A STUDY OF BARBITURATE INTOXICATION BY AN ULTRA-VIOLET SPECTROPHOTOMETRIC TECHNIQUE

BY

J. T. WRIGHT AND R. G. S. JOHNS

From the London Hospital, E.1

(RECEIVED FOR PUBLICATION AUGUST 23, 1952)

The diagnosis of barbiturate intoxication is usually made from circumstantial evidence coupled with a characteristic, if non-specific, clinical picture. The need for a confirmatory laboratory test is often felt.

Riley, Krause, Steadman, Hunter, and Hodge (1940) have described the shortcomings of the commonly used cobalt color reactions. The extraction of barbiturate crystals and the determination of their melting point must remain the most satisfactory method of analysis for medico-legal work, but this technique is limited in clinical practice by the large quantities of biological material required and the time involved in the extraction and purification processes.

During the past decade research has proceeded along a number of lines, including ultra-violet and infra-red spectroscopy, x-ray crystallography, and paper chromatography. Most attention has been directed to ultra-violet spectrophotometry, which is at present the most practicable procedure in view of available suitable equipment. The details of procedure and the form of the absorption curve are similar for all but the thio-barbiturates, which absorb in a different region of the spectrum, but the general shape of the absorption curve is of some, though limited, qualitative value in identifying the particular barbiturate present.

The ultra-violet absorption spectra of barbiturate compounds have of course been known for many years. In 1940 Stuckey studied the absorption of a number of derivatives in acid and alkaline solution and came to important conclusions about their characteristics from which he suggested that the technique might be applicable to the analysis of tissues containing barbiturate. At the same time Elvidge (1940) assayed barbiturate tablets by this method. Jailer and Goldbaum (1946) and Walker, Fisher, and McHugh (1948) subsequently applied the principle to medical problems. Born (1949) in London, and Lous (1950) in Copenhagen, developed similar methods. All these methods depend on the fact that both the position and height of the absorption maximum shown by barbiturate is a function of pH, and also that most barbiturates show negligible absorption at wavelengths greater than 235 m. for pH values below 6.0.

The technique which we have employed is based on published methods, and aims to combine a reasonable degree of accuracy with a minimum of procedure. By its use it is possible to estimate the barbiturate content of four samples of blood or urine in about two hours.

Method

A Hilger "uvispek" spectrophotometer was employed in the present work. Measurements of pH were made with a lithium glass electrode.

Five ml. of heparinized whole blood is placed in a 50-ml. glass-stoppered flask and shaken for three minutes in a mechanical shaker with 25 ml. of distilled pure chloroform. The mixture is filtered through a Whatman No. 1 paper into a similar flask and the residual sludge washed with a further 5 ml. of chloroform, the washings being filtered and combined with the main extract. The filtrate, which is clear but may be slightly yellow, is shaken for three minutes with 5 ml. of 0.05% aqueous sodium hydroxide. The mixture is transferred to a separating funnel; the chloroform layer is discarded and the aqueous layer collected into a test tube. After allowing time for chloroform droplets to settle, 4 ml. of the solution is pipetted into a clean tube containing 2 ml. of 0.5 M borate buffer, pH 10. As a further precaution against chloroform contamination a fine stream of nitrogen is blown through the sample for 30 seconds.

The absorption curve of the sample is obtained over the range 220 to 300 m. against a blank consisting of 4 ml. of 0.05% sodium hydroxide and 2 ml. of borate buffer. A 10-mm. cell is usually suitable, and particular attention is paid to the range 235 to 245 m. The test and blank solutions are then returned to their respective test tubes and 0.5 ml. of concentrated hydrochloric acid added to each. After mixing, the absorption curve is again obtained over the same wavelength range.
BARBITURATE INTOXICATION BY SPECTROPHOTOMETRY

The extraction procedure for urine, cerebrospinal fluid, and gastric contents is similar to that for blood except that the specimens are adjusted to approximately pH 6 before extraction, and the separation of the two phases after the initial chloroform extraction is performed by the use of a separating funnel, with subsequent filtration of the extract. For the estimation of barbiturates in tissues we have used 1 to 2 g. of wet sample homogenized with 10 ml. of phosphate buffer, pH 6, in a large glass homogenizer. The total volume is made up to 15 ml. and a 5-ml. aliquot extracted in the usual way. Troublesome emulsions frequently occur; we have not been able fully to overcome this difficulty by the use of other solvents as suggested by Goldbaum (1948).

