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Note

Thin-layer chromatographic analysis of phenytoin and its hydroxy metabolites

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Phenytoin (5,5-diphenylhydantoin) is a widely used anticonvulsant agent that undergoes extensive metabolic oxidation in the body prior to its excretion in urine[1]. The major metabolites of phenytoin in humans and most experimental animals are 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin (Dihydrodiol), and 5-(3,4-dihydroxyphenyl)-5-phenylhydantoin (Catechol)[2–6]. These metabolites are excreted in the urine primarily as glucuronide conjugates. However, glucuronide of 5-(*m*-hydroxyphenyl)-5-phenylhydantoin (*m*-HPPH) is identified as the major urinary metabolite of the drug in dogs[5]. The presence of *m*-HPPH in the urine of humans and other laboratory animals is also reported[1–6] which is believed to be an artifact arising from the labile dihydrodiol metabolite during the isolation process.

While several gas[1,3,6] and liquid[7,8] chromatographic methods are reported for the simultaneous analysis of phenytoin and its hydroxy metabolites, there appears to be no convenient thin-layer chromatographic (TLC) method currently available for the simultaneous analysis of these compounds. The present paper describes a simple and rapid TLC method that utilizes neutral solvent systems for the analysis of phenytoin and its major hydroxy metabolites. Also, application of the TLC procedure in the study of phenytoin metabolism in the rat is reported.

EXPERIMENTAL

Thin-layer chromatographic procedure

Silica gel 60 F_{254} pre-coated TLC plates (E. Merck, Darmstadt, G.F.R.), 20 × 5 or 20 × 20 cm, layer thickness 0.25 mm, were utilized after activation at 105° for 5 min. Appropriate amounts of test compounds in ethyl acetate or methanol were spotted on TLC plates and developed in solvent system A, B or C (Table I) by the ascending technique. The resolved compounds on chromatograms were detected by either viewing under short-wavelength ultraviolet (UV) light (254 nm) or by exposing to iodine vapors.

Administration of [^{14}C]phenytoin to rats

Two male Sprague-Dawley rats, weighing 180–200 g, were housed in individual metabolism cages and were given rat chow (Ralston Purina, St. Louis, Mo., U.S.A.) and tap water *ad libitum*. [4-^{14}C]Phenytoin (spec. act. 50.5 mCi/mmole, New England

TABLE I

TLC OF PHENYTOIN AND ITS HYDROXY METABOLITES

Solvent systems: A, chloroform–ethyl acetate–methanol (5:1:1); B, chloroform–acetone–methanol (6:1:1); C, chloroform–methanol (3:1).

Compound	R_F*			*Color observed (Detection limit, μg)*	
	Solvent system				
	A	*B*	*C*	*UV*	*Iodine*
Phenytoin	0.49 ± 0.02	0.51 ± 0.03	0.70 ± 0.03	Blue (2)	Brown (10)
m-HPPH	0.34 ± 0.01	0.30 ± 0.01	0.57 ± 0.02	Blue (1)	Brown (1)
p-HPPH	0.32 ± 0.01	0.29 ± 0.01	0.56 ± 0.02	Blue (1)	Brown (1)
Catechol	0.29 ± 0.01	0.18 ± 0.01	0.37 ± 0.01	Blue (2)	Brown (5)
Dihydrodiol	0.15 ± 0.01	0.09 ± 0.01	0.17 ± 0.01	Blue (5)	Brown (5)

* Mean of six determinations ± standard error.

Nuclear, Boston, Mass., U.S.A.) was diluted with appropriate amount of non-radioactive phenytoin (Eastman-Kodak, Rochester, N.Y., U.S.A.) in distilled water containing a few drops of 0.01 *N* sodium hydroxide and administered to the rats by the intraperitoneal route at a dose of 50 mg/kg, 50 μCi/kg. Urine samples were collected for 24 h in brown glass bottles and stored at −17° until time of analysis.

Isolation of urinary metabolites

The general procedure described by Rao *et al.*[9] was followed. The rat urine samples (15 ml) were chromatographed on Amberlite XAD-2 column (Drug-Skreen adsorbent cartridges, Brinkmann, Westbury, N.Y., U.S.A.). The methanol eluate (40 ml) was evaporated to dryness *in vacuo*, and the residue was taken up in 20 ml of distilled water containing 1 g of sodium acetate and pH adjusted to 7.4 with glacial acetic acid. The enzymatic hydrolysis of conjugated phenytoin metabolites was carried out at room temperature for 24 h by the addition of 2000 units of glucuronidase-sulfatase (Sigma, St. Louis, Mo., U.S.A.). The reaction mixture was then extracted 6 times with 10 ml of ethyl acetate and each extract was dried over anhydrous sodium sulfate and filtered. The pooled ethyl acetate extracts were evaporated to dryness *in vacuo* and the residue analyzed by TLC for phenytoin and its hydroxy metabolites as described above.

Quantitation of phenytoin and its hydroxy metabolites excreted in the rat urine was performed by their isolation and measurement of radioactivity. To isolate the compounds, chromatograms were developed in solvent system A and the spots corresponding to reference compounds (visualized under the UV light) were scraped into scintillation vials. A 15-ml volume of liquid scintillation cocktail (Insta-gel, Packard, Downers Grove, Ill., U.S.A.) was added to each vial and radioactivity measured in a liquid scintillation spectrometer.

RESULTS AND DISCUSSION

The TLC data on the resolution and detection of phenytoin and its four hydroxy metabolites are given in Table I. When necessary, better resolution of *m*-HPPH and *p*-HPPH can be achieved by separate analysis of these two isomers on TLC plates developed in 4% methanol in diethyl ether (solvent system D, R_F 0.32 and 0.28, respectively).

The earlier TLC systems reported for the separation of phenytoin and its

hydroxy metabolites have used acidic solvent systems for the development of chromatograms[2,10]. Since the instability of the dihydrodiol metabolite of phenytoin under acidic conditions to form phenolic products is well recognized[2,6], the neutral solvent systems described in the present paper are particularly suited for phenytoin metabolism studies. In addition, the TLC procedure reported here is rapid since the chromatographic development time for solvent system A, B or C is about 1 h.

The urinary excretory profile of [4-^{14}C]phenytoin in the rat based on the above TLC procedure is given in Table II. The identity of *p*-HPPH was confirmed by its isolation (1% methanol in ethyl acetate was used for its extraction from TLC plates) and re-chromatography in solvent system D. These results are in general agreement with the earlier phenytoin metabolism studies in the rat[1–6].

Currently, we are utilizing this TLC procedure in the studies involving oral metabolism and gingival tissue adverse reactions induced by prolonged administration of phenytoin[11].

TABLE II

METABOLISM OF [4-^{14}C]PHENYTOIN IN THE RAT

Percentage urinary excretion of ^{14}C-labeled phenytoin and its hydroxy metabolites 24 h after a single intraperitoneal dose of [4-^{14}C]phenytoin (50 mg/kg; 50 μCi/kg). The metabolite conjugates were subjected to enzyme hydrolysis prior to the analysis. The recovery efficiency for the various compounds from urine were: Phenytoin, 92%; *p*-HPPH, 90%; Catechol, 85%; Dihydrodiol, 88%.

Compound	*Percent of dose*		
	Rat No. 1	*Rat No. 2*	*Average*
Phenytoin	1.9	1.7	1.8
p-HPPH	55.8	59.6	57.7
Catechol	2.1	1.9	2.0
Dihydrodiol	26.2	24.4	25.3

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