CHROM. 6049

Determination of low levels of amobarbital in serum by gas–liquid chromatography

A variety of methods is available to measure amobarbital in serum[1–7]. Most of these lack sensitivity and require high concentrations of drug in the serum. These levels are seen only in cases of barbiturate poisoning. Methods to measure low levels of amobarbital in serum demonstrate a precision of only ± 0.5 μg/ml[2,7]. This degree of imprecision can be tolerated if the values obtained serve only to correlate serum levels with therapeutic effect. If, as in the present case, it is desired to study the pharmacokinetics of amobarbital elimination in individuals ingesting a single 65-mg dose of the drug, a method displaying greater accuracy is needed. The present paper describes a method developed for this purpose. Levels of amobarbital, in the range of 0.3–1.5 μg/ml of serum, can be measured with a possible error of less than ± 0.05 μg/ml. It must be emphasized that the method to be described was developed as a research tool to measure low levels of amobarbital without interference from endogenous materials. No guarantee is offered that other drugs will not interfere. For the purpose of our study this was not necessary. Subjects participating in the project were carefully controlled and received only amobarbital.

Materials and methods

Gas–liquid chromatography. A F & M Model 402 gas–liquid chromatograph with a flame ionization detector (Hewlett-Packard) was used. The U-shaped glass column (120 cm × 4 mm I.D.) was packed with 3% SE-30 on 60–80 mesh, AW–DMCS Chromosorb W (Varian Aerograph) and conditioned for 16 h at 280°. Operating conditions: column temperature, 200°; injector port at 240°; detector at 280°. Gas flow: carrier gas helium, 60 ml/min; hydrogen, 38 ml/min; air, 310 ml/min. Electronic integrator: Model 3370-A (Hewlett-Packard).

Reagents and materials. Amobarbital, free acid, was kindly provided by Eli Lilly Co., Ltd., Toronto, Canada; [2-^{14}C]amobarbital by Amersham/Searle Corp.; QF-1 and OV-17 by Varian Aerograph; methyl stearate by Sigma Chemical Co.; and Reacti-Vial, 1 ml, by Pierce Chemical Co.

Standard curves. A standard containing 0.020 to 0.140 mg of amobarbital per ml of chloroform together with 0.200 mg of methyl stearate per ml was prepared; 1 μl of the standard was injected into the column. Before the injection, the active sites of the column were saturated with 10 μg of amobarbital. The retention times of amobarbital and methyl stearate were 0.8 min and 2.9 min, respectively. The peak heights of both amobarbital and methyl stearate were measured and the ratios were plotted against the amobarbital concentration.

Extraction procedure. One milliliter of serum was placed in a test tube together with 3 ml of diethyl ether and a solution containing 2.00 μg of methyl stearate in 0.5 ml of chloroform. The liquids were mixed with the aid of a Vortex mixer. If the mixing resulted in emulsion, the mixture was centrifuged. The organic layer was transferred to another test tube. The extraction was repeated twice with 3 ml of ether containing 20% chloroform. The extraction was checked with [^{14}C]amobarbital and found to be 98.3 ± 0.6% (S.D.). The combined organic layers were

washed with 0.5 ml of 0.01 *N* hydrochloric acid and then reduced to a small volume by passing a stream of nitrogen over it. This volume was transferred to a Reacti-Vial and the evaporation completed. Ten microliters of chloroform were added to the Reacti-Vial and 1 μl was injected into the column. After four to five determinations, the oven temperature was raised to 280° and kept at 280° for 20 min to flush out the high boiling substances extracted from serum.

Results and discussion

To obtain good sensitivity for amobarbital quantitation, it was found that unconventionally coarse solid support 60–80 mesh Chromosorb W, coated with SE-30, gave almost twice the peak height obtained with 80–100 or 100–120 mesh, without loss of resolution. Besides SE-30, other liquid phases such as OV-17 and QF-1 were also tried, but SE-30 gave the best separation of amobarbital from endogeneous materials in serum. Therefore, we chose 3% SE-30 on 60–80 mesh Chromosorb W, for the low level determination of amobarbital in serum.

Although attempts have been made to improve barbiturate quantitation by the formation of methyl or ethyl derivatives[4–6], an additional step in the analysis did not bring substantial gains. Only recently, a definite improvement was achieved by combining thin-layer (TLC) and gas–liquid chromatography (GLC)[8]. Unfortunately, this combination method is time-consuming and the double processing tends to give rise to inaccuracies, $\pm$ 0.2 μg/ml.

When a standard was injected into the column and the peak height ratios of amobarbital and methyl stearate were plotted against the amobarbital concentration, linearity was obtained between 0.02 and 0.14 μg of amobarbital as shown in Fig. 1. This linear relation was also obtained when the peak area ratios were plotted.

