# A NEW METABOLITE OF 5,5-DIPHENYLHYDANTOIN (DILANTIN+)

Tsun Chang, Ardis Savory and A. J. Glazko

Department of Pharmacology, Division of Medical and Scientific Affairs
Parke, Davis and Company, Ann Arbor, Michigan

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## SUMMARY

A new metabolite of 5,5-diphenylhydantoin (Dilantin+; DPH) present in rat and monkey urine, has been isolated and identified as 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin. Upon heating in acid, this metabolite is converted to a mixture of the 3- and 4-hydroxyphenyl derivatives of DPH. The new metabolite did not have significant protective effect vs. electroshock induced convulsive seizures in mice.

Kozelka and Hine (1) reported that small amounts of DPH were excreted unchanged in the urine of dog and man, together with small amounts of 2,2-diphenylhydantoic acid (DPHA), and large amounts of α-aminodiphenylacetic acid (ADPA). The presence of ADPA was not confirmed by subsequent investigations (2). Dill et al (3) in this laboratory identified unchanged DPH in rat and dog liver and in human plasma. Butler (4) and Maynert (2) identified the major urinary metabolite of DPH as the glucuronic acid conjugate of 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH). Woodbury (5) indicated that other probable metabolites in rat urine were the 3,4-dihydroxy and the 4,4'-dihydroxy derivatives of DPH, but no supporting evidence was presented.

## MATERIALS AND METHODS

A carbon-14 labelled preparation of DPH was obtained from New England Nuclear Corp., Boston, Mass. The label was in the 4-position of the hydantoin ring. This was administered perorally to 250 gm. male albino rats (Carworth) in doses of 40 mg/kg (0.2 μc/mg). Cage collections of urine were made over the next 24 hour period. Thin layer chromatography (TLC) was carried out with silica gel $GF_{254}$ (0.25 mm), using a solvent system containing $CHCl_3$:

+Dilantin is the Parke-Davis trade name for 5,5-diphenylhydantoin (phenytoin).

MeOH:glac.HOAc. After development, 0.5 cm bands of gel were scraped from the glass plates and transferred to counting vials. One ml of MeOH and 12 ml of a dioxane scintillation solution were added to each vial, and counting was performed in a Packard Tricarb Model 3375. Reference standards of DPH, HPPH and DPHA were chromatographed simultaneously on the same plates; their locations were determined by examination of the plates under ultraviolet light. Gas-liquid chromatography (GLC) was carried out on the trimethylsilyl (TMS) derivatives of DPH and its hydroxylated metabolites as described elsewhere (6).

Isolation of unknown metabolite: Urine obtained from 20 rats maintained on a drug-diet containing 0.2% DPH was "marked" by the addition of urine from rats receiving DPH-$^{14}$C. The pooled urine was adjusted to pH 5 and incubated with Glusulase (Endo Lab., Inc., Garden City, N.Y.) at 37°C for 16 hours. The urine was then extracted twice with equal volumes of isoamyl alcohol and the combined extracts were evaporated to dryness. The brown residue was then chromatographed on a Florasil F-100 column (Fischer Scientific Co., Fair Lawn, N.J.) using a mixture of $CHCl_3$ and MeOH (80:20). The fractions containing radioactivity were pooled and evaporated to dryness. The residue was dissolved in 100 ml n-butanol and run through a 30-plate countercurrent extraction vs. pH 10 carbonate-bicarbonate buffer as described by Butler (4). Each plate was then acidified, returning the metabolites to the alcohol phase. TLC indicated that HPPH was located in plates 14-23, and that the unknown fraction was present in plates 4-12. The latter fractions were combined, and the residue was run through a second 30-plate countercurrent extraction using isoamyl alcohol and tris buffer (2-amino-2-hydroxymethyl-1,3-propanediol; 0.1M solution adjusted to pH 8.5 with HCl). Each plate was then acidified, returning the unknown metabolite to the organic phase. Plates 13-22 containing a major radioactivity peak, were combined, evaporated to dryness, and the residue was dried under vacuum.

Infra-red spectra were run on KBr pellets with a Beckman IR9; nuclear magnetic resonance measurements were made in dimethylsulfoxide with a Varian

Model 60A; ultraviolet absorbance was measured with a Cary Model 11 Recording Spectrophotometer; optical rotation measurements were made in absolute methano with a Perkin-Elmer Model 141. Anticonvulsant activity was evaluated in mice using the technic described by Chen et al (7).

## RESULTS AND DISCUSSION

The results of TLC of urine from rats receiving carbon-14 labelled DPH are shown in Figure 1. A large polar fraction of low $R_f$ was observed (I), consisting mainly of conjugated HPPH. Small amounts of DPHA (II) and DPH (V) were also seen; their presence was confirmed by TLC in a different solvent system ($CHCl_3$:MeOH = 70:30). Free, unconjugated HPPH (IV), and a new, unknown fraction (III) were also observed. The amount of III represented about 28% of the total radioactivity in rat urine, and 30-37% in Rhesus monkey urine. Since the amount of III appeared to be fairly large in these species, attempts were made to isolate this fraction as described under Methods. The final product gave a single radioactive spot upon TLC, corresponding with III in its chromatographic characteristics.

