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DETERMINATION OF THE MAJOR URINARY METABOLITE OF DIPHENYLHYDANTOIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the determination of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), a major urinary metabolite of 5,5-diphenylhydantoin (DPH) in man. The assay involves acid hydrolysis, extraction and high-performance liquid chromatographic analysis using a column comprised of small-particle silica gel. This procedure has been used to determine *p*-HPPH levels in urine from healthy volunteers who ingested 400 mg of sodium DPH. The *p*-HPPH concentration was found to range from 10 to 300 μg/ml. The minimum detectable concentration of urinary *p*-HPPH was 1 μg/ml.

INTRODUCTION

The data on drug metabolites in urine are often a valuable addition to information derived from determinations of parent drug in plasma or urine. This experience led us to study the metabolites of diphenylhydantoin (DPH). For the determination of hydroxylated metabolites of DPH, several gas–liquid chromatographic (GLC) methods are available. However, these assays involve silylation[1–3] or methylation[4,5], which normally lengthens the analysis time and might introduce another variable. Since the concentrations of DPH metabolites in urine are generally high, it was thought that the use of high-performance liquid chromatography (LC) would be appropriate. In spite of decreased sensitivity, LC offers certain advantages over GLC. There is no need to derivatize the sample and the operation of the LC at room temperature eliminates fear of thermal decomposition of drugs often seen in GLC.

Anders and Latorre[6] reported that DPH and its major metabolite, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), could be separated by anion-exchange LC. However, they analyzed only standard samples and the resolution shown by chromatograms discouraged us from using the anion-exchange resin. Therefore, we decided to use small-particle silica gel (MicroPak™)[7] for the LC determination of urinary *p*-HPPH, since this material is versatile and offers good efficiency, high sample capacity and excellent resolution.

This LC assay was used to study the urinary excretion of *p*-HPPH in healthy volunteers who ingested 400 mg of sodium DPH.

MATERIALS AND METHODS

Liquid chromatography

A Varian Aerograph Series 4010-01 liquid chromatograph equipped with a stainless-steel MicroPak™ column[7] (silica gel 10 μ, 25 cm × 2.2 mm I.D.) was utilized. The ultraviolet (UV) detector of the instrument was operated at 254 nm and the disc integrator attached. The solvent, *n*-butyl chloride–dioxane (4:1), was delivered at 500 p.s.i. by compressed helium gas. The flow-rate of solvent through the column was 0.9 ml/min. The sensitivity range of the UV detector was set at 0.08–0.32 absorbance unit full scale (AUFS) depending on the sample in order to keep the *p*-HPPH signal on scale. This allowed proper integration of the peak.

Reagents and materials

Sodium 5,5-diphenylhydantoin in 100-mg capsules (Dilantin™; Parke, Davis and Co.) was administered to the volunteers. 5-(*p*-Hydroxyphenyl)-5-phenylhydantoin was obtained from Aldrich Chemical Co. All solvents used were reagent grade and supplied by Fisher Scientific Co. Phosphate buffer (pH 6.3) was prepared from 0.5 *M* potassium dihydrogen phosphate and 0.5 *M* disodium hydrogen phosphate solutions.

Standard curves

A standard solution containing 1.0 to 7.5 mg/ml of *p*-HPPH in methanol was prepared. A 1- or 2-μl aliquot of each standard was injected into the liquid chromatograph. The area of the *p*-HPPH signal for each injected sample was measured and plotted against the amount of the standard injected (Fig. 1). Urine samples containing known amounts of *p*-HPPH were processed by the method described below and a working standard curve was obtained.