Calculation.—This is made after Walker, Fisher, and McHugh (1948). The barbiturate concentration in μg. per ml. of the final extract (i.e., of the solution as measured in the spectrophotometer cell) is given by the expression

\[ \frac{M \times (E_{\text{alk}} - E_{\text{ac}}) \times 10^6}{\varepsilon_{\text{max}}} \]

where \( M \) = molecular weight of barbiturate; \( E_{\text{alk}} \) = optical density in alkaline solution at 239 m\( \mu \) and 10-mm. depth; \( E_{\text{ac}} \) = optical density in acid solution after correction for dilution due to addition of acid; \( \varepsilon_{\text{max}} \) = molecular extinction of the barbiturate at 239 m\( \mu \). The barbiturate concentration in the original specimen may readily be calculated from this value.

The values for \( M \) and \( \varepsilon_{\text{max}} \) in the above expression clearly depend upon the particular barbiturate present. Where the actual compound is unknown we prefer to calculate its concentration provisionally in terms of barbital; since this substance has a low molecular weight and a high molecular extinction a conservative estimate is thereby obtained. In the special case of phenobarbitone a correction is necessary, since this derivative has an abnormally high absorption in acid solution.

Results

Fig. 1 represents the absorption spectra in alkaline and acid conditions of 5 ml. samples of blood from 10 normal subjects. In the absence of barbiturate it is seen that the absorption is practically the same in both acid and alkali at 239 m\( \mu \). (This wavelength is the normal position of the barbiturate peak.)

These blood samples were used to determine the percentage recovery of varying concentrations of barbital added to 5 ml. samples. Over a concentration range of 100 to 250 μg. per 5 ml. (2 to 5 mg. per 100 ml.) the mean recovery in 17 estimations was 60% with a standard error of the mean of ±1. With concentrations below 100 μg. per 5 ml. it was not always possible to determine the precise amount of barbital because an absorption peak at 239 m\( \mu \) was not always observed owing to the relative preponderance of other chromogens. However, with experience of the method it is possible to make a fair estimate of barbiturate down to levels of 50 μg. per 5 ml. At concentrations above 250 μg. per 5 ml. a lower recovery was obtained; thus the recovery is 55% at 600 μg. per 5 ml., and about 50% with concentrations of 1,000 μg. per 5 ml. (20 mg. per 100 ml.).

Since 60% is the recovery obtained over the range found in cases of intoxication, we have felt justified in correcting the final figure for this recovery (i.e., the experimental value is multiplied by 1.67). Using this correction factor, significant over-estimation of the blood level of barbiturate is unlikely. Recovery experiments have not been made on other compounds because it would appear from the work of Walker, Fisher, and McHugh (1948) that no significant variations exist. Urine and gastric contents are so variable in composition that we prefer to give the uncorrected estimate of barbiturate content.

Clinical Data

Full details of the clinical investigation will be published elsewhere. A brief summary has been made here for correlation with the laboratory findings.

