THE ESTIMATION OF BARBITURIC ACID DERIVATIVES IN BIOLOGICAL MATERIAL FOR MEDICO-LEGAL PURPOSES

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In medico-legal determinations of barbiturates in biological materials the usual method, the gravimetric Stas-Otto process, is time-consuming. A difficulty attending this type of examination is that the short-acting barbiturates may be almost completely metabolised (Brodie, Burns, Mark, Lief, Bernstein and Papper[1]). Hence, apart from those occasions when the examination may show the presence of such a small amount of barbiturate that it cannot account for the death, there are numerous occasions when this lengthy process gives negative results. Thus in its medico-legal application any method should possess the advantage of being rapidly conducted and, as its use may be extended to forensic cases admitted to hospital in a probable barbiturate coma, should be applicable to small volumes of body fluids.

Experience in this laboratory has confirmed the recognised disadvantages of some of the existing methods. Additional processes introduced in extraction techniques by some workers to eliminate the interfering factors have so lengthened the time of performance that, for medico-legal purposes, the methods have no advantages over large-scale procedures such as that of Valov[2].

Nature of Interfering Factors

Gould, Mayo and Bowman[3] used an ether extraction process with a semi-micro continuous extractor and determined barbiturates spectrophotometrically by absorption in the ultra-violet region. They found that their method extracted other ultra-violet absorbing materials from serum, and for 100 samples of normal human serum (that is, serum supposedly free from drugs) the optical densities of the extracts varied from 0·040 to more than 0·200 at 240 mμ. As the majority of sera gave these blank values in the range 0·100 to 0·150 with a mean at 0·130, these workers selected this last figure as the standard absorption value for serum. Walker, Fisher and McHugh[4] drew attention to the large amount of material, absorbing at 240 mμ, which was extracted by the process of Jailer and Goldbaum[5] from 1 ml. of serum. This method involved a chloroform extraction of buffered plasma with subsequent transference of the thiopentone into sodium hydroxide solution. As these extracts from normal sera showed variation in absorption when they were in acid and alkaline medium, Walker *et al.*[4] discarded the method of direct extraction of the blood with chloroform in favour of a procedure giving a blank absorption which was independent of *p*H. In their method a tungstic acid filtrate of blood was extracted with chloroform and the latter was then extracted with 0·05 per cent. aqueous sodium hydroxide. The *p*H of the alkali was

adjusted to within a range of 9·0 to 10·5 and the absorption spectrum in the ultra-violet determined. The barbiturate was reported as the difference in absorption at 240 mμ between *p*H 10 and *p*H 2. The average recovery of pentobarbitone added to 2·5 ml. blood was 69 per cent. by this method, and determinations with other barbiturates returned a similar figure.

Tests were conducted in this laboratory with the various methods for the estimation of barbiturates in small samples of blood. Direct extraction using chloroform followed by sodium hydroxide was tried but was found highly unsatisfactory. On barbiturate-free material the absorption varied from sample to sample, the optical densities reaching values similar to those found by Walker *et al.*[4]. Their protein precipitation method was then tried both on normal sera and on sera to which phenobarbitone had been added. While the absorption in the case of normal sera was reduced so that maximum optical densities were in the vicinity of 0·10, the process was a long one and had the disadvantage that rather large losses of barbiturate were incurred, probably in the precipitation stage. It will be agreed by most of those engaged in the practice of forensic chemistry that calculations of concentrations which allow for losses incurred during analysis may not be well received in courts of law. In this country, at least, toxicological chemists prefer to report the actual amount estimated as the "minimum concentration" and sacrifice the amount lost in the extraction process rather than be placed in the position of explaining to a jury, uninformed in chemical procedure, why a large percentage has been added to the actual figure obtained in the estimation. In the method described by Walker *et al.*[4] the average recovery of barbiturate was 69 per cent., so that almost half this amount has to be added in each case. With phenobarbitone—probably the most commonly used barbiturate—a further correction of 13·6 per cent. is made for its own absorption in acid solution. For purposes other than forensic the method of Walker *et al.*[4] is reliable for the estimation of barbiturates but, to the forensic chemist, the above objection is real.

