Colorimetric Assay of 5,5-Diphenylhydantoin (Dilantin[1]) and 5-(*p*-Hydroxyphenyl)-5-phenylhydantoin (35644)

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HPPH[2] has been recognized as the principal metabolic product of DPH (1). It is excreted in urine mainly in the form of a conjugate with glucuronic acid (2). Butler (1) used countercurrent extraction and UV absorbance to measure HPPH excretion. He also described a color reaction of HPPH with 4-aminoantipyrine and ferricyanide, later applied to the assay of urine by Panalaks (3). However, the high blanks encountered with urine made this procedure of little practical value. Maynert (2) separated HPPH by paper chromatography and coupled the product with diazotized sulfanilic acid to produce a bright orange spot, which could be eluted for colorimetric estimation. Huissman (4) separated the nitrated derivatives using TLC. Oleson (5) also separated HPPH by TLC, and estimated concentrations by elution and UV absorbance measurement. Chang and Glazko (6, 7) first reported a highly specific GLC assay procedure for HPPH, based upon the formation of the trimethylsilyl derivative. Grimmer *et al.* (8) prepared the 3-*N*-methyl derivative of HPPH using diazomethane as a reagent, and estimated its concentration by GLC. On-column methylation technics with tetramethylammonium hydroxide (9) or trimethylanilinium hydroxide (10) have been described for DPH assays, and should be applicable to HPPH. Although GLC methods inherently provide a high degree of specificity, they are generally too slow for routine use in the clinical laboratory.

The present report is concerned with a colorimetric procedure for DPH and HPPH, based upon separation of the two compounds by solvent extraction technics, followed by application of the nitration procedure of Dill *et al.* (11) to each component. It has been applied to the measurement of DPH and HPPH in normal and uremic subjects, reported elsewhere (12, 13).

Materials. Reagents. Sodium hydroxide, 1 and 5 *N* solutions; acetate buffer, 0.1 *M*, pH 5.0; phosphoric acid, 2.85 and 5.7 *M*; phosphate buffer, 1 *M*, pH 6.8; EDC (purified), chloroform (reagent grade, Baker Chem. Co., Phillipsburg, N.J.); ethyl acetate (nanograde, Mallinckrodt Chem., St. Louis, Mo.); EHX (Union Carbide Corp., So. Charleston, West Va.); Glusulase (Endo Lab., Inc., Garden City, N.Y.).

Analytical Procedure. (*a*) *Plasma.* Extraction may be carried out directly on plasma for "free" drug assays, or after incubation with Glusulase for "total" drug. In the *free drug* assays, 4 ml of plasma are transferred to a 20 × 150-mm glass-stoppered test tube, 0.4 ml of 1 *M* phosphate buffer (pH 6.8) is added, and the mixture is extracted by shaking with 14 ml of a 2:1 mixture of EDC and EHX. For *total drug* assays, 4 ml of heparinized plasma are transferred to the 20 × 150-mm glass-stoppered test tubes, 0.5 ml of 0.1 *M* acetate buffer (pH 5.0) and 1 drop of glacial acetic acid are added, followed by 0.15 ml of Glusulase and 4 drops of chloroform. The mixture is incubated at 37° overnight (16 hr). The pH is then raised by the addition of 0.1 ml of 5 *N* NaOH and 0.5 ml of 1 *M* phosphate buffer (pH 6.8), and the mixture is extracted by shaking with 14 ml of

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

[2] Abbreviations used: DPH, 5,5-diphenylhydantoin (Dilantin; phenytoin); HPPH or *p*-HPPH, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin; *m*-HPPH, 5-(*m*-hydroxyphenyl)-5-phenylhydantoin; EDC, 1,2-dichloroethane; EHX, 2-ethylhexanol; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; UV, ultraviolet.

a 2:1 mixture of EDC and EHX.

Ten ml of the EDC-EHX extract are transferred to a second test tube and shaken with 3.5 ml of 1 *N* NaOH. The aqueous phase is separated by centrifugation and 3 ml are transferred to a smaller glass-stoppered test tube (15 × 150 mm). The solution is neutralized by addition of 0.6 ml of 2.85 *M* phosphoric acid, and the mixture is extracted by shaking with 5 ml of chloroform. After separation of the phases, the chloroform layer is washed twice by shaking with two 7.5-ml portions of 0.1 *M* phosphate buffer (pH 6.8) to remove any traces of HPPH. Four ml of the chloroform are evaporated to dryness in a test tube prior to application of the colorimetric procedure (DPH fraction). Three ml of the chloroform-extracted aqueous residue are extracted again by shaking with 5 ml of ethyl acetate, and 4 ml of this extract are evaporated to dryness in a test tube prior to application of the nitration procedure (HP PH fraction).

