Gas Chromatographic Assay of Underivatized 5,5-Diphenylhydantoin (Dilantin) in Plasma Extracts

Dian Sampson, Irene Harasymiv, and W. J. Hensley

A simple, rapid, and accurate method is described for determining diphenylhydantoin concentrations in plasma. An internal standard, primidone (primaclon), is added to 2 ml of acidified plasma, and primidone and diphenylhydantoin are extracted into chloroform. The extract, washed with hexane and concentrated, is injected into a gas chromatograph. Extraction and measurement of a single sample requires about 30 min, and as many as 40 samples can be assayed in a working day. Both therapeutic and overdose concentrations of diphenylhydantoin in plasma can be detected and measured. Nine other acidic drugs, added to plasma samples, did not interfere with the analysis.

Additional Keyphrases  phenobarbital identification • emergency determinations

5,5-Diphenylhydantoin (DPH) is one of the most commonly used anticonvulsant drugs, and as such a routine method is needed for its assay at the concentrations found in both therapeutic (up to 20 µg/ml plasma) and overdose (over 20 µg/ml) concentrations. It has previously been identified by both paper and thin-layer chromatography and assayed by colorimetric (1), spectrophotometric (2–5), and gas chromatographic methods. Of these, the most useful is gas chromatography—interference from the presence of other drugs is much easier to minimize and recognize.

Current methods available for gas chromatographic analysis of DPH (6–10) are cumbersome: they require either derivatization of DPH, a lengthy extraction procedure, or both. Assay of DPH by any of these methods would take at least twice as long as the method described here. The extraction method used by Evenson et al. (11) does not incorporate a clean-up step to remove unnecessary material such as lipids from the chloroform extract of acid plasma; we find this method gives rise to high serum blanks that interfere with the accuracy of the estimation, especially in the low-concentration therapeutic range.

We use an internal standard of primidone throughout, so that a known amount of internal standard added can be compared with an unknown amount of DPH present. This technique compensates for slight variations in the volume injected into the gas chromatograph and for individual variations in the injection technique.

Materials and Methods

Apparatus

Chromatography was performed on a 7300 series gas chromatograph (Packard Instrument Co., Downers Grove, Ill. 60515) equipped with a flame ionization detector and a series 17 recorder (Honeywell Inc., Minneapolis, Minn. 55408) with a span of 0 to 1 mV. The column used was 1.8 m long and 4 mm i.d. It was packed with 3% OV-17 on 60/80 mesh Gas Chrom Q (prepacked, from Applied Science Laboratories, Inc., State College, Pa. 16801). The flow rates used were: hydrogen, 60 ml/min; air, 600 ml/min; and nitrogen carrier, 60 ml/min. The pressure of the carrier gas was $166 \times 10^3$ N/m² (24 lb/in.²). The injector temperature was maintained at 300°C, the column at 290°C (isothermal operation), and the detector at 290°C. The sensitivity setting was at range 3 × $10^{-10}$ A. The use of 3% OV-17 at 290°C has not had any detrimental effect on column efficiency over a period of six months (the supplier's maximum recommended temperature is 350°C).
Reagents

"Nanograde" chloroform and hexane were obtained from Mallinckrodt Chemical Works, St. Louis, Mo. 63160.

2-Desoxyphenobarbital (primidone, primaclone), used as an internal standard, was kindly supplied by Imperial Chemical Industries of Australia and New Zealand. The drug was dissolved in distilled water for addition to plasma, or in chloroform for direct injection into the gas chromatograph.

DPH was kindly supplied as dilantin base by Parke, Davis and Co., Caringbah, N.S.W., Australia, and was freshly dissolved in absolute ethanol for addition to plasma, or in chloroform for direct injection into the gas chromatograph. The maximum amount of ethanol added to plasma was 0.15 ml/ml of plasma—this caused no apparent precipitation of protein and no observable difference in the behavior of the final plasma extract on gas chromatography.

Procedure

To 2.0 ml of plasma were added 1.0 ml of 0.5M HCl and 0.2 ml of primidone internal standard (40 μg/ml of distilled water). The acidified plasma was extracted with 25 ml of chloroform. The organic phase was filtered and evaporated to dryness on a rotary evaporator. In order to extract plasma lipids and other hexane-soluble material, the residue was dissolved in 0.5 ml of absolute ethanol and extracted with 2.0 ml of hexane. On addition of one drop of distilled water, an interface formed and the hexane layer was removed and discarded. The ethanol layer was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 100 μl of chloroform; 5 μl of this was injected into the gas chromatograph.

In patients on therapeutic dosage, in whom the DPH concentration was less than 5 μg of DPH per ml of plasma, 4.0 ml of plasma were extracted in order to obtain a more accurate measurement. In cases of DPH overdose, in whose plasma there was more than 40 μg of DPH per ml, 1.0 ml of plasma was extracted so that the DPH/primidone ratio fell on the standard curve. The volumes and concentrations of reagents used on these plasmas containing smaller and larger amounts of drug were identical to those used previously in this method. This did not change the recovery of either DPH or primidone.

