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SIMULTANEOUS DETERMINATION OF 5,5-DIPHENYLHYDANTOIN AND 5-(*p*-HYDROXYPHENYL)-5-PHENYLHYDANTOIN IN SERUM, URINE AND TISSUES BY GAS–LIQUID CHROMATOGRAPHY AFTER FLASH-HEATER METHYLATION

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SUMMARY

A gas–liquid chromatographic method is described for the simultaneous determination of diphenylhydantoin and free and conjugated *p*-hydroxyphenylphenylhydantoin in biological fluids and tissues. Diphenylhydantoin and *p*-hydroxyphenylphenylhydantoin, after hydrolysis, are extracted with diisopropyl ether containing 5-(*p*-methylphenyl)-5-phenylhydantoin as the internal standard, transferred into a tetramethylammonium hydroxide solution, then quantitatively converted by flash-heater methylation in the injection port of the chromatograph into 1,3-dimethyl-5,5-diphenylhydantoin and 1,3-dimethyl-5-(*p*-methoxyphenyl)-5-phenylhydantoin, respectively. Separation and measurement of these derivatives are accomplished on a 3% OV-17 column, using a flame ionization detector. The method has the advantages of high specificity, sensitivity and rapidity and appears to be suitable for the routine monitoring of blood and urine concentrations in patients receiving multi-drug therapy.

INTRODUCTION

Diphenylhydantoin (DPH) is at present one of the most effective anticonvulsant agents. However, the adequate treatment with this drug of patients suffering from epilepsy requires critical levels of administration and individual therapy is necessary. There is only a narrow interval between the optimum beneficial and toxic plasma concentrations of DPH[1]. Also, certain variations in the clinical response have been demonstrated that are related to DPH bioavailability and to individual differences, either congenital or induced by other drugs, in the absorption and metabolism rates of DPH[2–8]. It has been shown that a correlation exists between DPH intake, levels of the drug in the blood, seizure control and signs of toxic side-effects[9,10]. Considerable attention has therefore been paid in recent years to the determination of blood and urine concentrations of DPH and its major

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metabolite, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (HPPH). In addition to sensitivity, the selected method should be highly specific, in order to eliminate interferences from other drugs administered concurrently, and suitable for routine clinical assays. In this paper, a gas–liquid chromatography procedure is described which permits the simultaneous identification and quantitative determination of both DPH and HPPH, following the conversion of the parent compounds into their methyl derivatives by flash-heating. It constitutes an extension and an adaptation of the methods of Grimmer *et al.*[11], MacGee[12] and Hammer *et al.*[13]. Improvements in sample preparation and experimental parameters have resulted in a method for which the accuracy, sensitivity, specificity and rapidity have been optimized. In addition, identification of the methylation products of DPH and HPPH has been carried out.

EXPERIMENTAL

Reagents

All chemicals were of reagent grade. DPH, HPPH and 5-(*p*-methylphenyl)-5-phenylhydantoin (TPH) were purchased from Parke, Davis & Co (Detroit, Mich., U.S.A.) and used without further purification. Tetramethylammonium hydroxide solutions (about 24% in MeOH) were obtained from BDH Chemicals Ltd. (Poole, Dorset, Great Britain) and protected from moisture.

Solutions

Phosphate buffer. $NaH_2PO_4 \cdot H_2O$ saturated solution, pH 6.5.

Extraction solvent. Diisopropyl ether containing 2.5 μg/ml of TPH as the internal standard.

DPH and HPPH standard solutions. Aqueous standard solutions were prepared to contain 5, 10, 15, 20 and 25 μg/ml by diluting a concentrated ethanolic stock solution with distilled water. They had to be used within 1 h from the time of preparation.

Apparatus

Gas chromatography was carried out by using a Varian Aerograph Model 2100 instrument equipped with a hydrogen flame ionization detector and a Philips PR 3500 recorder with a range of 0 to 1 mV and a chart speed of 800 mm/h. U-shaped glass columns, 6 ft. long × 0.25 in. I.D., were used. Comparison of four different stationary phases, 3% OV-1, 3% OV-17, 3% SE-30 and 2% XE-60, resulted in the selection of columns containing 3% OV-17 on 100–120 mesh Gas-Chrom Q. These columns were pre-conditioned by heating them for 18–24 h at 250° with carrier gas flowing.

The operating conditions were as follows: carrier gas, purified nitrogen at 38 ml/min, hydrogen produced by an Elhygen Milton-Roy generator at 30 ml/min and dry compressed air at 300 ml/min; injector temperature, 250°; linearly programmed oven temperature, 110–230° at the rate of 10°/min; detector temperature, 250°; electrometer sensitivity setting, 10^{-11} A/mV with, as a rule, an attenuation at × 16.