Table I shows some of the data relating to 17 cases of poisoning so far investigated. Previous authors have commented on the lack of any
close correlation between blood level and clinical condition, and particular stress has been laid on the fact that epileptic and other barbiturate habitues may regain consciousness while their blood levels are still high. In Table I, Case 8 was a known epileptic, Case 4 may have been, and Cases 12 and 16 were known to be habituated. Nevertheless, from Table I it would appear that, for a given barbiturate, a correlation between blood level and clinical state does exist when all factors are taken into account. The time given for full recovery is very approximate, and signifies full return to consciousness rather than cessation of vertigo and diplopia, since these symptoms invariably last for several days after severe intoxication. Alcohol ingestion was only presumptive in three cases, but if indeed present it has a significant bearing on the prognosis. The relatively weak hypnotic action of barbital is evident from the high blood levels compatible with consciousness; on the other hand, it appears that patients suffering from intoxication by rapidly acting barbiturates may be dangerously depressed even with blood barbiturate levels at the lower limit of reliable estimation. Barbital is freely excreted, and the urine output and blood level show an excellent correlation. A relatively good correlation is found with the rapidly acting barbiturates. In our limited experience pheno- barbitone is poorly excreted, and high blood levels may occur with a relatively low urine output.

In two cases, 4 and 11, there was no circumstantial evidence of ingestion of barbiturate. In these cases crystals were obtained from chloroform extracts of urine; the crystals gave no depression of melting point with pure samples of phenobarbital and barbitone respectively. As additional confirmation the absorption curves of equal weights of the urine crystals and the known compounds compared very favourably (Figs. 2 and 3).
BARBITURATE INTOXICATION BY SPECTROPHOTOMETRY

Post-mortem material was obtained from Cases 3 and 4, these patients dying in hospital. Table II indicates the minimum barbiturate concentrations in some tissues examined by our method, and Fig. 4 illustrates the spectra obtained with extracts of blood and liver from Case 4.

Where interfering chromogens are present in high concentration, particularly with urine extracts,

**Table II**

<table>
<thead>
<tr>
<th></th>
<th>P.M. Blood* (mg./100 ml.)</th>
<th>Liver (mg./100 g.)</th>
<th>Kidney (mg./100 g.)</th>
<th>Suprarenal Gland (mg./100 g.)</th>
<th>Spleen (mg./100 g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 3</td>
<td>3.3</td>
<td>3.5</td>
<td>2.8</td>
<td>2.6</td>
<td>2.1</td>
</tr>
<tr>
<td>Case 4</td>
<td>7.0</td>
<td>9.2</td>
<td>2.8</td>
<td>2.6</td>
<td>2.1</td>
</tr>
</tbody>
</table>

* The blood level is corrected on the basis of 60% recovery; other values are uncorrected and are therefore minimum levels.

no peak may be found in the absorption curve, or a peak may be apparent at a wavelength lower than the 239 mμ, which is usually the case with barbiturates. A relatively low absorption in acid solution may be suggestive of the presence of barbiturate, but even if there is definite evidence of the ingestion of barbiturate no reliable estimate of amount can be made, although a maximum value may be surmised. In the absence of a peak at 239 mμ the presence of barbiturate cannot be inferred without risk, for at least one common group of substances (phenol derivatives) absorbs radiation in alkaline solution but not in acid solution at this wavelength.

In Case 17 the presence of a preponderance of interfering substances in a urine extract can be inferred from the shift of the barbiturate peak to 235 mμ and from the high absorption in acid solution (Fig. 5). Amytal crystals were obtained.
from chloroform extracts of this urine in sufficient quantity to allow a macroscopic melting point determination to be made. A solution of these crystals showed the normal peak at 239 mμ.

**Discussion**

The method described above is similar to that developed by Lous (1950), which is in turn based on both the procedures of Goldbaum (1948) and Walker et al. (1948). We, however, use whole blood rather than serum, and in this respect, if heparin is the particoagulant, it is essential to use the pure product. Heparin as supplied for intramuscular use contains phenolic preservatives which absorb in alkaline solution over the critical range (Giotti and Maynert, 1951). Oxalate and fluoride do not appear in the final extracts and may be used in place of heparin. In the method described by Lous barbiturate is removed from the chloroform extract by direct shaking with buffer; we have used weak alkali, as there is some evidence that this is more efficient. The pH is subsequently brought to a value of 10 by the addition of buffer. Lous did not define the lower limit of sensitivity of his technique, and it is impossible to assess his recovery rate as he estimates unknown blood levels by comparison with calibration curves based on the extraction of known amounts of barbiturate added to normal serum.

