Liquid-Chromatographic Method for Simultaneous Determination of Phenytoin and 5-(4-Hydroxyphenyl)-5-phenylhydantoin in Plasma and Urine

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We describe a liquid-chromatographic assay for phenytoin and its para-hydroxylated metabolite, 5-(4-hydroxyphenyl)-5-phenylhydantoin, in plasma and urine. Unlike previously reported methods, this procedure is sensitive enough to allow quantitation of the unconjugated metabolite in plasma at concentrations observed clinically (50–500 μg/L). Simultaneous measurements of the drug and its metabolite in plasma and urine may be helpful in explaining changes in the apparent clearance of phenytoin, and in characterizing its nonlinear disposition, which is assumed to occur through its metabolite. The method may also be used, without modification, for analysis for phenobarbital and carbamazepine in plasma. The accuracy of our method for these three drugs was assessed for 25 months in a quality-assurance program for determining drug concentrations. Regression slopes for measured vs reported values for phenytoin, phenobarbital, and carbamazepine were 1.02, 0.99, and 0.99, respectively.

Additional Keyphrases: “high-pressure” liquid chromatography · phenobarbital · carbamazepine · anticonvulsant drugs · bioavailability of phenytoin

The importance of monitoring concentrations of phenytoin in plasma in the treatment of epilepsy has been well established (1–3). Because of the concentration-dependent clearance of this drug, the determination of its plasma concentration is necessary for individualizing phenytoin dosage regimens. Even minor adjustments in daily doses of this drug can greatly affect its steady-state concentration in plasma and the patient’s response.

At least two mechanisms have been proposed to explain the “dose-dependent,” or nonlinear, elimination of phenytoin. First, its major metabolite, 5-(4-hydroxyphenyl)-5-phenylhydantoin (HPPH), may inhibit the metabolism of parent phenytoin. This occurs in vitro (4) and in rats in vivo (5), but the results of a recent study (6) suggest that unconjugated HPPH, even in extremely high concentrations, does not alter the clearance of phenytoin in humans. The second and more widely addressed (7–9) possible mechanism relates to the ability of phenytoin in the therapeutic range to saturate the hepatic enzymes that metabolize it in vivo; this would decrease the efficiency with which the liver metabolizes the drug as concentration increases in plasma.

Because the dose-dependent metabolism of phenytoin is assumed to occur through the pathway that gives rise to HPPH (i.e., hydroxylation), an analytical method for simultaneously measuring the parent drug and its metabolite would be most useful in characterizing the complex behavior of phenytoin in individual patients. Analysis of plasma or urine samples for HPPH may be particularly helpful in the following two areas. In cases where alterations of steady-state concentrations of phenytoin in plasma are observed within an individual patient, it may be useful to know whether this change has occurred as a result of a change in the extent of absorption of the drug by the patient, or whether alterations in metabolic clearance, which may or may not be related to the addition or withdrawal of other drugs, are responsible. The second situation is that in which phenytoin bioavailability is estimated from data on phenytoin concentration in plasma vs time. Generally, the plasma concentration–time integral (“area under the curve”) is used as an index of bioavailability. Phenytoin bioavailability studies include the assumption of first-order elimination kinetics; plasma concentration data for either HPPH or its glucuronide conjugate would be helpful in supporting or refuting this assumption.

Because concentrations of HPPH observed in the plasma of patients undergoing chronic therapy with phenytoin (10) or receiving single doses of the drug (11) are relatively low (50 to 500 μg/L), methods for measuring it must be quite sensitive. Recently, a gas–liquid chromatographic method of analysis for phenytoin and total HPPH was reported (12), which allows quantitation of 150 μg of phenytoin per liter and 125 μg of HPPH per liter; however, 2.0 mL of plasma and on-column derivitization with trimethylammonium hydroxide are required. Another report (13) describes analysis for phenytoin and HPPH in plasma by “high-pressure” liquid chromatography; however, the presence of an interfering peak limits the method to the assay of HPPH in moderately high concentrations, and phenobarbital interferes with the analysis for HPPH. A more recent publication (14) describes the simultaneous analysis of phenytoin, phenobarbital, and their para-hydroxylated metabolites in urine by high-pressure liquid chromatography; the limit of detection of HPPH is 2.0 mg/L, however, thus precluding the use of this method for measuring plasma concentrations of unconjugated HPPH.

We describe a procedure routinely used in our laboratory to quantitate phenytoin and HPPH in concentrations as low as 100 and 50 μg/L, respectively, in only 1.0 mL of plasma or urine. The assay, which does not involve the formation of derivatives, is also being used without modification in this laboratory for the simultaneous analysis for two other anticonvulsant drugs, phenobarbital and carbamazepine.

