# DETERMINATION OF SMALL AMOUNTS OF BARBITURATES IN BLOOD SERUM

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It was demonstrated that the present determination of small amounts of barbiturates in blood serum is hampered by the presence of higher fatty acids which are normal components of the serum. During the methylation of barbiturates the fatty acids are converted into corresponding methyl esters and appear on chromatograms as strong peaks which may interfere with the peaks of the substances investigated. The disturbing effect can be eliminated by a suitable adjustment of the samples in which fatty acids are converted to poorly soluble barium salts and filtered off. A procedure was elaborated enabling the determination of barbiturates in the 0—5 μg/ml concentration range in serum which is substantially more sensitive than earlier methods. The reproducibility of the method is very good; the coefficient of variation is lower than 2·3%.

In literature a series of various modifications of gas chromatographic determination of barbiturates in blood serum has been described. Usually an acidified sample of the serum is extracted with ether or some other organic solvent, and the extract obtained is worked up either directly[1–7] or after previous purification by which the predominant part of accompanying impurities is eliminated[8–17]. The isolated barbiturates are then chromatographed either as such[1,2,4,7,8,10,11,17] or they are converted (especially when trace amounts have to be analysed) to corresponding 1,3-dimethyl derivatives which have more advantageous chromatographic properties[3,5,6,9,13–19]. In the analyses of blood sera the peaks of unknown substances often appear on the chromatographic curves of methylated samples, and sometimes even overlap the peaks of the barbiturates under analysis. A quantitative evaluation of the chromatograms is thus greatly hampered[16].

We have proposed a new method of purification of the extracts, by which the amount of the interfering substances passing into the final solution is decreased. In order to achieve a smooth methylation we used trimethylphenylammonium acetate which converts barbiturates into corresponding 1,3-dimethyl derivatives without formation of by-products[18]. As internal standard we chose di-n-amylbarbituric acid, *i.e.* a substance of analogous properties, which, however, is not used for therapy and the retention time of which is between the retention times of common barbiturates. These modifications brought about an important improvement in the accuracy of the analyses and enabled the determination of barbiturates at very low concentrations.

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## EXPERIMENTAL

### Chemicals and Apparatus

*Trimethylphenylammonium acetate*, approx. 0·1M solution: 0·263 g of trimethylphenylammonium iodide were weighed into a 100 ml conical flask with a ground-glass neck and dissolved in 5 ml of methanol. 0·200 g of solid silver acetate were then added, the flask closed with a glass stopper and the mixture stirred with a magnetic stirrer for 2 hours. For control several drops of supernatant were withdrawn, acidified with dilute nitric acid, and mixed with 2–3 drops of silver nitrate solution. If no precipitate was formed the reaction mixture was filtered and the filtrate diluted with 5 ml of acetone. The reagent should be stored in a well closed flask of brown glass; it is stable for several months.

*Stock solutions* of allobarbital, amobarbital, aprobarbital, cyclobarbital, phenobarbital, hexobarbital and pentobarbital were prepared by dissolution of 0·125 g of corresponding barbituric acid in 50 ml of methanol.

*Standard solutions of barbiturates in blood serum*: 50 µl of the stock solution of barbiturate were measured with a microsyringe and transferred into a volumetric flask and made up with the serum (plasma) to 25 ml volume. From this solution increasing volumes (0 to 5 ml) were pipetted and diluted with serum (plasma) to 5 ml volume. The resulting solutions contained in 1 ml volume 0 to 5 µg of barbiturate. They were stored in a refrigerator and used within maximum 14 days.

*Solution of internal standard*: 0·100 g of diamylbarbituric acid (obtained by condensation of ethyl ester of di-n-amylmalonic acid with urea[19] m.p. 120·5°C) were dissolved in methanol and the solution diluted with methanol to 10 ml volume. By a five-fold to ten-fold dilution with methanol working solutions were obtained, containing in 1 µl 2 or 1 µg of diamylbarbituric acid.

*Other chemicals used*: distilled diethyl ether, 0·05M barium hydroxide solution, hydrochloric acid 0·05M and concentrated, anhydrous sodium sulfate, redistilled water (after addition of a small amount of potassium hydroxide and potassium permanganate). Glass wool digested with nitric acid, washed with water, dried, silanized for 24 hours by submerging it in a 5% hexamethyldisilazane solution in toluene, and dried at 110°C.

