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METHOD FOR THE ESTIMATION OF BARBITURIC AND THIOBARBITURIC ACIDS IN BIOLOGICAL MATERIALS

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In preliminary work on the fate and distribution of barbiturates in the animal body several known methods of determining them were tried (Levvy, 1940; Delmonico, 1939; Anderson and Essex, 1943), but none was completely satisfactory. With these methods the recoveries of known amounts of barbiturates added to samples of blood and tissues were low.

A method has been developed for the estimation of barbituric acids based on Koppanyi's colour reaction (1934) and for the estimation of thiobarbituric acids based on Cowan's colour reaction (1939). The main feature of this method is the purification of the extracts containing barbituric and thiobarbituric acids by chromatography, which has the additional advantage of permitting the determination of both types of compounds when present together in the same sample.

The method involves the following stages: (i) extraction of the drugs from the biological material; (ii) purification of the extracts by chromatography; and (iii) determination of the drug content in the eluates.

REAGENTS

1. Peroxide-free ether. Ether (technical) was treated overnight with ferrous sulphate, washed with water, dried with anhydrous calcium chloride and distilled over sodium.
2. 10 per cent (w/v) solution of sodium dihydrogen phosphate.
3. Crystalline sodium dihydrogen phosphate.
4. Anhydrous sodium sulphate.
5. Chloroform free from alcohol: chloroform (B.P.) was washed with water and then with a saturated solution of calcium chloride. After drying with anhydrous calcium chloride it was distilled and kept in a dark bottle.

6. Methanol A.R. quality.

7. Benzene A.R. quality.

8. Activated alumina: 1,200 g. activated alumina, "grade O," supplied by Messrs. Peter Spence, Manchester, were boiled for 2 hours with 1,800 ml. 10 per cent (v/v) acetic acid. The alumina was filtered and the excess of acetic acid removed by washing with at least 20 l. of hot distilled water. The alumina was dried and reactivated by heating until the temperature reached 360° C.; it was then partly deactivated by adding water (2.5 per cent w/v).

9. For the estimation of barbituric acids: (*a*) 1 per cent (w/v) cobalt acetate in methanol and (*b*) 5 per cent (v/v) *iso*propylamine in methanol.

10. For the estimation of thiobarbituric acids: (*a*) a saturated solution of anhydrous copper sulphate in methanol and (*b*) 10 per cent (v/v) diethylamine in methanol.

PROCEDURE

EXTRACTION—*Blood.*—10–20 ml. volumes of oxalated blood are mixed with equal volumes of water and of the sodium dihydrogen phosphate solution and extracted with ether in a continuous extractor (at 45–50° C.) for 8–10 hours. The ether extract is evaporated to dryness.

Urine.—The total or an aliquot of the urine is acidified with conc. HCl to pH 5. It is then extracted with ether in a continuous extractor (at 45–50° C.) for 8–10 hours and the extract evaporated to dryness.

Tissues.—Ether extracts of tissues are difficult to purify by chromatography, but the following method of extraction has been used with success.

Samples of about 10–20 g. of tissues are ground in a mortar with sand and then mixed with solid sodium dihydrogen phosphate (1 g. for every 10 g. of tissue) and allowed to stand for 5–10 minutes. Anhydrous sodium sulphate (20 g. for every 10 g. of tissue) is then added slowly, with continuous grinding, to give a fine homogeneous powder. The whole is transferred to a desiccator and left over anhydrous calcium chloride for one hour. The dry powder is extracted for 2–3 hours with 50 ml. benzene in a well-stoppered 100 ml. conical flask. The benzene extract is then filtered and the residue and flask washed three times with about 10–15 ml. of benzene. The filtrate and washings are pooled and concentrated to about 5 ml. in a distillation flask at 50° C. under reduced pressure. This method of extraction can also be applied to blood.

PURIFICATION OF THE EXTRACTS AND SEPARATION OF BARBITURIC FROM THIOBARBITURIC ACIDS

This is based on the work of Kondo (1937), who separated barbitone from phenazone by chromatography on alumina columns.

