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Simultaneous determination of *p*-hydroxylated and dihydrodiol metabolites of phenytoin in urine by high-performance liquid chromatography

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ABSTRACT

Accurate urinary measurements of the two major metabolites of phenytoin, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH) and 5-(3,4-dihydroxy-cyclohexa-1,5-dienyl)-5-phenylhydantoin (dihydrodiol, DHD), are necessary for pharmacokinetic and drug-interaction studies of this commonly used antiepileptic drug. We describe a simple, rapid, acid hydrolysis, with liquid–liquid extraction and simultaneous isocratic reversed-phase high-performance liquid chromatography of *p*-HPPH and 5-(*m*-hydroxyphenyl)-5-phenylhydantoin (*m*-HPPH) (hydrolytic end product of DHD). *p*-HPPH and *m*-HPPH were quantitated against their separate respective internal standards of alphenal and tolylbarb. The mobile phase consisted of water–dioxane–tetrahydrofuran (80:15:5, v/v/v) at 2 ml/min and at 50°C, with detection at 225 nm. Baseline separation was achieved by use of a 16 cm × 3.9 mm Nova-Pak C_{18} column and total analysis time of 12 min. *p*-HPPH and *m*-HPPH concentrations ranged from 10 to 200 and from 2 to 30 μg/ml, respectively, with between-day coefficients of variation of 3.3–4.5% and 2.2–5.1% for controls. All standard curves were linear with r values > 0.993. The DHD concentration was determined by multiplying *m*-HPPH concentrations by 2.3.

INTRODUCTION

Phenytoin (PHT) is one of the most widely used and extensively studied drugs for the treatment of epilepsy. It is well established that PHT has non-linear "dose-dependent" pharmacokinetic properties. Therefore, relatively minor variations in the daily dose of the drug can significantly affect steady-state plasma concentrations, metabolism and elimination of PHT. Drug absorption can vary with drug formulation [1].

In a study in which the bioavailability of a generic extended-release form of Na PHT was compared with the brand name Na PHT (Dilantin) it was necessary to make accurate and precise measurements of PHT and its major metabolites in plasma and urine. Steady-state plasma PHT concentrations (for area under the curve (AUC) values) and clearance were measured by our method described elsewhere [2]. The major hydroxylated metabolite of phenytoin in urine (*p*-HPPH) occurs in unconjugated and conjugated (glucuronide) form. For quantitation of total urinary

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p-HPPH it was necessary to convert conjugated *p*-HPPH to its unconjugated form. This can be accomplished by either enzymatic [3,5–14] or acid hydrolysis [4,5,13,15–21] of the glycosidic bond in the glucuronide. Enzymatic hydrolysis is time consuming, and was considered unreliable by the sponsors of the clinical study. Acid hydrolysis was fast and reproducible; however, it was known [4,5,13,22] to complicate *p*-HPPH measurements with the dehydration of the dihydrodiol PHT metabolite (DHD) to produce derived *p*-HPPH and derived *m*-HPPH (Fig. 1). The problem was that existing high-performance liquid chromatographic (HPLC) procedures [7,14,17–21] could not resolve the *m* and *p* forms of HPPH and so could not accurately quantitate the metabolites. We developed a HPLC method that separates and measures against an internal standard total *p*-HPPH and derived *m*-HPPH. Accurate quantitation of urinary *p*-HPPH and DHD concentration can be accomplished with a few simple calculations (eqns. 1–4).