Extraction and analysis procedure

The described extraction procedure is a modified method of Chang and Glazko[1]. A 2-ml aliquot of urine specimen was placed in a 50-ml centrifuge tube together with 2 ml of 12 *N* hydrochloric acid. The acidified aqueous solution was heated for 1 h at 95°. After cooling, the hydrolysate was neutralized with concentrated sodium hydroxide followed by the addition of 2 ml of phosphate buffer (pH 6.3). The buffered mixture was shaken for 10 min with 15 ml of cyclohexane, which was discarded. The aqueous phase was then shaken with two 10-ml portions of ethyl acetate for 10 min each time. The organic layers were transferred and combined in a second tube and then the solvent was removed with the aid of a stream of nitrogen. The residue was diluted with 50 μl of ethyl acetate and a 2-μl aliquot was injected into the liquid chromatograph. The retention time for *p*-HPPH in the standard solutions and in the urine standards was 2.7 min.

Urinary excretion of p-HPPH in healthy subjects

Five healthy male subjects took a single 400-mg dose of sodium DPH orally. Pre-drug urine and four consecutive 12-h urine samples were collected. Known amounts of *p*-HPPH at five different concentrations were added to the pre-drug

urine to obtain a working standard curve. The urine samples were worked up as described above.

RESULTS AND DISCUSSION

Some of the GLC procedures for the determination of *p*-HPPH excreted in the urine involve the use of the *meta* isomer as the internal standard[1,2]. This would tend to yield low total *p*-HPPH readings as approximately 8 % of the total HPPH in the urine is *m*-HPPH[5]. In our procedure this presents no problem as *m*-HPPH is not utilized as an internal standard.

It is possible that some *p*-HPPH and *m*-HPPH might be formed from degradation of the dihydrodiol metabolite of DPH during the acid hydrolysis step[8]. However, only a trace of the dihydrodiol has been detected in adult urine and the contribution to the overall level of HPPH would probably be very small. In the case of the urine from the newborn human, it must be cautioned that an appreciable amount of the dihydrodiol might be present.

The LC measurements using the solvent *n*-butyl chloride–dioxane (4:1) yielded little differentiation between *m*- and *p*-HPPH peaks (retention times 2.5 and 2.7 min, respectively), while DPH at 1.8 min was clearly separated from both HPPHs. The *m*- and *p*-HPPH isomers could be resolved by reducing the polarity of the eluting solvent from 4:1 to 9:1 (retention times 9.1 and 11.1 min, respectively). However, by this method *m*-HPPH could not be detected in the urine samples from the subjects who ingested DPH. This may be partly due to the weaker LC response to *m*-HPPH than to *p*-HPPH (peak area ratio 31:100). Therefore, it is still possible

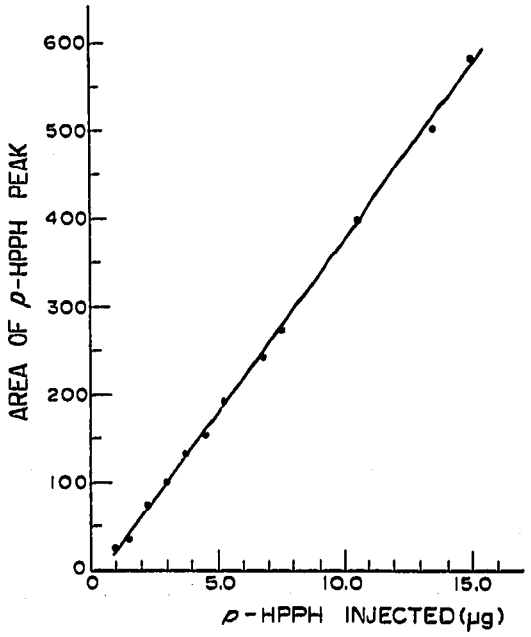

Fig. 1. Standard curve of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH).

that *m*-HPPH represents 8 % of the total HPPH in the human urine as reported by Atkinson *et al.*[5].

