Concurrent Assay of Phenobarbital and Diphenylhydantoin in Plasma by Vapor-Phase Chromatography

J. C. Van Meter, H. S. Buckmaster, and L. L. Shelley

The weakly acidic phenobarbital and diphenylhydantoin, separated from interfering substances by simple solvent partition, are estimated concurrently by vapor-phase chromatography. Solid sample injection and internal standard (primidone) are used. The entire procedure can be completed within 2 h after venipuncture. Both drugs could be measured after therapeutic doses have been administered.

Since the pioneer work of Goldbaum (1, 2), a profusion of methods for estimating phenobarbital in biological fluids have appeared. In 1956, Dill et al. (3) presented a colorimetric method for assay of diphenylhydantoin, and Plaa and Hine (4) published a spectrophotometric method for simultaneous determination of phenobarbital and diphenylhydantoin. In 1963, Svensmark and Kristensen (5) improved the spectrophotometric method by modifying the solvent separation of these two drugs. Numerous authors have used paper and thin-layer chromatography to identify and roughly quantify them. Recently, Wallace (6) assayed diphenylhydantoin spectrophotometrically as the benzophenone, formed by permanganate oxidation of the drug. Sandberg et al. (7) methylated diphenylhydantoin with diazomethane and estimated the methyl ether by vapor-phase chromatography (vpc). Chang and Glazko (8) estimate the p-hydroxylated metabolic product of diphenylhydantoin by vpc of the trimethylsilyl derivative. Recently Sunshine et al. (9) evaluated methods for determination of barbiturates in biological materials.

A rapid, specific assay of phenobarbital and diphenylhydantoin in body fluids is needed. This encouraged us to examine the possible use of vpc for this purpose. The vpc of drugs in crude extracts of biological specimens is subject to gross interference by both normal and drug constituents. By appropriate solvent partition this interference is eliminated. In our method, phenobarbital and diphenylhydantoin are extracted from plasma or serum that has been purified by partitioning between chloroform and neutral, alkaline, and acid water; the extract is assayed by vpc.

Procedure

Specimen Preparation

(a) Add 2.0 ml of plasma or serum to 1.0 ml of 1.0M phosphate buffer, pH 6.8, and 15 ml of freshly redistilled chloroform in a stoppered centrifuge tube. Gently agitate the mixture for 15 min on a mechanical shaker. Centrifuge and separate the phases. Discard the aqueous phase.

(b) Transfer 13 ml of the chloroform phase to 5 ml of 0.4M phosphate buffer, pH 11, in a stoppered centrifuge tube. Shake mixture as before for 10 min. Centrifuge and separate phases. The drugs being assayed here are in the aqueous phase; the extracted chloroform phase may be held for further examination of other drugs.

(c) Adjust the alkaline extract to pH 1 with 0.5 ml of 8N hydrochloric acid and extract with 15 ml of freshly redistilled chloroform as before. Discard the aqueous phase.

(d) Evaporate 13 ml of the chloroform extract to dryness in a nitrogen stream at 60°C in a water bath. Suspend the residue in 5 ml absolute ethanol and again evaporate to dryness as before.

(e) Dissolve the solvent-free residue from Step d in 0.5 ml of redistilled methanol containing 50 μg of primidone (2-desoxyphenobarbital) as an internal standard and quantitatively transfer the solution to a stoppered polyethylene tube of 1.5 ml ca-
capacity. Evaporate the transferred solution to dryness in a nitrogen stream at 60°C.

(f) Dissolve the specimens prepared in Step e above in 50 μl of redistilled methanol, place 5-μl aliquots of the solution in the depressions of a Teflon spot plate, and add to each about 50 μl of redistilled dichloromethane. In the center of each depression place a clean stainless steel gauze ring (#S22-950/05, Griffin & George Ltd., London). Permit the solvent to evaporate at room temperature. Add an additional 50 μl of dichloromethane to each specimen on the spot plate and again allow the solvent to evaporate.

