A RAPID METHOD OF IDENTIFYING PARTICULAR BARBITURATE DERIVATIVES IN SMALL SAMPLES OF BLOOD BY PAPER CHROMATOGRAPHY

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The quantitative estimation of barbiturates in blood by ultra-violet spectrophotometry (Goldbaum, 1948; Walker, Fisher, and McHugh, 1948; Born, 1949; Lous, 1950; Wright and Johns, 1953) is of limited value unless the particular barbiturate derivative is known or can be identified. Goldbaum (1952) described an ultra-violet spectrophotometric technique by which he claimed to make such identification, but many workers with experience in this field will prefer to rely on more direct methods of qualitative analysis. For general purposes the micro-crystalline method of Turfitt (1948) is suitable if urine or gastric contents are available, though even then it is always best to confirm the identification by extraction and mixed melting point determination. Cases of barbiturate poisoning frequently occur, however, where such material is not available for analysis, or if available, does not contain adequate amounts of barbiturate, as in poisoning by short-acting derivatives. It is in these cases that a qualitative method applicable to small blood samples is of particular value. Paper chromatography seems suitable for this purpose.

Wickström and Salvesen (1952) described three satisfactory solvent systems and were able to separate 10 barbiturate derivatives by paper chromatography. Algeri and Walker (1952) were apparently the first to apply paper chromatography to blood extracts, and they explored at least 27 solvents before settling on a butanol-ammonia system which enabled them to identify 10 barbiturate derivatives. These authors used chemical methods to reveal the spots, though Algeri and Walker employed ultra-violet light to show up thiopentone. Grieg (1952) sprayed the chromatogram with alkali and demonstrated the spots by holding the paper up to a fluorescent screen illuminated by ultra-violet light.

Paper chromatography is a relatively slow process if excellent results are to be obtained, and the

present method sacrifices certain refinements in the interests of speed, since a delayed report is of little use to the clinician.

Principle

Barbituric acid derivatives are selectively distributed between suitably buffered filter paper and a chloroform moving phase.

Materials

Re-distilled chloroform, Whatman No. 1 filter paper strips, stock solutions of M-20 sodium carbonate and M-20 borax for making suitable buffers, and a "hano-via chromatolite" lamp are required.

Method

Descending chromatograms are run in a glass tank measuring 25×25×60 cm. fitted with a ground-glass lid and kept in a dark cupboard. The atmosphere inside the tank is kept saturated with water and chloroform vapour, but owing to the liability of chloroform to decompose, the tank is washed out frequently. Stock mixtures of 0.05 M-borax and 0.05 M-sodium carbonate are used to make up buffers of pH 10 and pH 10.6 as required (Clark, 1928) and the pH of each buffer is checked on a lithium glass electrode before use. Rectangular strips of No. 1 Whatman filter paper 15×50 cm. are then sprayed with the appropriate buffer solution, usually pH 10.6, about 20 ml. of buffer being required to saturate this area of paper. The paper is left suspended in air by the edge which will ultimately hang lowest in the tank, and is used as soon as it feels reasonably dry. Paper which appears to have become thoroughly dry may be used provided that, after the application of the spots, it is allowed to hang in the moist atmosphere of the tank for at least an hour before the moving phase is added. Barbituric acid derivatives in 0.25% alcoholic solution are applied to the base line 2.5 cm. apart with a capillary pipette graduated to contain 0.01 ml. Small spots are obtained by intermittently applying the pipette to filter paper under an infra-red lamp. The paper is then suspended in the tank with its upper end immersed in re-distilled chloroform.

Key to Chromatogram	Official Name	Some Proprietary Names
1 Starting line		
2 Phenyl methyl barbituric acid		Rutonal (May & Baker)
3 Diethyl barbituric acid	Barbitonum (B.P.)	Veronal (Bayer)
4 Phenyl ethyl barbituric acid	Phenobarbitonum (B.P.)	Medinal (Schering)*
5 Diallyl barbituric acid	Allobarbitonum (B.P.C.)	Gardenal (May & Baker)
6 Allyl isopropyl barbituric acid		Luminal (Bayer)
7 Ethyl-cyclo-hexenyl barbituric acid	Cyclobarbitonum (B.P.C.)	Dial (Ciba)
8 Allyl isobutyl barbituric acid		Allonal (Roche)†
9 Butyl ethyl barbituric acid	Butobarbitonum (B.P.C.)	Phanodorm (Bayer)
10 Isoamyl ethyl barbituric acid	Amylobarbitonum (B.P.C.)	Sandoptal (Sandoz)
11 Ethyl (1-methylbutyl) barbituric acid	Pentobarbitonum (B.P.)	Soneryl (May & Baker)
12 Allyl (1-methylbutyl) barbituric acid	Quinalbarbitonum (B.P.)	Neonal (Abbott)
13 Ethyl (1-methylbutyl) thio barbituric acid	Thiopentonium (B.P.)	Amytal (Lilly)
14 Methyl cyclohexenyl-N-methyl barbituric acid	Hexobarbitonum (B.P.)	Nembutal (Abbott)*
15 Solvent front		Seconal (Lilly)
		Pentothal (Abbott)
		Cyclonal (May & Baker)
		Evipan (Bayer)

* These products are sodium salts.