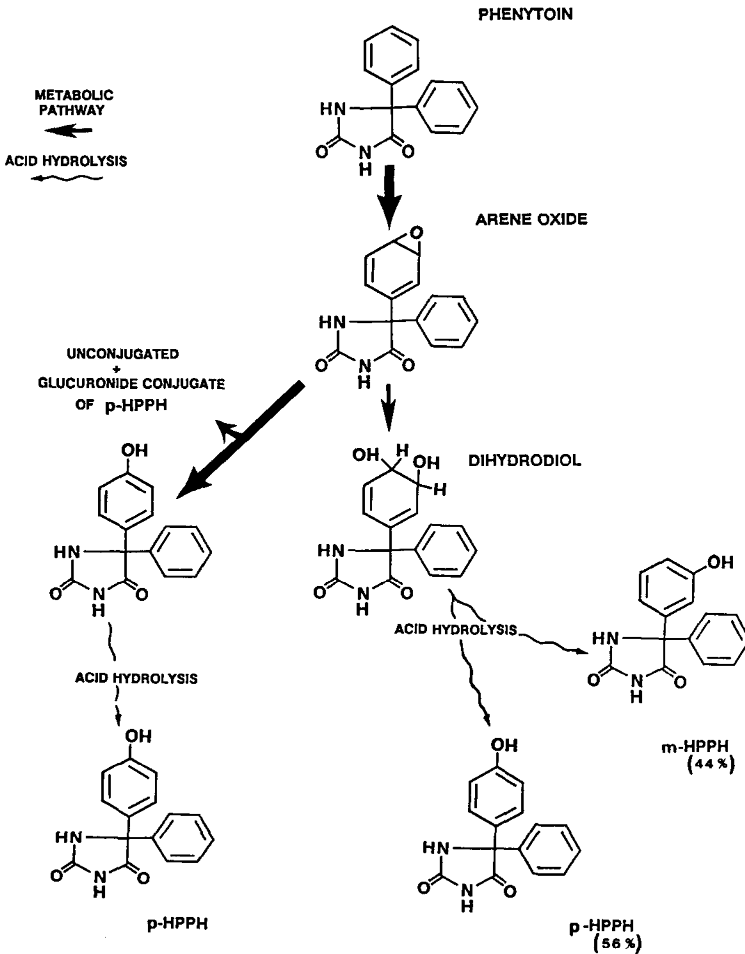

Fig. 1. Metabolic fate of phenytoin and its principal analytes after acid hydrolysis.

$$\text{Corrected total } p\text{-HPPH} = (\text{24-h urine volume} \times \text{measured } p\text{-HPPH}) - \left[\text{total measured } m\text{-HPPH} \times \left(\frac{56\% \text{ derived } p\text{-HPPH}}{44\% \text{ derived } m\text{-HPPH}}\right)\right] \quad (1)$$

or

$$\text{Corrected total } p\text{-HPPH} = \text{total measured } p\text{-HPPH} - (\text{total measured } m\text{-HPPH} \times 1.3) \quad (2)$$

$$\text{Corrected total DHD} = (\text{24-h urine volume} \times \text{measured } m\text{-HPPH}) \times \left(\frac{100\% \text{ DHD}}{24\% \text{ derived } m\text{-HPPH}}\right) \times \left[\frac{\text{mol.wt. DHD (286)}}{\text{mol.wt. } m\text{-HPPH (268)}}\right] \quad (3)$$

or

$$\text{Corrected total DHD} = \text{total measured } m\text{-HPPH} \times 2.3 \times 1.07 \quad (4)$$

EXPERIMENTAL

Instrumentation

The HPLC system consisted of a Model 2350 solvent pump and Model V4 variable-wavelength UV detector, both from ISCO (Lincoln, NE, U.S.A.) with an autosampler WISP Model 710B from Waters Assoc. (Milford, MA, U.S.A.). Chromatography was performed on a Nova-Pak C_{18} (4 μm particle size, 15 cm $\times$ 3.9 mm I.D.) stainless-steel column from Waters Assoc., heated with a water circulator pump, Model FE, Haake Instruments (Saddlebrook, NJ, U.S.A.). Detector output was monitored by a Recordall Series 5000 strip-chart recorder, Fisher Scientific (Pittsburgh, PA, U.S.A.) and peak quantitation was performed on a Canon AS-100 computer from Binary Systems (Newton, MA, U.S.A.), with an analog-to-digital converter from Quasitronics (Houston, PA, U.S.A.) and software designed by Binary Systems.

Standards and reagents

The analytical standards, D,L-5-(4-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), 5-(3-hydroxyphenyl)-5-phenylhydantoin (*m*-HPPH) (Fig. 1) and internal standard 5-ethyl-5-(*p*-methylphenyl)-barbituric acid (tolylbarb) were obtained from Aldrich (Milwaukee, WI, U.S.A.). The other internal standard, 5-allyl-5-phenylbarbituric acid (alphenal) was obtained from Applied Science Labs. (State College, PA, U.S.A.). Disodium hydrogenphosphate, potassium dihydrogenphosphate, disodium hydrogenphosphate heptahydrate, hydrochloric acid, HPLC-grade diethyl ether, 1,4-dioxane, tetrahydrofuran (THF) and methanol reagents and solvents were obtained from Fisher Scientific. Stock analytical and internal standard solutions for *p*-HPPH, *m*-HPPH, alphenal and tolylbarb were prepared by dissolving 10 mg of each

compound in 10 ml of methanol. Methanolic working standards at varying concentrations were prepared from stock solutions for construction of standard curves and urine controls. Working internal standard solutions were prepared in methanol–distilled water (1:1) at concentrations of 500 μg/ml and 50 μg/ml for alphenal and tolylbarb, respectively.

