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Note

Analysis of the toxicologically relevant metabolites of phenytoin in biological samples by high-performance liquid chromatography

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Phenytoin (5,5-diphenylhydantoin, Dilantin®) is one of the most efficacious and widely prescribed anticonvulsants for the treatment of epilepsy. The association of phenytoin with teratological sequelae in animals and humans has been reviewed along with the proposed mechanisms [1]. Phenytoin teratogenicity has been established and characterised in animals, and strongly implicated in humans. Teratologic sequelae are thought to result from the cytochrome P-450-mediated bioactivation of phenytoin to a reactive arene oxide intermediate which, if not detoxified, can bind covalently to essential fetal cellular macromolecules, thereby causing developmental aberrations. The arene oxide can be detoxified by rearrangement via an NIH shift to 5-(4-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), or can be hydrated by epoxide hydrolase to a *trans*-dihydrodiol and subsequently to a catechol and then a methoxycatechol metabolite. Mechanistic studies of phenytoin teratogenicity require the ability to measure all these metabolites to evaluate the balance of phenytoin bioactivation and detoxification. Several assays have been reported using high-performance liquid chromatography (HPLC) [2—6]; however, they fail to detect or separate all toxicologically relevant metabolites, and generally require long analysis time to complete. We describe herein a sensitive HPLC method for the detection within 15 min of phenytoin and its metabolites. Results from a number of HPLC columns and solvent systems are compared.

METHODS

Materials and reagents

Sodium phenytoin (5,5-diphenylhydantoin, sodium salt), 5-(4-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), and 5-(3-hydroxyphenyl)-5-phenyl-

hydantoin (*m*-HPPH) were obtained from Sigma (St. Louis, MO, U.S.A.). The α,α′-diphenylglycine was obtained from Aldrich (Milwaukee, WI, U.S.A.). The 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin (dihydrodiol) and 5-(3-4-dihydroxyphenyl)-5-phenylhydantoin (catechol) were gifts from Dr. J.H. Maguire (University of North Carolina), and 5-(3-methoxy-4-hydroxyphenyl)-5-phenylhydantoin (methoxycatechol) were gifts from Dr. T. Chang of Warner-Lambert Labs. (Ann Arbor, MI, U.S.A.). Metabolites were dissolved in HPLC-grade methanol (Fisher Scientific, Toronto, Canada). The 5,5-[phenyl-4-^{3}H(N)-diphenylhydantoin, 51.5 Ci/mmol, was obtained from New England Nuclear (Lachine, Quebec, Canada). Mephenytoin was obtained as a gift from Sandoz Pharmaceutical (East Hanover, NJ, U.S.A.). Purity of the radiolabeled phenytoin was > 99% as determined by HPLC. Solvents used were HPLC-grade 2-propanol, methanol, isopropyl alcohol and acetonitrile (Fisher Scientific); HPLC-grade water was prepared using a purification system (Milli-Q, Millipore, Mississauga, Canada). Prior to use, solvents were passed through a 0.45-μm filter (type HA® for aqueous solvents and type FH® for organic solvents, Millipore).

High-performance liquid chromatography

HPLC was employed using an automated system (Perkin-Elmer Canada, Toronto, Canada). Samples were injected from microvials using an autosampler (Model ISS-100). The pumping system (Model Series 4) consisted of a quaternary gradient system with on-line helium degassing and a controller. The detector was a double-beam, variable-wavelength, UV—VIS spectrophotometer (Model LC 85) equipped with a 2.4-μl flow cell, automatic baseline compensation (Autocontrol®) for gradient conditions, and stop-flow scanning accessories. The detector signal was integrated as the chromatographic peak area-under-the-curve by a 16-bit microcomputer (Model Sigma 15) and stored on-line on floppy disks using an 8-bit microcomputer (Model 3600).

