Identification of Barbiturates in Blood by Paper Chromatography

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A paper chromatographic method for the analysis of barbiturate in blood is presented. The Rf values and ratios of 8 standard barbiturates are given and compared with the values obtained from patients suspected of having taken barbiturate overdoses.

Statistics compiled by the Poison Control Center, New York City Department of Health, indicate that barbiturate intoxication in adults ranks first as a cause of poisoning (1). The need for a rapid and sensitive method for detecting and identifying barbiturates in body fluids for diagnostic purpose is obvious. Even though there have been many analytic methods described in recent years, the identification of commonly employed single or mixed barbiturates still remains uncertain. In the ultraviolet spectrophotometric method of Goldbaum (2), the identification and estimation of a particular barbiturate is determined by changes in absorption in the region 230–260 mμ under different pH conditions. Any extractable substance whose absorption changes with pH over this wave-length range may interfere to some extent with the identification of a particular barbiturate. Furthermore, it has been our experience that although the concentration of total barbiturate may be estimated by the ultraviolet method, the presence of more than one barbiturate in a sample yields composite absorption curves, making identification of the individual barbiturates extremely difficult.

Although gas chromatography has been used for the identification of barbiturates (3, 4), the applicability to routine analysis is complicated by the cost of the equipment and by variations in retention times when the apparatus is not operated continuously.

Published reports on thin-layer and paper chromatography methods (5–9) do not provide, in our opinion, the resolution and/or sensitivity required for the clinical analysis of body fluids.

The proposed technic, on the other hand, offers a degree of sensitivity

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suitable for clinical or toxicologic investigations, and a degree of resolution adequate for the identification of the commonly used barbiturates.

Procedure

Reagents and Equipment
1. Acetate buffer, pH 4.6  80 ml. of 0.1N sodium acetate + 1 ml. glacial acetic acid.
2. Standard sodium salts of barbituric acids  1 mg./1 ml. in 95% ethyl alcohol.
3. Na₂CO₃  0.5M.
4. AgNO₃  0.05N in 95% ethyl alcohol.
5. Mobile solvent* (a) Ethylene dichloride. (b) n-Butanol, NH₄OH, H₂O, 84:7:9. Mix 45 ml. of (a) + 1.5 ml. of (b); shake well to form clear solution.
6. Standard chromatographic tank
7. Spray bottles, chromatographic

Extraction
To 1 ml. of blood or serum, add 1 ml. of acetate buffer, 25 ml. of chloroform, and shake for 2 min. in a separatory funnel. Discard aqueous phase.
To the chloroform layer, add 25 ml. of 0.45N NaOH and shake for 2 min.; separate and discard chloroform layer.
Neutralize the aqueous layer with HCl and adjust the pH to 4.6 by adding 1 ml. of acetate buffer. The pH adjustments can be made by using pH indicator paper. Extract the barbiturate by shaking with 25 ml. of chloroform.
Discard the aqueous layer and filter the chloroform through Whatman No. 1 filter paper into a 100 ml. beaker. The chloroform extract can then be evaporated on a hot plate to a volume of approximately 1 ml. The beaker is then removed from the hot plate, and the remaining solvent is then evaporated with a current of air. The beaker should not be allowed to go to dryness on the hot plate, as this may cause charring of the residue. Dissolve the residue in 20 μl. of ethyl alcohol.

Chromatography
A sheet of 8 by 8 Whatman No. 1 paper is ruled and spotted with the unknown extract in 2 μl. portions in the conventional manner. Two μl. of

*All solvents are of reagent quality and are used without further purification.
the standard barbiturates are spotted at the same time. The paper is allowed to dry thoroughly and then is sprayed uniformly with the Na2CO3 0.5M reagent to a point where the paper is damp but not saturated.

The paper is immediately placed in the tank with the mobile solvent, and the front is allowed to rise to 1 in. from the top. The solvent front should be marked upon removal of the paper from the tank. The sheet is then allowed to dry and is sprayed with the AgNO3 reagent followed by a light spraying with water.

The paper is then placed while damp under the UV light until dark-gray spots, indicating the position of the barbiturate standards, appear on a light-gray background. These spots usually appear in about 10 min. The position of the standards and test material should be recorded at the completion of this stage because the paper becomes darker on standing and the spots may become more difficult to read.

Results

In Table 1, the Rf values, measured from the center of the spot, of the commonly prescribed barbiturates are presented. The ratio of the Rf values as compared to butabarbital are presented in the same table. The Rf ratios are preferred to the absolute Rf values because of their greater reproducibility. The values reported represent the average of approximately 20 determinations, each run on different days.

Figure 1 shows the results obtained with barbiturate standards and with serum extracts obtained from 5 patients suspected of suffering from acute barbiturate intoxication. Extracts A, B, C, and E contained 2 barbiturates each, while extract D contained 3 barbiturates. The ingestion of the particular barbiturates in these cases was confirmed by the patient upon recovery. Even though the analyses of the patients’ blood were conducted at different times, their agreement with the average values obtained with the standards demonstrates the reproducibility of the method.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rf values</th>
<th>Rf ratio</th>
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<tbody>
<tr>
<td>Barbital</td>
<td>0.04</td>
<td>0.11</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>0.09</td>
<td>0.25</td>
</tr>
<tr>
<td>Aprobarbital</td>
<td>0.18</td>
<td>0.50</td>
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<tr>
<td>Cycobarbital</td>
<td>0.32</td>
<td>0.89</td>
</tr>
<tr>
<td>Butabarbital</td>
<td>0.36</td>
<td>1.00</td>
</tr>
<tr>
<td>Amobarbital</td>
<td>0.62</td>
<td>1.72</td>
</tr>
<tr>
<td>Pentobarbital</td>
<td>0.70</td>
<td>1.94</td>
</tr>
<tr>
<td>Secobarbital</td>
<td>0.77</td>
<td>2.14</td>
</tr>
</tbody>
</table>
Fig. 1. Chromatograms of known barbiturates and extracts of blood from patients suspected of barbiturate poisoning: 1, barbital; 2, phenobarbital; 3, aprobarbital; 4, cyclobarbital; 5, butabarbital; 6, amobarbital; 7, pentobarbital; 8, secobarbital.

**Discussion**

When known amounts of barbiturates were added to normal control serum to determine the sensitivity of the method, the data revealed that 0.5 µg. of barbiturate in 1 ml. of serum could be detected without difficulty. This level of barbiturate is considerably less than that obtained when a patient ingests a therapeutic dose of barbiturate and is awake, competent, and only mildly sedated.

The sensitivity tests disclosed, in addition, that on the basis of the appearance of the spots obtained, the recovery of barbiturate from serum was approximately 50%.

In routine toxicologic cases, it is recommended that the described paper chromatographic procedure be employed in conjunction with UV spectrophotometric analysis. Using these 2 technics, the laboratory can provide the clinician with early identification and quantitation of the drug or drugs involved in a case of suspected barbiturate poisoning.

The presence of other organic acid drugs, such as salicylates, was found not to interfere with the detection of barbiturates by the described chromatographic procedure. As a time-saving device, it has been found that the barbiturate standards can be spotted on the paper in advance of the analysis and can be kept for weeks without any appreciable loss or change.
References