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Note

Analysis of *p*-hydroxyphenytoin in microsomal reactions by high-performance liquid chromatography with electrochemical detection

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5,5-Diphenylhydantoin (5,5-diphenyl-2,4-imidazolidinedione; phenytoin; Dilantin®) is an important and widely used drug [1]. Phenytoin is almost entirely metabolized (> 95%), and *p*-hydroxylation to 5-(*p*-hydroxyphenyl)-5-phenylhydantoin [5-(4-hydroxyphenyl)-5-phenyl-2,4-imidazolidinedione; *p*-hydroxyphenytoin] is the major pathway for its biotransformation in humans [1, 2]. Under clinical conditions, phenytoin exhibits a saturation (Michaelis-Menten) kinetic profile, and the excretion of *p*-hydroxyphenytoin is dose-dependent [2]. Because phenytoin elimination is subject to inhibition by other drugs [3], plasma phenytoin levels may become elevated, leading to various side effects, including nystagmus, ataxia, and mental changes [4]. Therefore, the use of phenytoin may present a clinical management problem.

Various analytical methods have been reported for determination of *p*-hydroxyphenytoin, including gas chromatography [5, 6], high-performance liquid chromatography (HPLC) using ultraviolet absorbance detection [7, 8], and gas chromatography—mass spectrometry (GC—MS) [9—11]. All of these methods have some drawbacks, and all were designed primarily for analyses of concentrations considerably greater than those generated in microsomal reactions. These methods are relatively laborious, requiring derivatization, sophisticated instrumentation, or the use of stable isotopes. As an alternative to the previously used radiometric methods employing ^{14}C [12—15], we describe a simple, rapid, and sensitive method for the quantitation of *p*-hydroxyphenytoin from in vitro metabolic reactions.

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EXPERIMENTAL

Materials

The reference standards, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (HPPH) and 5-(*m*-hydroxyphenyl)-5-phenylhydantoin, and the internal standard, 5-(*p*-hydroxyphenyl)-5-(*p*-tolyl)hydantoin (HPTH), were obtained from Aldrich (Milwaukee, WI, U.S.A.). Phenytoin was obtained from United States Pharmacopeial Convention (Rockville, MD, U.S.A.) and was used as a substrate. Sodium phenytoin, which was used for animal pretreatment, came from Parke-Davis (Ann Arbor, MI, U.S.A.). Water and diammonium hydrogen phosphate (both HPLC-grade) were obtained from J.T. Baker (Phillipsburgh, NJ, U.S.A.). Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH, Type X), and polysorbate 20 were purchased from Sigma (St. Louis, MO, U.S.A.). Baker analyzed reagents were obtained from J.T. Baker and organic solvents came from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Microsomal isolation

Male Sprague—Dawley rats (200—225 g) were pretreated with an aqueous sodium phenytoin suspension [50 mg/kg/day intraperitoneally in 0.5% (v/v) polysorbate 20] for three days to approximate the situation in epileptic patients undergoing long-term phenytoin therapy. The rats were then decapitated and their livers were excised quickly and washed sequentially in 0.25 *M* sucrose, 0.05 *M* Tris buffer, pH 7.50, containing 1.15% potassium chloride, and finally 0.25 *M* sucrose. Washings were performed at 4° C by gentle stirring with a magnetic stirrer for 10 min. All subsequent procedures were also performed at 4° C. The livers were then homogenized in four volumes of 0.25 *M* sucrose for 1—2 min, using a Waring blender. The homogenate was centrifuged at 9000 *g* for 25 min. The supernatant was poured into clean centrifuge tubes through two layers of cheesecloth to filter out lipid and large debris and was centrifuged at 105,000 *g* for 90 min. The resultant microsomal pellet was resuspended in 0.05 *M* Tris buffer, pH 7.5, containing 1.15% potassium chloride, using a hand-driven Potter-Elvehjem homogenizer. The resuspended pellet was again centrifuged at 105,000 *g* for 90 min. The resultant washed microsomal pellet was resuspended in 0.25 *M* sucrose as before and divided into 0.5—1.0 ml aliquots. The aliquots were stored under nitrogen at about —65° C. Protein determinations were made by minor modifications of the colorimetric assay of Lowry et al. [16].

