/liter, pH 8.0). After dialysis against 100 volumes of borate buffer (three changes), the binding site concentration and binding constant were determined by titration with DPH spin label. The binding site concentration was 1.9 × 10^{-4} mol/liter and the binding constant was 4.6 × 10^{6} liter/mol for the antibodies used in this experiment. The antibody was diluted with borate buffer to a binding-site concentration of 1.8 × 10^{-5} mol/liter.

The spin label was weighed out and dissolved in a
small amount of ethanol and diluted with distilled water to $1.58 \times 10^{-5}$ mol/liter. The final alcohol concentration was about 50 ml/liter. The correct spin label concentration was established by calibrating the DPH spin label solution against a morphine spin label solution of known concentration (by weight) with use of the low-field ESR signal for comparison.

To prepare the "spin-labeled-\gamma-globulin," we combined 5 \mu l of the antibody with 5 \mu l of the spin labeled DPH.

Ten microliters of the spin labeled-\gamma-globulin reagent was added to 40 \mu l of serum containing the unknown concentration of DPH. Blank samples were obtained by adding the 10 \mu l of antibody/spin labeled hapten complex to 40 \mu l of pooled human serum. Standards were prepared by adding various amounts of sodium 5,5-diphenylhydantoin (Sigma Chemical Co., St. Louis, Mo. 63178) to the same pooled human serum. Incubation time for the spin-labeled DPH-antibody complex with the DPH containing solution was 60–90 min, as previously described (1).

Binding of spin-labeled DPH to serum proteins. Binding of the \textsuperscript{N3}-substituted, spin-labeled DPH to serum proteins was assessed by adding an aliquot of the spin-labeled compound to human serum containing DPH (5,5'-diphenylhydantoin sodium, Sigma) at a final concentration of 10 \mu g/ml and comparing the amplitude of the low-field signal in the presence of serum to the amplitude of this peak for the same concentration of spin-labeled DPH in water. The decreased signal observed in the presence of serum is due to the binding of the spin-labeled drug to the serum albumin and the resultant immobilization of the free-radical species (1). The ESR spectrometer settings for these studies were the same as for the serum DPH assay except that the receiver gain was 2.5 \times 10^4.

Metabolism of DPH in rabbits. [4-\textsuperscript{14}C]-DPH (New England Nuclear, Boston, Mass. 02118; lot No. 648298; spec. act., 5.21 Ci/mol) was dissolved in saline and diluted with unlabeled sodium DPH in a final concentration of 25 g/liter (5 mCi/liter). One hundred milligrams of this dosing solution was injected intravenously into the marginal ear vein of a 4-kg female New Zealand white rabbit, and blood samples were withdrawn at various times from the opposite ear vein. The serum was separated and immediately frozen for later analysis. Aliquots of serum (0.10 ml) were dissolved in 10 ml of "Aquasol" scintillator fluid (New England Nuclear) and counted in a Beckman LSC 1000 scintillation counter, (Beckman Instruments, Inc., Fullerton, Calif. 92634).

Thin-layer chromatography. Serum samples containing [\textsuperscript{14}C]-DPH were extracted with chloroform: methanol (9:1), evaporated under reduced pressure, and the residue was chromatographed on silica gel GF plates with chloroform:methanol (9:1) as the developing solvent. The plate was scanned in a scanner (Packard Instrument Co., Downers Grove, Ill. 60515), and then 0.5-cm segments were scraped from it. These scrapings were counted in 10 ml of "Aquasol."

Ultrafiltration. Triplicate 5-ml aliquots of fresh human sera to which DPH was added (10 \mu g/ml, plus 1 \mu Ci of [\textsuperscript{14}C]-DPH) was ultrafiltered through a "Diaflow" membrane (QM 10; Amicon Corp., Lexington, Mass. 02173) at 483 kPa (70 psi). One-tenth milliliter aliquots of the filtrate were solubilized in "Aquasol" and the radioactivity was determined in a Beckman LSC 1000 scintillation counter. Nonspecific membrane binding of DPH was found to be 19% by the same procedure when saline was used instead of serum. The percentage of protein binding of DPH was corrected for this nonspecific membrane effect.