Materials and Methods

Instrumentation and Reagents

For high-pressure liquid chromatography we used a chromatography pump, Model M-6000A, a Model U6K injector, a Model 440 dual-beam ultraviolet-visible absorbance detector (all from Waters Associates, Inc., Milford, MA 01757), and a Series B5217-1 Omniscrbe dual-channel strip-chart recorder (Houston Instruments, Austin, TX 78753).

The separation is carried out on a 30 cm × 3.9 mm (i.d.) prepacked, microparticulate (10-μm av particle size) reversed-phase column (μBondapak C18, Waters), with use of a methanol/distilled water (55/45 by vol) mobile phase at a flow rate of 1.1 to 1.3 mL/min. The column effluent is monitored at 264 nm at 0.005 absorbance unit full-scale (Channel

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1) and 0.02 to 0.01 absorbance unit full-scale (Channel 2) for plasma analyses. Chart speed is ordinarily 0.25 cm/min.

Chemicals used were phenytoin and 5-(4-hydroxyphenyl)-5-phenylhydantoin (Aldrich Chemical Co., Milwaukee, WI 53200), 5-allyl-5-phenylbarbituric acid (alphalen; Applied Science Labs, Inc., State College, PA 16801), methanol distilled in glass (Burck Laubs, Inc., Muskegon, MI 49442), anhydrous ethyl ether (Mallinckrodt Chemical Works, St. Louis, MO 63160), and distilled water (Glenwood Inglewood, Minneapolis, MN 55405). The pre-mixed mobile phase (methanol:water) was passed, under reduced pressure, through a prefilter and a 0.4 μm polycarbonate filter (both from Nuclepore Corp., Pleasanton, CA 94566), thereby degassing and removing particulate material.

We used the following reagents:

Phosphate buffer, pH 7.5: 50 mL of potassium dihydrogen phosphate (0.1 mol/L) mixed with 41.1 mL of NaOH (0.1 mol/L).

Phosphate buffer, pH 11.2: 50 mL of disodium hydrogen phosphate (0.2 mol/L) mixed with 6.3 mL of NaOH (0.4 mol/L).

Phosphate buffer, pH 6.8: 6.81 g of potassium dihydrogen phosphate was dissolved in distilled water, then diluted to 500 mL (Solution A); 13.4 g of disodium hydrogen phosphate heptahydrate was dissolved in distilled water, then diluted to 500 mL (Solution B). Mix equal volumes of Solutions A and B to obtain the phosphate buffer, 0.1 mol/L.

Potassium phosphate buffer, pH 7.88: Mix equal volumes of dipotassium hydrogen phosphate trihydrate (5.0 mol/L) and potassium dihydrogen phosphate (1.0 mol/L).

To prepare a stock standard solution of phenytoin, we dissolved 100 mg of phenytoin in 10 mL of methanol. The working standard solution (0.1 g/L) was prepared by two consecutive 10-fold dilutions of the stock solution. A stock standard solution of HPPH was prepared by dissolving 100 mg of HPPH in 10 mL of methanol; working standards were prepared by diluting to 1.0, 0.1, and 0.01 g/L.

The internal standard solution was 10 mg of alphalen in 100 mL of methanol.

Procedure

Analysis for phenytoin and unconjugated HPPH in plasma and urine. To four 13-mL centrifuge tubes (no. 410050; Kontes, Evanston, IL 60204), add 50, 100, 250, or 500 μL of phenytoin standard solution and 5, 10, 25, or 50 μL of 0.01 g/L standard solution of HPPH (0.1 g/L solution for urine assays). Remove the methanol by evaporation (we used an Evapo-Mix; Buchler Instruments, Fort Lee, NJ 07024). Include a fifth tube, representing the blank, to prepare the standard curve.

Add 30 μL of the internal standard solution (50 μL for urine analysis) to each of the five tubes for the standard curve and to each sample tube. Add 1 mL of drug-free human plasma (or urine) to the tubes from which the standard curve will be prepared; add to the appropriate sample tubes 1.0-mL aliquots of the plasma (or urine) samples to be analyzed. From this point in the procedure, all tubes (standards and samples) are treated identically.

To each tube add 1 mL of phosphate buffer, pH 7.5, then 5.0 mL of anhydrous ether by automatic pipet (Repipet; Lab Industries, Berkeley, CA 94710). Stopper the centrifuge tubes with ground-glass stoppers, place them horizontally on a mechanical shaker (Eberbach Corp., Ann Arbor, MI 48106), and shake at 180 cycles per minute for 5 min. Centrifuge at 750 X g for 5 min.