Microsomal reactions

The reaction mixture contained 0.78 mg microsomal protein, 0.40 m*M* EDTA, 0.40 m*M* magnesium chloride, 1.0 m*M* potassium chloride, 0.05 *M* potassium phosphate buffer pH 7.50, 1.0 m*M* NADPH, and varying amounts of phenytoin (0.79—79.4 μM), the substrate. Methanolic stock solution of phenytoin was used, and the solvent was evaporated before the other reaction mixture components were added. The total reaction volume was 2.5 ml. Reactions were carried out at 37° C in air for 10 min; they were initiated by adding NADPH after a 1-min equilibration period at 37° C and were terminated by adding 5 ml of methyl *tert.*-butyl ether and vortex mixing. All reactions were run in duplicate.

HPLC

After the reaction was terminated, the internal standard (HPTH, 250 ng) was added to the microsomal extracts. The samples were then mixed for about 15 sec and centrifuged for 10 min at about 2000 *g* at 4°C. The methyl *tert.*-butyl ether phase was transferred to clean tubes and evaporated to dryness under nitrogen at 30—40°C. The residue was redissolved in 200 μl of mobile phase, and 5—25 μl of this mixture were injected into the chromatograph.

The chromatographic system consisted of a pump (Model 110A, Beckman, Palo Alto, CA, U.S.A.), an injector with a 50-μl loop (Model 7125, Rheodyne, Cotati, CA, U.S.A.), an amperometric detector with a TL-5 glassy carbon electrode (Model LC-4A, Bioanalytical Systems, West Lafayette, IN, U.S.A.), a standard strip-chart recorder, and a 30 cm × 3.9 mm I.D., 10-μm particle size μBondapak C_{18} column (Waters Assoc., Milford, MA, U.S.A.). A guard column (MPLC 5-μm C_{18} guard cartridge OD-GU, Brownlee Labs., Santa Clara, CA, U.S.A.) was also employed. The column was kept at ambient temperature. The mobile phase was acetonitrile—0.05 *M* diammonium hydrogen phosphate (30:70, v/v) and the flow-rate was 2.0 ml/min. The electrochemical detector was set at a potential of 1.05 V and a range of 10 or 20 nA.

For each analysis, a standard curve was generated by adding known, varying amounts of HPPH and a constant amount of the internal standard and the substrate to non-incubated reaction mixture. These standards bracketed the range of experimental values. Quantitation was achieved by using peak height ratios of HPPH to HPTH. As an additional cross-verification of the HPLC method, samples remaining after HPLC analysis were analyzed by selected ion monitoring (SIM), using a GC—MS—computer (GC—MS—COMP) system.

RESULTS AND DISCUSSION

This relatively simple method for the quantitation of in vitro metabolically generated *p*-hydroxyphenytoin involves a single extraction step, isocratic reversed-phase HPLC separation, and electrochemical detection. The large excess of substrate (phenytoin) does not create a problem since the electrochemical detector does not respond to phenytoin. Under the HPLC conditions described, the relative retention time of phenytoin to metabolite (HPPH) is about 2.10, as determined by interposing an ultraviolet detector between the column and the electrochemical detector. Since the electrochemical detector is transparent to phenytoin, it is not necessary to wait for phenytoin elution, and the analysis time is shortened considerably.

Fig. 1 shows representative chromatograms of extracts of a microsomal blank, a microsomal reaction, and two standards. The blank was run in the same manner as the actual reaction, but NADPH was added only after the reaction was terminated, and internal standard was omitted. An additional peak (I), which eluted in front of the internal standard, was present in the extract of the microsomal reaction but not in those of the standards or the blank. This peak was suggestive of a metabolite, especially since its peak height relative to that of HPTH increased with increased substrate concentration. The identity of this peak is unclear, but it is not the *m*-hydroxy isomer; *m*-hydroxyphenytoin was not generated under these conditions since the peak

