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A gas-chromatographic method is described for the quantitative determination of 11 barbiturates and glutethimide in blood. The extracted barbiturates are methylated and injected into a 7% DC-200 column. Use of methohexital as the internal standard, as well as the addition of standards to a drug-free sample of blood, compensates for losses and for differences in the distribution coefficient of the individual barbiturates, and makes the procedure highly precise and accurate.

Additional Keyphrases    drug abuse

Several gas-chromatographic methods for the separation and identification of free barbiturates (1–9) and glutethimide ("Doriden," Ciba) (10) have been reported. Although qualitatively useful, these methods are not well suited for quantitative work because of adsorption of these compounds on the column.1 Martin and Driscoll (11) converted the free barbiturates into 1,3-dimethyl derivatives before the sample was injected into the gas chromatograph; however, they reported that amobarbital and pentobarbital, two commonly ingested barbiturates, were not resolved.

On-column methylation as reported by Brochmann-Hanssen and Oke (12) shows a separation similar to that reported by us in 1968.2 In our judgment, it is better to methylate before injection because the derivatives form more consistently and quantitatively.

Improvements in sample preparation and experimental parameters, as outlined in the procedure below, have resulted in a highly accurate method for the separation and quantitation of the 1,3-dimethyl derivatives of 11 barbiturates and the 4-methyl derivative of glutethimide. Because the pharmacological actions of the individual barbiturates differ, with corresponding difficulties in interpreting results, a highly accurate method is essential.

Materials and Method

A Model GC-45 Gas Chromatograph with a flame ionization detector (Beckman Instruments, Inc., Fullerton, Calif. 92634) was used and operated with a carrier gas flow (helium) of 50 ml/min, a column temperature of 190°C, a detector temperature of 250°C, and injection port temperature of 230°C. The 365-cm (12 ft) columns (4 mm i.d.) were packed with 7% DC-200 (12,500 cs) on Gas Chrom Q. (80–100 mesh) (Applied Science Labs., Inc., State College, Pa. 16801) and conditioned by heating overnight at 250°C. These columns are extremely stable, and have been used routinely for this procedure for more than one year.

Reagents

1. Chloroform, AR or "Spectranalyzed." (This reagent must be checked for the presence of interfer-
ing substances by taking 20 ml of the chloroform through the procedure, beginning with step 5.)


4. Sodium sulfate, AR, anhydrous.

5. Stock barbiturate and glutethimide standard (0.5 mg each per milliliter of ethanol:water, 95:5 by vol). Place 50 mg each of pentobarbital, secobarbital, amobarbital, butobarbital, phenobarbital, and glutethimide into a 100-ml volumetric flask and dilute to volume with 95% ethanol. Except in our initial work, other barbiturates were not included in this standard mixture, because they are rarely encountered. If the presence of some other barbiturate is suspected, a standard mixture containing this compound can then be prepared.

6. Barbiturate working standard (0.1 mg each/ml). Dilute the stock standard fourfold with 95% ethanol.

7. Stock internal standard (1 mg of methohexital per ml). The solvent is 95% ethanol.

8. Working internal standard (0.1 mg of methohexital per ml). Dilute the stock internal standard 10-fold with 95% ethanol.

Procedure

To avoid possible interference, wash all glassware with acid. Plastic disposable tubes and pipets must not be used, because chloroform extracts interfering substances from the plastic.

1. Into a 50-ml glass-stoppered centrifuge tube pipet 1.0 ml of whole heparinized blood, and add 0.2 ml of the working internal standard solution.³

2. Into a second tube pipet 1.0 ml of a commercial serum control (e.g., "Metrix," Armour Pharmaceutical Co., Chicago, Ill.) or 1.0 ml of blood known to contain no barbiturates, and add 0.2 ml of the working internal standard solution and 0.2 ml of the working standard mixture. (This standard mixture is used for quantitation.)

3. Add 20 ml of chloroform to each tube, shake vigorously for 2 min, and centrifuge.

4. Transfer the chloroform layer into clean 50-ml glass-stoppered centrifuge tubes.

5. Add about 3 g of anhydrous Na₂SO₄, shake to dehydrate the chloroform layers (rotate the tubes until all Na₂SO₄ on the side of the tubes is released into the chloroform layer), and centrifuge.

