SCIENTIFIC SECTION

STUDIES ON BARBITURATES. II. CONTRIBUTIONS TO METHODS OF BARBITAL RESEARCH.*

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In a previous paper (1) we described methods of extraction of barbiturates from urine, blood and tissues; and also colorimetric procedures to estimate the quantity of barbiturates so recovered. The purpose of this paper is to describe certain improvements and modifications of our technique and some more recently developed methods, with a review of our original experimental procedures.

I. METHODS OF EXTRACTION.

Urine.

For ordinary routine procedures the urine is acidulated with dilute hydrochloric acid and shaken with ten volumes of chloroform. This usually furnishes sufficiently clear extracts for testing. Colored and concentrated urines, however, should be cleared. Twenty cc. of urine are shaken with an equal volume of a 10 per cent copper sulphate solution in a small separatory funnel after sufficient amounts of 5 per cent sodium hydroxide or potassium hydroxide solutions have been added to produce precipitation. The mixture is filtered, the filtrate acidulated with dilute sulphuric acid (not hydrochloric) and shaken with ten volumes of chloroform. The chloroform fraction is then filtered through a chloroform-moistened double hard filter to eliminate suspended water particles and their solutes. This chloroform extract may then be tested directly, or if it contains only small amounts of barbiturates it may be concentrated over a water-bath.

When dealing with urines in which the barbiturates are present in high dilutions it is advisable to add enough powdered copper sulphate crystals to make a 5 per cent solution and thus avoid an increase in the volume of urine to be extracted.

Blood.

1. Sodium Tungstate Method.—The Folin-Wu standard blood precipitation method, using sodium tungstate and $^2/_3$ normal sulphuric acid, can be successfully adapted for the extraction of barbiturates. It was found, however, that dilution hemolysis was not necessary. The procedure is as follows:

Ten cc. of blood, 10 cc. of 10 per cent sodium tungstate solution and from 10 to 20 cc. of $^2/_3$ normal sulphuric acid are shaken in a separatory funnel until a chocolate-brown color is obtained. The mixture is filtered with suction and an aliquot of the clear filtrate is shaken with ten volumes of chloroform.

Ten mg. of sodium barbital were added to 20 cc. of dog's blood. From 90.8 to 93.7 per cent of the barbital was recovered by this method.

2. Myers and Wardell's Method (Adopted).—The method used by Myers

* Scientific Section, A. PH. A., Washington meeting, 1934.

and Wardell (2) for the extraction of blood cholesterol can be used for the recovery of barbiturates from blood.

A convenient amount of blood (1 to 2 cc.) is pipetted into a small mortar containing a sufficient amount of plaster of Paris to form a dry mixture. The mixture is pulverized and transferred to a paper extraction shell. This shell is inserted into a perforated glass tube enclosed in a flask and connected to a reflux condenser. Ten volumes of chloroform (10 to 20 cc.) are poured into the flask, and boiled over a water-bath for half an hour. The chloroform extract is then filtered and tested. If the test is negative the extract may be concentrated over a water-bath and again tested.

Ten mg. of sodium barbital added to 20 cc. of blood gave recoveries of from 88.0 to 92.7 per cent.

This method is particularly valuable when only 1 or 2 cc. of blood can be secured. It may also be used for small quantities of urine or for liquefied tissues.

Tissues.

1. *Copper Sulphate Precipitation Method.*—The weighed organs are ground and thoroughly mixed with a 5 per cent sodium hydroxide or potassium hydroxide solution of a volume sufficient to liquefy the organs within twenty-four hours. Then the alkalinized, liquefied organs, or known portions thereof, are shaken with an equal volume of 10 per cent copper sulphate solution and filtered until clear. The filtrate is acidulated with dilute sulphuric acid and shaken with ten volumes of chloroform.

Another equally simple procedure consists of mixing the ground organs with pepsin and a 3 per cent solution of hydrochloric acid, and allowing the mixture to stand for twenty-four hours. This mixture is shaken with an equal amount of copper sulphate solution (10 per cent) after its reaction has been made alkaline by adding sufficient amounts of potassium hydroxide or sodium hydroxide. It is then filtered and extracted with ten volumes of chloroform.

