13. THE ESTIMATION OF BARBITURATES IN BLOOD

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IN connexion with a study of the distribution and fate in the body of the shorter-acting barbiturates, evipan and pentothal, it was necessary to carry out estimations of these drugs in blood and other biological media. The quantitative extraction of these unstable compounds presents some difficulty, and none of the methods given in the literature for dealing with more stable barbituric acids [Brundage & Gruber, 1937; Fischer, 1939; Herwick, 1933; Linegar *et al.* 1935; Koppanyi *et al.* 1934] was found suitable. It was thus necessary to work out a satisfactory procedure.

In clinical work the need has long been felt for a simple method of determining barbiturate concentrations in blood. The method described here, in addition to giving satisfactory extraction of evipan or pentothal, has many advantages over previously described methods in the rapid, routine determination of any type of barbituric acid in blood. It was found that 20 ml. blood from a patient on only a moderate dosage of barbitone were quite sufficient for examination. A study has been planned of the use of the procedure as a routine clinical test. The method should also be of value in toxicology.

For quantities of 1 mg. or more of barbiturate, the observed maximum error for the complete procedure is 20 %, and for smaller quantities the error is correspondingly greater. This large experimental error is to be expected, since the colorimetric estimation after extraction is itself only accurate to within 10 %, and since the drugs in question are unstable and difficult to obtain absolutely pure. The method is considered sufficiently accurate to meet the requirements of the pharmacological research for which it was designed, and it is felt that efforts to determine evipan or pentothal more accurately would defeat their own end, owing to the instability of these compounds. Within the limits given, the method is very reliable; three determinations may be carried out in 5 hr., and require no attention for much of that time.

Some experiments have been carried out on the extraction of barbiturates from tissues, other than blood, with promising results. The technique may be applicable to the extraction of drugs other than barbiturates.

Method

Evipan and pentothal are completely insoluble in acid and neutral solutions, and in alkaline solution they are extremely unstable; hence no method of extraction involving a protein precipitation can be used. Methods of direct liquid extraction from acid media with large volumes of a fat solvent can hardly be considered suitable for a routine quantitative estimation. The technique finally adopted was to dry the blood with anhydrous sodium sulphate so as to give a fine powder which could be extracted in a Soxhlet apparatus with a fat solvent. After purification, the barbiturate was then estimated by Koppanyi's colour reaction with cobalt acetate and isopropylamine in chloroform

[Dille & Koppányi, 1934]. While this work was in progress, Griffon & Breton [1939] published a method for the detection of barbiturates, involving the use of sodium sulphate as a drying agent. These authors recommend as a qualitative test for barbituric acids a modification of Koppányi's reaction, using diethylamine in alcohol in place of isopropylamine in chloroform; this has been examined and found to be inferior to the original for quantitative work.

To acidify the blood before drying, sodium dihydrogen phosphate was added. The use of this has the advantage that no neutralization of the extract is necessary, nor is there any release of pigments from the blood as with strong acids. It was considered desirable to carry out the extraction at as low a temperature as possible, and ether was therefore at first used as solvent, but the fats extracted from the dried blood were found to interfere in the colour reaction. This difficulty was overcome by using the mixture of equal parts of ether and light petroleum (B.P. 30–40°), which Brundage & Gruber [1937] recommend for the elution of barbiturates from charcoal. With this solvent a crystalline residue practically free from fat was obtained.

The barbiturate thus extracted from blood could, in most cases, be taken up straight away in chloroform and estimated, but occasionally it was contaminated by a small quantity of phospholipins; this contamination always occurred with extracts of tissues other than blood. The contaminants must be removed to obtain a true colour match with the standard, and, as a routine precaution, all ethereal extracts were shaken with a mixture of charcoal and magnesium oxide [Fischer, 1939] for a few minutes before evaporation. Great care must be exercised in the use of an adsorbing agent for the purification of

Fig. 1. Recoveries of barbitone from blood for different times of extraction.

a barbiturate solution. Even under the conditions specified below for purifying extracts there was found to be a very slight, but quite definite, adsorption of the drug from a barbiturate solution in ether and light petroleum. Unfortunately, no alternative scheme of purification was found practicable.

All ether used in the estimation must be freed from oxides, as the smallest traces of these prevent the reaction with cobalt acetate, and they are not removed in the treatment with charcoal and magnesium oxide. Estimations can best be carried out serially.

Fig. 1 shows the relation found between time of extraction and percentage recovery of 10 mg. sodium barbitone added in 1-2 ml. water to 20 ml. citrated blood. From this it is seen that extraction is complete, within the experimental error of the colorimetric estimation, in 2.5-3 hr. Barbitone being one of the least soluble of the barbiturates in the solvent used, all extractions were continued for this time.