Spectra for the HPLC peaks of phenytoin and the authentic standards for its metabolites were analyzed individually by stop-flow scanning from 190 to 300 nm under chromatographic solvent conditions to determine the respective optimal wavelengths for maximal UV absorbance.

A number of columns and solvent conditions were evaluated. The columns included a 15-cm reversed-phase (RP) C$_{18}$ column with 5 μm particle size (Perkin-Elmer), three similar columns from Beckman Instruments (Toronto, Canada), a 5-cm RP C$_{18}$ (3 μm) column, a 7.5-cm RP C$_3$ (5 μm) column, a 25-cm RP C$_{18}$ (5 μm) microbore column, and a 3-cm RP C$_{18}$ (3 μm) column (Perkin-Elmer). The internal diameter was 4.6 mm for all columns except the microbore column, which was 2.0 mm.

The criteria for optimal HPLC separation include rapid analysis time and reproducible baseline resolution of all metabolites. These criteria are dependent on the efficiency and selectivity of the column, and on solvent conditions. These characteristics determine the ability of an HPLC system to resolve similar chemical species, giving narrow, symmetrical peaks with baseline separation. For phenytoin, the methoxycatechol and *p*-HPPH metabolites were particularly difficult to resolve under conditions favourable to all chemical species. The resolution of these two metabolites was a major factor in the final choice of column and solvent conditions.

Preparation of biological samples for HPLC analysis

Using CD-1 mice (Charles River Canada, St. Constant, Canada), phenytoin and its metabolites were measured in 100-μl blood and urine samples obtained respectively by tail-vein sampling and the use of a metabolic cage. The extraction procedure involved mixing plasma and urine samples with an equal volume of β-glucuronidase (Sigma), 20 000 U/ml in 0.2 M sodium acetate buffer, pH 4.9. This suspension was incubated at 37°C for 20 h, at which time 20 μl of a 100 μg/ml solution of mephenytoin, internal standard, was added. The mixture was extracted five times with 2 vols. of ethyl acetate. After each extraction the sample was centrifuged at 1000 g for 5 min (centrifuge Model TJ-6, Beckman) and the organic layer was removed. The ethyl acetate layers were pooled and blown to dryness under nitrogen. The sample was then reconstituted in 20 μl of methanol and 5 μl of this solution were injected into the HPLC system equipped with a 15-cm RP C_{18} (5 μm) column (Beckman).

RESULTS

High-performance liquid chromatography

Stop-flow spectral scans from the HPLC detector under optimal chromato-
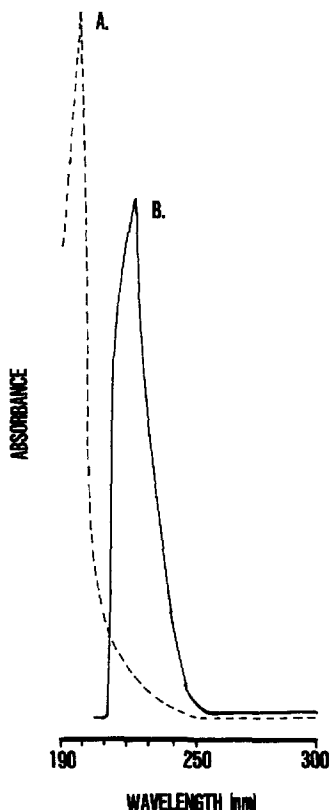

Fig. 1. Stop-flow UV spectral scans of phenytoin. Scans were obtained under different chromatographic conditions using a variable-wavelength, spectrophotometric HPLC detector. (A) water—isopropyl alcohol (80:20). This was the optimal solvent system used in gradient mode for subsequent analyses. (B) Methanol—1% acetic acid (30:70).

graphic conditions indicated a maximal UV absorbance for phenytoin at 195 nm (Fig. 1A), and within ± 2 nm for its metabolites. Subsequent analyses were performed at 225 nm to minimize the confounding absorbance of endogenous compounds. Changes in solvent system produced a marked displacement in the absorbance maximum (Fig. 1B).