Results and Discussion

Figure 1 (left) shows the result of the extraction and injection of 2.0 ml plasma with internal primidone standard but without added DPH (blank). Figure 1 (right) shows the result of the extraction and injection of plasma with internal primidone standard and with DPH added to give a concentration of 10 μg of DPH per milliliter plasma (final concentration 1 μg of DPH per 5 μl of chloroform injected). Figure 2 shows the chromatogram obtained with an extract of plasma from a patient who had received therapeutic dosages of DPH. In this case the concentration of DPH was 17 μg of
DPH per milliliter of plasma. The peak adjacent to the solvent peak is phenobarbital. The peak following DPH in this chromatogram may correspond to a DPH metabolite; we do not yet know.

Retention time of DPH was 3.45 ± 0.27 min; that of primidone 2.75 ± 0.14 min (standard deviation for 12 observations in each case). Because these retention times varied as the gas pressure changed slightly (and would also differ from column to column), the retention time of DPH was calculated relative to that of primidone:

Relative retention time for DPH =

\[
\frac{\text{retention time for DPH}}{\text{retention time for primidone}}
\]

The relative retention time of DPH was 1.26 ± 0.05 (standard deviation for 12 observations). Similarly, the retention time of phenobarbital relative to that of primidone was found to be 0.45 ± 0.03 (standard deviation for seven observations). Identification of phenobarbital under these conditions is useful, as many patients treated with DPH are also receiving phenobarbital.

Several minor substances were extracted from the plasma by this procedure. These are indicated in Figure 1 (left) and designated X, Y and Z. Peak Z (retention time relative to primidone, 3.31 ± 0.06, standard deviation for seven observations) is the remainder of a large peak found in all plasma samples tested, of which more than 95% was extracted into the hexane layer. Peaks X and Y were found in about a fourth of the plasma samples tested. Their retention times relative to primidone were 1.19 ± 0.03 (standard deviation for 7 observations), and 1.70 ± 0.05 (standard deviation for 7 observations), respectively. No matter how small the DPH peak is, no confusion arises between DPH, X, and Y when the relative retention times are calculated. As indicated in Figure 1 (left), X, Y, and Z are minor contributors, giving rise to very small peaks on the chromatogram; at sensitivities greater than \( 3 \times 10^{-19} \) A, these peaks could not be distinguished from the baseline.

The background resulting from this extraction of plasma compared favorably with that generated by other extraction methods. When there was no cleanup step, as in the method of Evenson et al. (11), a much higher background resulted and the DPH peak was difficult to distinguish, especially at therapeutic dosages of DPH. A similar background could be obtained by extracting DPH from acidified plasma into chloroform, then re-extracting it into an aqueous alkaline solution so that lipids remained in the organic layer. The aqueous layer containing DPH must then be acidified so that DPH can again be extracted into chloroform. The chloroform is then removed under reduced pressure, and the residue resuspended in 0.1 ml of chloroform for injection into the gas chromatograph. Although this procedure gave results as reliable as those obtained by the method described earlier in this paper, more time was needed for the extraction. In cases of known or suspected DPH overdose it was decided to measure plasma DPH concentration is of extreme importance. Use of the longer extraction procedure also appreciably decreased the number of routine DPH assays that could be done each day.

Recovery of DPH from plasma by the procedure developed in this paper was 91% ± 5%, that of primidone 52% ± 3% (standard deviation for six observations in each case). The latter figure did not agree with the 98% given by Evenson et al. (11), because these authors did not wash their chloroform extract of serum with hexane. In our procedure, primidone partitioned equally between the ethanol and hexane phases. Interestingly, in the longer extraction procedure, involving re-extraction into aqueous base, DPH was almost completely recovered in the final chloroform fraction (81 ± 8%, standard deviation for three observations). However, only 10–20% of the primidone appeared in the final chloroform fraction; most remained in the initial, lipid-containing chloroform fraction and was not re-extracted into aqueous alkali because it is relatively insoluble in water.

The major source of error in measuring recoveries was the individual injection technique, and for this reason all standard curves have been expressed as a ratio of DPH to a known amount of internal standard.

The ratio of the peak heights of DPH and internal standard gave linear standard plots when graphed against the concentration of DPH in plasma for concentrations at least as high as 40 µg/ml (Figure 3).
The following drugs were tested and did not interfere with the procedure outlined, as their retention times were considerably less than those of DPH or primidone: glutethimide (Doriden), barbital, butobarbital, amylobarbital, pentobarbital, quinalbarbital, methylphenobarbital (meprobarmital), thiopental, and phenobarbital. Of these, only phenobarbital could be identified with certainty under the conditions used for DPH. Basic drugs such as diazepam (Valium) and chlordiazepoxide (Librium) are not extracted into chloroform by this procedure, but remain in the acidified aqueous layer.

The extraction and quantitation procedure outlined in this paper has been used to determine the DPH concentration in plasmas of 50 patients receiving therapeutic dosages of DPH, and one comatose patient suspected of DPH overdose. The concentrations after therapy were found to range from zero (less than 1 μg) to 22 μg of DPH per milliliter of plasma; the single overdose concentration assayed was 70 μg of DPH per milliliter of plasma. This latter patient was an epileptic who had previously been medicated on 100 mg of DPH three times per day and 200 mg of carbamazepine (Tegretol) twice daily. No further particulars concerning this patient were available.

We thank Dr. George Stathers of Goulburn, Dr. Brian Lucas of Parke, Davis and Co., and the Neurology Unit of Royal Prince Alfred Hospital for kindly making available plasma samples obtained from patients given therapeutic doses of diphenylhydantoin.

Dian Sampson is supported by the Marcella Yeo Bequest to Royal Prince Alfred Hospital.

References