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MICRO-DETERMINATION OF PLASMA DIPHENYLHYDANTOIN BY GAS—LIQUID CHROMATOGRAPHY

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SUMMARY

A selective, sensitive and precise gas—liquid chromatographic method for the determination of diphenylhydantoin in micro samples of blood plasma is described. After a double extraction with chloroform containing an analogue of diphenylhydantoin as an internal standard, the drug and standard are N,N-dimethylated in alkaline aqueous solution with methyl iodide followed by extraction into acetone. The methylated derivatives are separated gas chromatographically and measured using a flame-ionization detector. The lowest concentration of diphenylhydantoin in plasma which can be measured in a 100-μl sample is 1 μg/ml, which is well below the normal therapeutic concentration of 10—20 μg/ml in plasma. The methylated derivatives of diphenylhydantoin and the internal standard have been identified by their proton magnetic resonance spectra and mass spectra.

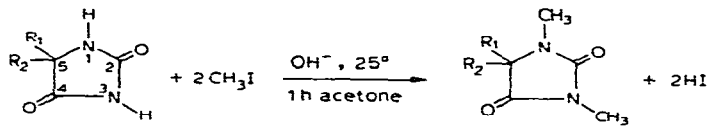
INTRODUCTION

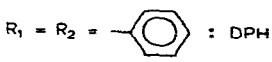
Diphenylhydantoin (5,5-diphenylhydantoin, phenytoin, DPH) is used in the therapy of epilepsy and it has been shown that knowledge of the blood level of this drug is helpful in the control of seizures in patients. Various methods for the determination of DPH and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (HPPH), the principal metabolite of DPH in man, as well as of other anticonvulsant drugs in biological material, have been reported [1—35]. Analyses using colorimetry, ultraviolet spectrophotometry and fluorimetry [1—4], thin-layer chromatography [1,5], radioimmunoassay [6,7], spin immunoassay [8] and polarography [9] have been carried out.

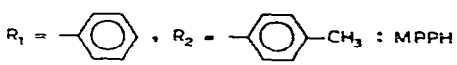
Gas—liquid chromatographic (GLC) determinations [10—35] have the advantage of being sufficiently sensitive and specific, so that several anticonvulsants can be determined simultaneously. In some of these procedures, the unchanged drugs are chromatographed [10—20], whereas in others deriv-

atives are prepared prior to GLC. Some workers [21] prepare trimethylsilyl (TMS) derivatives, but the most common approach appears to be the conversion of the antiepileptics into methylated products with diazomethane [22, 23], dimethyl sulphate [24,25] or the flash-methylating reagents tetramethylammonium hydroxide [26—29] and trimethylanilinium hydroxide [19, 30—35].

Recently, heptabarbital and cyclobarbital have been converted into their dimethyl derivatives by alkaline extraction of biological fluid with methyl iodide in acetone at room temperature [36]. In our work, an adaptation of this methylation procedure is used for the GLC determination of DPH in plasma. With known amounts of 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH) as an internal standard in the extraction solvent, there is no need for accurate aliquot measurements during extraction, derivative formation and chromatography. After a double extraction of 100 µl of plasma sample containing the drug, DPH and MPPH are dissolved in aqueous alkaline solution, methylated with methyl iodide in acetone and extracted into acetone.







These compounds are gas chromatographed and identified as the N,N-dimethylated derivatives of DPH and MPPH, 1,3-dimethyl-5,5-diphenylhydantoin (DPH-Me_2) and 1,3-dimethyl-5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH-Me_2), respectively.

OPTIMIZATION OF THE PROCEDURE

Extraction

DPH and MPPH are best extracted from plasma and aqueous buffer solutions into chloroform at pH 5—7 (for DPH pK_a' = 8.33 [37]). A peak of an unidentified physiological component with a retention time of approximately 12 min under the GLC conditions outlined below disappears when the plasma is extracted with chloroform (pH 6.8), the organic extract re-extracted into aqueous solution (pH 13) and the aqueous extract back-extracted into chloroform (pH 7.2) (Fig. 1).