6. Transfer part of the chloroform layer into a 15-ml glass-stoppered centrifuge tube and decrease the volume by heating the tube in a water bath at 70°C, under a stream of nitrogen, then transfer the rest of the chloroform layer into the same tube and continue the evaporation until the final volume is about 0.1 ml.⁴ (If too much chloroform remains, incomplete derivative formation results; however, complete drying must be avoided, as this results in a low recovery, probably because compounds adhere to the glass.)

7. Add 1 ml of methanol reagent 3 and 0.1 ml of dimethyl sulfate, mix, and heat this solution for 10 min at 70°C in a water bath.

8. Evaporate the solvent at 70°C under a stream of nitrogen. (The extent of evaporation is not critical; however, some evaporation of the solvent is necessary if the derivatives are to be effectively extracted in step 9.)

9. Add 1.0 ml of water to each of the tubes, and break up the residue by using a mixer. Add 12 ml of chloroform and shake vigorously for 2 min. Centrifuge, and aspirate and discard the aqueous layer.

10. Add about 1.5 g of Na₂SO₄, stopper, mix, and centrifuge.

11. Decant the chloroform layer into a clean 15-ml centrifuge tube. Evaporate to about 0.1 ml (70°C, under a stream of nitrogen).

12. Inject 1 μl of the sample containing the internal standard into the gas chromatograph. If, in addition to the internal standard, a peak with the retention time of a barbiturate is noted (Table 1), the procedure is continued by injecting the standard mixture contained in the control sample. The barbiturates are eluted in about 20 min; all other nonbarbiturate compounds are eluted by about an hour. (The column oven temperature may be increased to 220°C after the barbiturates are eluted, to hasten the complete elution of all compounds.)

13. Calculate as follows:

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\text{barbiturate, mg/100 ml} = \frac{\text{peak area of barb. in sample}}{\text{peak area of int. stand. in sample}} \times \frac{\text{peak area of int. stand. in stand.}}{\text{peak area of corresp. barb. in stand.}} \times 2.0
\]

Results

The results of the separation of the 11 methylated barbiturates and of glutethimide, each in a concentration of 2.0 mg/100 ml, is shown in Figure 1. Figure 2A and 2B shows patients' samples that are negative for barbiturates and glutethimide;

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³ An additional tube containing 1 ml of the patient's whole blood, without added internal standard, may be taken through the procedure to rule out the presence of methohexital. Thus far, we have not encountered such interference.

⁴ The concentrated chloroform extract may be injected into a column such as 2% DC-200 or 3% QF-1 before derivative formation, to screen for the presence of the barbiturates or to confirm the identity of a barbiturate.
Table 1. Relative Retention Times for Methyl Derivatives of Some Barbiturates and Glutethimide

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time*</th>
<th>Duration of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barbital</td>
<td>0.31</td>
<td>long</td>
</tr>
<tr>
<td>Probabital</td>
<td>0.40</td>
<td>intermediate</td>
</tr>
<tr>
<td>Aprobabital</td>
<td>0.47</td>
<td>intermediate</td>
</tr>
<tr>
<td>Butababital</td>
<td>0.56</td>
<td>intermediate</td>
</tr>
<tr>
<td>Amobarbital</td>
<td>0.64</td>
<td>intermediate</td>
</tr>
<tr>
<td>Pentobabital</td>
<td>0.72</td>
<td>short</td>
</tr>
<tr>
<td>Vinbarbital</td>
<td>0.77</td>
<td>intermediate</td>
</tr>
<tr>
<td>Secobarbital</td>
<td>0.83</td>
<td>short</td>
</tr>
<tr>
<td>Methohexital</td>
<td>1.00</td>
<td>ultrashort (iv)</td>
</tr>
<tr>
<td>Hexobarbital</td>
<td>1.38</td>
<td>short (iv)</td>
</tr>
<tr>
<td>Glutethimide</td>
<td>1.41</td>
<td>short</td>
</tr>
<tr>
<td>Phenobarbital, mephobarbital</td>
<td>1.53</td>
<td>long</td>
</tr>
<tr>
<td>Heptabarbital</td>
<td>2.22</td>
<td>short</td>
</tr>
</tbody>
</table>

* Relative to that of methohexital (about 11 min).