2. *Liquid Air Method.*—It has been found possible to extract barbiturates from tissues which have been pulverized after being frozen with liquid air.

The organ or tissue is weighed, placed in a pyrex beaker, liquid air is poured over the tissue until it is frozen. It is then pulverized, and a convenient amount of this pulverized material is weighed and placed in a separatory funnel, and shaken with 10 cc. of chloroform per Gm. of tissue. The chloroform extract must be filtered before it is tested. If necessary, the chloroform extract may be concentrated over a water-bath.

Frogs were injected with 1 mg. of barbital per Gm. of body weight. After ten minutes they were frozen in liquid air and the barbital extracted as described. Recoveries were from 83.0 to 91.0 per cent.

This liquid air method is of special value where rapidity is desired. It can be satisfactorily applied to every organ and tissue except the brain and spinal cord. The brain and spinal cord must be liquefied with alkali and precipitated with copper sulphate before they can be successfully extracted with chloroform. With brain tissue as with other tissues, excess alkali causes pigments to be carried over into the chloroform extract. These pigments may interfere with the colors formed in the test.

II. METHODS OF COLORIMETRY.

The Macro Test.—In all experiments we extracted the appropriately treated unknown solution by shaking with ten volumes of chloroform in a suitable separatory funnel. All chloroform extracts are filtered through a chloroform-moistened filter until clear. The colorimetric estimation of barbiturates is always carried out with portions of the chloroform extract.

To perform the test 6 cc. of an extract are divided into three equal volumes and placed in test-tubes called A, B and C. To A is added 0.05 cc. of 1 per cent cobaltous acetate solution in absolute methyl alcohol and 0.1 cc. of 1 per cent barium hydroxide solution in absolute methyl alcohol. To B are added 0.1 cc. of the cobalt reagent and 0.2 cc. of the barium hydroxide reagent. To C are added 0.2 cc. of the cobalt reagent and 0.4 cc. of the barium reagent. A blue color indicates a positive test.

If malonyl-urea derivatives are present in the extracts a blue color appears in A, may appear in B or even in C. If only A gives a blue color the solution contains more than 0.09 mg. but less then 0.2 mg. of barbiturate per cc. If B also yields a blue color, the extract contains 0.3 mg. per cc. or less; if A, B and C turn blue, the extract contains 0.3 mg. per cc. or more. However, if one allows these colors to stand for five minutes the color of C will fade if the extract contains less than 0.35 mg. per cc. Chloroform extracts containing less than 0.2 mg. per cc. become positive, but turn quickly negative when treated as B, but remain positive (blue) when treated as A. Also chloroform extracts containing 0.25 to 0.30 mg. of barbital per cc. when treated like C turn positive but fade from blue to greenish yellow. However, they remain positive if treated like A and B. Chloroform extracts containing 0.35 mg. of barbital per cc. or more if treated as C, remain positive.

Positive tubes are then compared with standards, within the range of the positive tubes, treated similarly.

Not only alcoholic solutions of barium hydroxide, but also any anhydrous alkaline solution, as sodium, magnesium or potassium hydroxide will provide a medium for the test. We have investigated these, as well as sodium, magnesium and calcium methoxide; and also ammonia in methyl alcohol. The colors produced in these solutions are unstable and unsuited for finer colorimetric comparisons. Solutions of calcium and magnesium methoxide cannot be prepared in suitable concentrations for the test. The best alkalies are barium and lithium hydroxide.[1]

The test using barium hydroxide is directly sensitive to one part of barbital in ten thousand parts of solutions. We shall call this the *macro test.* The test using lithium hydroxide we shall call the *micro test.* This test is sensitive to one part of the barbital in one hundred thousand parts of solution. Since chloroform extracts can be concentrated at least twenty times, the micro method can detect one part in two million.

Amines were found to provide a suitable alkaline medium for the test. The colors produced were unusually stable, fading only after several days. Thus they are well adapted for use in standard colorimeters. We investigated isobutyl amine, isoamylamine, normal butylamine, propanol amine, isopropylamine, hexamethylenetetramine and naphthylamine. Only the primary saturated amines were found to be of value; of these isopropylamine produced the most readable color.