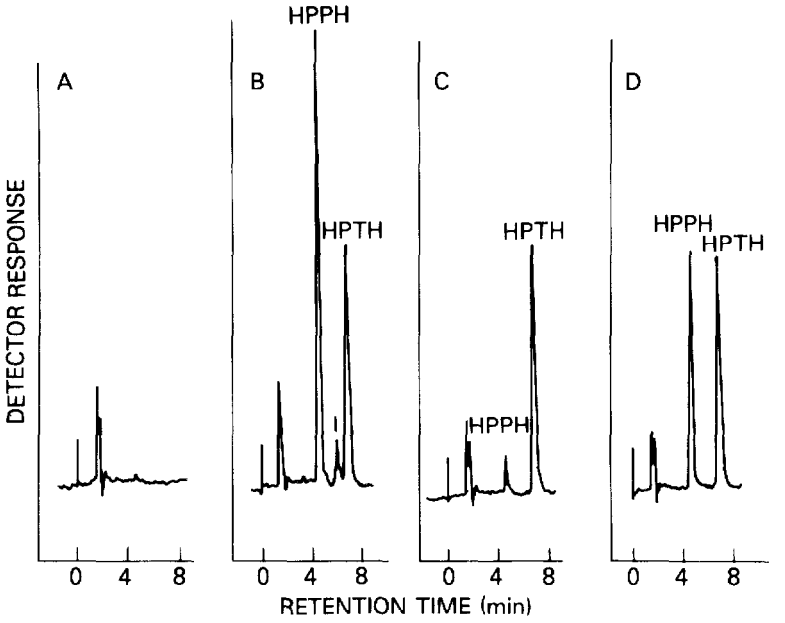

Fig. 1. High-performance liquid chromatogram of extracts from microsomal blank (A), microsomal reaction (B), standard containing 25 ng of HPPH (C), and standard containing 200 ng of HPPH (D). All samples initially contained 23.8 μM phenytoin. In all instances, 25 μl of each extract was injected into the chromatograph and the electrochemical detector range was set at 20 nA. See text for details.

corresponding to it (using a reference compound) was not detected by HPLC or SIM analysis. Further analysis of samples by GC—MS—COMP confirmed that phenytoin was not present in microsomes and that HPPH was not present and was not generated unless phenytoin was added to the reaction mixture.

The extraction efficiency was determined by comparing peak height ratios of HPPH to HPTH for samples spiked with HPPH and extracted relative to non-extracted samples. The recovery of HPPH was about 98%.

Standard curves were generated routinely by using eight standards covering a range of 25—1000 ng HPPH. Good linearity and acceptable Y-intercept were found routinely. By least-squares linear regression analysis, representative equation of the line and the regression coefficient (r^2) were $Y = 0.00537X - 0.00008$, $r^2 = 0.998$. The r^2 value was always at least 0.998.

The HPLC method was independently validated by SIM analysis of the same microsomal reaction extracts. The comparison of findings from 47 samples tested by both methods gave a correlation coefficient (r) of 0.998 and the equation of the least-squares linear regression was $Y = 0.994X - 0.79$, showing a good correspondence between the methods.

In the microsomal reactions, the substrate (phenytoin) concentration ranged from 0.79 to 79.4 μM. Fig. 2 shows a representative plot of the reaction velocity (v) versus substrate concentration (s). The data were replotted as the standard double reciprocal (Lineweaver-Burk) plot ($1/v$ versus $1/s$) (Fig. 3). A biphasic kinetic profile was observed, suggesting high- and low-affinity metabolic sites. This finding is consistent with recent findings of Tsuru et al. [15].