The 15-cm RP C_{18} (5 μm) column from Perkin-Elmer was evaluated using two solvent systems; 70—76% phosphoric acid (1%) and 30—24% methanol, or 20—30% acetic acid (1%) and 80—70% methanol. Under isocratic elution the methoxycatechol and p-HPPH metabolites were unresolved. Peaks were broad with considerable tailing, and retention times were long.

The 5-cm RP C_{18} (3 μm) column from Beckman provided adequate metabolite resolution with sharp peaks and short retention times using a step-wise linear gradient at 2 ml/min, with a ternary solvent system which consisted of methanol (15—25%), acetonitrile (0—10%) and 1% acetic acid (85—65%). Binary solvent conditions caused drifting baselines with fair resolution.

The 7.5-cm RP C_3 (5 μm) column from Beckman provided fair resolution of all metabolites with fast retention times (less than 10 min). The solvent system used was 17% methanol and 83% water with a flow-rate of 1.5 ml/min.

The 25-cm RP C_{18} (5 μm) microbore column from Beckman could not resolve the methoxycatechol and p-HPPH metabolites to baseline, but there was a dramatic increase in sensitivity. A ternary solvent system was used, ranging from 70% to 77% phosphoric acid (0.5%), 5% to 15% acetonitrile and 15% to 18% isopropyl alcohol. Retention times were long, with phenytoin eluting at 26 min, and peak shapes were broad even under optimal conditions. Gradient methods proved to be impractical owing to the very low flow-rates (0.2 ml/min) and long equilibration times.

Gradient Solvent Conditions

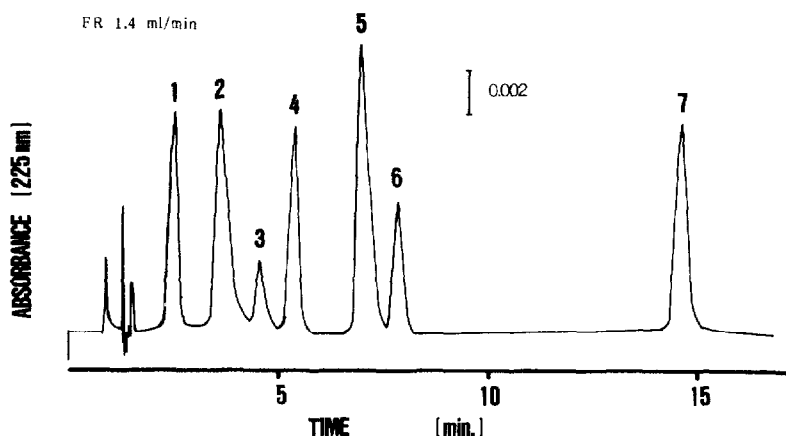| Time (min)	Water (%)	2-Propanol (%)	Gradient Curve
3	80	20	–
7	75	25	1
3	80	20	1

Fig. 2. HPLC chromatogram of metabolite standards. Dihydrodiol (1); catechol (2); methoxycatechol (3); p-HPPH (4); m-HPPH (5); mephenytoin, internal standard (6); phenytoin (7). The α,α'-diphenylglycine metabolite (not shown) elutes with retention time of 2.60 min. The injection volume was 5 μl and UV detection was performed at 225 nm for this and all subsequent HPLC analyses. The HPLC column was a 15-cm RP C_{18} column with a particle size of 5 μm (Beckman).

The 3-cm RP C_{18} (3 μm) column from Perkin-Elmer could not completely resolve the methoxycatechol and p-HPPH peaks, however, peak retention times were rapid and metabolite peak shapes were excellent; only the phenytoin peak was broad. The optimal solvent system under isocratic conditions was 88% phosphoric acid (1%) and 12% isopropyl alcohol at a flow-rate of 1.0 ml/min. This column was too short for gradient techniques. The sensitivity for metabolites was superior to all other columns except the microbore column.