The UV spectra were recorded in chloroform with a Unicam SP 800 spectro-

photometer. The IR spectra (KBr pellet) were run on a Perkin-Elmer Model 237 apparatus. The proton magnetic resonance (PMR) spectra were obtained by using a JEOL Model C 60 spectrometer; the solvent was deuterated chloroform and the internal reference tetramethylsilane, A Varian CH 5 instrument with a direct inlet system was used for recording the mass spectra (MS) at ionizing potentials of 20 and 70 eV.

Synthesis of reference methyl derivatives of DPH

3-Methyl-5,5-diphenylhydantoin. An ethereal solution of diazomethane, prepared from *p*-tolylsulphenylmethylnitrosamide, was distilled in a solution of 0.5 g (2 mmoles) of DPH in 30 ml of Et_2O maintained at +10°, until nitrogen evolution stopped. The reaction mixture was evaporated under reduced pressure and the white solid residue purified by recrystallization from EtOH (yield, 98%). The compound had the following properties:

m.p.: 210–211° (Tottoli); 213° (Kofler Heizbank apparatus); lit.[11], 217–218°.

UV $\lambda_{max.}$: 241 nm (ε 1860).

IR: 3300 cm^{-1}, N(1)–H, lit.[14]; 1775 cm^{-1}, CO(4), lit.[15]; 1712–1695 cm^{-1}, CO(2), lit.[15].

PMR: 3.05 p.p.m. (δ), N(3)–CH_3; 7.40 p.p.m., $>C<^{C_6H_5}_{C_6H_5}$; 8.25 p.p.m., N(1)–H.

MS: *m/e* 266 (31%), M^+. Significant peaks at *m/e* 209 (11.2%), M–CH_3NCO; *m/e* 180 (37.2%), $\overset{+}{N}=C<^{C_6H_5}_{C_6H_5}$; *m/e* 104 (24%), $HN=\overset{+}{C}-C_6H_5$.

1,3-Dimethyldiphenylhydantoin. A 10-ml volume (106 mmoles) of dimethyl sulphate was added dropwise with magnetic stirring to a solution of 0.25 g (1 mmole) of DPH in 50 ml of 2*N* NaOH. The reaction was allowed to proceed for 90 min at 25° and the white precipitate was collected by filtration and recrystallized from EtOH. This material was still impure, as shown by the presence of two spots on silica gel thin-layer chromatography (TLC). Preparative TLC (silica gel plates, Merck F_{254}; solvent, chloroform–acetone, 19:1, v/v) afforded the dimethyl derivative (yield, 35%). The compound had the following properties:

m.p.: 189–190° (Tottoli); 192° (Kofler Heizbank apparatus); lit.[11], 193°; lit.[16], 197°.

UV $\lambda_{max.}$: 241 nm (ε 2350).

IR: 1762 cm^{-1}, CO (4); 1718–1708 cm^{-1}, CO (2).

PMR: 2.82 p.p.m. (δ), N(1)–CH_3; 3.15 p.p.m., N(3)–CH_3; 7.35 p.p.m. (multiplet), $>C<^{C_6H_5}_{C_6H_5}$.

MS: *m/e* 280 (7.1%), M^+. Significant peaks at *m/e* 223 (21%), M–CH_3NCO; *m/e* 194 (10.7%), M–CH_3NCO–CO–H; *m/e* 118 (20%), CH_3–N=C^+–C_6H_5.

The mass spectra of these mono- and dimethyl derivatives were in agreement with those reported by Grimmer *et al.*[11] and Sabih and Sabih[17].

Extraction of DPH and HPPH from biological samples

The analytical procedure involved the extraction of DPH and HPPH from biological samples with an organic solvent containing a known concentration of an internal standard, transfer into the methylating reagent, derivative formation in the injection port of a gas chromatograph and chromatography.

Extraction of free DPH and HPPH from serum or urine

A serum or urine sample (1.0 ml) and phosphate buffer (1.0 ml) were mixed in a 20-ml stoppered glass centrifuge tube. The resulting solution, in which, for serum, a white precipitate formed, was extracted with diisopropyl ether (5.0 ml) containing 2.5 μg/ml of TPH. The tube was shaken vigorously by hand for 30 sec and then, if necessary, centrifuged for 10 min at 3000 r.p.m. in order to break any emulsion. About 4 ml of the upper organic layer were transferred into a dry, stoppered 10-ml test-tube and 100 μl of the tetramethylammonium hydroxide solution added. After vigorous shaking for 30 sec, the two phases were allowed to stand for 5 min until separated. From the lower aqueous layer 2–5 μl were removed and injected on to the gas chromatograph.

