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Note

Determination of plasma phenytoin by capillary gas chromatography with nitrogen—phosphorus detection and with selective ion monitoring

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Phenytoin (5,5-diphenylhydantoin, DPH) is an important drug used mostly for the control of grand-mal epilepsy. It has the disadvantage of a narrow therapeutic range and thus it is important for individual therapy to monitor plasma concentrations. The half-life of the drug in plasma is variable being influenced by a number of factors such as dose, degree of tissue saturation and duration of administration [1]. Pregnancy also may affect the pharmacokinetics of the drug [2, 3] but in a way that is not completely understood. As part of a study of DPH therapy in pregnancy we have developed a reliable method of assay based on the use of capillary gas chromatography (GC) with nitrogen—phosphorus detection (NPD) for determining DPH in patients plasma, which is accurate down to concentration levels of 0.5 μg/ml. The pharmacokinetics of the drug can be investigated in the steady state by using the mass spectrometer as a detection system [4]. In this study a single oral dose of isotopically labelled [$^2H_5$]DPH was given to chronically dosed pregnant epileptic women and the unlabelled and labelled drug were measured by capillary GC with selective ion monitoring (SIM).

EXPERIMENTAL

Standards and reagents

DPH, 5-(*p*-methylphenyl)-5-phenylhydantoin (PTH) and [$^2H_6$]benzene (99.5 atom% deuterium) were obtained from Aldrich Chemical (Gillingham, U.K.). 5-[$^2H_5$]Phenyl-5-phenylhydantoin ([$^2H_5$]DPH) was prepared by a method described [5] for the unlabelled compound from the corresponding labelled benzophenone. The reaction under Friedel-Craft's conditions in the

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presence of aluminium chloride of benzoyl chloride with [$^2H_6$]benzene gave [$^2H_5$]benzophenone. Stock standard solutions of the reference compounds prepared in ethyl acetate at a concentration of 1 mg/ml were stored at 0°C. These solutions were diluted in ethyl acetate (1:20) before use. Solvents (ethyl acetate, methylene chloride, hexane) were BDH (Poole, U.K.) AnalaR grade and were used without further purification. Methyl iodide (laboratory reagent grade) was also obtained from BDH. Tetrabutylammonium hydrogen sulphate (TBAH, Aldrich Chemical) was prepared as a 0.5 *M* solution in 1.0 *M* sodium hydroxide.

Gas chromatography

The instrument used for the GC determinations was a Carlo Erba Fractovap 4160 equipped with an electrically heated alkali-bead nitrogen–phosphorus detector. Separations were made on a 20 m × 0.3 mm OV-1 (cross-linked) glass capillary column prepared according to the procedure of Grob et al. [6]. The column was maintained at 100°C for 3 min, then programmed at 30°C/min to 320°C. Helium was used as carrier gas at a linear flow-rate of 2 ml/min measured at 100°C. Samples were injected using a split injection system (10:1 split ratio). The nitrogen–phosphorus detector was operated at an air flow-rate of 120 ml/min and a hydrogen flow-rate of 4 ml/min. Quantitation was by determining peak heights computed with an electronic integrator (Hewlett-Packard, Model 3390A).

Gas chromatography–mass spectrometry

Analysis by gas chromatography–mass spectrometry (GC–MS) was carried out on a 70-70 VG Micromass double-focusing mass spectrometer interfaced to a Pye Unicam Series 204 gas chromatograph. The column used was a 25 m × 0.3 mm SE52 (cross-linked) fused-silica capillary. The outlet end of the column was located up to the entrance of the ion source. Samples were introduced into the capillary using an on-column injector or a split injection system (10:1 split ratio). Helium was used as carrier gas at a flow-rate of 2 ml/min. The column was maintained at 100°C for 30 sec followed by a 16°C/min programme to 250°C. The temperatures of the injection port, GC–MS interface and ion source were 250, 300 and 220°C, respectively. The mass spectrometer was operated in the electron impact mode with an ionisation potential of 70 eV and a trap current of 200 μA. For SIM the ions at *m/z* 280, 285 and 294 were measured (50 msec per mass channel) using as lock mass the ion *m/z* 295 from a constant septum bleed of heptacosafluorotributylamine. The ion intensity was recorded using a Rikadenki Series DBE-6 Multi-Pen recorder. The resolution of the mass spectrometer (10% valley) was 650.