[1] The sodium salt of evipal (*N*-methyl-cyclohexenyl-methyl-malonyl-urea) is fairly soluble in chloroform, and will yield a positive test with cobaltous acetate in anhydrous media without the otherwise necessary addition of the alkaline reagent. However, *N*-methyl-cyclohexenyl-methyl-malonyl-urea itself can be tested in the usual way, but about ten times as much must be used as of the other barbiturates, both in the macro and micro tests. Thus it seems that the substitution of alkyl radicals on the nitrogen considerably desensitizes the test.

THE MICRO TEST.

Reagents.

Cobaltous Acetate 0.20%.—Two hundred mg. of the salt is weighed accurately and placed in a volumetric flask and enough absolute methyl alcohol is added to make 100 cc. of solution.

Lithium Hydroxide 0.20%.—Two hundred mg. of the chemically pure, dry material is placed in a 100 cc.-volumetric flask and about 50 cc. of absolute methyl alcohol added to it. The flask is gently warmed on a water-bath to facilitate solution. It is allowed to cool and the volume made up to 100 cc. A small amount of insoluble material is present. This amounts to about 0.5%. It does not interfere with the test and may either be left in the reagent or filtered out. Due to the insoluble material, this reagent is not quantitatively 0.20% but is about 0.199%. This difference is not serious.

The Standards.

Barbital is dissolved in chloroform to give solutions of the following concentrations:

0.002% 0.003% 0.004% 0.005% 0.006% 0.007%

These are prepared by first making a 0.1% solution of barbital in chloroform and then, by proper dilutions, preparing solutions of the above concentrations.

The standards are kept in glass-stoppered bottles and evaporation avoided.

Procedure.

Six cc. of the chloroform extract are divided in three equal parts A, B and C.

The cobaltous acetate reagent is added to each of the three test-tubes, 0.05 cc. to A, 0.1 cc. to B and 0.15 cc. to C.

The tubes are shaken and the lithium hydroxide reagent is added to the tubes, 0.05 cc. to A, 0.1 cc. to B and 0.15 cc. to C. The tubes are shaken and the colors noted against a plain white background. The tubes should be observed for one minute before a final reading is made.

TABLE I.—COLORS APPEARING IN THE DIFFERENT TUBES WITH DIFFERENT CONCENTRATIONS OF BARBITAL.

Concentration of Barbital Mg. per Cc.	A. 0.05 Cc. of Each Reagent.	B. 0.1 Cc. of Each Reagent.	C. 0.15 Cc. of Each Reagent.
0.02	Positive	Negative	Negative
0.03	Positive	Positive, fading in 30 seconds	Fades immediately
0.04	Positive	Positive	Fades in 30 seconds
0.05	Positive	Positive	Fades in about 2 minutes
0.06	Positive	Positive	Permanent for more than 2 minutes

If a permanent blue (color persisting more than 2 minutes) is secured in Tube C, and the other tubes have also shown blue, the concentration is above the range of the test. The chloroform extract should then be diluted and the test repeated.

If no color is secured with the original extract, a convenient amount should be evaporated to dryness in an evaporating dish on a water-bath and the residue dissolved with chloroform. The test is repeated with this concentrated solution.

The blue colors are faint, but quite distinct. We recommend a series of experiments with the standards before actual extracts are used.

The principal error is in reading the different intensities of color. For example, if a blue is obtained in Tubes A and B but not in Tube C the actual concentration may be within a range between 0.035 and 0.045. It is possible, however, by standard solutions, to estimate concentration between the extremes of the range.

ISOPROPYLAMINE TEST.

Reagents.

Cobaltous Acetate 1.00%.—One Gm. of cobaltous acetate is weighed accurately and placed in a volumetric flask. Enough absolute methyl alcohol is added to make 100 cc. of solution.

Isopropylamine 5.00%.—Five cc. of isopropylamine (Research Laboratories, Eastman Kodak Company) are dissolved in sufficient absolute methyl alcohol to make 100 cc. of solution.

