High-Pressure Liquid-Chromatographic Determination of 5-(4-Hydroxyphenyl)-5-Phenylhydantoin in Human Urine

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We describe a sensitive and precise high-pressure liquid-chromatographic method for measurement of total 5-(4-hydroxyphenyl)-5-phenylhydantoin, a metabolite of phenytoin, in urine. An aliquot of urine, containing 5-(4-methylphenyl)-5-phenylhydantoin as an internal standard, is processed and chromatographed. The metabolite and internal standard are identified from their retention times and quantitated from their relative response factors. The metabolite is separated from normal urine constituents and internal standard in less than 8 min. The sensitivity of the method is such that after the usual dose it can be measured in 0.5 ml of urine; the lower limit of detection is 300 ng.

Additional Keyphrases: drug assay • monitoring therapy

Chang and Glazko (7) used a gas-chromatographic procedure for the trimethylsilyl derivative of HPPH and applied it to clinical studies. Their use of 5-(3-hydroxyphenyl)-5-phenylhydantoin as an internal standard for this assay, is no longer recommended, because it was found in dog urine. Although only traces of it are found in human urine, it is also formed from the dihydrodiol metabolite of phenytoin during sample preparation for HPPH determination. Grimmer et al. (8) developed a methylation procedure with diazomethane for use in HPPH assay by gas-chromatography. Recently, Hoppel et al. (9) have described a mass-fragmentographic method for determination of phenytoin and HPPH concentrations in human plasma.

Inaba and Brien (10) used a high-pressure liquid chromatographic method to determine HPPH concentrations in human urine. However, they reported a recovery rate for HPPH of only 50 to 78%, and no provision was made for the use of an internal standard. Additionally, they gave no data on precision or interference. In contrast, our method offers: (a) a more consistent and quantitative recovery of HPPH from urine, (b) use of 5-(4-methylphenyl)-5-phenylhydantoin as an internal standard, (c) easy adaption to the simultaneous determination of both HPPH and phenytoin in the same urine specimen, and (d) no interference from other commonly used anticonvulsant drugs. Total chromatographic time is 8 min.

Materials and Methods

Chromatography

We used a Model 601 (Perkin-Elmer Corp., Norwalk, Conn. 06856) high-pressure liquid chromatograph equipped with a variable-wavelength ultraviolet detector (Perkin-Elmer LC 55), a 1-MV Honeywell Electronic 194 recorder, and a 30 cm × 4.0 mm (i.d.) column containing silica beads chemically bonded with octadecyl trichlorosilane (μ-Bondapack C18; Waters Associates Inc., Milford, Mass. 01757). The column was eluted with acetonitrile/water (37/63 by vol) at the rate

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of 1.5 ml/min, and the column effluent was monitored at 254 nm.

Reagents and Standards

1,2-Dichloroethane and ethyl acetate, AR grade (Mallinckrodt, Inc., St. Louis, Mo. 63147).

Acetonitrile, ACS grade (Eastman Kodak Co., Rochester, N. Y. 14650).

Water, glass-distilled.

Brown's buffer: To 80 g of sodium bicarbonate, add 150 ml of 5 mol/liter NaOH and dilute to 1 liter with water. Adjust to pH 7.0.

5-(4-Hydroxyphenyl)-5-phenylhydantoin (HPPH) and 5-(4-methylphenyl)-5-phenylhydantoin (Aldrich Chemical Co., Milwaukee, Wis. 53233).

5,5-Diphenylhydantoin (Eastman Organic Chemicals, Rochester, N. Y. 14650).

Drug reference standard: A standard solution of HPPH (25 mg), phenytoin (5,5-diphenylhydantoin 25 mg), and 5-(4-methylphenyl)-5-phenylhydantoin (25 mg) was prepared by dissolving all of them in 100 ml of ethanol. This solution was stable for at least one month at 4 °C.

A stock internal standard of 5-(4-methylphenyl)-5-phenylhydantoin (100 mg/liter) was prepared in ethanol. The solution was stable for at least one month at 4 °C.

A working internal standard was prepared by diluting the stock internal standard threefold.

Procedure

Urine collection. We collected 24-h urine samples from patients who were receiving phenytoin. The specimen was preserved by adding 10 ml of concd HCl. The total volume of the 24-h urine was measured.

