

Barbiturate Detection using Thin-layer Chromatography

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Thin-layer chromatography has been applied to the analysis of barbituric acids. The ratio of the R_f of 23 different barbituric acid samples to that of simultaneously run phenobarbital is given. This ratio is reproducible and characteristic. By using this technic the average laboratory can provide a barbiturate analysis of biologic material in about 2 hr.

MEDICATIONS are one of the leading causes of fatal and nonfatal poisonings. Of all medications, salicylates (in children) and barbiturates (in adults) are most frequently involved in these poisonings. When these occur, the physician treating the victim may request a chemical analysis of a blood sample to confirm his clinical impression. A salicylate analysis is simple and rapid (1). However, a barbiturate determination is nonroutine, fairly elaborate, and beyond the facilities of most clinical laboratories (2). Thin-layer chromatography is suggested as a new technic that will permit the average laboratory to analyze biologic materials for barbiturates with relative ease and economy.

Procedure

Reagents

Chloroform Acetone Mixture 9:1 (v/v).

HgSO₄ spray Suspend, with mixing, 5 gm. of HgO in 100 ml. H₂O. Add, while mixing, 20 ml. conc. H₂SO₄. Cool, dilute to 250 ml. with H₂O.

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Diphenylcarbazone spray Dissolve 5 mg. in 50 ml. CHCl_3 . Store in dark bottle.

Fluorescein spray To 500 ml. H_2O add 20 gm. NaOH and 20 mg. sodium fluorescein.

KMnO₄ spray 0.1% aqueous solution.

Standard barbiturate acids 10 mg./100 ml. (w/v) in CHCl_3 .

Silica gel "G" According to Stahl.

Equipment

1. Standard apparatus for coating glass plates with a 250- μ layer of silica gel "G"
2. Rectangular jars, $8\frac{1}{2} \times 4 \times 8\frac{1}{2}$ in. with ground-glass cover
3. Spray bottles
4. Tuberculin syringes with size 24 $\frac{1}{4}$ -in. needles
5. Mineralite ultraviolet hand lamp with 254 $m\mu$ filter

Method

Plates (20 \times 20 cm.) were coated with Silica gel G using standard thin-layer chromatography technics (3). These coated plates were dried in a ventilated oven for 20 min. at 100°. The dried plates were then stored at room temperature for subsequent use. Prior to an analysis, lines $\frac{1}{2}$ in. apart were ruled on the plate, forming a series of parallel columns for concurrent analyses of many samples. Two drops of a chloroform solution of each sample were applied at a point 1 in. from the bottom of the plate in the center of a column. The spots were air dried and then the plate was placed in the rectangular jar containing 110 ml. of the 9:1 chloroform-acetone mixture. The mixture should be placed in the jar at the beginning of the preparation for this analysis to insure saturating the jar with the mixture's vapor. This jar can be reused and its solvent mixture need not be changed for 2 days.

In this solvent system, the solvent front will rise 10–12 cm. in 45–60 min. The plate is then removed and the solvent front marked immediately. Then the plate is air dried at room temperature. When dry, the plate is sprayed first with the HgSO_4 (4), allowed to dry partially, and then sprayed again with the diphenylcarbazone. In each instance the size, position, and color intensity of each spot is noted. An excess of the diphenylcarbazone is used. The overall colored background resulting slowly disappears and purple or violet spots remain to allow accurate determination of their respective R_f values.

A second identification system that has proved effective consists of an initial spray with alkaline fluorescein. When viewed under 254 m μ light the barbiturates give a purple spot against a yellow-green background. After completing this observation, the plate may then be sprayed with the KMnO₄ (5). All unsaturated substituted barbiturates yield yellow spots against a purple background. The same KMnO₄ spray can be used without the fluorescein to give similar data.

Since 12-14 columns can easily be ruled on one plate, the same sample and suitable standards can be applied to consecutive columns, and then a second or third series can be placed on the next group of columns on the same plate. Several different sprays can then be used on one plate by suitably masking all but one series of columns for each spray. On each plate, known barbiturates, singly and in mixtures, should be run.

Sample Preparation

Samples for analysis are isolated by direct CHCl₃ extraction at pH 7. Blood and tissue samples require no pH adjustment, but urine, stomach content, and drugs should be made alkaline or dissolved in NaOH. The alkaline solution should be filtered and a 3-5 ml. sample taken. These are then made mildly acid with dilute mineral acid and extracted with 50 ml. CHCl₃.