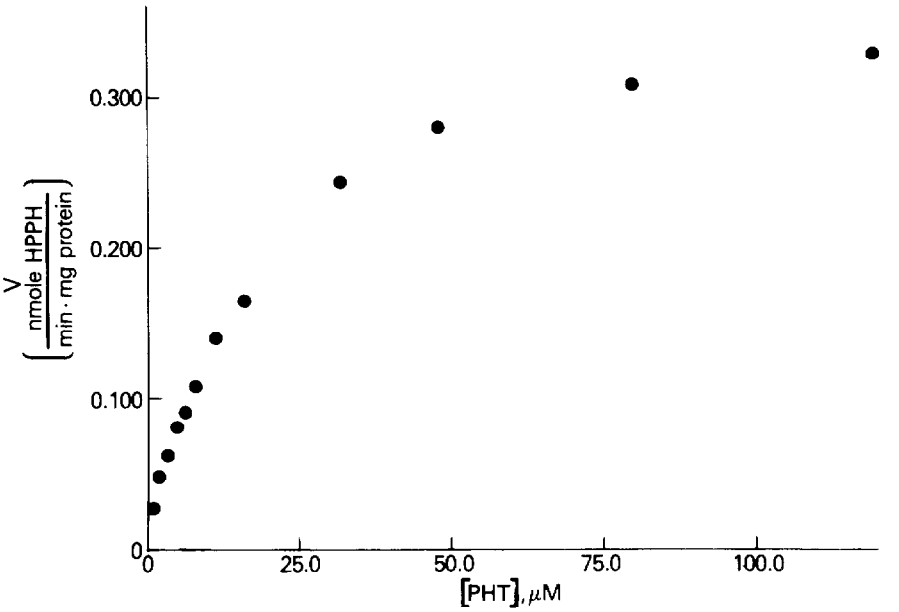

Fig. 2. Reaction velocity—substrate concentration profile for microsomal *p*-hydroxylation of phenytoin. See text for details.

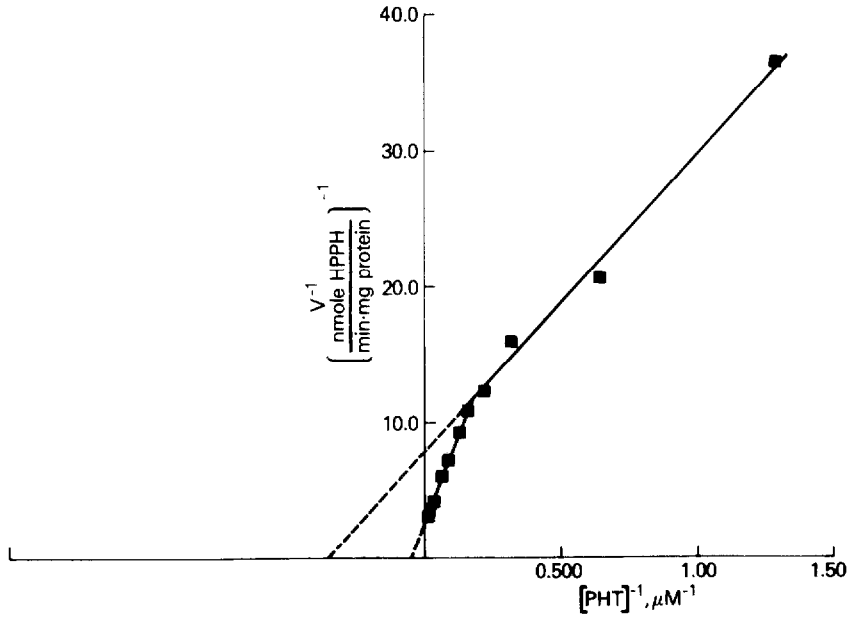

Fig. 3. A double reciprocal plot for microsomal *p*-hydroxylation of phenytoin. The lines were drawn based on least-squares linear regression. See text for details.

Therefore, the method is well suited for kinetic studies of in vitro *p*-hydroxylation of phenytoin and is currently being employed to study drug—drug interactions. Microsomes from untreated rats were used in subsequent studies, and the results were comparable to those described here for phenytoin-treated rats.

The method provides good sensitivity. The smallest amount of HPPH injected was about 3 ng. A further increase in sensitivity can be achieved by injecting a large fraction of the sample and/or increasing the sensitivity setting on the detector. Specificity is offered by the electrochemical detector, which preferentially detects the compound of interest, in this case the metabolite and not the substrate. The method should be suitable for in vitro studies of other *p*-hydroxylation reactions since the detector is selective for and relatively sensitive to phenolic structures.

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