Fig. 1. Separation of 11 barbiturates and of glutethimide (2 mg of each per 100 ml) as their methyl derivatives

1, barbital; 2, probabital; 3, aprobabital; 4, butababital; 5, amobarbital; 6, pentobabital; 7, vinbarbital; 8, secobarbital; 9, methohexital (internal standard); 10, glutethimide; 11, phenobarbital; and 12, heptabarbital. (Numbering of peaks is same in Figures 2 and 3)

Fig. 2B differs in that it contains added internal standard. All chromatograms (Figures 2 and 3) show peaks with relative retention times of 1.97, 3.73, and 4.16, which are thought to be those of the methyl esters of palmitic, oleic, and stearic acids, respectively. Other peaks with relative retention times of 0.94, 1.34, 1.83, 3.63, and 6.37 were frequently noted but not identified.

Twenty-five blood samples from patients for whom barbiturate analyses were requested were analyzed by the ultraviolet procedure of Broughton (13), and by the gas-chromatographic procedure presented here. The coefficient of correlation for the barbiturates by these methods was 0.98. Three of the samples were found to contain no barbiturates, two being cases of glutethimide ingestion in which the drug was identified by gas chromatography but not detected by the ultraviolet method, and a case in which diazepam was identified by the ultraviolet method but no peak was noted for this compound by the present method. Figure 3 shows the gas chromatograms of

(a) a specimen from an unconscious patient with a blood secobarbital concentration of 1.0 mg/100 ml; (b) a “negative” sample to which a standard mixture was added and which served as the basis for quantitation; and (c) a conscious patient with a blood phenobarbital concentration of 4.3 mg/100 ml.

Discussion

Possible interference by strongly acidic and alkaline compounds was minimized by extracting the barbiturates and glutethimide from whole blood at a neutral pH. The lack of interference by other compounds or drugs was demonstrated by analyzing more than 100 blood samples from hospital patients who had received various drugs, including diphenylhydantoin, meprobamate, salicylic acid, and diazepam.

Glutethimide was included among the compounds to be analyzed by this method because it is a common nonbarbiturate sedative that is frequently taken in overdose. Readily extracted by chloroform at the neutral pH of the sample, it forms a 4-methyl derivative.

Hexobarbital, a short-acting barbiturate that is rarely used orally, forms a methyl derivative with a retention time near that of glutethimide (Table 1). Although there is some overlap of peaks if
both compounds are present, they can be differentiated and measured by subjecting the sample and a standard mixture containing hexobarbital and glutethimide to the given procedure, except that the column temperature is increased to 220°C and the carrier gas flow adjusted to 80 ml/min.

Phenobarbital and mephobarbital (1-methylphenobarbital) form identical 1,3-dimethyl derivatives and therefore cannot be separated by this technique. Both are long-acting barbiturates and mephobarbital is converted to phenobarbital in the body; thus their differentiation is not clinically important.

Measurements were made by calculating the peak areas of the barbiturate and the internal standard in the sample and relating these to the peak areas obtained after subjecting a standard mixture (added to a known negative specimen) to an identical procedure. Methohexital was used as an internal standard because this ultrashort-acting barbiturate is not used orally and because the peak for this compound, by the method given, is located centrally in the chromatogram. We found palmatic acid methyl ester, used by Martin and Driscoll (11), to be unsuitable for use as an internal standard because palmatic acid derived from the sample interferes positively.

Recovery of the individual barbiturates and glutethimide, added in a concentration of 2 mg/100 ml to separate blood samples, varied from 96% to 104%. Adding the internal standard to the sample before extraction and adding a standard mixture to a control sample, respectively, compensate for losses and for the individual differences in the distribution coefficients of each barbiturate and glutethimide, and are in part responsible for the high degree of accuracy of this method.

We thank the following companies for supplying the barbiturates used in this investigation. Eli Lilly & Co. (barbital, amobarbital, pentobarbital, methohexital, and phenobarbital); A. H. Robins Co., Inc. (secobarbital); McNeil Laboratories, Inc. (butabarbital); Geigy Pharmaceuticals (heptabarbital); Roche Laboratories (apobarbital); Merck, Sharp & Dohme (vinobarbital); E. R. Squibb & Sons (probabral); and Gaines Chemical Works, Inc. (hexobarbital).

References