(*b*) *Urine.* Assays for DPH are generally not needed in urine since the excretion of free unchanged drug is extremely low (11). However, the excretion of conjugated HPPH accounts for a large fraction of the total dose (14). *Acid hydrolysis* at elevated temperatures was found to be the best method to achieve complete hydrolysis of the conjugates in urine. The procedure is to take 1 ml of urine in a 20 × 150-mm glass-stoppered tube, add 1 ml of 12 *N* HCl, and heat on a steam bath for 1 hr. After cooling, 2 ml of 1 *M* K_2HPO_4 and 2 ml of 5 *N* NaOH are added, and the mixture is extracted by shaking for 15 min with 6 ml of the 2:1 mixture of EDC and EHX. Under certain conditions described below, acid hydrolysis may yield high results; for this reason *enzymatic hydrolysis* is usually preferred. Using 20 × 150 mm glass-stoppered test tubes, 1 ml of urine is mixed with 1 ml of pH 5 acetate buffer (0.2 *M*), 0.15 ml of Glusulase, and 4 drops of chloroform. The mixture is incubated overnight at 37° and then is extracted directly by shaking with 6 ml of the 2:1 EDC–EHX mixture. The organic phase is washed by shaking with an equal volume of 5% $NaHCO_3$ solution to remove interfering substances. Four ml of the organic phase are transferred to another glass-stoppered test tube containing 2.5 ml of 1 *N* NaOH, and shaken to extract DPH and HPPH. Two ml of the NaOH layer are transferred to another tube and partially neutralized to pH 7.4 with 0.2 ml of 5.7 *M* phosphoric acid. This mixture is extracted with 2.5 ml of ethyl acetate, bypassing the chloroform-extraction step because of the low concentration of DPH present in the urine. Finally, 2 ml of the ethyl acetate layer is evaporated to dryness in a test tube prior to nitration.

(*c*) *Colorimetric.* The standard procedure of Dill *et al.* (11) is used for subsequent handling of DPH and HPPH residues, with a few minor changes. Evaporation of the solvent is carried out on a steam bath to insure complete removal of the solvents. Nitration is carried out with 0.2 ml of the nitric–sulfuric acid reagent, in place of the 0.25 ml originally recommended. The alcohol mixture has been modified to contain *n*-butanol:isopropanol in a 40:60 (v/v) ratio, instead of in equal volumes. The 3-plate countercurrent extraction step for the separation of phenobarbital prior to the colorimetric assay cannot be used in the present procedure, since it eliminates HPPH from the assay. All assay results are expressed in terms of DPH-equivalents, to facilitate comparison with the administered dose.

Results and Discussion. Extraction Methods. Preliminary experiments indicated that the nitration procedure applied to HPPH produced about 75% of the color obtained with DPH, and the absorbance at 550 nm was found to be directly proportional to the amount of HPPH present. Subsequently, this procedure was used for the study of the extraction characteristics of HPPH in various organic solvents. Our earlier observations with DPH (11) indicated that extraction into chloroform was nearly complete at pH 8; only small amounts of HPPH were extracted under these conditions. Good extraction was obtained from acidified solutions using ethyl acetate, isobutyl alcohol, *n*-butanol, and 2-ethylhexanol; incomplete extraction resulted with dichloromethane, EDC,