Derivative formation

The methylation reaction of DPH and MPPH in a mixture of water (buffer) and methyl iodide in acetone followed by extraction of the methylated derivatives into the organic phase is pH-dependent. By shaking 20 µg of DPH with 0.5 ml of aqueous buffer solution of variable pH and 1 ml of a 4% (v/v)

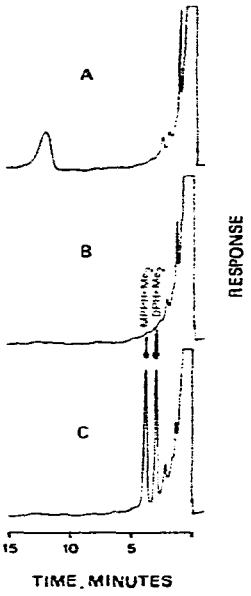


Fig. 1. Gas chromatograms of 100-μl plasma extracts subjected to the procedure described. A, Drug-free plasma, single extraction; B, drug-free plasma, double extraction; C, peaks of DPH-Me_2 and MPPH-Me_2 from plasma containing 15 μg/ml of DPH (2 μg of MPPH per sample). GLC conditions: 3% OV-225 on Chromosorb W HP; nitrogen flow-rate, 35 ml/min; column temperature, 234°; a 3-μl aliquot of 200 μl of chloroform extract was injected.

solution of methyl iodide in acetone at room temperature for 1 h followed by GLC analysis of the evaporated organic phase at pH > 13, only one methylated derivative is produced. At pH 11.5, two reaction products are observed, whereas at pH < 11 DPH is not methylated. A single chromatographic peak appears after methylation of DPH and MPPH with buffer of pH 13 (0.47 *M*). These reaction products have been identified as the N,N-dimethylated derivatives of DPH and MPPH (DPH-Me_2 and MPPH-Me_2, respectively). The rate of formation of DPH-Me_2 and MPPH-Me_2 is not increased by shaking the reaction mixture in a water bath at elevated temperature (22—70°) and is almost completed within 30 min at room temperature. Optimal reaction yields are obtained with a reaction time between 1 and 2 h. The ratio of aqueous buffer solution to acetone in the reaction mixture does not seem to be critical. For the methylation of micro amounts (0—3 μg of DPH and 2 μg of MPPH) there is no change in reaction yield and reproducibility when using 50—300 μl of buffer of pH 13 (0.47 *M*) and 0.6—2 ml of a 4% solution of methyl iodide in acetone (10^5—10^6-fold in excess).

Recovery

GLC response curves which correlate peak area with the amount of DPH-Me_2 and MPPH-Me_2 per sample offer the possibility of determining total yields (double extraction and derivative formation) for DPH and MPPH in our procedure. For 2 μg of DPH in 100 μl of plasma and 2 μg of MPPH extracted,

methylated and chromatographed under standard optimal conditions, the total recoveries are 64% (coefficient of variation, CV = 3.5%) for DPH and 68% (CV = 4.5%) for MPPH (nine determinations).

Stability

A freshly prepared plasma standard solution of 20 μg/ml of DPH was compared with a standard solution of the same drug level, kept at —18° for 2 months and with a similar standard solution produced by diluting an aqueous alkaline (0.1 *N* NaOH) solution of DPH, which had been refrigerated for 2 months, with fresh plasma. The variations in the GLC peak area ratios were insignificant.

Solutions of DPH-Me_2 and MPPH-Me_2 in chloroform awaiting GLC were examined by repeated injection of aliquots of the same extract. It was found that these extracts, refrigerated when not in use, are stable for several days.

Interference from other drugs and the metabolite HPPH

So far, no interference has been observed from other antiepileptic drugs, including phenobarbital and primidone, by analyzing plasma samples of patients receiving anticonvulsant therapy. A mixture of plasma from 50 patients receiving a large number of commonly prescribed drugs has been examined by our procedure, and no drug has been found to interfere.

In addition, micro amounts of barbital, allobarbital, allylisobutylbarbital, cyclobarbital, heptabarbital, phenobarbital and HPPH together with DPH and MPPH were methylated with methyl iodide. The methylated derivatives of the compounds investigated did not interfere in the DPH assay and were separated chromatographically from each other by using a temperature programme. Therefore, a simultaneous quantitative determination of these drugs and the metabolite HPPH should be possible.

Extraction from urine

The method for the determination of DPH in plasma was applied to urine samples. Volumes of 100 μl of drug-free urine and 100 μl of urine containing 2 μg of DPH (2 μg of MPPH per sample in the extraction solvent) were extracted, methylated and chromatographed. There was no interfering peak in the blank and the peak area ratio was approximately unity.

MATERIALS AND METHODS

Human blood and plasma

Fresh human blood, mixed with CPD anticoagulant (Fenval Division, Travenol Labs., Brussels, Belgium) is centrifuged for 20 min at 3000 rpm(1250 *g*). Plasma and plasma standard solutions of DPH are stored at —18°.

