Journal of Chromatography, 130 (1977) 422–425
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 9465

Note

Assay of the major (4-hydroxylated) metabolite of diphenylhydantoin in human urine by reversed-phase high-performane liquid chromatography

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(First received May 3rd, 1976; revised manuscript received June 21st, 1976)

We recently described[1] an assay of the major metabolites of diphenylhydantoin (DPH), *i.e.*, 5-(4-hydroxyphenyl)-5-phenylhydantoin (4-OH-DPH) and its glucuronic acid conjugate by gas chromatography after flash methylation with trimethylanilinium hydroxide. Extraction with isoamyl alcohol was used in order to remove the metabolite 5-(3,4-dihydroxycyclohexa-1,5-dienyl)-5-phenylhydantoin[2], which occurs to the extent of about 10–20% in the urine of male human volunteers under steady-state conditions of DPH[3]. When the conjugate of 4-OH-DPH is cleaved with hydrochloric acid, this diol-metabolite is dehydrated to give equal amounts of 3- and 4-OH-DPH, and the latter interferes in the assay.

The glucuronide conjugate of 4-OH-DPH has also been analyzed directly by gas chromatography after permethylation[4], but the method was only qualitative.

Liquid chromatographic methods for the assay of 4-OH-DPH and its glucuronide conjugate have also been published. Inaba and Brien[5] used a silica gel column (Micro-Pak) to estimate 4-OH-DPH in human urine, but the chromatograms showed that the 4-OH-DPH peak was badly resolved from background peaks originating from the urine. By employing an ion-exchange column (DEAE-cellulose), Albert *et al.*[6] were able to resolve the uncleaved glucuronide conjugate of 4-OH-DPH from 4-OH-DPH itself and DPH in plasma. Radioactively labelled DPH was used and the metabolites were detected in the eluate by liquid scintillation counting. No mention was made, however, of its applicability to unlabelled material in plasma or urine. Also by using an ion-exchange column, Anders and Latorre[7] achieved the qualitative separation of 4-OH-DPH and DPH. They analyzed only standard samples and the resolution shown by chromatograms discouraged us from using an anion-exchange resin.

We believe that a direct analysis of the conjugate without derivatization can be accomplished in a simpler manner by means of high-performance liquid chromatography (HPLC). As a first step in achieving this aim, we report here an HPLC method using a μBondapak C_{18} column for the analysis of 4-OH-DPH glucuronide after hydrolysis with hydrochloric acid.

EXPERIMENTAL AND RESULTS

Chemicals

4-OH-DPH was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and the other chemicals used were obtained from Kebo-Grave Labcenter (Stockholm, Sweden). All chemicals were of analytical-reagent grade.

Apparatus

The pump was a Waters Model 6000 solvent delivery system and the injector was a Waters Model U6K. A Waters Model 440 absorbance detector was used. The column was a Waters stainless-steel pre-packed μBondapak C_{18} column (300 $\times$ 4 mm), particle size 10 μm.

Chromatographic system

The influence of pH and the polarity of the mobile phase on the resolution of 4-OH-DPH from urine background peaks was studied. The best resolution was obtained in a mobile phase consisting of 27% ethanol in 0.1 *M* acetate buffer, pH 3.2 (Fig. 1). The background pattern and retention time of 4-OH-DPH remained constant in the pH range 2.4–6.0.

The efficiency of the chromatographic system expressed as the height equivalent to a theoretical plate, *H*, at different flow-rates of the mobile phase was estimated for 4-OH-DPH. With the column used, an *H* value of less than 0.6 mm was obtained with flow-rates up to 1.8 ml/min. In urine, the separation from background peaks was also very good at the highest flow-rates.

Determination of conjugated 4-OH-DPH

To 2.00 ml of urine was added 1.00 ml of 3 *M* tris(hydroxymethyl)aminomethane hydrochloride(Tris-HCl) buffer (pH 7.5). After two extractions with 10.0 ml of isoamyl alcohol (saturated with water) for 15 min, the organic phases were discarded. One millilitre of the aqueous phase was transferred into another tube and 1.0 ml of 12 *N* hydrochloric acid was added. The conjugate was cleaved by treatment in a water-bath at 100° for 150 min. The cooled acidic phase was carefully neutralized with 1.0 ml of 12 *M* sodium hydroxide solution followed by the addition of 0.5 ml of 3 M Tris-HCl buffer (pH 7.5). 4-OH-DPH was then extracted with 8.00 ml of diethyl ether, and 7.00 ml of the extract were re-extracted with 0.50 ml of 0.1 *M* sodium hydroxide solution. An exact volume (*e.g.*, 10.0 μl) was then injected into the chromatograph. The mobile phase consisted of 27% ethanol in 0.1 *M* sodium acetate buffer at pH 3.2 with a flow-rate of 1.2–1.5 ml/min, depending on differences in the background patterns between patients.