Equilibrium dialysis. Equilibrium dialysis was performed in triplicate by dialyzing 5 ml of pooled human serum against 50 ml of saline (9 g/liter) that contained 10 \mu g of DPH per milliliter, plus 1 \mu Ci of [\textsuperscript{14}C]-DPH. After 24 h, the difference in [\textsuperscript{14}C]-DPH activity in the serum and in the dialysate was determined by scintillation counting.

Cross reactivity of the anti-DPH antibodies. Drugs examined for possible cross reactivity with this assay were gifts of: primidone, Ayerst Laboratories, New York, N.Y. 10017; ethosuximide, Parke-Davis & Co., Minneapolis, Minn. 55441; ethotoin, Abbott Laboratories, North Chicago, Ill. 60064; mesantoin, Sandoz Pharmaceuticals, East Hanover, N.J. 07936; chlor Diazepoxide and diazepam, Hoffmann-La Roche Inc., Nutley, N.J. 07110; and glutethimide, USV Pharmaceutical Corp., Tuckahoe, N.Y. 10707. Phenobarbital sodium was purchased from Merck Chemical Co., St. Louis, Mo. 63116; and 5-(p-hydroxyphenyl)-5-phenylhydantoin was obtained from Aldrich Chemical Co., Milwaukee, Wis. 53233.

DPH specimens. Sera from patients receiving DPH for seizure control were obtained from the clinical services, VA Hospital, Minneapolis, Minn., for comparison of the spin immunoassay determination with the DPH concentration as determined by the gas-chromatographic method.

Determination of DPH by gas chromatography. The concentration of DPH in serum was determined essentially by the method of Kupferburg (5). In this method 5-(4'-methylphenyl)-5-phenylhydantoin was added to the serum (1 ml) as an internal standard to give a final concentration of \mu g/ml. This was followed by the addition of potassium phosphate buffer (1 ml, 0.25 mol/liter, pH 7.4). The drug was extracted into 6 ml of ethylene dichloride. Hexane (5 ml) was added and the organic phase extracted with K_2PO_4 (0.2 mol/liter, 4.5 ml). Of the aqueous phase, 3.5 ml was acidified with HCl (5 mol/liter, 0.3 ml) and finally extracted into 10 ml of ethylene dichloride. The extract was dried in a stream of nitrogen and redissolved in 50 \mu l of a 0.1 mol/liter solution of trimethylammonium hydroxide in methanol. The mixture was then injected into a Perkin-Elmer Model 881 gas chromatograph (Perkin-Elmer Corp., Norwalk, Conn. 06856), with a six-foot glass column packed with 3% OV-1 Gas Chrom Q (60–80 mesh; Applied Science Labora-
tories, State College, Pa. 16801). The carrier gas was argon at 103 kPa (15 psi). The oven temperature was programmed from 150–200 °C at 10 °C/min after 5 min at the initial temperature. The peaks were detected with a flame ionization detector and the areas integrated with an Autolab 6300-2 digital integrator (Spectra-Physics, Mt. View, Calif. 94040).

**Results**

For determination of DPH in serum we prepared a standard curve (Figure 1) with use of sera supplemented with DPH in various concentrations. Figure 1 shows the peak-to-peak amplitude of the low field nitroxide triplet peak (after subtraction of the non-DPH-containing serum blank) for various DPH concentrations between 1.0–50.0 μg of DPH per milliliter. A new standard curve was prepared daily for routine DPH analysis.