Standards.

The barbiturate is dissolved in chloroform to give solutions of the following concentrations:

0.02% 0.04% 0.06% and 0.08%.

These standards are kept in glass-stoppered bottles and evaporation is avoided.

Procedure.

One cc. of the chloroform extract is placed in a test-tube and 0.05 cc. of the cobaltous acetate reagent and 0.3 cc. of the isopropylamine reagent is added. If barbiturates are present in the extract a reddish violet color develops. This is then compared in the micro-cups of a colorimeter with the color produced under the same conditions by one of the standards.

A simple formula giving the concentration in mg. of the barbiturate per cubic centimeter of chloroform extract is:

$$\frac{\text{Reading of standard} \times \text{Concentration of standard (mg. per cc.)}}{\text{Reading of unknown}} = \text{Milligrams of barbiturate per cubic centimeter of extract.}$$

III. DISCUSSION.

The three tests described above are identical as far as their specificity is concerned. Of chloroform-soluble substances only theobromine, theophylline and thymine show positive tests. Other compounds of similar configurations, such as uracil, alloxan and tricarbonimid, are automatically eliminated due to their insolubility in chloroform. No substance has been found which in the urine, blood or tissue either positively or negatively interferes with these tests, with the exception of lecithin or lecithin-like substances (3).

It is noteworthy that the lithium test can be performed only in chloroform solution. If the barbiturates are dissolved in absolute methyl alcohol and treated with the cobaltous acetate and lithium hydroxide reagents the color does not develop. The barium hydroxide and isopropylamine are effective in absolute alcohol, chloroform and ether.

REFERENCES.

(1) Koppanyi, Murphy and Krop, *Arch. intern. Pharm. Therap.*, 46 (1933), 76.
(2) Myers and Wardell, *J. Biol. Chem.*, 36 (1918), 147.
(3) Koppanyi, Dille, Krop, *J. Pharmacol. and Exper. Therap.*, 52 (1934), 121.

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STUDIES ON BARBITURATES. III. CHEMICAL ASSAY OF BARBITURATES.*

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In a previous paper Koppanyi, Murphy and Krop (1) presented a modification of their original quantitative barbiturate test (2) using cobaltous acetate and isopropylamine dissolved in absolute methyl alcohol as reagents. This test was utilized in the following work as a method of chemical assay of various preparations containing barbiturates.

EXPERIMENTAL.

The test depends upon the formation of a bluish or reddish purple color produced by cobaltous acetate in an alkaline medium when barbiturates are present. The barbiturate to be assayed is either directly dissolved in chloroform or extracted with chloroform from an aqueous solution. To 2 cc. of this chloroform solution are added 0.1 cc. of 1.00 per cent cobaltous acetate ($CO(CH_3COO)_2.4H_2O$) in absolute methyl alcohol and 0.6 cc. of a 5.00 per cent (by volume) solution of isopropylamine in absolute methyl alcohol. A reddish violet color develops which is compared in a standard colorimeter with the color produced by a standard made up of the barbiturate under consideration.

As is generally the case with colorimetric tests, the concentration of the standard must be near that of the unknown. In order to select such a standard a rough approximation of the strength of the unknown is first made. Two cc. of each of the following solutions of the barbiturate in chloroform are placed in test-tubes: 0.040%, 0.060%, 0.080%, 0.100% and 0.120%. To each tube 0.1 cc. of cobaltous acetate reagent and 0.6 cc. of isopropylamine reagent are added. The tubes are then stoppered and set aside. The unknown solution treated similarly is then compared with the color of the standards, and a standard close to the unknown is chosen. The series of color standards can be kept for several hours in stoppered test-tubes.

If the concentration is above the range of the standards, the unknown solution may be diluted to bring it within the range covered by the series of standards. If it is weaker, it may be concentrated by evaporation on a water-bath.

After a standard has been chosen, 2 cc. each of the standard solution and the unknown are placed in two test-tubes. The reagents are added and the standard and unknown are compared in the microcups of a standard colorimeter.

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* Scientific Section, A. PH. A., Washington meeting, 1934.

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