Simultaneous Gas-Chromatographic Analysis for Phenobarbital, Diphenylhydantoin, Carbamazepine, and Primidone in Serum

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We describe a simple, sensitive determination of phenobarbital, diphenylhydantoin, carbamazepine, and primidone in serum, by use of gas-liquid chromatography with temperature programming. The methylated derivatives of these anticonvulsants are well resolved, as was 5-(p-methylphenyl)-5-phenylhydantoin, the internal standard. The proposed procedure requires only 0.20 ml of serum and can be done in less than 30 min. The lower limit of detection for each of the drugs is 0.5 mg/liter. Analytical recoveries of drug from serum were excellent and peak height and concentration were linearly related up to twice the toxic concentration for serum.

Gas-liquid chromatography is widely used for determining antiepileptic drugs (1-3). Its sensitivity and specificity allows simultaneous measurement of anticonvulsant drugs. Here, we describe a simple method for simultaneously determining phenobarbital, diphenylhydantoin, primidone, and carbamazepine in 1 ml of plasma or serum in less than 30 min.

Materials and Methods

Reagents and standards

Drugs used as standards were diphenylhydantoin (Dilantin; Parke-Davis & Co., Detroit, Mich. 48232), carbamazepine (Tegretol; CIBA Pharmaceutical Co., Summit, N. J. 07901), phenobarbital (Eli Lilly and Co., Indianapolis, Ind. 46206), primidone (Ayerst Laboratory Inc., New York, N. Y. 10017) and 5-(p-methylphenyl)-5-phenylhydantoin (Aldrich Chemical Co., Milwaukee, Wis. 53200).

Trimethylphenylammonium hydroxide, 0.2 mol/liter. This was prepared from trimethylphenylammonium iodide (Fisher Scientific Co., Silver Spring, Md. 20910) that had been recrystallized three times from absolute ethanol as follows. Into a 250-ml glass-stoppered Erlenmeyer flask with a Teflon stirrer, add 6.94 g of silver oxide, 10.52 g of trimethylphenylammonium iodide, and 200 ml of absolute methanol. Stir (magnetic stirrer) for 2.5 h and store at 4 °C. The supernatant solution is stable for at least a month. Filter appropriate amounts of it through Whatman No. 1 filter paper immediately before use.

Standards

Internal standard. Dissolve 18 μg of 5-(p-methylphenyl)-5-phenylhydantoin per milliliter of chloroform, and use 5 ml of this solution for the extraction.

Drug standards. The stock drug standards were prepared in the following concentrations: phenobarbital, 2 g/liter; carbamazepine, 0.5 g/liter; primidone, 1 g/liter; and diphenylhydantoin, 2 g/liter. The stock drug standards were added to a drug-free pool of serum to obtain five standards with the concentrations listed in Table 1. All four drugs were added to the same serum. These standards were kept frozen in screw-cap, Teflon-coated tubes until use.

Standard curve. Standard curves were prepared for each drug. Each serum standard was extracted and chromatographed in duplicate as if it were a patient sample. The mean value of the relative peak height ratios from the duplicate analyses of each standard were plotted against the concentration of the respective standard (Figure 1).

Instrumentation

We used a Model 2400 gas chromatograph (Fisher Scientific Co., Pittsburgh, Pa. 15219) with dual hydrogen flame-ionization detectors and borosilicate glass columns (200 cm x 2 mm i.d.) packed with 3% SP 2250 on 100/120 mesh “Supelcoport,” type 50-50 Me-Ph (Supelco, Inc., Bellefonte, Pa. 16823). Before use, the columns were heat conditioned at 310 °C for 24 h with a carrier gas (nitrogen) flow rate of 50 ml/min.

| Table 1. Concentration of Working Standards in Serum |
| Standard | Phenobarbital | Diphenylhydantoin | Primidone | Carbamazepine |
| 1 | 5 | 2.5 | 2.5 | 1 |
| 2 | 10 | 5 | 5 | 2.5 |
| 3 | 20 | 10 | 10 | 5 |
| 4 | 40 | 20 | 15 | 7.5 |
| 5 | 80 | 40 | 20 | 10 |

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Received Nov. 21, 1975; accepted Feb. 2, 1976.
Oven temperature was maintained at 190 °C during periods of inactivity, with a carrier-gas flow rate of 50 ml/min. The injection ports were sealed with 10-mm septums (Supelco). Detectors and injection ports were heated to 310 and 250 °C, respectively. Electrometer output was monitored with a Fisher Model 5000 series dual-pen recorder, at a chart speed of 25 mm/min. The electrometer was operated at a range of $10^{-11}$ A/mV and the amplifier output was attenuated at 128.