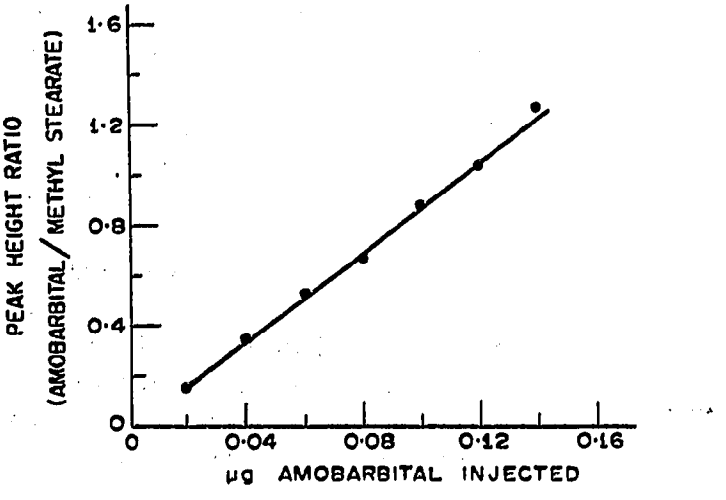
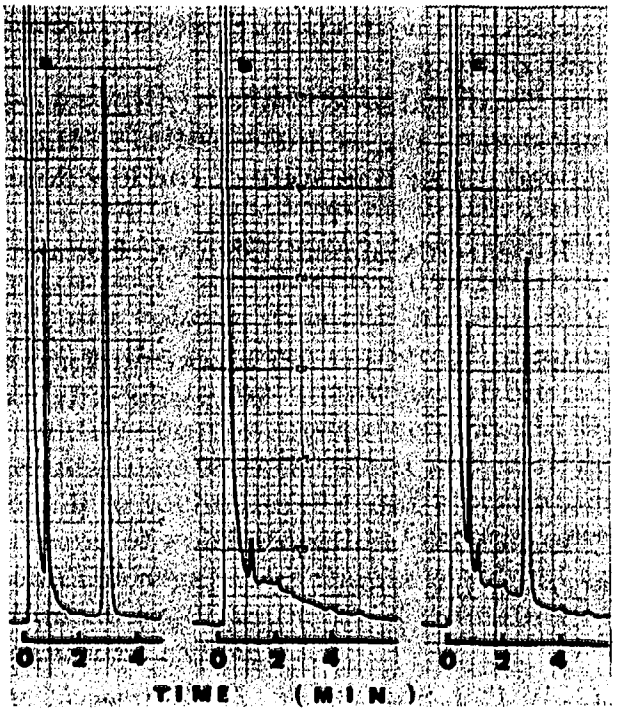


Fig. 1. Standard curve.

For the GLC micro-determination, it is advisable to use an internal standard to check the extraction procedure and the amount injected into a column. Although barbiturates have been used as an internal standard[6–8], the peak tailing due to their thermal decomposition[3] tends to lead to inaccuracies. Therefore, we chose a stable compound, methyl stearate, which is as freely soluble in organic solvent as is amo-



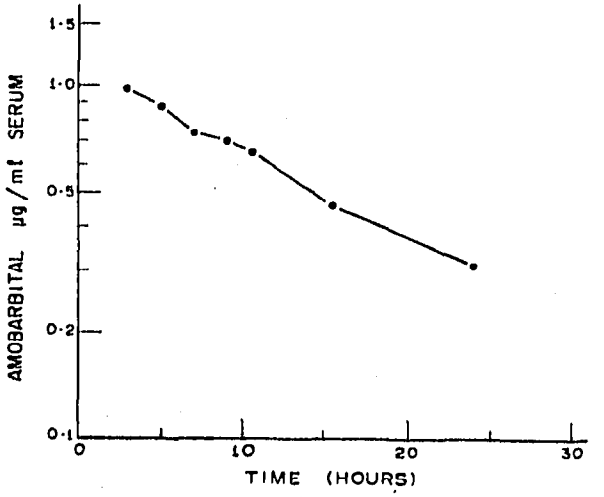


Fig. 2. Chromatograms from amobarbital analysis. (a) A standard: amobarbital, 0.07 µg, 0.8 min; methyl stearate, 0.20 µg, 2.9 min. (b) Blank serum extract. (c) A standard added to serum: amobarbital, 0.7 µg/ml; methyl stearate, 2.0 µg/ml.

Fig. 3. Serum amobarbital level after an oral dose of 65 mg.

barbital. There was no interference from the endogeneous serum contents with the methyl stearate peak even at highly sensitive GLC settings. It can be seen from Fig. 2 that no visible interfering substances are present in serum and that a known amount of amobarbital added to serum was fully recovered.

In order to check the reproducibility of the GLC measurement, two different concentrations of amobarbital, 0.40 and 1.40 µg/ml, were added to aliquots of human serum and the two samples were then subjected to the extraction procedure. From each sample, three GLC determinations using peak height ratios were performed. As one might expect, Table I shows that the error of the GLC measurement is approximately ± 0.05 µg/ml regardless of the amount added. This determines the

TABLE I

RECOVERY OF AMOBARBITAL FROM SERUM

Amount added (µg/ml)	*Found by GLC (µg/ml)*	*Mean ± S.D. (µg/ml)*
0.40	0.45 0.36 0.43	0.41 ± 0.05
1.40	1.33 1.41 1.41	1.38 ± 0.05

limits of the method. As stated above, the error associated with the extraction process is almost negligible (± 0.6% regardless of amount of drugs).

To test the method under working conditions, amobarbital concentrations were measured in the serum of a volunteer who had ingested 65 mg of amobarbital sodium. As can be seen in Fig. 3, drug levels could be followed for 24 h in spite of the low dose.

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