In Nilsson's (1951) series Lous determined the blood barbiturate levels of 58 cases. Unfortunately most of the patients were poisoned by ally-propymal (alurate), a drug of which we have no experience, but four cases of phenobarbitone poisoning are described. The most severely affected was a man of 58 with an initial blood level of 17.1 mg. per 100 ml. who died after eight days from anuria and hyperpyrexia. The three other cases were men between the ages of 31 and 52 who recovered after initial blood levels of 15.5 mg. per 100 ml. (deep coma); 9.9 mg. per 100 ml. (deep coma, epileptic); and 5.3 mg. per 100 ml. (light coma).

The method described by Walker, Fisher, and McHugh (1948) has theoretical advantages, but despite the elaborate steps taken to procure a relatively chromogenic-free alkaline solution for spectroscopy, their normal blood extracts appear to absorb nearly as much radiation as ours and their recovery rate is only 70%. They claim a sensitivity of 0.4 mg. per 100 ml. of blood, and using this technique, Fisher, Walker, and Plummer (1948) found two cases of coma following ingestion of seconal and phenobarbital with blood levels as low as 1.0 and 0.8 mg. per 100 ml. respectively.
In two fatal cases blood pentobarbital levels of 0.6 and 0.7 mg. per 100 ml. were found, but both patients were chronic alcoholics. Walker, Fisher, and McHugh discuss in some detail the identification of other drugs, notably salicylates and sulphonamides, which may interfere with the spectrophotometric estimation of barbiturate, and they mention the advisability of scanning the whole range between 220 and 300 m\(\mu\). They also state that picrotoxin, benzedrine, and caffeine do not interfere. This has also been our experience, but it is possible that xanthine derivatives contribute to the high levels of chromogens in urinary extracts.

Goldbaum (1948) also claimed a sensitivity of 0.4 mg. per 100 ml. of blood. He summarizes barbiturate analyses on seven fatal and three non-fatal cases. It is of some interest that in two fatal cases poisoned by seconal and pentobarbital respectively he found a concentration of barbiturate in the liver five times that in the blood. Born (1949) uses an ethanol-ether-alkali extraction technique; he mentions that the method is suitable for blood, but gives no clinical data. Giotti and Maynert (1951) used an ether extraction method for studying the renal clearance of barbital in dogs. By treating their ether extracts of blood and urine with activated charcoal they achieved considerable reduction in interfering chromogens without significant loss of barbital. It is doubtful whether this refinement can be applied in the case of all barbiturates, particularly where chloroform is the solvent.

Summary

A modification of existing methods for the ultra-violet spectrophotometric estimation of barbiturates in biological fluids and tissues is described.

Seventeen cases of barbiturate intoxication are recorded; blood and urine barbiturate levels appear to be related to the degree of depression of the nervous system.

Blood barbital levels up to 10 mg. per 100 ml. and phenobarbitone levels up to 5 mg. per 100 ml. may be compatible with consciousness, whereas coma is usually produced with levels of short-acting barbiturates of only 1 to 2 mg. per 100 ml. Alcoholic intoxication may be a complicating factor.

Urinary clearance is low for phenobarbitone, good for quick-acting barbiturates, and high for barbital.

We wish to express our gratitude to Professor Clifford Wilson for encouragement and criticism, to Dr. and Mrs. Morris and Drs. Holiday and Beavan for advice, to the physicians of the London Hospital, Dr. Alastair Hunter of St. George's Hospital, and Dr. F. E. Camps for allowing us to take specimens, to Professor J. R. Marrack for making available the Hilger "uvispek" spectrophotometer, and to Mr. C. H. Sykes, chief pharmacist at the London Hospital, for his constant co-operation.

References