A number of hydroxylated derivatives of DPH were available for comparison with III. The $R_f$ values obtained by TLC, and the retention times of the TMS

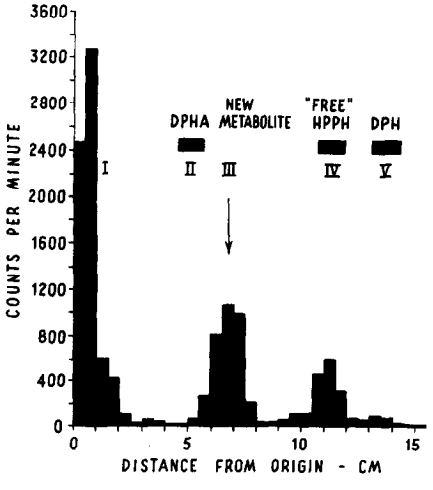



Fig. 1. TLC of rat urine following peroral administration of DPH-$^{14}C$.

Table I

CHROMATOGRAPHIC CHARACTERISTICS OF METABOLITE III COMPARED WITH SOME PHENOLIC DERIVATIVES OF 5,5-DIPHENYLHYDANTOIN (DPH)

| DPH DERIVATIVE+ | TLC ($R_f$) | GLC (Min.) |
|---|---|---|
| DPH | 0.66 | 4.5 |
| III | 0.34 | 8.8 |
| 3-OH | 0.54 | 7.8 |
| 4-OH (HPPH) | 0.53 | 9.8 |
| 3,4-diOH | 0.38 | 12.5 |
| 2,4-diOH | 0.41 | 13.5 |
| 3,4'-diOH | 0.41 | 16.5 |
| 4,4'-diOH | 0.39 | 21.5 |

+Compounds designated by position of hydroxyl groups on the phenyl rings

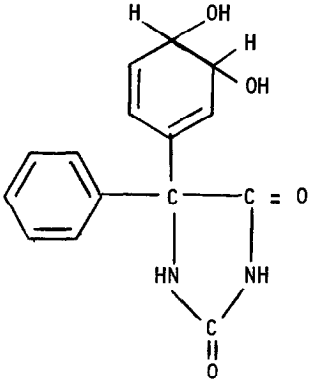
derivatives obtained by GLC are shown in Table I. A combination of these technics provided good evidence for the separate identity of III.

An examination of the infra-red spectrum of III revealed major structural changes. III showed a greatly reduced absorbance between 1400-1600 $cm^{-1}$, indicating possible loss of aromaticity. Two distinct peaks were observed between 1000-1100 $cm^{-1}$ indicating the possible presence of aliphatic alcohol groups. However, the hydantoin ring appeared to be intact, with typical imide absorption present at 1725 $cm^{-1}$. This was supported by titration data in 50% methanol: the apparent pKa' for III was 8.8, similar to that of DPH (9.0); HPPH showed two inflection points (8.8, 10.6), due to ionization of the phenolic group as well as the hydantoin ring.

The nuclear magnetic resonance spectrum of the 4,4'-dihydroxy derivative of DPH showed two resonance peaks at $\delta$ 7.4 and 6.4, due to the two phenolic groups. III showed only one resonance peak at $\delta$ 7.4 (5 protons); three

broader peaks were seen at $\delta$5.8, 5.1 and 4.5, due to 3, 2, and 2 protons, respectively. Another major difference was seen in optical rotation; the $[\alpha]_{25}^{D}$ for DPH was zero; for HPPH isolated from rat urine, -18°; for III, -163°, indicating the presence of additional asymetric carbon centers.

Structure: On the basis of these observations the structure proposed for the new metabolite of DPH is 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin:




The isolated preparation was a white powder with a melting point of 291-295°C (dec.). Elemental analysis showed good agreement with theoretical values. Analysis: calculated for $C_{15}H_{14}N_2O_4$ (Mol. Wt. 286.29): C, 62.9; H, 4.9; N, 9.8. Found: C, 62.6; H, 5.2; N, 9.6. Ultraviolet absorbance in methanol-HCl showed three identical peaks, with $E^{1\%}_{1cm}$ = 103 at 259 mμ, 251 mμ and 247 mμ. No protection was observed vs. electro-shock induced convulsive seizures in albino mice with I.P. doses of 64 mg/kg, whereas DPH was effective at 10 mg/kg.

Supporting evidence for the diol structure came from observations indicating that equal amounts of the 3- and 4-hydroxyphenyl derivatives of DPH were formed from III by heating in 3N HCl at 90°C; heating the dry powder at 250°C also resulted in formation of the same products. These were identified by gas chromatography of the TMS derivatives. The conversion was accompanied by changes in I.R., U.V., pKa' and optical rotation, characteristic of phenolic products. The instability of dihydrodiols in acid to form phenolic products

has been reported previously (8). Since assay procedures for HPPH are usually preceded by treatment of urine with acid to hydrolyze glucuronic acid conjugates, it is now evident that the presence of III may interfere with HPPH assays. The present observations suggest that appropriate corrections can be introduced by GLC assay of the 3-hydroxyphenyl derivative. In addition, DPHA is known to undergo ring closure when heated with acid, resulting in the formation of DPH (1). Treatment of urine with β-glucuronidase or Glusulase to hydrolyze glucuronic acid conjugates did not result in the conversion of III to phenolic products, nor did it increase the amount of III, indicating absence of conjugated derivatives.

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