Vapor-Phase Chromatography

Apparatus. A Barber-Colman Chromatograph was used, with a Model 5072-2 U Column Bath, Model 5081-1 Column Temperature Controller, Model 5102-1 Injector Detector Controller, Model 5124-400 Flame Detector, Model 5003 Detector Bath, Model 5043-100 Electrometer, and a Sargent Recorder, 0 to 5 mV input (Model SR with Disc Integrator).

Column and packing. Four-millimeter i.d. glass column 1.8-m long, containing 3% dimethylsilicone (OV-1) 100/120 Supelcort (Supelco, Inc., Bellefonte, Pa.).

Operational parameters. The conditions of operation were: column temperature, 220°C isothermal; injector temperature, 280°C; detector temperature, 300°C; detector sensitivity, 3 X 10−10 A; detector attenuation, variable; carrier gas, purified nitrogen, 120 ml/min; compressed air, purified, 250 ml/min; hydrogen, purified, 50 ml/min. Column is preconditioned for 18 to 24 h at 260°C with carrier gas flowing.

Chromatographic procedure. (a) When the solvent in the depressions of the spot plate containing the specimen extracts has completely evaporated, transfer the gauze rings to the turntable of the specimen injection apparatus.

(b) Reseal the injection apparatus and purge it with carrier gas for 5 min. Check for gas leaks with the Snoopy Leak Detector (Nupro Co., Cleveland, Ohio).

(c) Ignite the flame detector and establish a steady baseline on the recorder; adjust to zero response and use the parameters listed under operational parameters above.

(d) At the start of each run inject in duplicate the following standards:

<table>
<thead>
<tr>
<th></th>
<th>1st, μg</th>
<th>2nd, μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenobarbital</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Diphenylhydantoin</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Primidone</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Note baseline, contamination areas if any, retention time, and integral values of each peak (Fig. 1).

Compare the results with previous runs. If the column has been properly prepared and conditioned, and if the chromatograph is operated according to the parameters given above, the three drug standards will completely clear the column in 10 to 12 min. The baseline will have returned to zero. A new specimen may be injected every 15 min. Plasma and serum specimens prepared according to the above directions will also exit from the column in 12 min. The source of any extraneous contamination introduced into the system should be eliminated before proceeding with the assay.

(e) If the chromatographic response of the injected standards appears normal as to location, resolution, and area of eluted peaks, continue to inject successive assay specimens every 12 to 15 min. If abnormal responses are noted, stop the chromatograph, locate and eliminate the source of difficulty, and retest with another series of injected standards.

(f) At the end of each run, determine the recovery of standards containing 50 and 100 μg each of phenobarbital and diphenylhydantoin added to 2.0 ml of normal human plasma treated as assay specimens.

Calculation

Standard curves. Plot the ratio of the area of the phenobarbital (or diphenylhydantoin) peak
divided by that of the 5-μg primidone internal standard vs. the quantity of the drug injected into the chromatograph (Fig. 2).

Compute drug/primidone peak-area ratios for each specimen. From the standard curve read the amount of drug in the chromatographed specimen. Multiply the drug concentration found by the factor 6.6 to obtain μg drug/ml plasma:

\[
\text{μg drug} \times \frac{(50/5) \times (15/13)^2}{2} = \text{μg drug} \times 6.6 = \text{μg drug/ml plasma}
\]

Discussion

Few patients with neuropsychiatric disorders are treated with a single drug; most are on multiple therapy such as phenobarbital and (or) diphenylhydantoin in combination with various other medicaments. This poses a difficult analytical problem, since one drug often interferes with the assay of another, causing results to be of doubtful value. Current assay methods for phenobarbital and diphenylhydantoin which depend upon colorimetry or differential spectrophotometry, lack specificity and are subject to interference by accompanying drugs (5, 10). Paper and thin-layer chromatography are useful for identifying drugs in blood, but the methods reported to date are only roughly quantitative. Methods based on the preparation of derivatives—e.g., methyl ethers, aromatic amines, alkaline hydrolysis, and oxidation products—are time-consuming and often misleading. For instance, we find that chlorpromazine interferes with the assay of diphenylhydantoin by the method of Dill et al. (5).