The standard curve, derived from plotting the area of the *p*-HPPH peak against the amount of standard analyzed, displayed linearity between 1 and 15 μg of *p*-HPPH as shown in Fig. 1. In order to determine the percent recovery using this procedure, known amounts of *p*-HPPH were added to pre-drug urine obtained from each volunteer. The recovery values for the extraction and analysis using urine from five subjects were: 78.9 ± 1.7 (% ± S.E.), 72.7 ± 6.7, 54.1 ± 4.9, 50.7 ± 5.9, and 55.7 ± 3.6. Blank urine samples collected in succession from a given individual over a period of 48 h showed only minor fluctuations in recovery values. Therefore, it was decided to use a standard curve derived from each subject's urine sample spiked with known amounts of *p*-HPPH. The LC tracings for two *p*-HPPH, drug-free urine and urine containing *p*-HPPH are shown in Fig. 2. The minimum detectable concentration of urinary *p*-HPPH was 10 μg/ml, quantitatively, and 1 μg/ml, qualitatively.

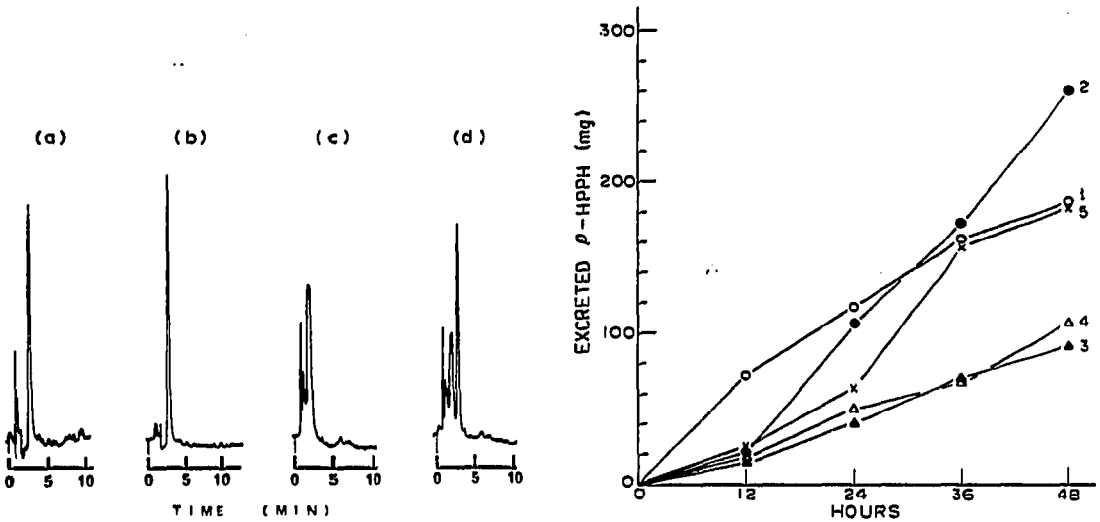

Fig. 2. Chromatograms from *p*-HPPH analysis. Column: MicroPak™, 25 cm × 2.2 mm I.D.; carrier: *n*-butyl chloride–dioxane (4:1); flow-rate: 0.9 ml/min; pressure: 500 p.s.i.; temperature: ambient. (a) A standard, *p*-HPPH 1.0 μg, 2.7 min, UV × 0.04 (AUFS). (b) A standard, *p*-HPPH 5.25 μg, 2.7 min, UV × 0.16. (c) Blank urine extract, UV × 0.16. (d) A standard added to urine, *p*-HPPH 130 μg/ml, 2.7 min, UV × 0.16.

Fig. 3. Cumulative urinary excretion of *p*-HPPH in five healthy subjects following a single oral dose of 400 mg of sodium DPH.

This method has been applied to the determination of *p*-HPPH in four consecutive 12-h urine samples collected from five healthy male volunteers who had ingested a single 400-mg dose of sodium DPH. The concentration of total *p*-HPPH in urine ranged from 10 to 300 μg/ml and Fig. 3 shows the individual differences in the excretion of *p*-HPPH. The percentage dose excreted as *p*-HPPH in 48 h was found to vary between 23.2 and 66.6 %. This LC assay could also be used for the routine analysis of urinary *p*-HPPH in epileptic patients[9].

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