Urine and blood.—The residues left by evaporation of the ether extracts are dissolved in 5 ml. chloroform and dried by shaking with about 1–2 g. anhydrous sodium sulphate. The solutions are then chromatographed on alumina columns (3/8″ × 4″). The chloroform solutions are filtered directly on to the columns; the

flask and filter are washed three times with 5 ml. of chloroform and the washings poured on to the column. The column is then washed with chloroform until the eluates are free from pigment.

Tissues.—The benzene tissue extracts are passed through alumina columns, with slight suction. The flasks are washed three times with benzene and the washings added to the column. The column is then washed with benzene until the eluates are free from pigment and finally with 20 ml. chloroform.

The chloroform and benzene eluates are discarded. If the extracts contain more than 2 mg. of a thiobarbituric acid, the latter can be seen under ultraviolet light as a dark band at the top of the column.

Separation.—Thiobarbituric acids are recovered from the columns by elution with 50 ml. 2 per cent methanol in chloroform (v/v). Barbituric acids are not eluted by methanol and chloroform in this proportion, but they can be recovered by further elution with 50 ml. 10 per cent methanol in chloroform (v/v). These eluates are kept for estimation.

The separation of thiobarbituric acids from barbituric acids is complete and the recoveries of both fractions are almost theoretical. Mixtures containing 0.25–0.5 mg. thiophenobarbitone and 0.25–1 mg. phenobarbitone were added to alumina columns, and the average recoveries were: thiobarbituric acid 102 per cent and barbituric acid 98 per cent.

ESTIMATION.—The eluates from the columns are evaporated to dryness in distillation flasks under reduced pressure at 40–50° C. The residues are dissolved in chloroform and their barbituric or thiobarbituric acid content estimated by the following reactions.

Thiobarbituric acids.—Thiobarbituric acids are estimated by a modification of the reaction demonstrated by S. L. Cowan at the Physiological Society in 1939. An aliquot of the final chloroform solution is taken in a test tube and for every 2 ml., 0.2 ml. of the diethylamine solution and 0.5 ml. of the copper sulphate solution are added in that order. A green coloration develops at once which is stable for about two hours. The samples are compared in a colorimeter, photoelectric or otherwise, with a series of similarly treated standard solutions of the thiobarbituric acid to be estimated, containing from 0.03–0.5 mg./ml. These are prepared by diluting a fresh solution containing 0.5 mg./ml. of the thiobarbituric acid in chloroform.

Extracts of tissues, such as brain and liver, give a slight blank with the copper reaction for thiobarbituric acids. This can be as high as 1 mg./100 g. of tissue and it is necessary to subtract this blank from the estimations.

The reaction is fairly specific. According to Cowan (personal communication) it is not given by malonic acid, theophylline, theobromine, thiourea, caffeine, guanine, uric acid, urea, creatinine, oxamide, succinic acid, lecithin, cholesterol, cystine or glutathione.

Barbituric acids give a faint bluish colour under the conditions described above. The intensities of the colours given by standard solutions of phenobarbitone and thiophenobarbitone are compared in Fig. 1.