Chromatographic conditions

The isocratic mobile phase consisted of a ternary mixture of HPLC-grade water–dioxane–THF (80:15:5, v/v/v). The flow-rate was 2 ml/min, and column temperature was stabilized at 55°C. Eluents were UV monitored at a wavelength of 225 nm with the detector set at 0.05 absorbance units full scale (a.u.f.s.).

Sample preparation

Patient urine samples collected for this study consisted of pre-study blank urine and a specimen 24 h after the last dose from each phase of the cross-over design. Urines were kept frozen at −20°C until assayed. The hydrolysis and extraction of conjugated and unconjugated urinary metabolites of phenytoin were previously described by Sawchuk and Cartier [19], with only slight modifications.

A 500-μl volume of methanolic standard solution was added to 125 × 16 mm disposable glass culture tubes (for standard curve samples only) and evaporated to dryness. To this tube, 0.5 ml of drug-free urine were then added.

In similar fashion, 0.5 ml of control or patient urine was added to clean tubes. Next, to all tubes 0.5 ml of concentrated (12 *M*) hydrochloric acid were added. Tubes were gently mixed and placed in a preheated oven for 1.5 hours at 100°C. Solutions were allowed to cool and 100 μl of working internal standard solutions were added, followed by 5.0 ml of diethyl ether. For extraction, tubes were capped with polyethylene Tainer Tops (Fisher Scientific) and horizontally shaken on a mechanical shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 10 min at 180 cycles per min. Next, tubes were centrifuged for 5 min at 650 *g*. The ether layer was then transferred to a clean 125 × 16 mm culture tube that contained 1.0 ml of phosphate buffer pH 11.2. Back extraction was then performed. Tubes were recapped, shaken and centrifuged as before, then the ether layer was aspirated off. To the remaining aqueous fraction 1.0 ml of phosphate buffer pH 6.8 was added. The resulting solution was mixed, 5 ml of ether was added and re-extracted as in the previous steps. The ether layer was then transferred to a clean culture tube. The ether fraction was evaporated under a gentle stream of dry air at 40°C in the Organomation Meyer N-VAP (Berlin, MA, U.S.A.). In the final step the dried residue was reconstituted with 200 μl of methanol. The tube is then vortexed (15 s) and the solution is transferred to a WISP vial for 15 μl injection on LC system.

RESULTS

Precision and accuracy

Separate stock standards and working standards of *p*-HPPH and *m*-HPPH were prepared for each of the three days of the precision study. A fourth set of stock standards was used to prepare the controls in blank human urine. Three separate replications of a six-point standard curve and three sets of quality-control urine at low,

mid and high concentrations were assayed on each day of the precision study. The control samples had between-day coefficients of variation (C.V.) of 2.2–5.1% (Table I). Accuracy of the standard-curve points was evaluated by a comparison of the experimentally measured values (back-calculated from regression analysis) to the expected concentrations. The ratio of observed to expected concentrations was expressed as a relative percent of the expected concentrations. The percents for both compounds ranged from mean values of 96 to 106% at standard-curve concentrations for *p*-HPPH 10.0–200.0 μg/ml and *m*-HPPH 2.0–30.0 μg/ml (Table 1).

CALCULATIONS

PHT-metabolite concentration values were calculated from peak-height ratios of *p*-HPPH to alphenal and *m*-HPPH to tolylbarb *versus* standard values by least-square linear regression analysis. All curves were linear with r values > 0.993. Patient urine *p*-HPPH and DHD concentrations required corrections from derived *p*-HPPH and derived *m*-HPPH produced from the acid hydrolysis. As described by Maguire *et al.* [4], and reproduced in our laboratory, pure DHD reproduces 56% derived *p*-HPPH and 44% derived *m*-HPPH upon treatment with acid and heat. In a patient's urine sample, the corrected total *p*-HPPH concentration is equal to the ratio of derived *p*-HPPH percent (56%) to derived *m*-HPPH percent (44%) multiplied by the measured *m*-HPPH concentration and subtracted from the measured *p*-HPPH (eqns. 1 and 2). In a patient's urine sample the total corrected DHD concentration is equal to the ratio of DHD percent (100%) to derived *m*-HPPH percent (44%) multiplied by measured *m*-HPPH times the molecular-weight ratio of DHD (286) to *m*-HPPH (268) (eqns. 3 and 4).