Further, gas chromatograph Chrom 4 with FID was used, as well as stainless steel columns 250 cm long, 3 mm internal diameter, packed with 5 % GE XE 60 or 5% Carbowax 20M on silanized Chromosorb W 100/120 mesh. Nitrogen flow was 30 ml/min, hydrogen flow 25 ml/min, air flow 500 ml/min. Working temperature was 180 or 200°C, the temperature of the injection port 250°C. Record sensitivity 1/20, chart shift 10 mm/min, injected sample volume 3 µl. Separation funnels (50–100 ml), conical test tubes (10 ml) and further glass vessels were purified with sulfuric acid–chromic acid mixture and thoroughly rinsed with distilled water.

### Procedure

a) *Extraction of barbiturates*: 2 ml of blood serum, 4 ml of 0·05M hydrochloric acid, 5 µl of internal standard solution (= 10 or 5 µg of diamylbarbituric acid), and 15 ml of ether were measured into a separation funnel and the mixture was shaken thoroughly for 3 minutes. The aqueous layer was drained into another separation funnel and extracted again with 15 ml of ether. The ethereal layers were combined and washed three times with 2 ml volumes of water.

b) *Purification of the extract*: 5 ml of a 0·05M barium hydroxide solution were added to the ethereal extract and shaken thoroughly for 3 minutes. The aqueous phase was filtered through a funnel in the stem of which a plug of silanized glass wool was inserted, about 1 cm long and wetted with 0·05M barium hydroxide (0·5 ml). The filtered liquid was collected in a clean separa-

tion funnel. The remaining ethereal layer was shaken with another 5 ml portion of barium hydroxide solution. The combined filtrates were acidified with 0·2 ml concentrated hydrochloric acid and shaken with 10 ml of ether for 5 minutes. Then the aqueous phase was discarded and the ethereal extract washed twice with 2 ml portions of water and dried by shaking with 1 g of anhydrous sodium sulfate. The ether solution was transferred into a conical test tube and evaporated there under mild heating by introducing a stream of nitrogen into the upper part of the tube.

c) *Chromatographic determination*: The residue was dissolved in 50 μl of trimethylphenylammonium acetate solution and 3 μl of the solution obtained were injected into the gas chromatograph. On the chromatogram the heights of the peaks of the analysed barbiturate and of the internal standard were measured, their ratio was calculated and the concentration of the barbiturate in the serum (μg/ml) was read from the calibration curve.

d) *Calibration curve*: 2 ml of the standard barbiturate solution in blood serum were worked up in the same manner as above. The heights of the peaks of the barbiturate and the internal standard were measured on the record and their ratio calculated (internal standard = 1). The value obtained was plotted in a diagram against the concentration of the barbiturate in the sample analysed (μg/ml).

## DISCUSSION

### *Extraction and Purification of Extracts*

A number of various solvents has been proposed for extraction of barbiturates from blood serum, as for example chloroform[1,3,6,16,17], diethyl ether[7,8,11], dichloroethylene[10], toluene[13,15] and others[7]. In our work we gave preference to ether because barbituric acids are mostly well soluble in it and because untractable emulsions are not formed when the samples are shaken with it. With some barbiturates (pentobarbital, amobarbital) satisfactory yields were achieved (about 96%) even after a single extraction with ether, but with others the yield of extraction was lower. Therefore we used double extraction which guarantees a quantitative isolation without regard to the type of barbiturate analysed.

When sera are extracted, not only barbituric acids pass into the non-aqueous layer but other substances (impurities) of neutral or acid nature as well. Therefore the extracts are usually purified by shaking with sodium hydroxide solution. Neutral substances remain in the non-aqueous layer, while barbiturates and other acid substances pass into the alkaline aqueous layer; after their separation and acidification they may be extracted again. If a sample purified in this manner is submitted to methylation with trimethylphenylammonium hydroxide or better acetate, additional peaks appear on the chromatograms as well as those of barbiturates, which greatly hinder a quantitative evaluation[20,21]. We have found that this difficulty is caused by higher fatty acids (myristic, palmitic, stearic, linoleic and oleic – Fig. 1*b*) which cannot be eliminated by this method of purification and which are converted to methyl esters during methylation; on chromatograms which were obtained in the

same manner, but without previous methylation, these additional peaks are not present. Very high concentrations of free fatty acids were usually found in sera stored for longer periods.