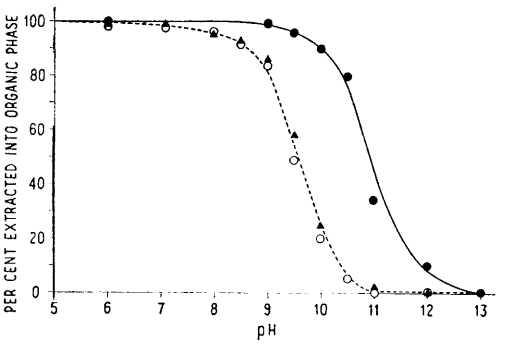

FIG. 1. Extraction of DPH and HPPH from aqueous buffers with a 2:1 mixture of EDC and EHX: drug conc. 100 μg/ml; 15 ml of aqueous phase extracted with 75 ml organic phase. Buffers consisted of 0.2 *M* phosphate in pH range 6.0–7.1 and 10.5–12.0; 0.2 *M* borate in pH range 8.0–10.0; 0.1 *N* NaOH for pH ~13. Colorimetric assays were carried out on the aqueous residue after extraction of an acidified aliquot with ethyl acetate, evaporation to dryness, and application of the colorimetric procedure of Dill *et al.* (11) to the residue. (●) DPH; (○) *p*-HPPH; (▲) *m*-HPPH.

and chloroform. Ethyl acetate proved to be satisfactory for the extraction of both DPH and HPPH in our GLC assay procedure (7), because of the high degree of specificity inherent in this technic. However, ethyl acetate extracts of acid-hydrolyzed urine specimens produced high blank values in the colorimetric assay procedure. A more satisfactory solvent system consisted of a mixture of 2 parts EDC and 1 part EHX (v/v). The extraction characteristics of DPH and HPPH with this solvent system are shown in Fig. 1. Both compounds were extracted quantitatively at pH<7. However, a clean separation of DPH and HPPH could not be achieved in a single extraction step.

Specificity. Since a number of DPH metabolites produce color in the nitration reaction, this possible source of interference was examined more closely. Synthetic DPH, *m*-HPPH, and *p*-HPPH were employed, together with the ^{14}C-labeled dihydrodiol metabolite of DPH (15), isolated from rat urine. Chloroform solutions containing 25-μg amounts of these compounds were evaporated to dryness and carried through the standard procedure of Dill *et al.* (11). The absorbance of the final solutions at 550 nm relative to DPH (100%) were: *m*-HPPH, 74%; *p*-HPPH, 76%; and dihydrodiol, 52%. When subjected to the EDC–EHX solvent extraction procedure, *m*-HPPH appeared in the same fraction as *p*-HPPH. The dihydrodiol metabolite did not interfere with DPH assays (chloroform extract); but 37% of the original concentration appeared in the HPPH fraction (ethyl acetate). Phenobarbital also produced a large amount of color in the nitration procedure; about 25% of the original concentration appeared in the DPH fraction (chloroform extract), and 15% in the HPPH fraction (ethyl acetate extract). Consequently, the current procedure for DPH and HPPH cannot be used in the presence of phenobarbital.

The presence of *m*-HPPH in urine specimens as a result of DPH administration has been reported by Atkinson *et al.* (16). We found only traces of *m*-HPPH in the urine of normal adult subjects by GLC procedures, but we have confirmed the presence of large amounts of *m*-HPPH in dog urine, together with lesser amounts of *p*-HPPH. Other sources of error following acid hydrolysis of urine at elevated temperatures include ring closure of diphenylhydantoic acid to form DPH (17), and conversion of the dihydrodiol metabolite to a mixture of *meta*- and *para*-HPPH (15). Although these metabolites have been observed in animal urine, no significant amounts have been found in the urine of normal adult human subjects.

The cross-interference of DPH and HPPH in the extraction procedure was minimal. Using standard solutions containing 10 μg of each compound, the DPH in the ethyl acetate extract (HPPH assay), and the HPPH in the (washed) chloroform extract (DPH assay), represented less than 1 % of the original amount added. To demonstrate further the low degree of cross-interference in this procedure, DPH and HPPH were added to human plasma in concentrations of 0, 2.4, 8.0, and 24.0 μg/4 ml. The known mixtures were then assayed for DPH and HPPH by the extraction procedure described above. The results are shown in Table I. No significant interference was encountered with HPPH because of the wash step in the final chloroform extract. The interference due to

TABLE I. Assay of DPH and HPPH Added in Known Amounts to Human Plasma.[a]

Standards added		Colorimetric assay (μg)	
DPH (μg)	HPPH (μg)	DPH	HPPH
0.0	0.0	0.0	0.0
	2.4	0.0	2.4
	8.0	0.0	7.7
	24.0	0.0	23.1
2.4	0.0	2.5	0.1
	2.4	2.4	2.4
	8.0	2.4	8.3
	24.0	2.7	23.1
8.0	0.0	8.6	0.2
	2.4	8.3	—[b]
	8.0	8.6	7.9
	24.0	8.3	23.2
24.0	0.0	25.6	0.4
	2.4	23.9	3.0
	8.4	24.3	8.8
	24.0	24.8	23.3

[a] μg DPH-equivalents/4 ml of plasma.
[b] Sample lost.