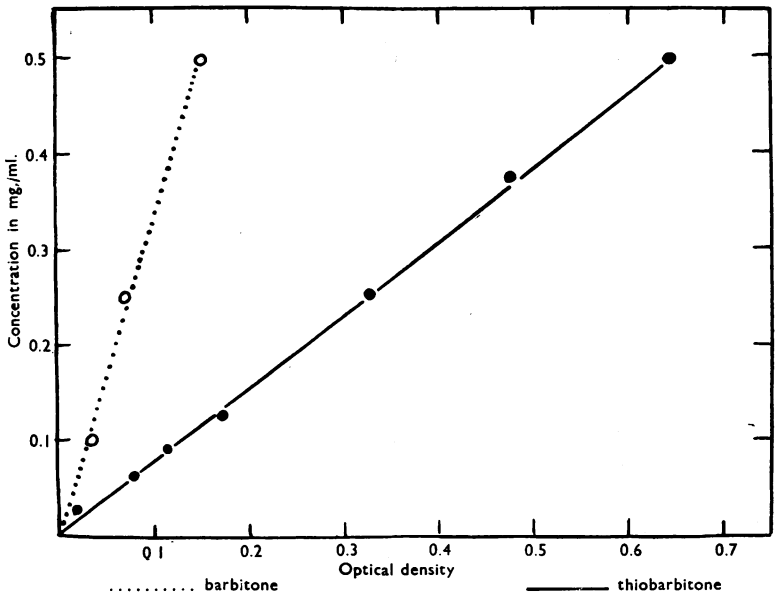

FIG. 1.—Intensity of the colour given by solutions of barbitone and thiobarbitone in Cowan's reaction. Measurements made in the Pulfrich photometer with filter 66, 6/3.5, and 10 mm. cells.

The Pulfrich photometer was used in these estimations. With filter S.66, 6/3.5, maximum transmission at 679 mg. and 1 cm. cells, the reaction is sensitive to concentrations of 0.03 mg./ml., but the sensitivity can be increased to 0.005 mg./ml. if 5 cm. cells are used.

Barbituric acids.—These are estimated by Koppanyi's reaction (1934). For every 2 ml. of the final chloroform solution, 0.6 ml. *iso*propylamine and 0.1 ml. cobalt acetate reagents are added. The reddish colour given by the sample is compared in a colorimeter with a series of similarly treated standards containing 0.1–1.0 mg. of the appropriate barbituric acid per ml. chloroform.

Recovery.—The method was tested in a series of control experiments in which known amounts of sodium kemithal (5-$\Delta^{2:3}$-*cyclo*hexenyl-5-allyl-2-thiobarbituric acid) were added to samples of blood and tissue and treated as described above. The results of these experiments are summarized in Table I.

The recovery of known amounts of barbituric or thiobarbituric acids added to samples of blood and tissues is approximately complete, except when the amount in the 10 ml. sample is less than 0.3 mg., when the recovery may fall below 95 per cent.

TABLE I

RECOVERIES OF 5-$\Delta^{2:3}$-*cyclo*HEXENYL-5-ALLYL-THIOBARBITURIC ACID (KEMITHAL) FROM BLOOD AND TISSUES

Tissue	Tissue in g. Blood in ml.	Na kemithal added mg.	Equivalent to kemithal acid. mg.	Kemithal acid found mg.	Per cent recovery
Blood	10	8.0	7.15	6.5	91
	10	8.0	7.15	6.9	96
	10	8.0	7.15	7.2	103
	10	1.125	1.0	0.98	98
	10	1.125	1.0	1.01	101
	10	1.0	0.89	0.86	97
	10	1.0	0.89	0.86	97
	10	0.5	0.445	0.43	96.5
	10	0.5	0.445	0.42	94.5
	10	0.5	0.445	0.45	101
Liver	10	0.884	0.788	0.69	89
	10	0.884	0.788	0.75	97
	10	1.768	1.576	1.42	91
	10	1.768	1.576	1.47	95
	10	1.125	1.0	0.96	96
	10	1.125	1.0	0.98	98
	10	1.125	1.0	0.97	97
	10	1.125	1.0	0.96	96
Brain	10	1.0	0.89	0.86	97
	10	1.0	0.89	0.91	102
	10	1.0	0.89	0.84	94.5
	10	1.0	0.89	0.94	105
	10	1.0	0.89	0.84	97
	10	2.0	1.78	1.70	95.5
	10	2.0	1.78	1.88	105
	10	2.0	1.78	1.80	101

Average recovery per cent 97.3 $\pm$ 4

SUMMARY

A method for the estimation of barbituric acids and thiobarbituric acids in tissues and animal fluids is described.

The method enables both types of barbiturates to be separated and estimated when they are present together.

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