The three 15-cm RP C_{18} (5 μm) columns from Beckman provided the best results, with baseline resolution of all metabolites, sharp peak shapes, good sensitivity and short retention times. A total analysis time of 15 min was obtained using a stepwise gradient elution (Fig. 2). Column pressures varied between 24 and 27 MPa at the beginning and end of the gradient. The variability observed with these three columns over four months was low, as demonstrated by the retention time for phenytoin. The intra-day retention time was 14.68 ± 0.13 min (mean $\pm$ S.D., $n = 19$), and the inter-day time was 14.50 ± 0.54 min ($n = 144$).

Biological samples

The extraction efficiency from urine and plasma was 94.7% with a 4.8% coefficient of variation. The limit of UV detection sensitivity, taken as two times the baseline excursion, was 5 ng, and the standard curve was linear up to at least 5 μg. However, the use of radiolabeled phenytoin extends the lower limit of sensitivity by 10 000-fold, to 0.1 pg. Fig. 3 and 4 show the UV chromatograms of urine and plasma samples taken from mice treated with native phenytoin together with radiolabeled phenytoin. Analysis of the fractions of the column eluent by liquid scintillation spectrometry revealed several critical metabolite peaks below the limit of sensitivity by UV detection, or peaks masked or obliterated by endogenous components. Table I shows the percent urinary recovery of phenytoin and its metabolites following each successive ethyl acetate extraction. The total recovery of each metabolite represents the
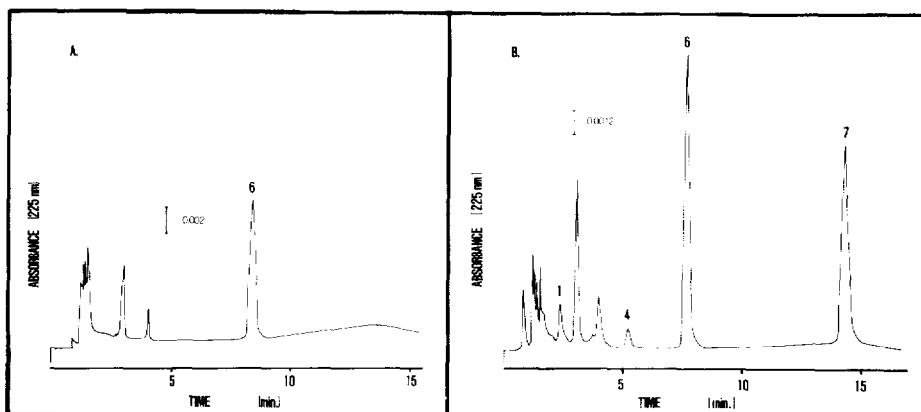