Transfer a 4-mL aliquot of the ether phase by pipet to a clean 13-mL centrifuge tube into which 1.0 mL of phosphate buffer, pH 11.2, has already been added; shake the mixture for 5 min, and centrifuge for 5 min as before. Aspirate and discard the ether layer, and add 1.0 mL of phosphate buffer, pH 6.8, to each tube. Then add (Repipet) anhydrous ether (5.0 mL), and shake and centrifuge the tubes as before. Transfer a 4.0-mL aliquot of ether to a clean 13-mL centrifuge tube, and evaporate (Evapo-Mix) without added heat. Reconstitute all samples with 100 μL of the mobile phase before injecting into the liquid chromatograph.

Analysis of phenytoin and total (conjugated plus unconjugated) HPPH in plasma and urine. To four centrifuge tubes, add 25, 50, 100, or 200 μL of phenytoin standard solution and 5, 10, 25, or 50 μL of HPPH standard solution (0.1 g/L); for assays of urine add 25, 50, 100, or 200 μL of 1 g/L HPPH solution. Remove the methanol by evaporation. To prepare the standard curve, add a fifth tube, representing the blank.

Add 0.5 mL of drug-free human plasma or urine to the standard curve tubes, and 0.5-mL aliquots of the plasma or urine samples to be analyzed to the appropriate sample tubes. Under a fume hood, add 0.5 mL of concentrated HCl; vortex-mix the contents briefly, then place the tubes into a boiling water bath for 30 min. Allow the tubes to cool and add 0.5 mL of 12 mol/L NaOH. Vortex-mix again briefly, and allow the tubes to cool once more. Add 1 mL of potassium phosphate buffer, pH 7.88, and vortex-mix.

For analysis of plasma samples, add 50 μL of internal standard solution; for urine samples, add 50 μL of a 4.0 g/L solution of internal standard. Vortex-mix, and proceed with the extraction procedure as described for analysis of unconjugated HPPH in plasma and urine (above).

Chromatography. Inject into the liquid chromatograph approximately 10 μL of the samples reconstituted with the mobile phase and chromatograph, using conditions as described above. Measure peak heights manually and calculate the ratios of peak heights of phenytoin and HPPH to the internal standard.

Analytical Recovery

Recoveries of phenytoin, HPPH, and phenobarbital were determined by comparing their peak-height ratios after extraction of plasma samples containing known amounts with those peak height ratios measured in unextracted samples supplemented with known amounts of compound. For purposes of these calculations, we added the internal standard to the samples just before injection into the chromatograph.

Regression Analysis

Concentrations of phenytoin, HPPH, and phenobarbital are determined from the regression equations relating measured peak-height ratios of the standards to their concentrations. We did not force the regression lines to pass through the origin.

To assess the accuracy of the method for phenytoin, phenobarbital, and carbamazepine, we analyzed, at monthly intervals, samples containing known amounts of anticonvulsant drugs supplied to this laboratory during participation in a nationally conducted program of quality-control assessment (15).

Results and Discussion

Chromatograms

No interfering peaks were noted in control plasma blanks. Chromatograms resulting from the analysis of blank plasma and patient’s plasma are shown in Figure 1. Table 1 gives retention times for phenytoin and HPPH, as well as for other drugs taken through the extractions and chromatographic procedure. If interfering peaks were suspected, we monitored
Table 1. Relative and Absolute Retention Times at a Flow Rate of 1.2 mL/min

<table>
<thead>
<tr>
<th>Drug</th>
<th>Absolute Retention Times, min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relative</td>
</tr>
<tr>
<td>Caffeine</td>
<td>3.60</td>
</tr>
<tr>
<td>HPPH</td>
<td>4.10</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>4.60</td>
</tr>
<tr>
<td>Internal standard (alphenal)</td>
<td>5.25</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>6.56</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>7.72</td>
</tr>
</tbody>
</table>

Analytical recovery. Recovery for phenytoin, HPPH, and phenobarbital was calculated to be 41, 79, and 67%, respectively. Although recovery of phenytoin is significantly decreased by including the buffer wash in our procedure, this step is necessary to avoid peaks that would otherwise interfere with the determination of HPPH in plasma.