A filtered 40-ml. aliquot of the chloroform is then evaporated to dryness under forced air and mild heat. The residue is dissolved in 0.5 ml. CHCl₃ and portions of this solution are applied to the plate along with suitable standards and mixtures thereof.

Results and Discussion

Data are presented for 23 barbiturates in Table 1. The absolute R_f values were not recorded because these vary from one run to the next. However, when the ratio of the R_f of any sample to that of phenobarbital is determined, these ratios are reproducible and significant. Those given in Table 1 represent the average of 15-20 determinations, each run on different days. Ninety per cent of these determinations gave R_f ratios that were within ± 0.10 of those reported in Table 1.

The R_f ratio is enough to indicate the relative activity of the barbiturates from 1.0 for those of long activity to over 2.0 for the very short-acting. Additional data from the reaction to the spray reagents allow more specific identification, although for clinical purposes this is

Table 1. R_f RATIOS OF BARBITURIC ACID TO PHENOBARBITAL (SILICA GEL G SOLVENT: CHLOROFORM-ACETONE, 9:1)

R_f ratio		Drug	Sprays*		
			HgO	DPC	KMnO ₄
1.00		Phenobarbital	W	V	
1.00		Barbital	W	V	
1.19	U†	Vinbarbital	W	V	Y
1.28		Butabarbital	W	V	
1.30	A†	Allobarbital	None	B	Y
1.30	A	Aprobarbital	None	B	Y
1.32	U	Heptabarbital	W	V	Y
1.38	U-A	Cyclopal	None	B	Y
1.41		Butethal	W	V	
1.41	A	Talbutal	W	B	Y
1.42		Pentobarbital	W	V	
1.44		Amobarbital	W	V	
1.44	A	Allylbarbituric acid	W	B	Y
1.50	A	Butallyonal	W	B	Y
1.56		Hexethal	W	V	
1.67	A	Secobarbital	W	B	Y
1.77	A	Sigmodal	W	V	Y
2.06	U	Hexobarbital‡	None	V	Y
2.35		Metharbital‡	None	V	
2.37		Mephobarbital‡	W	V	
§		Thiopental	W		Y
§	A	Thiamylal	W	B	Y
§	U	Cyclobarbital	W	B	Y

*B indicates blue; V, violet; Y, yellow; W, white.

†A indicates allyl substituent present in 5 position; U, unsaturated side chain present in 5' position.

‡N methyl barbiturates.

§Run with solvent front.

||Thiobarbiturates.

not essential. The yellow spots following a KMnO₄ spray identified all thio-barbiturates as well as those with unsaturated substituents. The diphenylcarbazone spray gives a dark-blue color with those barbiturates containing an allyl group and with cyclobarbital. All these spots are also semiquantitative and comparison with standards permits an educated guess as to their concentration.

In addition to pure drugs this thin-layer technic has been successfully applied to biologic samples from over 100 victims of barbiturate intoxication. In 90-120 min. after starting the extraction, one could report whether or not one or more barbiturates were present, which drugs were probably implicated, and an educated guess as to how much

was present. This is a reasonable answer to the question "was a barbiturate ingested by my patient?" Most important, it is an answer which can be given by any laboratory which has the simple and inexpensive equipment required. This involves only a \$300 investment compared to the \$3000 required for a more precise answer based on ultraviolet spectrophotometry. It has an advantage over the spectrophotometric procedure in that one can easily ascertain whether more than one barbiturate is involved.

Unfortunately it does not permit a precise quantitative estimation, but this is a small price to pay for a heretofore unavailable determination.

References

1. Trinder, P., *Biochem. J.* **57**, 301 (1954).
2. Sunshine, I., *Standard Methods of Clinical Chemistry*. Academic, New York, 1961, pp. 46-55.
3. Wollish, E. G., Schmall, M., and Hawrylshyn, M., *Anal. Chem.* **33**, 1138 (1961).
4. Zaar, B., and Gronwall, A., *Scand. J. Clin. Lab. Invest.* **13**, 225 (1961).
5. Wichstrom, A., and Salresen, B., *J. Pharm. Pharmacol.* **4**, 98 (1952).