Fig. 3. HPLC chromatogram of mouse plasma. (A) Control plasma. (B) Plasma sample taken 17 h after administration of phenytoin, 55 mg/kg intraperitoneally. The volume injected into the chromatograph was 5 μl and the metabolite concentrations were 11.28 μg/ml dihydrodiol (1), 9.68 μg/ml p-HPPH (4), 200 μg/ml mephenytoin internal standard (6), and 181.6 μg/ml phenytoin (7).
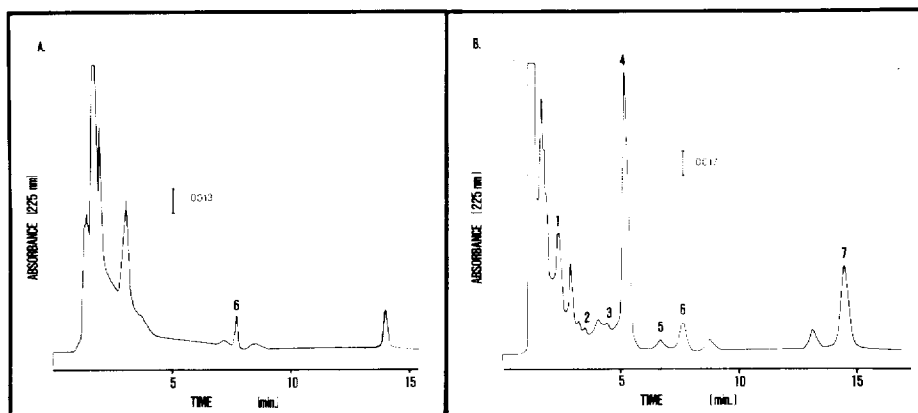
Fig. 4. HPLC chromatogram of mouse urine. (A) Control urine. (B) Urine sample collected over 16 h following administration of phenytoin, 55 mg/kg intraperitoneally. The volume injected into the chromatograph was 5 μl and the metabolite concentrations were 260 μg/ml dihydrodiol (1), 2.7 μg/ml catechol (2), 3.9 μg/ml methoxycatechol (3), 741 μg/ml p-HPPH (4), 84 μg/ml m-HPPH (5), 200 μg/ml mephenytoin, internal standard (6), and 369 μg/ml phenytoin (7).

TABLE I

URINARY RECOVERY (%) OF PHENYTOIN AND ITS METABOLITES FOLLOWING SUCCESSIVE EXTRACTIONS

Radiolabeled phenytoin (DPH), 2 μCi/g, was administered intraperitoneally to pregnant CD-1 mice on gestational day 12 and urine was collected over 48 h. Phenytoin and its metabolites were extracted from the urine with ethyl acetate.

Metabolite*	Extraction No.					Total
	1	2	3	4	5	
DHD	6.28	4.81	4.10	0.95	0.96	17.10
CAT	—	—	—	—	—	—
MET	0.29	0.13	0.03	—	—	0.45
p-HPPH	32.97	9.50	2.55	0.70	0.18	45.90
m-HPPH	—	0.56	0.28	0.07	—	0.91
DPH	20.25	5.81	1.82	0.83	0.45	29.16
						93.52

*Abbreviations: DHD, dihydrodiol; CAT, catechol; MET, methoxycatechol; p-HPPH, para-hydroxyphenyl-5-phenylhydantoin; m-HPPH, meta-hydroxyphenyl-5-phenylhydantoin; DPH, unmetabolized phenytoin.

percent contribution of each route of phenytoin's biotransformation. The use of radiolabeled phenytoin was essential for quantification of toxicologically relevant metabolites generated as products of minor metabolic pathways undetectable by UV absorbance.

DISCUSSION

The optimal column for the resolution and detection of phenytoin and its

toxicologically relevant metabolites was a 15-cm RP C_{18} column with a particle size of 5 μm. It is worth noting that even this type of column with identical specifications from several suppliers provided inadequate metabolite resolution or peak shape. In addition, there is some variation in the performance of different lots of the same column from the same supplier. For a given assay, columns from the same production lot will ensure optimal column reproducibility. The optimal solvent conditions are given in Fig. 2. While the columns with a particle size of 3 μm resulted in sharper peaks, increased detection sensitivity, and shortened retention times, the methoxycatechol and p-HPPH were not completely resolved, and this is a disadvantage if effluent peak fractions are to be collected for further analysis. The 3-μm columns also incur higher back-pressures and degenerate more rapidly than 5-μm columns with repeated injections of biological samples. For detection of the toxicologically relevant trace metabolites (dihydrodiol, catechol and methoxy-catechol) which reflect the arene oxide pathway, sensitivity is increased 10 000-fold with the use of radiolabeled phenytoin and HPLC-fraction collection followed by scintillation spectrometric analysis.

The assay reported herein will permit more complete quantification of the critical pathways of phenytoin biotransformation and should prove useful to investigators concerned with the toxicology of phenytoin.

ACKNOWLEDGEMENTS

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