Extraction of free DPH and HPPH from tissues

Tissue sample (about 1 g wet weight of brain, intestine, kidney and liver) was mixed with 10 ml of 0.85% sodium chloride solution and homogenized in a Potter–Elvehjem type of apparatus. An aliquot (1.0 ml) of the suspension was then transferred into a 20-ml stoppered glass centrifuge tube and treated in exactly the same manner as the serum or urine samples.

Extraction of total HPPH from serum or urine

Total HPPH was determined following the hydrolysis of the glucuronic acid conjugate which constitutes the major metabolite of DPH. This was accomplished by mixing 1 ml of serum or urine sample with 1 ml of 12 *N* hydrochloric acid in a stoppered test-tube and heating for 90 min in a steam-bath. An aliquot (0.1–1.0 ml) was then transferred into a 20-ml stoppered glass centrifuge tube and neutralized with 2 ml of phosphate buffer. The following steps were identical with those described for free DPH and HPPH.

Gas chromatography

A 2–5 μl aliquot of the lower tetramethylammonium hydroxide phase was injected into the chromatographic unit. The injection port temperature (250°) ensured the completeness of the methylation for DPH, HPPH and the internal standard. The column was kept at 110° for about 7 min in order to eliminate the solvent peak, then the programming was started at the rate of 10°/min up to 230° and this temperature was maintained for 5 min. Under the conditions described,

the peak representing DPH appeared at the end of the programming step, *i.e.*, about 19 min after injection, the internal standard peak 30 sec and the HPPH peak 90 sec later. A typical elution pattern is shown in Fig. 1.

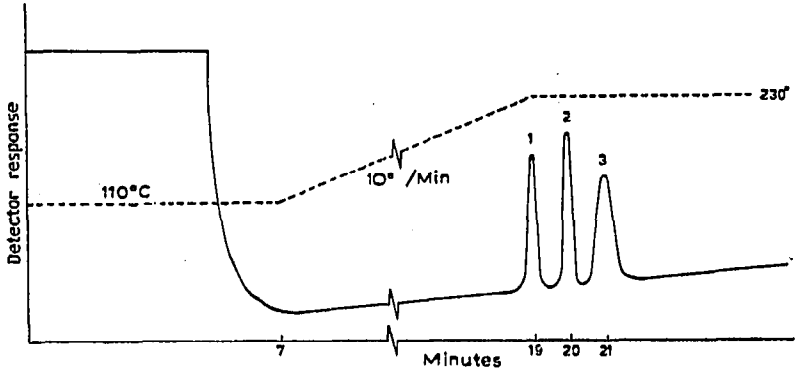

Fig. 1. Typical gas–liquid chromatogram of methylated derivatives of DPH (1) and HPPH (3) extracted from human urine. The intermediate peak (2) represents TPH, the internal standard. Chromatographic conditions are given in the text.

The time for the total analysis was about 40 min for a single assay. The column could be used for about 15 injections, after which it had to be reconditioned overnight at 290°.

Calibration

The calibration graphs were constructed using DPH and HPPH standard solutions of concentration 5, 10, 15, 20 and 25 μg/ml. Other reference standards

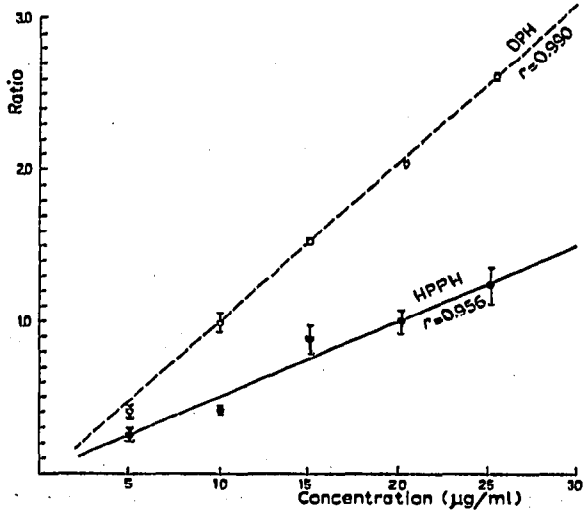

Fig. 2. Standard calibration curves for DPH and HPPH. ○, Peak height ratio of methylated DPH to methylated internal standard plotted against concentration; ●, peak area ratio of methylated HPPH to methylated internal standard plotted against concentration. The best straight lines for each set of data were calculated by the method of the least squares. Values of the correlation coefficient, *r*, are given. Vertical bars indicate confidence intervals at the 0.05 level estimated from the statistical analysis of seven separate samples at each concentration.

were prepared by the addition of known amounts of DPH and HPPH to extraction solvent and to normal serum; they gave similar curves. Peak heights were measured from the baseline with a millimetre scale and areas under the peaks were measured by multiplying the peak height by the width at half-height. In actual practice, peak height measurements proved to be entirely satisfactory for DPH assays, whereas the shape of the peak corresponding to HPPH indicated that the measurement of peak area would be preferable for its determination. Linear working curves (Fig. 2) relating DPH:TPH and HPPH:TPH mean ratios to their concentrations in biological samples were obtained over the range 1–30 μg/ml.