DPH may be appreciable in normal plasma specimens, where HPPH concentrations are extremely low. This situation is reversed with urine specimens, where DPH concentrations are low in comparison with the "total" HPPH levels.

Recovery. The recovery of DPH and HPPH from plasma in comparison with recovery from aqueous solutions was established by replicate assays with samples containing known concentrations of these compounds (25 μg/4 ml). Using 8 samples/series, the mean recovery of DPH from plasma was 99.8% of that obtained from water, with a standard error of less than 1%. Approximately 0.3% of the added DPH appeared in the HPPH assay. Similarly, eight replicate assays with known amounts of HPPH added to water or plasma (25 μg/4 ml) showed no significant difference between the means, by Student's *t* test. The recovery from plasma was 101.6% of that from water, with a standard error of less than 1%. Less than 2% of the added HPPH appeared in the DPH assay. The absolute recovery of DPH and HPPH by the extraction procedures employed represented about 90% of the starting material. The major loss of HPPH occurred in the chloroform-extraction step. All analytical results are based upon aqueous standards which were carried through the same extraction steps as the unknowns.

Comparison with Established Methods. A comparison of DPH plasma levels obtained by the procedure of Dill *et al.* (11) and by the current modified procedure are shown in Table II, together with data for "total" HPPH obtained in the same specimens. The subject was from a clinical trial described elsewhere (14). The modified procedure showed good agreement with the earlier colorimetric procedure. The plasma levels of "free" HPPH were less than 0.1 μg/ml in this series of assays; whereas significant levels of "total" HPPH were observed after treatment with Glusulase. Peak HPPH levels (0.84 μg/ml) occurred at the 8-hr sampling period, as shown in Fig. 2. The delayed peak in HPPH plasma levels corresponded with the delayed urinary excretion of HPPH noted in our earlier work (14). The apparent half-life of HPPH in the plasma was about 36 hr, estimated from the semilogarithm plot

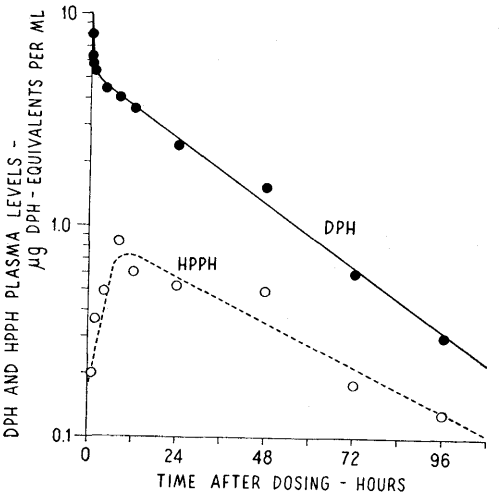

FIG. 2. Plasma levels of DPH and HPPH in a normal adult subject following a 250 mg IV dose of DPH. Specimens obtained from study described previously (14). Colorimetric assays for DPH and "total" HPPH obtained by the present procedure: (●) DPH; (○) HPPH. Lines of regression calculated by method of least squares from plasma level data in the 8- to 96-hr postdose period.

TABLE II. Plasma Levels in an Adult Human Subject Following a 250-mg Intravenous Dose of Dilantin; a Comparison of Different Assay Procedures.

Time after dosing	DPH assays (μg/ml)		Total HPPH assays[b] (μg/ml)
	Dill procedure[a]	Modified procedure[b]	
Pre-Rx	(0.1)	(0.2)	(0.0)
(min) 5	7.9	7.9	0.01
20	6.3	6.4	0.10
35	5.8	6.1	0.13
(hr) 1	5.3	5.0	0.20
2	4.6	5.0	0.36
4	4.4	4.2	0.49
8	4.0	3.8	0.84
12	3.6	3.1	0.60
24	2.4	2.4	0.53
48	1.5	1.1	0.50
72	0.6	0.4	0.18
96	0.3	0.4	0.13

[a] See Ref. (11).
[b] Procedure described in this paper. All values corrected for pre-Rx blanks.

of the analytical data; DPH half-life in this subject was about 24 hr.