In order to suppress this disturbing effect of fatty acids we made use of the fact that they form poorly soluble barium salts. For the purification of extracts barium hydroxide solution was used instead of sodium hydroxide and the barium salts formed were separated by filtration through glass wool. This procedure is very efficient, but it still does not guarantee a perfect elimination of fatty acids; trace amounts usually pass into the final solution and appear on the chromatograms as small peaks which, however, do not disturb the determination under certain conditions given below (Fig. 1*a*).

The use of barium hydroxide has another important advantage. It is known that the majority of barbiturates is decomposed to a greater or lesser extent under the effect of sodium hydroxide, not only at elevated, but also at laboratory temperature[22]. Therefore uncontrolled losses may occur during the purification of the extracts, the magnitude of which is dependent on the type of barbiturate and on the time of contact with sodium hydroxide. This important fact is quite overlooked in the majority of analytical procedures. We made a few tests and found that in 0·1M sodium hydroxide solutions at room temperature the initial content of hexobarbital decreases by 36% after one hour, the content of phenobarbital by 15% and of amobarbital by 9%. Analogous experiments with equally concentrated barium hydroxide

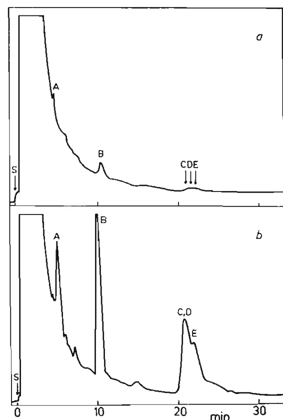


FIG. 1

Purification of Extracts from Plasma

*a*) With barium hydroxide solution, *b*) with sodium hydroxide solution. Methyl esters of acids: A myristic, B palmitic, C oleic, D stearic, E linoleic; S sample injection; column packing 5% GE XE 60 on Chromosorb W 100/120 mesh, column temperature 180°C, sensitivity 1/20.

solution showed that the decomposition takes place considerably more slowly; in none of the mentioned barbiturates did the loss exceed 2%. Hence, the use of barium hydroxide is advantageous not only because it enables the elimination of disturbing fatty acids, but also because the losses of barbiturates are smaller during the purification.

*Choice of Internal Standard and Conditions of Chromatographic Separation*

As a rule in the determination of barbiturates some other commercially available barbiturate is employed as internal standard[5,8,11,13-15]. The standard is added to the sample of serum and in consequence of its analogous properties it accompanies the analysed substance during the whole extraction and purification procedure. Of course, when a sample of unknown composition is investigated it is important to determine by a preliminary analysis which barbiturates are present in the sample. Only then a suitable standard can be selected and the process of extraction and purification repeated. The addition of the internal standard at a later stage (for example into the final extract)[6,10,17] may facilitate the work, but it decreases the accuracy of determination, because the possible losses occurring during the extraction and the purification are not compensated.

An ideal internal standard would be such a derivative of barbituric acid which is not used in therapy; the possibility of its being present in the analysed serum sample would thus be avoided. We prepared several different compounds and found di-n-amylbarbituric acid most suitable. The retention time of this acid is between the retention times of common barbiturates; the mentioned acid may therefore well serve as a "universal" standard.

Special attention should be paid to the choice of the stationary phase. Therefore it is recommended to use always such a phase on which the retention time of the investigated barbiturate is sufficiently different from the retention times of methyl esters of higher fatty acids. In spite of the fact that the amount of fatty acids which might pass into the final solution is small, even a partial overlapping of peaks might cause considerable errors in determination. We found the silicone phase GE XE 60 and polyethylene glycol 20M to be best (Table I).

Couples of some barbiturates which are not well separated on the silicone phase (allobarbital–aprobarbital; cyclobarbital–hexobarbital) can be separated well on polyethylene glycol, and *vice versa*, couples which are poorly separated on polyethylene glycol (allobarbital–amobarbital) can be well separated on the silicone phase. If some of the mentioned couples are present in the sample it is recommendable to carry out the analysis on both phases (Table I).