Reagents

DPH and MPPH were obtained from Parke, Davis & Co., Detroit, Mich., U.S.A.; and chloroform, acetone and methyl iodide from Merck, Darmstadt, G.F.R. All of the solvents and reagents used were of analytical grade and were specially tested for purity by carrying out blank runs.

Buffer of pH 7 contained 35.22 g of KH_2PO_4 (0.26 *M*) and 72.65 g of $Na_2HPO_4 \cdot 2H_2O$ (0.41 *M*) in 1000 ml of distilled water (Titrisol, Merck).

Buffer of pH 13 (0.47 *M*) contained 37.28 g of KCl (0.50 *M*) and 18.84 g of NaOH (0.47 *M*) in 1000 ml of distilled water (Titrisol, Merck).

Buffer of pH 13 (0.047 *M*) was obtained by diluting buffer of pH 13 (0.47 *M*) 1:10 with distilled water.

0.1 *N* NaOH solution (Titrisol, Merck) was used.

OV-225, 3% on Chromosorb W HP, 100—120 mesh, was obtained from Supelco, Bellefonte, Pa., U.S.A.

Plasma standard solutions of DPH

Plasma standards containing 1—30 μg/ml of DPH are prepared by adding 0.25 ml of a solution of DPH containing 40—1200 μg/ml in 0.1 *N* NaOH solution to drug-free plasma to a total volume of 10 ml.

Extraction procedure and derivative formation

To 100 μl of plasma standard solution of DPH are added 100 μl of buffer of pH 7 and 2 ml of chloroform containing 2 μg of the internal standard (MPPH). The glass-stoppered tube (100 × 18 mm) is shaken for 10 min on a mechanical shaker at 200 rpm and centrifuged for 5 min at 4500 rpm (2700 *g*). The aqueous phase is removed by aspiration and discarded. The organic phase is transferred into a similar glass tube and shaken with 1 ml of buffer of pH 13 (0.047 *M*) for 10 min at 200 rpm. After centrifugation for 5 min at 4500 rpm, the aqueous phase is transferred into a third glass tube and the organic layer is discarded. The aqueous extract is neutralized by the addition of 600 μl of buffer of pH 7 and shaken for 10 min at 200 rpm with 4 ml of chloroform. The mixture is centrifuged for 5 min at 4500 rpm, the aqueous phase removed by aspiration and the organic extract transferred into a glass-stoppered conical centrifuge tube (100 × 9—22 mm) and evaporated to dryness under a stream of dry nitrogen at room temperature. To the dry residue are added 100 μl of buffer of pH 13 (0.47 *M*) and 600 μl of a 4% (v/v) solution of methyl iodide in acetone. The sample in the stoppered tube is mixed on a Vortex mixer for a few seconds and then shaken mechanically for 1 h at 200 rpm and room temperature. The supernatant organic layer is transferred into a similar centrifuge tube using a capillary Pasteur pipette and evaporated to dryness under a stream of dry nitrogen at room temperature. Chloroform (200 μl) is added to the dry residue and, after mixing for a few seconds, a 3-μl aliquot is injected "on-column" into the GLC unit.

Gas—liquid chromatography

A Pye Unicam GCV gas chromatograph with flame-ionization detector (FID), an Infotronics Model CRS 204 integrator and a W + W Model 1100 recorder were used. The column was a 5 ft. × 2 mm I.D. glass column packed with 3% OV-225 (phenylcyanopropylmethylsilicone) on Chromosorb W HP, 100—120 mesh, conditioned for 24 h at 245° with nitrogen at a flow-rate of 20 ml/min. The following flow-rates were used in the GLC analysis: nitrogen (carrier gas), 35 ml/min; hydrogen, 30 ml/min; air, 330 ml/min. Temperatures

were 234° in the column, 200° in the injector and 300° in the detector. Under these conditions, the retention times of N,N-dimethylated DPH and MPPH were approximately 3 and 4 min, respectively (Fig. 1C).

Quantitation

A standard curve for DPH in plasma was prepared by analyzing plasma standard solutions according to the procedure described above. Ratios of the area of the drug peak (DPH-Me_2) to that of the internal standard peak (MPPH-Me_2) were plotted against concentrations of DPH (Fig. 2). Peak areas were measured automatically with an Infotronics Model 204 integrator, which corrects for baseline variations. The standard curve was linear for DPH plasma concentrations in the range 1—30 μl/ml (2 μg of MPPH per sample) and therefore allows the use of peak-area ratios for the analysis of unknown samples.