Accuracy. The accuracy of this method for the analysis of phenytoin, phenobarbital, and carbamazepine was evaluated over a period of 25 months by analyzing, at monthly intervals, samples containing known amounts of anticonvulsant drugs supplied to this laboratory as part of a nationally conducted quality-assurance program (15). The results for phenytoin are given in Figure 3, along with the regression equations relating the measured value to the supplemented value for all three drugs. Performance Indices (PI) by our method for anticonvulsant analyses carried out over 25 consecutive months were 2.75, 2.78, and 3.45 for phenytoin, phenobarbital, and carbamazepine, respectively, calculated according to Pippenger et al. (16); values of PI <10 are considered to be exceptionally good. Because fortified HPPH samples were not available, we were unable to assess similarly the accuracy of the HPPH analysis.

The regression lines for the three drugs have slopes and intercepts close to unity and zero, respectively, indicating that the accuracy of the method is quite acceptable.

Phenytoin and HPPH in Samples of Patients' Plasma and Urine

We measured concentrations of phenytoin and HPPH in plasma after administering single intravenous and oral doses of sodium phenytoin (4.0 mg/kg of body weight) to the same subject on separate occasions. Unconjugated HPPH concentration was generally less than 0.1 mg/L, whereas that of the conjugated metabolite was between 0.4 and 2.0 mg/L. The sample containing a single dose of phenytoin,

Analytical Variables

Precision. Coefficients of variation for within-run precision (n = 10) for phenytoin were 2.6 and 2.3% for plasma concentrations of 5.0 and 20.0 mg/L, respectively; for HPPH, 4.3 and 2.5% for plasma concentrations of 0.05 and 0.50 mg/L, respectively; and for conjugated HPPH, 3.7 and 3.7% for urinary concentrations of 32.0 and 324.0 mg/L, respectively.

To assess among-run precision for phenytoin and phenobarbital analysis, we sequentially assayed aliquots of the same plasma samples containing added phenytoin and phenobarbital. We analyzed nine such samples four to eight times during six to eight weeks. Our results are shown in Table 2.

Fig. 2. Typical standard curves for phenytoin (DPH, 0) and HPPH (D) for plasma analysis; the internal standard is alphenal (AP)
Phenytoin/unconjugated HPPH area ratio was greater for the intravenous dose than for the oral dose (35.6 vs 25.4), demonstrating that kinetics are nonlinear in this concentration range for phenytoin. However, the ratio of the area under the curve for the metabolites (conjugated/unconjugated HPPH) was similar for the intravenous (21.7) and oral (18.4) doses, suggesting that the formation and elimination of the conjugate can be described by first-order kinetics. That the elimination kinetics of phenytoin are saturable in the concentration range observed here would invalidate the use of area ratios (oral to intravenous) in calculating the absolute bioavailability of phenytoin.

Simultaneous determinations of phenytoin and HPPH may be useful in elucidating the mechanism of changes in steady-state phenytoin concentrations associated with the addition or withdrawal of other medication from the regimen. Co-administered agents that inhibit or induce the formation of HPPH from phenytoin should theoretically affect the ratio of HPPH/phenytoin in the plasma at steady state.

Changes in the urinary output of total HPPH may help identify reasons for the alteration in steady-state concentrations of phenytoin in plasma. Decreased steady-state concentrations may result from an increase in metabolic clearance or a decrease in the extent of phenytoin absorption. Such a change was attributed to a short-term reversible increase in metabolic clearance of phenytoin in a patient suffering from infectious mononucleosis (17). Indeed, alterations in the urinary output of HPPH may help verify conclusions drawn on the basis of altered HPPH/phenytoin plasma concentration ratios.

Acid hydrolysis of the glucuronide conjugate of HPPH in human urine is accompanied by dehydration of a minor dihydrodiol metabolite, producing equivalent amounts of 5-(4-hydroxyphenyl)-5-phenyldihydropyridine and 5-(3-hydroxyphenyl)-5-phenyldihydropyridine (18). This result was overestimating the actual amount of HPPH present in the urine sample. However, the results of a recent study comparing acid and enzymatic hydrolysis of phenytoin metabolites in human urine suggest that acid-catalyzed degradation of the dihydrodiol metabolite contributes little if anything to total urinary HPPH (19).

The sensitivity of the method described in this report should allow its use in quantitating changes in the relative concentration of phenytoin, its conjugated metabolite, and unconjugated HPPH as well. Careful monitoring of these concentrations may assist in the investigation of altered phenytoin absorption and disposition. In addition, a knowledge of the relationship between phenytoin and HPPH concentrations may be helpful in assessing the extent to which nonlinear kinetics are involved in the metabolism of phenytoin.

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References