When barbiturates with shorter retention times are determined a 180°C operating temperature is suitable, while 200°C are used for the others. If it is necessary to determine alternately samples containing one or the other type of barbiturate it is

TABLE I

Relative Retention Times of Methylated Barbiturates and of Methyl Esters of Aliphatic Acids
Retention time of dimethyl ester of di-n-amylbarbituric acid = 1.

| Analysed component | Column packing | | Recommended stationary phase |
|---|---|---|---|
| | 5% GE XE 60 | 5% CX 20M | |
| Aprobarbital[a] | 0·250 | 0·358 | CX 20M, GE XE 60 |
| Allobarbital[a] | 0·260 | 0·381 | CX 20M |
| Amobarbital[a] | 0·366 | 0·387 | GE XE 60 |
| Pentobarbital[a] | 0·404 | 0·451 | GE XE 60, CX 20M |
| Thiopental[a] | 0·706 | 0·815 | |
| Myristic acid[a] | 0·268 | 0·279 | |
| Palmitic acid[a] | 0·566 | 0·587 | |
| Cyclobarbital[b] | 1·247 | 1·768 | CX 20M |
| Hexobarbital[b] | 1·269 | 1·851 | CX 20M |
| Phenobarbital[b] | 1·460 | 2·640 | GE XE 60 |
| Stearic acid[b] | 1·107 | 1·227 | |
| Linoleic acid[b] | 1·174 | 1·496 | |
| Oleic acid[b] | 1·102 | 1·292 | |

Column temperature: [a] 180°C, [b] 200°C.

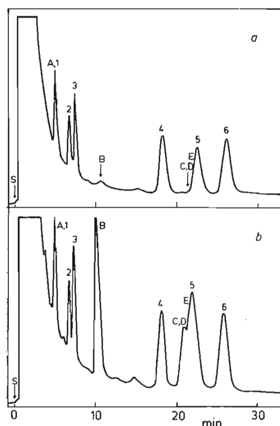


FIG. 2

Analysis of Barbiturates on a Column with 5% GE XE 60 on Chromosorb W 100/120 Mesh

The extract from plasma was purified with *a*) barium hydroxide solution, *b*) sodium hydroxide solution, S sample injection, A—E methyl esters of aliphatic acids (Fig. 1). Dimethyl derivatives of barbiturates: *1* aprobarbital (2 μg/ml), *2* amobarbital (2 μg/ml), *3* pentobarbital (3 μg/ml), *4* di-n-amylbarbituric acid (5 μg/ml), *5* hexobarbital (5 μg/ml), *6* phenobarbital (5 μg/ml); column temperature 180°C, sensitivity 1/20.

advantageous if two columns of various lengths (250 and 120 cm) are built into the chromatograph, and the first used at the same temperature for the determination of lower boiling and the other for the determination of higher boiling derivatives. This saves the time necessary for the temperation of the columns when passing from one to the other operating temperature.

In addition to the mentioned phases neopentyl glycol succinate and diethylene glycol succinate were also studied. In these phases the retention time of the internal standard (*i.e.* di-n-amylbarbituric acid) interferes with the retention times of methyl esters of stearic and oleic acid. These phases may be used for the determination of barbiturates only if another internal standard is used (for example some of the commercial barbiturates).

*Disturbing Effects, Scope of the Method*

The proposed method of purification of extracts permits the elimination of all neutral components of the serum and the predominant part of higher fatty acids which could impair the determination of barbiturates (Fig. 2). However, the procedure does not guarantee that under special conditions (for example after administration of barbiturates in combination with other drugs) interfering substances do not appear in the final solution. The conditions of determination and the method of elimination of the disturbing effect must then be chosen from case to case.

During the determination of some labile barbiturates (allobarbital, hexobarbital) care should be taken that the final ethereal extract is perfectly dry. If this condition is not fulfilled an aqueous solution remains after evaporation of ether, during the heating of which a partial decomposition of the mentioned compounds could take place. In other barbiturates the decomposing effect of water is negligible.

The procedure described enables the determination of individual barbiturates in the 0–5 μg/ml of serum concentration range; hence, the sensitivity is several times higher than in earlier methods. In cases when still lower concentrations have to be determined the recommended volume (2 ml) of the sample may be increased to 5 ml. Under such conditions the combined ethereal extracts must be washed at least five times with 2 ml portions of water; if this is not followed emulsions are formed during the subsequent shaking with barium hydroxide solution. The calibration curves of all barbiturates investigated have a strictly linear character. The reproducibility of the results is very good; 20 analyses of the same serum sample to which aprobarbital, pentobarbital and phenobarbital were added (as representatives of substances with a short, medium and long retention time) in 3 μg/ml concentration gave the following variation coefficients: 2·31, 1·86, 2·16%

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