To establish the reliability and validity of this assay for the determination of serum DPH, we gave a female rabbit [4-14C]5,5-diphenylhydantoin (25 mg/kg orally), and serum DPH concentrations were determined by liquid scintillation counting, the spin immunooassay technique, and gas-chromatography at several time intervals from 20 min to 24 h after administration (Figure 2). The DPH dosage was selected to produce serum DPH concentrations similar to those produced in man by therapeutic doses of DPH (6, 7). The distribution of DPH was apparently complete 20 min after the intravenous administration. Between 1.0 and 40 μg of DPH per milliliter of serum, the agreement between the 14C activity, the gas-chromatographic determination, and the spin immunooassay was quite good; the half-life for this linear phase of drug disappearance was 4.0 h. At serum concentrations of DPH greater than 40 μg/ml the spin immunooassay tended to give slightly higher values than did measurement of 14C-activity or the gas-chromatographic determination. This discrepancy disappeared when the samples of higher concentration (>40 mg/liter) were diluted with an equal volume of non-DPH-containing serum and then assayed for 14C-activity and by spin immunooassay. These data for the spin immunooassay determination also indicate a high degree of intra-assay precision on triplicate determinations.

Thin-layer chromatography of a chloroform:methanol extract of rabbit serum obtained 6 h after DPH administration indicated that 96% of the 14C activity was present in the serum as unmetabolized DPH. The residual 4% of the 14C activity was in a compound that corresponded to an authentic standard of p-hydroxydiphenylhydantoin, indicating that only a small fraction of the serum concentration of 14C activity was present as the hydroxylated DPH metabolite. Thus, the 14C activity evidently provided a good estimate of the true concentration of DPH in serum.

We checked the possibility of cross reactivity in the spin immunooassay of several compounds that are frequently used with DPH therapy. Human serum containing 10 μg of DPH per milliliter (36.5 μmol) was used as the reference standard. To this serum was added each of the indicated compounds (Table 1) at an equimolar concentration (36.5 μmol), and at 10 (365 μmol) and 100-fold excess of the DPH (3.65 mmol). The serum sample was then spin-immunoassayed and the results were compared to a serum sample containing 10 μg (36.5 μmol) of DPH per milliliter. Table 1 shows the percent response for each compound at each concentration studied as compared to 10 μg of DPH per milliliter (100% response). Of the therapeutic compounds, only primidone and the highest phenobarbital concentration showed significant binding to the DPH-antibody. Because the phenobarbital concentration that gave cross reactivity in the assay is far in excess of that which is found in clinical situations (8), it would be very unlikely that this false-positive reaction would be encountered.
Table 1. Cross Reactivity of Diphenylhydantoin in Spin Immunoassay

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<thead>
<tr>
<th>Compounda</th>
<th>Percent responseb</th>
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<tr>
<td>DPH</td>
<td>100</td>
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<tr>
<td>p-Hydroxyphenylhydantoin</td>
<td>&gt;1000&lt;sup&gt;c&lt;/sup&gt; &gt;1000&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>106</td>
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<tr>
<td>Primidone</td>
<td>150&lt;sup&gt;c&lt;/sup&gt; 400&lt;sup&gt;c&lt;/sup&gt; ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethosuximide</td>
<td>95</td>
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<tr>
<td>Ethotoin</td>
<td>98</td>
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<tr>
<td>Mesantoin</td>
<td>95</td>
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<tr>
<td>Chlorazepoxide</td>
<td>96</td>
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<tr>
<td>Diazepam</td>
<td>102</td>
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<tr>
<td>Glutethimide</td>
<td>104</td>
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Compounds added to human serum containing 10 μg (36.5 μmol) of DPH per milliliter. Values represent the percent response as compared to 10 μg of DPH per milliliter determined in each sample by spin immunoassay.

* Drugs added at indicated concentration to serum containing 10 μg/ml DPH (36.5 μmol).

<sup>b</sup> Values are percent response as compared to a solution containing only DPH (10 μg/ml).

<sup>c</sup> Significant cross reactivity.