† This product also contains amidopyrine.

FIG. 1.—Paper chromatogram showing resolution of 13 barbituric acid derivatives. The purity of these compounds was confirmed by melting point determination. (Paper buffered to pH 10.6, chloroform run for six hours at temperature 21° C. Total length of run 45 cm.)

Adequate development of the chromatogram takes between four and seven hours; owing to the relatively slow progress of the solvent front as it nears the lower end of the paper, development may usually be allowed safely to continue overnight if this is more convenient. At the end of this time the paper is removed from the tank, dried in air, and is ready within five minutes to be viewed under ultra-violet light. A "hanovia chromatolite" lamp emitting maximal radiation at 253.7 mμ is used, and the spots, which appear dark on a bright background, are for ordinary purposes ringed in pencil, though permanent records can be made by suitable exposure to ultra-violet light of photographic paper placed in direct contact with the chromatogram. The point of maximal density of the spot (usually its centre) is used for making measurements. Should there be any doubt regarding their identity as barbiturate the spots are eluted with 0.05% sodium hydroxide, buffered to pH 10, and the solution submitted to ultra-violet spectrophotometry. Experience showed that less material is required to give well-defined spots of slow running barbiturates such as barbital, phenobarbital, and methyl-phenyl-barbital. These are therefore applied in 0.083% solution. Well-defined spots are thus obtained with amounts of barbiturate varying from about 10 to 25 μg. For chromatography of blood extracts the final acid solution used for ultra-violet

spectrophotometry is extracted with ether three times, the ether phase is evaporated to dryness and sufficient alcohol added to give a concentration of barbiturate estimated at about 0.25%. Crude extracts of blood made by direct shaking with ether have also been used with success, though it is difficult to obtain spots of suitable size unless the approximate quantity of barbiturate present is known.

Results

Fig. 1 shows the distribution of 13 barbiturate derivatives run simultaneously on the same piece of paper. It will be seen that in general there is a relationship between the distribution of the various derivatives on the chromatogram and their relative speeds of clinical action; the slow acting barbiturates travel slowly, medium acting barbiturates more rapidly, and fast acting barbiturates most rapidly. The chromatogram thus parallels Tatum's (1939) classification of barbiturates, the only notable exception being ethyl cyclohexenyl barbiturate acid (cyclobarbitone "phanodorm") which this author classifies as short acting and yet runs relatively slowly on the chromatogram.

TABLE I
R VALUES RELATIVE TO PENTOBARBITAL (pH 10.6
CHLOROFORM)

	Mean	S.D.	Range	No. of Observations
Barbital ..	0.12±	0.06	0.06-0.21	10
Phenobarb ..	0.13±	0.01	0.10-0.17	11
Allyl isopropyl	0.61±	0.05	0.53-0.70	10
Butobarbital ..	0.69±	0.04	0.63-0.75	13
Amylobarbitol	0.93±	0.03	0.88-0.98	15
Quinalbarbital	1.06±	0.02	1.01-1.08	9

This parallelism between the chromatographic distribution and clinical action is most useful from the diagnostic standpoint, since even if a given barbiturate derivative cannot immediately be identified, its position on the paper roughly indicates its speed of action so that an estimate can be made of the significance of blood levels already obtained by spectrophotometry.

As might be expected in a chromatographic system involving the use of buffered paper with chloroform as the moving phase, in which no great pains are taken to ensure uniform temperature or humidity, absolute R_f values are too variable to be useful in identifying particular derivatives. R values relative to marker barbiturates are of more use, particularly in differentiating derivatives which lie close to the marker. Table I shows some R values relative to pentobarbital. It is clear that

amylobarbitol and quinal barbitol may readily be differentiated whereas the ranges of allyl-isobutyl barbitol and butobarbital overlap as also do those of barbital and phenobarbital.

In practice the identification of an unknown barbiturate is best done by running it on the same sheet of paper as known representatives of slow, medium, and quick acting derivatives. Two alcoholic solutions of markers are used here, one containing phenobarbital, butobarbital, and pentobarbital and the other barbital, allyl-isobutyl barbitol, and amylobarbitol.