Calibration graphs

Calibration graphs were prepared by adding different amounts of 4-OH-DPH to drug-free human urine, which had first been extracted twice with isoamyl alcohol. The peak heights of 4-OH-DPH were plotted against concentration in the range 10–60 μg/ml (Fig. 2). The concentration of conjugated 4-OH-DPH was calculated by multiplying the value obtained from the calibration graph by a factor of 1.08 to adjust for the loss of the conjugate to the isoamyl alcohol phase. This factor was obtained

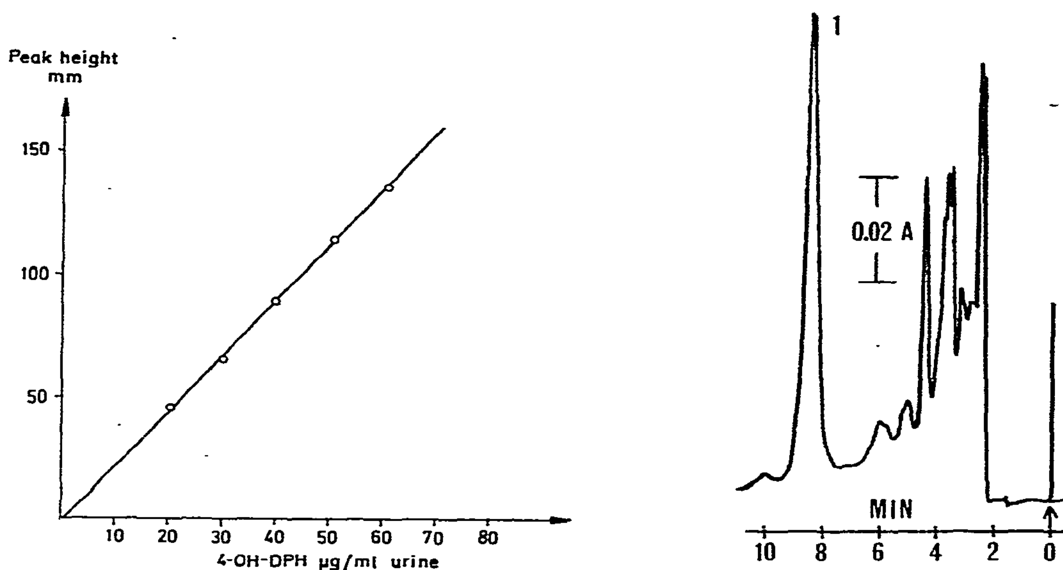

Fig. 1. Chromatogram of an extract from the urine of a patient treated with DPH. The depicted peak (1) corresponds to 4-OH-DPH at a concentration of 41.6 µg/ml. Chromatographic conditions: 27% ethanol in 0.1 *M* acetate buffer, pH 3.2, 1.3 ml/min, 1800 p.s.i., room temperature, UV detection 254 nm).

Fig. 2. Calibration graph for conjugated 4-OH-DPH in urine; attenuation 0.02.

by dividing the 100% theoretical recovery by the 92.5% obtained. The partition coefficient of the conjugate between isoamyl alcohol and buffered urine has been calculated[1] to be 0.012.

Recovery and precision of the method

The recovery of 4-OH-DPH in the extraction steps was calculated to be 97.6% by employing the partition coefficient (17.8) between diethyl ether and buffered urine[1]. The final extraction into 0.1 *M* sodium hydroxide solution was quantitative. The recovery in the hydrolysis step was checked by analyzing urine from patients treated with DPH. The urine was boiled with hydrochloric acid for different times and the hydrolysis was found to be complete after 150 min[1].

The precision of the assay was determined by the analysis of six replicate samples from the same patient's urine. The relative standard deviation for this urine was 1.8%. When duplicate urine samples from five different patients in the concentration range 40–100 µg/ml were analyzed, the standard deviation was found to be 1.4%.

DISCUSSION

The method has been used to analyze 4-OH-DPH conjugate in the urine from a few patients. The results showed that it can be used with good reproducibility and the precision is very good in comparison with gas chromatographic methods. An

advantage of HPLC is that 4-OH-DPH can be chromatographed directly without derivatization. By employing reversed-phase chromatography, the most of the urinary constituents are not retained on the column and are eluted with the front. This will probably allow the use of the column for a long time without noticeable contamination or deterioration of its separation properties.

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