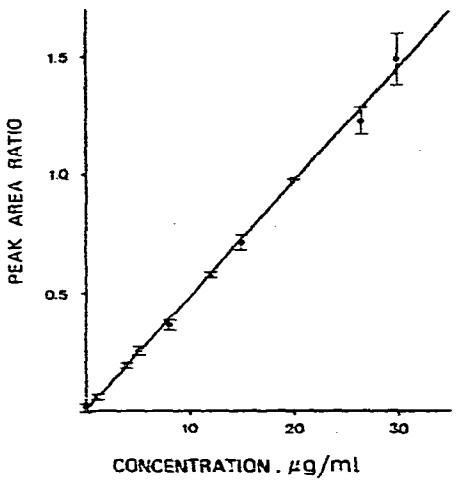


Fig. 2. Standard curve for DPH in plasma (2 μg of MPPH per sample): peak-area ratio of DPH-Me_2 to MPPH-Me_2 as a function of DPH plasma concentration. Points and vertical bars represent the mean ± standard deviation of three separate determinations at each concentration. The straight line was calculated by the method of least squares (correlation coefficient = 0.9988).

RESULTS

Identification of the derivatives

Milligram amounts of DPH and MPPH were methylated separately with methyl iodide in aqueous alkaline solution at room temperature, followed by extraction of the reaction products into acetone.

The recrystallized compounds were identified by their proton magnetic resonance (PMR) spectra and mass spectra (MS) as 1,3-dimethyl-5,5-diphenylhydantoin (DPH-Me_2) and 1,3-dimethyl-5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH-Me_2). The PMR spectra of DPH-Me_2 and MPPH-Me_2 in deuterated chloroform were run on a Varian HA-100 spectrometer with tetramethyl-

silane as internal reference. Mass spectra were recorded on an AEI-MS 30 double-beam mass spectrometer with a direct inlet probe at 50° and electron energy 75 eV with perfluorokerosene as mass marker.

DPH-Me_2 : m.p. 190—192°.
PMR: 2.81 ppm (δ), N(1)-CH_3 ; 3.14 ppm, N(3)-CH_3 ; 7.2—7.5 ppm, aromatic protons.
MS: *m/e* 280, M^+; peaks for $M - CH_3NCO$, $M - C_6H_5$, $M - CH_3NCONCH_3$ and $C_6H_5CNCH_3^+$.

MPPH-Me_2 : m.p. 114—116°.
PMR: 2.36 ppm (δ), *p*-C_6H_4-CH_3 ; 2.80 ppm, N(1)-CH_3 ; 3.12 ppm, N(3)-CH_3 ; 7.1—7.45 ppm, aromatic protons.
MS: *m/e* 294, M^+; peaks for $M - C_6H_5$, $CH_3C_6H_4CNCH_3^+$ and $C_6H_5CNCH_3^+$.

These data are in good agreement with those reported by other workers for methylated derivatives of DPH [23,25,29,38].

Response curve

The linearity of the detector (FID) response was demonstrated by injecting mixtures of various amounts of both DPH-Me_2 and MPPH-Me_2 into the gas chromatograph. Peak-area ratios of DPH-Me_2 to MPPH-Me_2 are plotted against the ratio of the amount of DPH-Me_2 to that of MPPH-Me_2 in the sample. The response curve was linear in the ranges 0.26—2.6 μg of DPH-Me_2 and 0.58—2.3 μg of MPPH-Me_2 in 200 μl of chloroform (3 μl injected).

Standard curve and working standard curve: precision and reproducibility

A standard curve prepared by analyzing plasma standard solutions of DPH is shown in Fig. 2. For three independent determinations at each level, the coefficient of variation was less than 10% in the concentration range of 1—30 μg/ml of DPH in plasma (Fig. 2). The reproducibility of standard curves over the course of 2 months was examined by performing control analyses with concentrations of 5, 15 and 30 μg/ml of DPH in plasma simultaneously with the determination of unknown samples. Each individual working standard curve showed a linear relationship between the peak-area ratio and DPH plasma