<sup>d</sup> ND, not determined, owing to insolubility.

Fig. 3. Correlation between serum diphenylhydantoin concentrations determined in a series of patients by gas chromatography (GLC) and by spin immunoassay

Linear regression of slope: 0.94 ± 0.04 (mean ± 1 SD). Correlation coefficient: 0.97

However, the cross reactivity of primidone, which has a therapeutic range of 5–15 μg/ml (24–72 μmol) (9), would probably lead to an erroneously high value for DPH if both DPH and primidone were present in serum at roughly equal concentrations. Since antibodies produced in different animal species are known to have different reactivities toward the same compound, the possibility of utilizing a different antibody source to circumvent this problem is currently being investigated.

The marked cross reactivity of the p-hydroxydiphenylhydantoin indicates that hydroxylation of the pa-<sup>α</sup> position on one of the phenyl rings of DPH does not significantly alter binding by the DPH-specific antibody. The cross reactivity of this DPH metabolite would not greatly affect the accuracy of the assay, however, because it is rapidly cleared by the kidneys and thus does not constitute a significant proportion of the total DPH in the serum. As indicated in the previously discussed rabbit study, only 4% of the total DPH was present in the serum as this compound; thus, its contribution to the total drug concentration in serum would be negligible.

To establish the reliability of this assay in the clinical situation, we obtained serum from 28 patients chosen without conscious bias, who were receiving DPH for seizure control, and simultaneously examined these sera by the spin immunoassay and gas chromatography. As Figure 3 shows, the two methods agreed very well (r = 0.97, P < 0.001). Furthermore, the slope of the regression line was not significantly different from 1.0 (slope ±1 SD = 0.94 ± 0.04), indicating that the spin immunoassay measures serum DPH as precisely as does gas-chromatography.

Addition of spin-labeled drug without antibody to human serum results in a decreased peak amplitude as compared to spin-labeled drug in water, a decrease attributable to binding of the spin-labeled drug to...
serum proteins (Figure 4). In our study on morphine (1) we found that the decreased ESR signal agreed well with ultrafiltration data. On the other hand, for DPH both ultrafiltration and equilibrium dialysis gave a value of protein binding of 88% (Table 2), in agreement with previous workers (10). The spin-labeled DPH gave a markedly smaller percentage of binding to protein (61%). This difference in binding can be explained by the charge characteristic of the two spin-labeled haptenps. In the case of spin-labeled morphine the charge of the molecule is essentially unaltered when compared with morphine. Spin-labeled DPH has a positive charge in the amidine linkage, which is nonexistent in DPH. Therefore, the spin-labeled DPH is more hydrophilic and binds less to the hydrophobic binding sites occupied by DPH.

Discussion

These studies indicate that the technique of spin immunooassay is readily applicable to the determination of serum diphenylhydantoin, and offers several advantages over its assay by either gas chromatography or radioimmunoassay. Perhaps the single most attractive advantage of this method is the speed and simplicity of operation—the entire assay requires only the mixing of two solutions and then, after an equilibration period during which other samples can be prepared, the concentration can be directly determined by a 2–4 min scan on the ESR spectrometer. In contrast, with radioimmunoassays it is necessary to include a step for separating the free label from that bound to antibody. If only for this reason, the spin immunooassay is faster, simpler, and less prone to errors resulting from multiple manipulations.

The sensitivity required for routine clinical monitoring of serum DPH (1–50 μg DPH per milliliter) is 100-fold greater than the lowest concentration that can be measured by this technique (1). Therefore, this technique is readily adaptable for long-term monitoring of DPH disappearance in man or in laboratory animals.

Finally, the mechanical configuration of the microwave cavity in the ESR spectrometer should readily facilitate the complete automation of this technique. A quartz solution-cell can be easily incorporated into a flow-through system, and serve as the detector unit in any one of a number of commercially available automated mixing and incubation flow systems.

References