Extraction of the Chloroform

In view of the foregoing it was decided to investigate whether the advantage of the Walker method—elimination of interfering substances—could be gained from a direct extraction process, thus reducing the loss of barbiturate. As indicated, the chloroform—sodium hydroxide extraction process is unsuitable for quantitative application. In addition, the *p*H of the extracting sodium hydroxide solution must be strictly controlled for reasons outlined by Walker *et al.*[4], and for further reasons described below.

In an effort to simplify the extraction procedure by elimination of the close control of the *p*H of the aqueous extracting phase, aqueous ammonia solution was tried as the transferring agent of the barbiturate from the chloroform. It was thought that the use of this weak base might also extract less of the interfering "chromogens". Tests showed that ammonia behaved in a manner similar to sodium hydroxide in the regions below *p*H 9 (Fig. 1). Above this value the behaviour of the two bases differed. In sodium hydroxide solution between *p*H 9 and 10·5 the extinction for a

given concentration of barbiturate is constant; above *p*H 11 the maximum at 240 mμ gradually falls and the peak moves in the direction of the visible spectrum. Walker *et al.*[4] demonstrate a curve at *p*H 12 showing a maximum at 245 mμ with a decreased optical density, and they regard this as the barbiturate molecule having undergone a further change, probably due to a second stage of ionisation. Figure 2, however, shows that this is only the beginning of the change as the alkali concentration increases. The maximum moves through 245 mμ and 250 mμ to become stationary at 255 mμ. This maximum at 255 mμ is reached when the sodium hydroxide concentration is between 0·05 and 0·075N. On further increasing the alkali concentration through 0·1 and 0·5N the optical density at 255 mμ increases until at concentrations of 0·5N and above, the optical density is constant. This fact does not appear to have been previously reported, and the results described are shown in Table I.

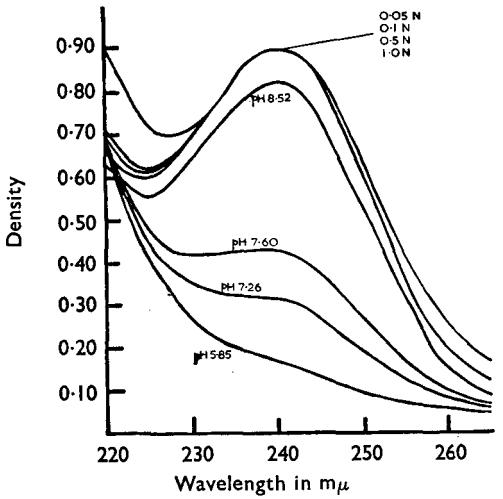

FIG. 1. Absorption curves for 20 μg./ml. of phenobarbitone. In all the curves above *p*H 8·52 the solutions are in ammonia at the concentration indicated.

Stuckey[6], in 1941, determined the absorption spectra of phenobarbitone and 1-methylphenobarbitone in 0·1N sodium hydroxide; he found the molecular extinctions for these compounds to be 8800 and 9000 respectively, but while in the case of phenobarbitone the maximum occurred at 256 mμ, that of 1-methylphenobarbitone occurred at 246 mμ. Methylation appears to have prevented the second ionisation stage and the consequent optical changes.

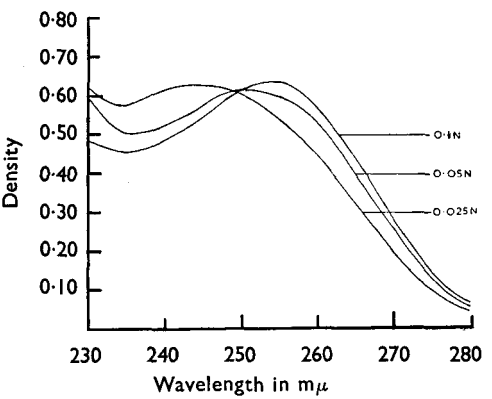

FIG. 2. Absorption curves for 20 μg./ml. of phenobarbitone in sodium hydroxide solution at the concentrations shown.