The urine specimens of the same subject were also available for assay by the new procedure. These were assayed for "total" HPPH after enzymatic hydrolysis with Glusulase, and also after heating with acid. The results are shown in Table III, together with GLC assay data obtained previously after acid hydrolysis (14). In most cases, the individual assays following enzymatic and acid hydrolysis showed good agreement, and the total recovery of drug over the 96-hr period also showed good correspondence. However, acid hydrolysis produced a higher blank with normal urine specimens, and the HPPH assays appeared to be slightly higher than those obtained after treatment of urine with Glusulase, possibly due to incomplete hydrolysis with this enzyme preparation. The peak excretion rate for HPPH (mg/hr) occurred in the 8- to 12-hr period after dosing, as noted in our earlier studies (12). In addition, the present observations reveal a direct relationship between the mean excretion rate for HPPH and the plasma levels of HPPH estimated at the midpoint of each urine collection period, shown graphically in Fig. 3. The

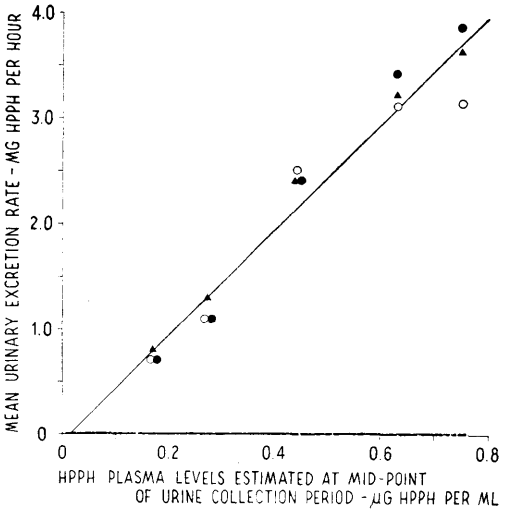

FIG. 3. Relationship between renal excretion of HPPH and plasma levels at midpoint of urine collection periods: Plasma levels of HPPH estimated from line of regression shown in Fig. 2. Urinary excretion rate (mg HPPH/hr) calculated from data in Table III for periods beyond 8 hr after dose; (●) acid hydrolysis, GLC; (○) Glusulase hydrolysis, colorimetric assay; (▲) acid hydrolysis, colorimetric assay. Line of regression calculated by method of least squares using data from all three assay procedures.

TABLE III. Colorimetric Assay of Urine for HPPH After Treatment with Glusulase or Heating with Acid; Comparison with GLC Assays After Heating with Acid.

Urine collection period (hr after dosing)	Total HPPH excreted (mg)[b]		
	Glusulase hydrolysis	Acid hydrolysis	
	Colorimetric	Colorimetric	Gas chromatography[a]
Pre-Rx (24 hr)	(8.1)	(15.0)	(0.0)
0–2	1.4	1.4	1.8
2–4	4.0	4.6	3.7
4–6	5.2	6.0	5.8
6–8	8.0	4.6	4.2
8–12	12.8	15.0	14.4
12–24	37.1	41.0	38.6
24–48	59.5	58.5	57.3
48–72	26.4	27.4	31.8
72–96	16.1	17.2	19.4
Total (mg) (0–96 hr)	170.5	175.7	177.0
% of dose	68.2	70.3	70.8

[a] Data are taken from Ref. (14). [b] All values corrected for pre-Rx blanks.

slope of the line of regression provides an index of the renal clearance of conjugated HPPH. In studies reported elsewhere (12, 13), a large increase in plasma HPPH was found in patients with impaired renal function, reaching values as high as 30 μg/ml. The analytical procedures reported above should be useful in evaluating the status of chronic uremic patients who require daily administration of DPH for the control of convulsive seizures.

Summary. The colorimetric procedure of Dill *et al.* (11), originally described for DPH, has been modified to permit assay of both DPH and HPPH in the same sample of plasma or urine. In a normal adult subject receiving a single 250 IV dose of DPH, the maximum plasma level of HPPH occurred about 8 hr after dosing. A direct relationship was found to exist between the plasma levels of HPPH and the rate of urinary excretion of this metabolite.

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