The slower running compounds can be more widely separated by running them on paper buffered to pH 10.0 or alternatively, using N butanol as solvent and paper buffered to pH 10.6.

Figs. 2, 3, and 4 are examples of unknown drugs identified by the above technique. Fig. 4 is of particular interest in that it shows how amylobarbitol and quinal-barbital may be separated in the blood of a patient who had taken a mixture of

FIG. 2.—(1) Base line, (2) phenyl methyl barbituric acid, (3) diethyl barbituric acid, (4) unknown blood extract, (5) phenyl ethyl barbituric acid, (6) unknown blood extract. Conclusion.—4 and 6=phenyl ethyl barbituric acid.

FIG. 3.—(1) Ethyl isoamyl barbituric acid, (2) ethyl (1-methylbutyl) barbituric acid, (3) unknown blood extract, (4) allyl (1-methylbutyl) barbituric acid, (5) solvent front. Conclusion.—Either the unknown blood contained both ethyl isoamyl and ethyl (1-methylbutyl) derivatives, or only one of these derivatives, the spot being too big. (Ethyl isoamyl barbituric acid was isolated from stomach contents.)

FIG. 4.—(1) and (2) Spots from extract of unknown blood, (3) ethyl isoamyl barbituric acid, (4) allyl (1-methylbutyl) barbituric acid, (5) and (6) spots from extract of stomach contents of same case as 1 and 2, (7) solvent front. Conclusion.—1 and 5=ethyl isoamyl barbituric acid; 2 and 6=allyl (1-methylbutyl) barbituric acid.

the two ("tuinal"). Altogether the barbiturates concerned in 20 cases of poisoning have been identified by this technique and in some cases successful identification was possible on blood specimens which had been stored in a refrigerator for periods as long as 18 months. Chromatographic identification of barbiturates present in urine, gastric contents, and tissues has also been made in a few cases.

Discussion

The distribution of barbituric acid derivatives in this system presumably depends on their individual pK_s and also on their solubility in chloroform. Bush (1937) gave pK values for five barbiturate derivatives as follows: phenobarbital 7.34, barbital 7.89, amylobarbitol 7.89, pentobarbital 8.04 and hexobarbital 8.23. These figures indicate a trend towards higher pK values in the more rapidly running compounds, as would be expected on theoretical grounds, though the fact that amylobarbitol and barbital have the same pK value clearly indicates that their wide separation on the chromatogram is due to another factor. Tabern and Shelberg (1933) found that the barbiturate derivatives decreased in water solubility and increased in oil/water distribution coefficient with increase in length of the 5.5 substituted alkyl side-chains, and their data were used by Fuhrman and Field (1943)

in reviewing the relationships between the physical properties and physiological actions of a number of barbiturate derivatives. Butler (1950), using mice, showed that the difference in the speed of action of a slowly acting derivative (barbital) and a rapidly acting one (ortal) could be accounted for by the relatively slow appearance of the former substance in brain tissue. He postulated that the essential difference in physiological action between barbiturate derivatives lies in their relative ability to transgress the brain cell membrane. Brodie (1952) has shown that the extreme rapidity of action of thionipental can be attributed to its high lipid solubility rather than to chemical instability.

These researches indicate that barbiturates obey the Meyer-Overton hypothesis, so long known to hold for volatile anaesthetics. The present observations on the chromatographic distribution of 15 barbituric acid derivatives suggests that their relative solubilities in chloroform parallel their lipid solubilities. The position of ethyl-cyclohexenyl barbital (cyclobarbital) is interesting, for this substance was classified by Tatum as rapidly active. According to the chromatogram it should have a slow medium action akin to butobarbital, and this is supported by clinical experience of this drug. In contrast, however, the analogue of this substance N methyl cyclo-hexenyl-methyl barbituric acid ("evipal") is one of the quickest acting barbiturates and indeed it runs almost with the solvent front with the system described here.

Algeri and Walker (1952) mentioned that with their system the spots obtained in some cases from blood samples of patients who have taken known barbiturates did not correspond with the position of the pure substance run on the same piece of paper. They interpret such differences as possibly due to metabolic products of barbiturate in the blood samples. With the present method no convincing differences of this nature have been obtained, the slight divergences which are sometimes observed being readily attributable to the size of the spot, irregularities in the texture of impregnation of the paper with buffer, or to slight asymmetry in suspension of the paper.

Summary

A simple, relatively rapid, paper chromatographic system is described for the separation and identification of barbiturates in small blood samples.

At least 13 of the common barbiturate derivatives can be separated by this means and their relative distribution correlated with their speed of clinical action.

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