VPC analysis of unchanged barbiturates in biological specimens has been reported by many investigators (11-16), who are largely concerned with qualitative identification of a variety of barbiturates, not with specific assay of the two most commonly used anticonvulsant drugs, phenobarbital and diphenylhydantoin. VPC of crude extracts, though qualitatively informative, can hardly be relied upon to give trustworthy quantitative data. VPC of undegraded derivatives certainly produces dependable assays in which a single product is formed, but the preparation of the derivative is a time-consuming and sometimes unpleasantly hazardous task—e.g., methylation of diphenylhydantoin with diazomethane in the Sandberg method (7). Street (17) has shown primidone forms both mono- and disilylated derivatives; this complicates the assay of these drugs. VPC of degradation products is nonspecific since inactive metabolites may appear as if they were unchanged drug.

The weakly acidic, chloroform-soluble phenobarbital and diphenylhydantoin are selectively separated from other compounds and interfering substances by solvent partition. Since the compounds being assayed must have the same retention time and characteristic response in vpc as intact phenobarbital and diphenylhydantoin under rigidly controlled conditions, specificity is further ensured.

On solvent partition as described, primidone, a frequently used anticonvulsant drug, appears in the neutral fraction and phenobarbital and diphenylhydantoin in the weakly acidic, chloroform-soluble fraction. Therefore, primidone can be used as an internal standard for the assay of these drugs and, conversely, phenobarbital can be used as an internal standard for the assay of primidone. Under the conditions of VPC, the primidone peak is well separated from the phenobarbital peak, which precedes it, and the diphenylhydantoin peak, which follows it (Fig. 1).

Sibert (18) used 4.5% cyclohexanediethanol succinate (HI-EFF 8BP) effectively as an adsorbent in the vpc of barbiturates in pharmaceuticals, but phenobarbital required 33 min to exit.
from the column. Pippenger and Gillen (16) used 1% of the same adsorbent under similar conditions and reported a retention time of about 2 min for this drug. Although Pippenger and Gillen (16) favored cyclohexanedimethanol succinate over dimethyl silicone as a liquid phase in the extraction of anticonvulsant drugs, we find the latter to be superior and we use 3% dimethyl silicone columns at temperatures of 200° to 250°C. By adjusting the temperature, one may change retention time and distance between peaks at will. With cyclohexanedimethanol succinate, it was necessary to heat the column to 250°C, the maximum recommended temperature, to achieve adequate resolution and response for these compounds.

A solvent peak was eliminated from our chromatograms by applying the sample to stainless steel gauze specimen holders on a Teflon spot plate, by the technique of Menini and Norymberski (19). After evaporating the solvent, we placed the gauze holders in the automatic sample injection magazine of the Barber-Colman gas chromatograph. Unfortunately, the size of the sample that could be absorbed by the gauze holders, as judged by replicate injected standards, varied from 50 to 200% of the mean peak area response. Without an internal standard added to the specimen just before chromatography, the response data are almost meaningless.

Calculation of the ratio of the peak area response of the drug divided by the internal standard gives a curve such as shown in Fig. 2. The slope of this curve varies with changes in operating parameters such as column temperature, carrier gas flow, and signal amplification, but is highly reproducible when these factors are held constant. Our present data indicate recovery of extracted standards to be 90 to 95% of the expected. Since any deviation from this is also shown by standards run concurrently with patient specimens, this poses no problem.

In the short time our procedure has been in use at this hospital, the therapeutic concentrations observed for phenobarbital and diphenylhydantoin have been in the range of 20 to 40 and 5 to 15 μg/ml plasma, respectively. Higher values have been associated with signs of toxicity (nystagmus and/or ataxia); those below were considered ineffective in seizure control at both this hospital and elsewhere (20).

The authors are indebted to Drs. W. E. Murray, J. T. Brock, H. W. Gillen, E. T. Mertz, and J. K. Penny for encouragement and advice.

References