EXPERIMENTAL

Reagents

Extraction mixture. Oxide-free ether is prepared weekly by washing with ferrous sulphate solution and water, and distilling into a bottle containing clean copper wire. It is mixed before use with an equal volume of light petroleum (B.P. 30-40°) in a measuring cylinder, the walls of which retain any water that may separate.

Charcoal and magnesium oxide mixture. The charcoal (Merck's "Ultra-carbon") is washed with oxide-free ether containing a few drops of glacial acetic acid, and then with ether till free from acid. Three parts prepared charcoal are mixed with one part magnesium oxide.

Cobalt acetate solution. 1% in absolute methyl alcohol.

isoPropylamine solution. 5% by volume in absolute methyl alcohol.

Standard solutions for colorimeter. Solutions of the barbituric acid (not the sodium salt) under examination are prepared in chloroform in the following concentrations: 0.02, 0.04, 0.06, 0.08, 0.10, 0.12%. These are stored in dark, glass-stoppered bottles.

Procedure

In a mortar of 250 ml. capacity, 20 ml. blood (coagulated or citrated) is mixed with 2 g. NaH_2PO_4 . There are then added, in small portions with constant grinding, 40 g. anhydrous Na_2SO_4 . After thorough mixing of blood and sulphate, the mortar is placed in a desiccator for 10-15 min. to allow complete drying to occur. The dry cake obtained is broken up and packed into a 100 ml. Soxhlet thimble. It is advisable to place a filter paper in the top of the filled thimble to prevent displacement of the powder. Extraction is carried out for 2.5-3 hr. at 50° with 80 ml. of ether-petroleum mixture. The addition of a few glass beads to the ether is necessary to facilitate even boiling.

At the end of the extraction the contents of the flask are shaken for a few minutes with about 0.25 g. charcoal and magnesium oxide. This is then removed by filtration directly into a distilling flask through a Jena sintered glass funnel, washing with small quantities of ether-petroleum, and the filtrate is distilled at 50°. The crystals of barbiturate which remain in the flask are taken up in a small quantity of chloroform and transferred to a 10 ml. graduated centrifuge tube, the solution being made up to a convenient volume. Owing to the difficulty of measuring chloroform, the loss of accuracy involved in the use of graduated tubes for this purpose was considered to be justified by the gain in rapidity of working.

To 2 ml. of the chloroform solution are added 0.1 ml. cobalt acetate solution and 0.6 ml. iso-propylamine solution, and the colour is compared in a micro-

colorimeter with that given by 2 ml. of one of the standard solutions similarly treated. It may be necessary to dilute or concentrate the unknown solution to bring it within the range of concentrations covered by the standards.

Results

Figures are shown in Table 1 for the recoveries of four barbituric acids added, as their sodium salts in 1 ml. water, to 20 ml. citrated blood. Blank determinations were carried out, using double quantities of citrated or fresh coagulated blood to which no barbiturate had been added. After distilling off the ether-petroleum mixture, the flask was washed out either with 2 ml. chloroform or with one of the standard barbiturate solutions. No colour was produced with cobalt acetate and *isopropylamine* in the former case, and in the latter no change was observed in the intensity or shade of the colour given by the standard solution. From the latter series of observations it appears that, under the stated conditions, there is extracted from blood no substance which could cause a false result through an additive or potentiating effect on the reaction between barbiturates and cobalt acetate.

Table 1

Barbiturate	Added mg.	Found mg.	Recovery %
Barbitone	10.4	9.5	91
	8.9	8.9	100
	4.5	4.6	102
	1.04	1.05	101
	0.45	0.56	124
Phenobarbitone	10.3	8.5	83
	5.1	4.3	84
	0.51	0.41	80
Pentothal	9.3	7.8	84
	4.6	4.3	94
	0.46	0.59	128
Evipan	9.9	9.3	94
	8.7	7.2	83
	8.7	8.8	101

Samples of blood (supplied by Dr H. Tod) from three patients, to whom barbitone had been regularly administered for years, were examined by this method (Table 2). The dosage was such as had been found to give the same degree of depression in each case.

Table 2

Case	Daily dose mg./kg.	Barbitone found mg./20 ml.
Mrs G.	20.2	1.1
Miss D.	16.5	1.1
Miss S.	16.0	1.0

SUMMARY

A simple procedure has been developed for the extraction of unstable barbiturates from blood. It has been found satisfactory for use in the routine determination of any type of barbiturate.

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