Urine, 0.5 ml, was combined with 0.5 ml of the diluted internal standard and 0.5 ml of concd HCl in a loosely stoppered test tube, and heated at 110 °C for 1 h. The hydrolysate was cooled and poured into a 40-ml centrifuge tube. The hydrolysate was neutralized with 4 ml of Brown's buffer, 20 ml of ethyl-acetate/dichloroethane (1/2 by volume) was added, and the mixture was mechanically shaken for 5 min and then centrifuged for 5 min at 210 × g (2000 rpm). The aqueous layer was removed and the organic layer was washed with 15 ml of sodium bicarbonate (50 g/liter). The resulting aqueous layer was then removed; the organic layer was dehydrated with sodium sulfate and then evaporated at 70 °C under reduced pressure with a rotary evaporator. The residue was redissolved in 75 μl of methanol, and 20 μl was injected into the high-pressure liquid chromatograph.

Results

Analytical Variables

Figure 1 illustrates representative chromatograms of a drug reference standard and an extract of urine from a patient receiving phenytoin.

Detection and sensitivity. HPPH is detected at 254 nm. The lower limit of detection for pure HPPH standard is 300 ng by our method. HPPH in 0.5 ml of urine can be measured.

Linearity. Urine-based standards were prepared containing various amounts of HPPH (equivalent to 50 to 500 mg/liter). A constant amount of the internal standard was added to each sample. For HPPH, concentration and absorbance were linearly related from 50 to 500 mg/liter.

Recovery. Known amounts of HPPH and internal standard were added to normal urine specimens, which were then hydrolyzed with acid, extracted, and assayed as described. The mean analytical recovery of HPPH was 97.0% ± 5.0% for the entire range of 50 to 500 mg/liter (Table 1). HPPH and the internal standard appeared to be equally stable under the hydrolytic conditions.

Precision. Data on within-run precision were obtained by taking 10 aliquots of a urine sample known to contain HPPH, and processing them separately through the complete procedure during a single day. The mean value was 131.2 ± 4.0 (range) mg/liter (CV, 3.0%).

Day-to-day precision was calculated from values for a single sample assayed on 10 consecutive days. The mean was 133.8 ± 4.7 (range) mg/liter (CV, 3.5%).

Quantitation and identification of HPPH. We evaluated unknowns by measuring their peak height relative to that of the internal standard. HPPH and internal standard were identified by retention time, 4.0

| Table 1. Analytical Recovery of Added HPPH from Human Urine |
|---------------|----------------|---------|
| Added         | Recovered      | Recovery (%) |
| mg/liter      |                |          |
| 50.0          | 48.0           | 96       |
| 100.0         | 102.0          | 102      |
| 200.0         | 184.0          | 92       |
| 300.0         | 294.0          | 98       |
| 500.0         | 473.0          | 95       |

Fig. 1. High-pressure liquid chromatography of 5-(4-hydroxyphenyl)-5-phenylhydantoin
Trace A shows a typical chromatogram for the reference standard containing 10 μg of HPPH and phenytoin (DPH). Trace B shows the chromatogram for a 24-h urine sample from a patient excreting (in this specimen) 138 mg of HPPH per 24-h
and 8.0 min, respectively. The 24-h excretion of HPPH was calculated from the total volume of urine.

Measurement of HPPH in Human Urine

Twenty-four-hour urine specimens were obtained from hospitalized patients who had been receiving phenytoin together with various other drugs for periods ranging from several months to years. The specimens were assayed for total HPPH by our procedure. HPPH excretion ranged from 80 mg to 244 mg per 24 h. Drugs such as phenobarbital, antacids, and dexamethasone, which were administered concurrently with phenytoin did not interfere. Sometimes an unidentified peak preceded the HPPH peak in our chromatogram, but we encountered no difficulty in identifying and measuring HPPH in these samples.

Discussion

Our method easily provides the sensitivity to determine HPPH concentration in 0.5 ml of urine from a person who is receiving therapeutic doses of phenytoin. High-pressure liquid chromatography does not require derivatization as do most gas-chromatographic methods. Total chromatographic time is 8 min, and the method requires only a single extraction. This procedure could also be used to simultaneously determine phenytoin in the same sample.

With this assay, the utility of HPPH determinations in monitoring treatment with phenytoin can be determined.

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References