Extraction and derivatization procedure

All operations were carried out in Eppendorf disposable microcentrifuge tubes. A 200-μl aliquot of plasma was spiked with 1 μg of the internal standard PTH and 500 μl hexane were added. After vortex mixing and centrifugation the hexane layer was removed and discarded. The plasma was then extracted twice by vortex mixing and centrifugation with 500-μl aliquots of ethyl acetate. The solvent extracts were combined and blown to dryness under

nitrogen. The sample was then derivatized by extractive methylation according to the procedure of Hoppel et al. [7]. To the dried extract were added 100 μl of 1.0 *M* sodium hydroxide, 50 μl TBAH solution, 500 μl methylene chloride and 25 μl methyl iodide. The mixture was vortexed for several seconds and the tubes then placed on a rotary mixer for 45 min. After centrifugation the upper aqueous layer was removed and the remaining solvent layer washed with 500 μl water. The solvent extract was transferred to a small glass tube, then taken to dryness and redissolved in 100 μl ethyl acetate for analysis by GC—NPD and GC—MS.

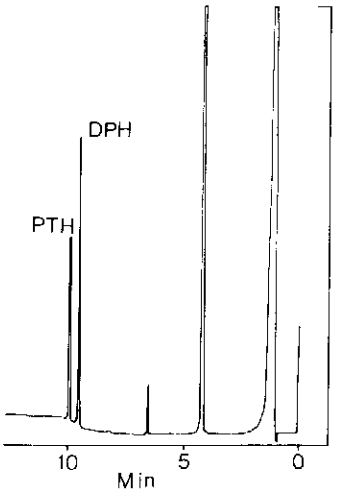
Quantitation of the drug in the plasma samples was made by reference to a standard calibration curve constructed with each batch of samples analysed. The standard curve for the GC—NPD determinations was obtained from the analysis of 200-μl aliquots of drug-free plasma to which had been added 1 μg PTH and various amounts of DPH (500 ng to 2 μg). The ratio of the peak heights (DPH/PTH) was plotted against the concentration of DPH in the plasma. The standard curves for the GC—MS analysis with SIM were constructed similarly but with PTH (2 μg per sample) and [$^2H_0$]DPH (0—4 μg) and [$^2H_5$]DPH (0—0.2 μg). The ratios of the peak heights of the molecular ions of [$^2H_0$]DPH (*m/z* 280) or of [$^2H_5$]DPH (*m/z* 285) to the molecular ion of PTH (*m/z* 294) were plotted against the concentration of DPH in the plasma. The standard curves for both the GC and GC—MS determinations typically had regression coefficients > 0.99.

RESULTS AND DISCUSSION

High-resolution capillary GC, usually with a selective GC detection system or combined with MS, is now an established method for quantifying drugs and their metabolites in biological samples [8]. We have previously demonstrated the advantage of capillary GC with NPD for determining the concentration of psychotropic drugs in plasma at the therapeutic level [9, 10] and have now successfully applied this technique to the measurement of DPH in plasma.

Extractive alkylation has been used previously in the GC and GC—MS analyses of a number of drugs including DPH [4, 7, 11, 12]. The permethylated derivatives of both DPH and the internal standard PTH were chemically very stable and had good GC properties. Fig. 1 shows a typical GC—NPD tracing from the plasma of a patient receiving a therapeutic dose of the drug. No interference from endogenous compounds was found in the analysis of over 100 samples from six women taken at various times during their pregnancies. Fig. 2 shows a SIM trace from a chronically dosed patient who had been given a single dose of [$^2H_5$]DPH.