When aqueous ammonia solution was used as the extracting phase for chloroform it was found that for concentrations up to 15N the maximum

did not show any tendency to move from 240 mμ; the concentration of the extracting ammonia solution could thus safely vary between 0·1N (*p*H 11·1) and 1N (*p*H 11·4) without affecting either the magnitude of the optical density or the position of the maximum. In addition, these extracts could be left overnight without significant alteration.

TABLE I

Concentration of sodium hydroxide solution	Wavelength of maximum (mμ)	Optical density
0·025N	245	0·654
0·05N	250	0·638
0·1N	255	0·662
0·25N	255	0·680
0·5N	255	0·694
N	255	0·694

Quantitative Estimation

Since aqueous ammonia solution was a satisfactory extracting phase in the above respects, the relation between the maximum absorption values and the concentration was determined. Distilled water was put through the extraction process outlined below for blood, and the final 0·5N ammonia so obtained was used as the dissolving medium for phenobarbitone to produce curve 1 of Figure 3. This shows that the magnitude of the absorption at 240 mμ is proportional to the concentration of the dissolved barbiturate at least to a concentration of 25 μg./ml. With these solutions still in the silica cells of the spectrophotometer, 8N sulphuric acid was added to reduce the solutions to *p*H 2. After stirring, curve 2 of Figure 3 was obtained which, when corrected for dilution in the cells, showed that the acid absorption of phenobarbitone was regularly 15 per cent. of the alkaline value. It thus followed that aqueous ammonia solution was more suitable than sodium

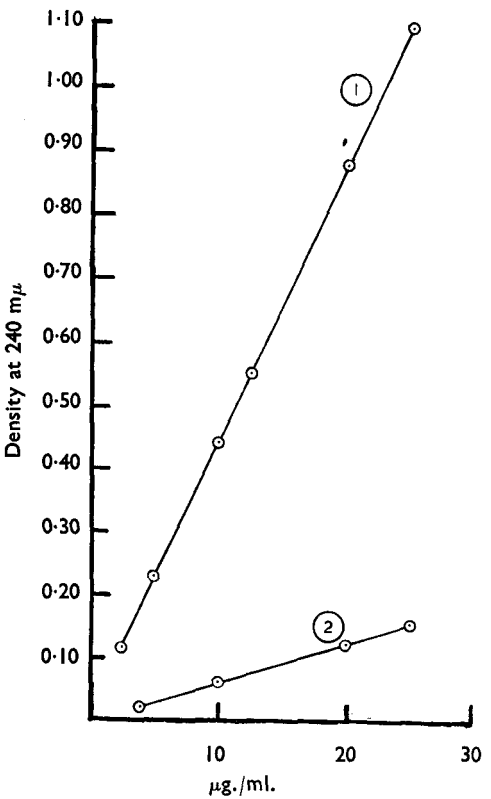

FIG. 3. Curve 1 shows the relationship between optical density and concentration of phenobarbitone in 0·5N ammonia. Curve 2 shows the same relationship when solutions are at *p*H 2.

hydroxide as the extracting phase for chloroform since the derivative is stable in the former solvent and the necessity to observe close control of *p*H is eliminated.

Differential Extraction

The barbiturates are not extracted by the usual organic solvents from aqueous solutions of sodium hydroxide because they exist as salts. An attempt was therefore made, in order to avoid the precipitation loss found in the method of Walker *et al.*[4], to remove interfering substances by a process of differential extraction. Hence samples of barbiturate-free serum and urine were first made alkaline with sodium hydroxide and extracted with chloroform. This chloroform was discarded. The serum and urine samples were then acidified with hydrochloric acid and again extracted with chloroform.

The chloroform extract of the acidified material was then washed with a very dilute solution of sulphuric acid. Substances which partition fairly evenly between the organic and aqueous phases, and which would therefore be partially transferred to the chloroform during the acid extraction, were thus partially removed from the chloroform.

This chloroform from which nearly all interfering substances had, in the case of serum, been removed, was now extracted with aqueous ammonia solution and read on the spectrophotometer over the range 225 to 290 mμ. In a series of 40 extractions of different barbiturate-free sera by this method, the optical densities varied from 0·012 to 0·078 at 240 mμ. The majority were between 0·05 and 0·06 and only 2 were above 0·065. It was therefore evident that with such low and constant absorption in blank determinations there was no necessity with this method to read serum samples at *p*H 2 to obtain barbiturate absorption. Consequently in subsequent determinations the standard normal absorption of 0·050 was adopted for sera and was subtracted from the optical density of the barbiturate absorption at 240 mμ.