**Chromatographic conditions.** Oven temperature was programmed from 190 to 300 °C at a rate of 15 °C/min, the program being started immediately after the sample extract was injected. Gas-flow rates were: nitrogen, 160 ml/min; hydrogen, 30 ml/min; and air, 300 ml/min.

**Extraction Procedure**

One milliliter of serum or plasma was combined with 0.5 ml of 0.25 mol/liter hydrochloric acid in a 13 x 150 mm test tube (Teflon-lined screw cap). Five milliliter of chloroform containing 90 μg of the internal standard was added to the screw-cap tube, which was then shaken for 1 min on a vortex-type mixer. The aqueous (upper) phase was aspirated and the organic phase filtered through a Whatman No. 1 filter paper (the filter paper was rinsed before use with few milliliters of chloroform) into a conical centrifuge tube and evaporated in a warm water bath (40 °C) under a stream of nitrogen. The tubes were closed tightly with a cork until the residues were to be injected into the gas chromatograph.

**Table 2. Between-day Analytical Recoveries**

<table>
<thead>
<tr>
<th>Drug added to serum</th>
<th>Mean concn Added (mg/liter)</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenobarbital</td>
<td>20</td>
<td>19.8</td>
</tr>
<tr>
<td>Diphenylhydantoin</td>
<td>10</td>
<td>9.9</td>
</tr>
<tr>
<td>Primidone</td>
<td>15</td>
<td>15.1</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>10</td>
<td>10.4</td>
</tr>
</tbody>
</table>

**Chromatography**

The residue in the centrifuge tube was reconstituted with 100 μl of the trimethylphenylammonium hydroxide solution. One to two microliters of this extract was chromatographed at a programmed temperature on the SP 2250 column. Peaks were identified by comparing their retention times relative to the retention time for the internal reference peak to such data for known standards. Drug concentrations were calculated from the standard curve.

The precision of the proposed method was checked by using aliquots of drug-supplemented serum pools, which were stored frozen. The serum pool contained all four drugs.

**Results**

Figure 1 shows standard curves for primidone, diphenylhydantoin, carbamazepine, and phenobarbital, prepared by the peak-height ratio technique. All four curves are linear to approximately twice the toxic concentration. Analytical recovery of the drugs from the serum was measured by adding known amounts of these four drugs to the same sample and analyzing it for 31 consecutive days. Recovery ranged from 99 to 104% (Table 2).

Chromatograms of serum specimens extracted and methylated according to our procedure are presented in Figure 2. Chromatogram A, with the internal standard, is a typical pattern for serum from a normal individual not receiving anticonvulsants. The three predominant peaks marked S are attributable to normal serum constituents. Chromatogram B is from a patient who was receiving all four of the antiepileptic drugs. The concentrations calculated from this serum sample were: primidone, 4 mg/liter; phenobarbital, 20 mg/liter; carbamazepine, 3 mg/liter; and diphenylhydantoin, 10 mg/liter. Chromatogram C is our standard 4 (Table 1), prepared by adding the drugs to pooled serum. All four drug peaks were well separated and there were no interfering peaks.

**Precision**

The between-day precision of the proposed method was evaluated (Table 2). The standard deviation varied from 0.4 to 1.1 mg/liter. Analytical recoveries varied from 99 to 104%.
The usual sample volume used was 1 ml of serum or plasma. In pediatric cases, similar results can be obtained by decreasing the amounts of serum, chloroform, and reagent proportionately. In some instances we successfully used as little as 0.2 ml of serum.

Drug concentrations as low as 0.5 mg/liter were easily measured by adjusting the sensitivity of the analysis, e.g., injecting a more concentrated extract, increasing the volume of injection, or increasing the electrometer output.

Discussion

Rose et al. (4) emphasize that determinations of antiepileptic drugs are not readily available to most physicians who prescribe these medications. Laboratories are reluctant to establish anticonvulsant assays because of the considerable capital investment and commitment of experienced technical personnel. However, such monitoring is important to better treatment.

Our method is simple and well-suited for the clinical laboratory. A single extraction and chromatographic system is used to analyze for all four of the anticonvulsant drugs. Use of a single internal standard and one temperature programming reduces the technical difficulties and standardization procedure, and gives well-separated peaks for all of the four drugs. The on-column methylation, and the use of glass column and SP 2250 gives good peak characteristics and a linear standard curve.

The five standards that contain all the four drugs were prepared on the basis of a report on observed concentrations in serum (5). In our proposed method the concentration in standard 5 is about twice the toxic concentration, standard 1 is less than a third the therapeutic concentration.

With this procedure one can determine the most commonly used antiepileptic drugs in a single operation within 30 min.

References