The recovery of the drug from plasma by ethyl acetate extraction was determined by spiking two sets of drug-free plasma with DPH (1, 4, 8, 12 or 16 μg/ml). The PTH internal standard was added to one set of plasma before ethyl acetate extraction and to the ethyl acetate extracts from the other set. The mean recovery of DPH as measured by GC—NPD over the concentration range 1—16 μg/ml was 96.6 ± 3.01% S.D. The accuracy and precision of the GC—NPD method was determined from replicate recovery experiments of authentic DPH added to drug-free plasma at concentration levels of 1, 4, 8,



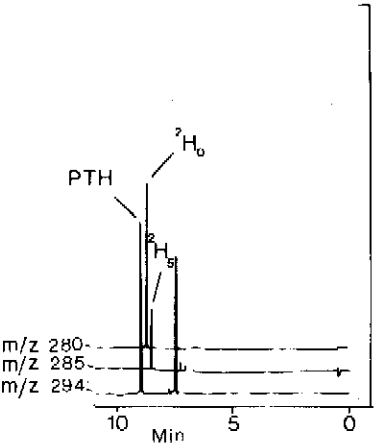


Fig. 1. GC—NPD trace of an extract from a plasma sample calculated to contain 3.6 μg of DPH per ml plasma. For conditions see text.

Fig. 2. SIM trace (*m/z* 280, *m/z* 285) of an extract of plasma from a pregnant epileptic woman dosed with [$^2H_0$]DPH and [$^2H_5$]DPH. For conditions see text.

TABLE I

PRECISION AND ACCURACY OF THE METHOD FOR DPH

DPH added (μg/ml)	DPH found mean ± S.D. (μg/ml, n = 6)	Percent recovery (mean ± S.D.)
1	1.07 ± 0.03	107.0 ± 2.65
4	4.01 ± 0.17	100.25 ± 4.19
8	7.75 ± 0.13	96.81 ± 1.59
12	12.17 ± 0.18	101.44 ± 1.49
16	16.32 ± 0.33	101.99 ± 2.07

12 and 16 μg/ml. The calculated recoveries from six replicate analyses at each of these levels are presented in Table I.

A number of plasma samples from patients receiving both the unlabelled and labelled drug were assayed by GC—NPD and by GC-MS. The sum of the concentrations of [$^2H_0$]DPH and [$^2H_5$]DPH determined by GC—MS was plotted against the total DPH concentration measured by GC—NPD. Taking the latter results as the standard the regression line of the GC—MS results on the GC—NPD results was calculated. The regression line (see Fig. 3) was $y = 1.042x - 0.087$. The slope of the line is 1.042 ± 0.037 which is not significantly different from 1.00 and the intercept is −0.087 ± 0.092 which is not significantly different from zero, x^2 = 96.8%.

Although in this work PTH has been used as the GC—MS internal standard in order to allow the simultaneous use of the MS and NPD detection systems, the GC—MS approach has also been satisfactorily applied using the analogue [$^2H_{10}$]DPH as an internal standard.

In conclusion the GC method described in this paper can be performed on

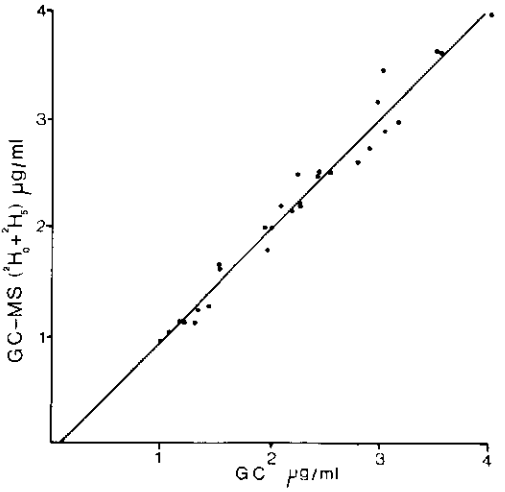


Fig. 3. The sum of [$^2H_0$]DPH and [$^2H_5$]DPH concentrations in plasma determined by GC—MS plotted against total DPH concentrations determined by GC—NPD.

small plasma volumes, and is both reliable and accurate down to the 0.5 μg/ml DPH level. This assay is suitable for routine clinical application and derivatized samples can if required be stored at 0° C for several months. The use of SIM has allowed pharmacokinetic studies to be made of a pulse dose of [$^2H_5$]DPH administered to patients who are taking a steady-state dose of the drug. The clinical implications of the pharmacokinetic studies carried out on pregnant epileptic women will be reported shortly.

ACKNOWLEDGEMENTS

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