TABLE I

PEAK-AREA RATIO OF DPH-Me_2 TO MPPH-Me_2, STANDARD DEVIATION AND COEFFICIENT OF VARIATION OF WORKING STANDARD CURVES PREPARED BY ANALYZING PLASMA SAMPLES OF 5, 15 AND 30 μg/ml OF DPH OVER THE COURSE OF 2 MONTHS (2 μg OF MPPH PER SAMPLE)

Concentration (μg/ml)	Peak-area ratio (mean)	Number of determinations	Standard deviation	Coefficient of variation (%)
5	0.251	17	0.030	12.0
15	0.728	18	0.041	5.6
30	1.459	17	0.096	6.6

TABLE II

DETERMINATION OF DPH PLASMA LEVELS IN UNKNOWN SAMPLES

Sample	Actual concentration (μg/ml)	Found concentrations (single analysis) (μg/ml)	Found concentration (mean) (μg/ml)	Coefficient of variation (%)	Difference between mean found and actual concentration (%)
1	15.0	13.8, 15.1, 15.1	14.7	5.1	−2
2	4.0	4.1, 3.8, 4.1	4.0	4.3	0
3	8.0	7.0, 7.7, 7.9	7.5	6.3	−6
4	33.1	31.5, 34.0, 34.7	33.4	5.0	+1
5	0.0	0.5, 0.0, 0.0	0.2		—
6	1.0	1.5, 1.5, 1.2	1.4	12.4	+40
7	26.5	23.6, 25.8, 25.4	24.9	4.7	−6
8	12.0	11.8, 12.0, 11.6	11.8	1.7	−2
9	20.1	19.8, 19.8, 19.8	19.8	0.0	−1

concentration, but there were minor day-to-day variations in this ratio. The results are given in Table I.

Sensitivity and accuracy

The lowest concentration of DPH that can be quantitatively determined in plasma using a 100-μl sample is 0.5—1 μg/ml. Nine test samples of DPH in plasma with concentrations unknown to the analyst were prepared in the same way as plasma standard solutions. Three separate determinations were made of the unknown samples and of three control samples with plasma levels of 5, 15 and 30 μg/ml of DPH. Plasma concentrations were evaluated by means of the simultaneously produced working standard curve. The results are listed in Table II.

Comparison of two GLC methods

The procedure described here for the determination of DPH levels in plasma was compared with another GLC method by analyzing plasma from nine patients undergoing treatment with DPH by means of the two methods. The results were in good agreement (Table III).

TABLE III

COMPARISON OF TWO GLC METHODS FOR THE DETERMINATION OF DPH PLASMA CONCENTRATIONS

Method 1 is a routine determination of the DPH plasma level in the management of epileptics performed at the Department of Clinical Pharmacology, University of Berne: double extraction of DPH and MPPH is followed by flash-heater methylation with trimethylanilinium hydroxide [39] and chromatography of drug and internal standard. Concentrations are rounded off. Coefficient of variation is less than 5% for three separate determinations of each sample in the range 4—40 μg/ml of DPH. Method 2 is the procedure described in this paper with three separate determinations on each sample.

Sample	Concentration determined by Method 1 (μg/ml)	Mean concentration ± standard deviation determined by Method 2 (μg/ml)
1	7	8.0 ± 0.5
2	32	31.4 ± 0.8
3	26	26.6 ± 0.8
4	10	10.6 ± 0.6
5	9	9.4 ± 0.3
6	42	39.7 ± 1.2
7	9	9.6 ± 0.8
8	22	20.8 ± 0.2
9	1	1.9 ± 0.1

CONCLUSIONS AND DISCUSSION

This procedure is sufficiently selective, sensitive and accurate for the measurement of DPH plasma levels of patients following therapeutic doses (therapeutic range 10—20 μg/ml). For a single assay, 100 μl of plasma are required, which is of great value in the control of antiepileptic therapy in children.

Forty samples can be extracted and chromatographed in 2 days. It is planned to make the method more rapid by means of automatic injection of extracts awaiting gas chromatography.

Most of the reported spectrophotometric, thin-layer and gas chromatographic methods of analysis for DPH and other anticonvulsant drugs use 1 ml or more of plasma for a single determination, whereas our method requires micro amounts of biological material. In addition to the aspect of sensitivity, "pre-column" derivative formation is performed in our procedure. Some of the advantages of "pre-column" compared with "on-column" methylation of DPH and MPPH are that the reaction conditions can be controlled, excess of reagents can be eliminated prior to GLC, extractive methylation with methyl iodide in acetone is a further clean-up step and chloroform extracts of the methylated derivatives awaiting GLC are stable.

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