Fluorometric Assay of Diphenylhydantoin in Plasma or Whole Blood

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A highly sensitive assay procedure for 5,5-diphenylhydantoin (DPH) involves oxidation with alkaline permanganate to form benzophenone. This is then extracted with heptane, allowed to react with concentrated sulfuric acid, and the fluorescence of the reaction product is measured. The procedure appears to be fairly specific for DPH; 5-(p-hydroxyphenyl)-5-phenylhydantoin (a DPH metabolite), phenobarbital, or other anticonvulsant drugs do not interfere. The sensitivity of the fluorometric procedure permits assays on finger-blood specimens. It provides a simple means for evaluating the effectiveness of a given dose schedule in different subjects.

We described a simplified procedure for assay of 5,5-diphenylhydantoin (DPH) in plasma (1), by using a modification of the benzophenone procedure originally developed by Wallace et al. (2). However, relatively poor sensitivity was obtained because ultraviolet absorbance was determined. When benzophenone is treated with coned H2SO4, a fluorescence develops that can be used for assay of DPH. In this report this sensitive modification is reported and applied.

Method

Using 12 × 100 mm glass-stoppered tubes, take 0.2 ml of heparinized blood or plasma, 1 ml of phosphate buffer (0.2mol/liter, pH 6.8), and 4 ml of ethylene dichloride (n-heptane, 1,2-dichloroethane, purified grade; J. T. Baker Chemical Co., Phillipsburg, N.J. 08865). Shake mechanically for 15 min and centrifuge.

Pipet 3 ml of the n-heptane (bottom) layer into a second glass-stoppered test tube (16 × 150 mm) containing 2.5 ml of 1 molar sodium hydroxide. Shake for 5 min and centrifuge.

Transfer 2 ml of the NaOH extract to a third glass-stoppered tube (16 × 150 mm), add 1.5 ml of NaOH solution (50g/100g; supplied by Baker), 4 ml of n-heptane (pure grade; Phillips Petroleum Co., Chem. Dept., Bartlesville, Okla. 74003), 0.5 ml of saturated aqueous potassium permanganate, and about 0.3 g of pulverized solid KMnO4. Mix the contents of the tube and insert the tube into a test-tube rack in a steam bath through a close-fitting gasket (aluminum foil), described previously (1). Immerse only the lower portions of the tubes, corresponding to the aqueous layer; the upper portions of the tubes are air-cooled. Alternatively, a metal heating-block can be used (1). Insert glass stoppers, loosely fitted, and heat for 30 min. Excess KMnO4 must be present during the oxidation step (1). After the heating period, cool all tubes to room temperature and shake mechanically to be certain that the benzophenone is completely extracted into the heptane.

Transfer 3 ml of the heptane layer to a 12 × 100 mm glass-stoppered test tube and add 1 ml of concentrated sulfuric acid.1 Shake the tubes mechanically for 3 min and centrifuge. Remove the heptane layer by aspiration and discard it. Pour the sulfuric acid layer into 10 × 75 mm disposable culture tubes (Kimble Products, Owens-Illinois, Toledo, Ohio 43601), and measure the fluorescence (details in Table 1).4

Standards

We prepared three series of DPH standards, in (a) water, (b) oxalated cow plasma, and (c) heparinized rat blood, to contain 0–50 μg/ml in 10 μg increments.

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### Table 1. Comparative Findings for Known Amounts of DPH Added to Control Blood, Plasma, or Water

<table>
<thead>
<tr>
<th>DPH concn, µg/ml</th>
<th>Whole blood</th>
<th>Plasma</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.1 ± 1.9</td>
<td>11.8 ± 1.6*</td>
<td>12.3 ± 1.8</td>
</tr>
<tr>
<td>10</td>
<td>33.0 ± 1.0</td>
<td>31.6 ± 0.5</td>
<td>38.9 ± 1.3*</td>
</tr>
<tr>
<td>20</td>
<td>55.6 ± 0.9</td>
<td>56.4 ± 1.4</td>
<td>55.9 ± 0.5*</td>
</tr>
<tr>
<td>30</td>
<td>72.5 ± 0.3</td>
<td>73.5 ± 1.3</td>
<td>73.0 ± 1.4</td>
</tr>
<tr>
<td>40</td>
<td>84.9 ± 0.8</td>
<td>83.9 ± 0.7</td>
<td>83.1 ± 1.1</td>
</tr>
<tr>
<td>50</td>
<td>95.4 ± 0.2</td>
<td>94.3 ± 0.2</td>
<td>94.5 ± 0.3</td>
</tr>
</tbody>
</table>

* Sample volume was 0.2 ml for each assay.
* Values given in Table are mean readings at 360 nm (activation)/490 nm (emission). Meter multiplier factor 0.003 (Amino-Bowman Spectrophotofluorometer). Standard errors calculated for small numbers of samples (n-1). No corrections were made for control blanks.

Using 0.2 X 0.01 ml serological pipets (Belloco Glass, Inc., Vineland, N. J. 08360), 0.2 ml volumes of the standards were rinsed out with 1 ml of phosphate buffer (0.2 mol/liter, pH 6.8) in 12 X 100 mm glass-stoppered glass tubes. The mixtures are extracted with ethyl ether, and handled as described previously. As we have reported elsewhere (4), blood-sugar pipets calibrated to contain 0.2 ml can be used in clinical work for the collection of finger-blood specimens.

### Results and Discussion

The fluorometer readings obtained when the standards were analyzed are shown in Table 1. The low standard errors indicate good reproducibility in replicate assays. Differences between the readings for whole blood, plasma, and water were not significant by Student's t-test except at the 10 µg/ml concentration, where the readings for recovery from aqueous solutions appeared to be high. In other trials, the fluorometer readings, corrected for blank, were directly proportional to the DPH concentrations over the range of 0 to 10 µg/ml (0 to 2 µg per sample). However, the readings began to be disproportionately low at 20 µg/ml. The present observations were extended to 50 µg/ml as a practical top limit for DPH assays. Since the fluorescence at higher concentrations is not directly proportional to DPH concentrations, a standard curve is needed to convert the readings to DPH concentrations.

This procedure has been extremely useful for the assay of DPH in laboratory animals where repeated blood samples are required. We have observed that the DPH concentrations in rat blood are nearly the same as in rat plasma (6), indicating rapid diffusion of DPH across the red cell membrane. Patients receiving daily doses of DPH showed nearly identical concentrations of DPH in whole blood taken simultaneously from the antecubital vein and from the fingertip. However, the whole blood concentrations of DPH in these patients averaged about 60 to 70% of the plasma concentrations, indicating relatively poor diffusion of DPH into human erythrocytes. This could be due to species differences in protein binding, or to differences in the permeability of the red cell membrane. The factors affecting the distribution of DPH between plasma and erythrocytes are poorly understood at this time, and further work will be needed to establish the relationship of whole blood to plasma DPH concentrations. However, there is no doubt concerning the greater sensitivity of the fluorometric benzophenone procedure when applied to plasma level determinations.

The benzophenone procedure is highly specific for DPH, with no known interference from barbiturates and other anticonvulsant drugs. We have confirmed that phenobarbital does not interfere in the fluorometric procedure, nor does 5-(p-hydroxyphenyl)-5-phenylhydantoin, the major metabolite of DPH.

### References