Although previously constructed calibration graphs could be used, more accurate results were obtained by determining the concentration of standard solutions simultaneously with the samples as there were minor day-to-day variations in the ratio values.

At the end of each run, the recovery was checked with standards containing 20 and 40 μg of DPH and HPPH added to 2.0 ml of human plasma treated as assay specimens.

RESULTS AND DISCUSSION

Derivative formation and identification of the peaks

Under the described conditions, the flash-heater methylation of both DPH and HPPH gave a single peak. Identical results were obtained by using four different stationary phases, 3% OV-1, 3% OV-17, 3% SE-30 and 2% XE-60, and DPH, HPPH samples extracted either from standard solutions or from biological materials. More particularly, no extra peak due to the unmethylated parent compounds could be detected at any time.

A tentative identification of the peak given by DPH was obtained by running reference methyl derivatives together with DPH samples. On all four stationary phases, the DPH peak was found to correspond to 1,3-dimethyl-5,5-diphenylhydantoin. In order to confirm this result and to extend it to HPPH, a combined gas chromatographic–mass spectrometric technique was used, which enabled the corresponding DPH peak to be definitively identified as the preceding 1,3-dimethyl derivative. This finding was in agreement with MacGee's report[12]. For HPPH, the mass spectra indicated that three methyl groups were introduced at the N-1 and N-3 atoms of the hydantoin ring and at the OH group of the 5-*p*-hydroxyphenyl substituent to produce 1,3-dimethyl-5-(*p*-methoxyphenyl)-5-phenylhydantoin: *m/e* 310 (17.4%), M^+; significant peaks at *m/e* 233 (23.2%), $M-C_6H_5$; *m/e* 224 (7.3%), $M-CH_3NCO-CO-H$; *m/e* 203 (2.4%), $M-C_6H_4OCH_3$; *m/e* 148 (10.5%), $CH_3-N=C^+-C_6H_4OCH_3$; and *m/e* 118 (4.5%), $CH_3-N=C^+-C_6H_5$.

Finally, it can be concluded that the present procedure for derivative formation ensures the rapid and complete methylation of both DPH and HPPH under widely different conditions.

Other methods[11,17] in which the conversion of DPH and HPPH was carried out before injection into the gas chromatograph enabled only partially methylated derivatives to be prepared, *i.e.*, 3-methyl-5,5-diphenylhydantoin and 3-methyl-5-(*p*-methoxyphenyl)-5-phenylhydantoin.

Recovery and accuracy

It is a well-known fact that DPH is extensively bound to plasma proteins[18–20]. In order to test the adequacy of the extraction procedure, working standards of DPH and HPPH in human and rat sera were assayed in comparison with aqueous standard solutions. At each concentration level used, 5, 10, 15, 20 and 25 μg/ml, no significant difference ($t_{0.05}$) was found between the means of seven replicate measurements. In addition, the extraction yield was directly estimated with [4-^{14}C]DPH added to serum. The average value of nine determinations at the 15 μg/ml level was 96.7 $\pm$ 3.6%. Further investigations showed that this yield was independent of the serum concentration over the whole range tested, 3–30 μg/ml. The efficiency of extraction was also examined for the serum of animals administered with DPH; the recovery of the radioactivity from the serum of four rats intravenously injected with a 5 mg/kg dose of [4-^{14}C]DPH was found to range from 83 to 86% ($\bar{x}$ 84.4%) before hydrolysis and from 92 to 101% ($\bar{x}$ 96.4%) after heating in 6 *N* hydrochloric acid for 90 min. These results indicate that DPH and probably HPPH, present in the serum in therapeutic concentrations, are quantitatively extracted and assayed.

In addition to recovery experiments, the accuracy was assessed from the effects of interfering drugs on the analytical procedure. This test was considered to be of the greatest importance as most of the patients with neuropsychiatric disorders are commonly treated with a multi-drug therapy. The lack of interference by other compounds was demonstrated by analyzing more than 33 blood samples from hospital patients. In particular, the following drugs were found not to interfere in the determination of both DPH and HPPH: allobarbital, hexobarbital, phenobarbital, prominal, mephenytoin, primidone, meprobamate and phenacetin.

Precision

The precision was determined by analyzing four serum samples containing between 5 and 20 μg/ml of DPH and four urine samples containing between 5 and 20 μg/ml of HPPH, at least seven times each. The relative standard deviation at each level was less than 4.2% for DPH and 6.3% for HPPH.

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