TABLE I

ACCURACY OF STANDARDS AND PRECISION OF CONTROLS

	Standards			Controls		
	Expected concentration (μg/ml)	Mean measured concentration[a] (μg/ml) (n = 9)	Mean relative (%) measured/expected[a] (n = 9)	Expected concentration (μg/ml)	Mean measured concentration (μg/ml) (n = 9)	C.V.
p-HPPH	10.0	10.6	106			
	20.0	19.9	100	30.0	30.7	4.5
	50.0	49.8	100			
	100.0	99.8	100	90.0	86.2	4.1
	150.0	150.2	100			
	200.0	199.2	100	180.0	178.1	3.3
m-HPPH	2.0	2.1	106			
	5.0	4.8	96	6.0	5.9	5.1
	10.0	10.1	101			
	15.0	14.9	99	12.0	11.6	2.2
	20.0	20.2	101			
	30.0	29.9	100	18.0	17.9	3.8

[a] See text for definition.

DISCUSSION

Sample preparation

The modified sample procedures of Sawchuk and Cartier [19], gave good recoveries of all compounds. We tried eliminating the back-extraction into pH 11.2 buffer; however, endogenous interfering peaks from some (but not all) acid-hydrolyzed urines necessitated the use of the clean-up step.

Chromatography

After experimenting with different combinations of organic solvent concentrations in the mobile phase, optimal resolution of *m*-HPPH from *p*-HPPH was obtained. Since between 50–75% of total phenytoin dose is recovered as *p*-HPPH [4], large amounts of *p*-HPPH could be expected in 24-h urine samples. This was especially critical in samples with low total-urine-volume output. In our procedure *m*-HPPH is well resolved from *p*-HPPH even as the concentration of *p*-HPPH rises (Fig. 2). This phenomenon of extreme concentration variation also necessitated the use of separate internal standards for each measured compound. The use of a heated column allowed for a relatively short run time under isocratic conditions and helped to reduce band spreading.

CONCLUSIONS

The method we describe for quantitation of urinary PHT metabolites has a simple, fast and reproducible sampling procedure, with demonstrated accuracy and

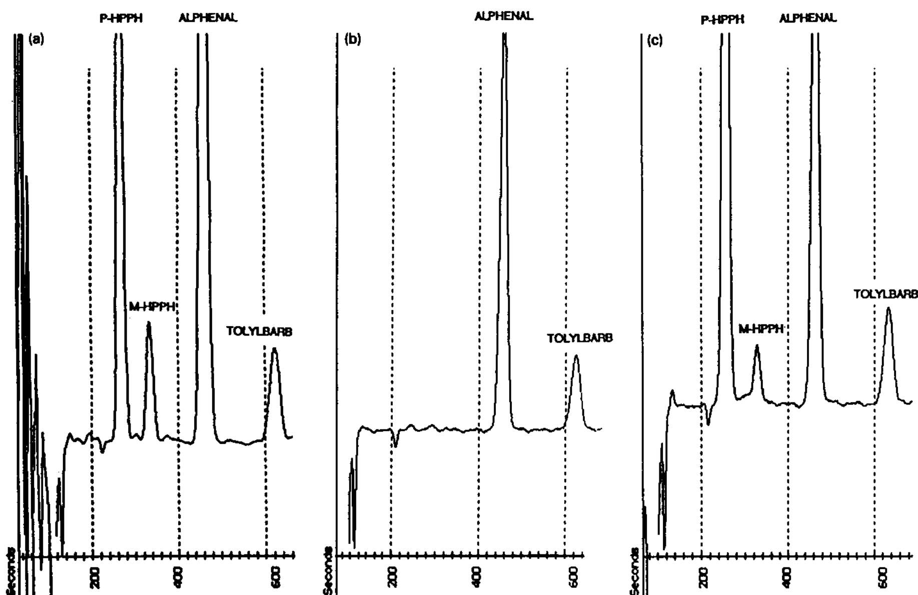

Fig. 2. Chromatograms of extracted urine samples. (a) Standard urine containing *p*-HPPH (50 μg/ml) and *m*-HPPH (10 μg/ml); (b) patient pre-study 24-h urine (blank) with internal standards; (c) patient 24-h urine containing *p*-HPPH (130.2 μg/ml) and *m*-HPPH (6.4 μg/ml).

precision over a representative concentration range. This method has proven suitable for bioequivalency studies. With minor alterations in the dynamic range, this method would also have use in pharmacokinetic and drug interaction studies where the measurement of alternative metabolic pathways is important.

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