TABLE II

Phenobarbitone added to 2 ml. serum µg.	Recovery per cent.
66·6	92, 90
133	88, 91
200	87, 88, 90, 92

The percentage recovery of barbiturate added to 2 ml. of serum was determined by simultaneously determining the absorption given by extracting 2 ml. of the same serum to which no barbiturate had been added. The recovery after subtraction of the blank absorption is given in Table II.

Urine Extraction

The spectrophotometric determination of barbiturates in urine has received less attention in the literature than of that in blood. An objection to urine estimations may be that extractable metabolic products in which the barbiturate ring is still intact may contribute to the absorption. In the forensic field this may be of assistance in those instances where the blood barbiturate level is inadequate to account for the death, and may serve to support an opinion that a large amount was originally ingested.

Extraction of 1 ml. samples of normal urine by the chloroform-sodium hydroxide method gave absorptions in many cases which were beyond the capacity of the spectrophotometer to measure. When the volume was reduced to 0·2 ml., urine from patients known to have consumed large amounts of barbiturates gave absorptions in which the characteristic curve of the barbiturate was present but obscured. By applying the method described below, and adopting the Walker principle of measuring between *p*H 10 and 2 it was found that normal absorption could be reduced to measurable amounts (*ca.* 0·200–p·450), and subtraction of the *p*H 2 values restored the barbiturate curve.

METHODS

Process for Blood

Procedure. Three 50-ml. separating funnels are clamped on a burette stand one beneath the other. The reference blank is prepared by using 2 ml. of distilled water and following through the method.

1. 2 ml. of serum or plasma is placed in the top separating funnel and 1 ml. of 0·25N sodium hydroxide is added. This is shaken for 3 minutes with 15 ml. of chloroform A.R. When the phases have separated this chloroform is run off and discarded.
2. 1·5 ml. of 0·5N hydrochloric acid and 20 ml. of chloroform A.R. are then added to the aqueous phase in the funnel and the whole shaken for 3 minutes. After separation the chloroform is run through cotton wool held in a small filter funnel into the separating funnel below. The original aqueous phase is re-extracted with 10 ml. of chloroform A.R. and transferred in a similar manner to the second separating funnel.
3. This chloroform in the second funnel is shaken with 7 ml. of 0·1N sulphuric acid for 2 minutes, and the organic phase is run into the third funnel through cotton wool. 10 ml. of 0·5N ammonium hydroxide is added to this funnel and shaken for 3 minutes. The aqueous phase is transferred to a centrifuge tube in which it is centrifuged at 2000 r.p.m. for 2 minutes. The solution is then read in the spectrophotometer, against the standard blank, over the range 225 to 290 mμ.

Process for Urine

The volume of urine to be tested may vary from 0·2 ml. to 2 ml., depending on the concentration suggested by the blood level.

The procedure as for blood is used, except that 20 ml. of 0·5N ammonium hydroxide is used to extract the chloroform. Into the absorption cells is put 3·5 ml. of the ammonia extract and the absorption is determined. 7 ml. of the extract is titrated with 8N sulphuric acid to determine the volume necessary to reduce the *p*H of the solution to *p*H 2. The solutions are then treated with the appropriate volume of acid and stirred; the absorption at *p*H 2 is then determined. Correction must be made for the dilution in the cells and for the fact that, with phenobarbitone, there is a regular absorption by the acid form amounting to 15 per cent. of the alkaline form. The latter correction is unnecessary with the other common barbiturates.

SUMMARY

1. A method is described for the estimation of barbiturates in biological material.

2. The method avoids the extraction of substances giving undesirable blank values inherent in chloroform-sodium hydroxide extraction procedures.

3. The method simplifies the spectrophotometric determination of those procedures using sodium hydroxide as the vehicle for the barbiturate in the spectrophotometer.

4. The percentage recovery is about 90 per cent. and thus avoids, in forensic determinations, mathematical calculations for material which is not actually measured.

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