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MICROTECHNIQUES FOR THE GAS CHROMATOGRAPHIC DETERMINATION OF BARBITURATES IN SMALL BLOOD SAMPLES

W. DÜNGES and E. BERGHEIM-IRPS

Pharmakologisches Institut der Universität, D 65 Mainz (G.F.R.)

H. STRAUB

Siemens-AG Gerätewerk, D 75 Karlsruhe (G.F.R.)

and

R.E. KAISER

Institut für Chromatographie, D 6702 Bad Dürkheim (G.F.R.)

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SUMMARY

A method for the determination of therapeutic levels of barbituric acids in 25 µl of whole blood is described. After extraction and controlled concentration of the extract to a volume of 5 µl, the barbituric acids are N,N'-dimethylated using a microrefluxer. Of the total extract 20—100% is injected into the gas chromatograph. Low blanks, recoveries of 70—80% and peak ratios that are comparable to those in calibration experiments are obtained provided the detailed working instructions are followed strictly. In addition, barbiturates were determined (1 ng in 25 µl blood) using column-switching devices and nitrogen-sensitive detection.

INTRODUCTION

Determination of barbituric acids at therapeutic and toxic levels in blood is required in the following cases. (A) After suicide attempts — the type and quantity of the drug must be determined for adequate treatment; (B) if barbiturates have been misused in the drug sence — their determination is required after accidental overdosage and for forensic purposes; (C) due to their extensive use as sedatives and narcotics, pharmacokinetic studies with laboratory animals and voluntary test persons are important. (D) Some barbiturates, like phenobarbital, are widely used antiepileptic drugs. To suppress epileptic

fits a minimal concentration of the drug has to be maintained in the central nervous system. The blood level can be influenced by other drugs. It can drop to low and ineffective levels or rise to high and toxic values. Consequently the regular determination of blood levels is required as a basis for long-term therapy.

It would be of great advantage if the blood volume required for the test could be about 20—50 µl since these amounts of blood can be drawn from the fingertip or the lobe of the ear. This would be especially convenient in pediatrics and for experiments with small laboratory animals.

Therapeutic levels of barbituric acids are between 1 and 20 µg/ml of blood. Toxic levels reach up to about 100 µg/ml. With gas chromatography (GC) 5 ng of a barbiturate can be determined after methylation of the acidic —NH groups [1]. Therefore a sample of 20 µl of blood should be sufficient for a GC determination even if only 25% of its barbiturate content can be injected into the gas chromatograph.

Using microtechniques [1—4] and methods that have been described for other purposes [5, 6] it was possible to develop techniques which fulfill these criteria.

Foundations of the microlitre techniques

The method is based on the following principles:

(A). Extraction by stirring avoids formation of a colloidal solution of the aqueous phase in the organic phase [5]. Colloid formation occurs during extraction by shaking. It leads to the formation of interfering peaks in the gas chromatograms.

(B). Concentration of the extracts under partial reflux [6] avoids loss of solutes by sublimation.

(C). After difficult and time-consuming experiments prechromatographic microlitre techniques have been developed for the gas chromatography of very volatile compounds (e.g. anisoles) for the purpose of studying their metabolism in liver microsomes [2]. In order to solve this complicated problem many details had to be worked out which are important for trace analysis

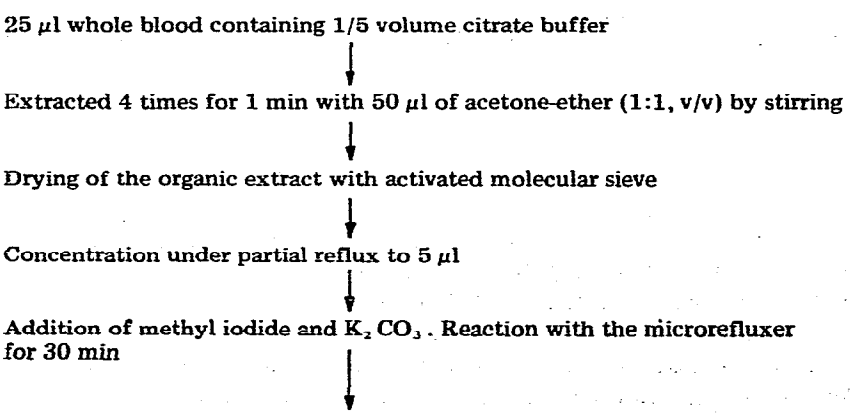

Fig. 1. Microlitre procedure.

in the μl range. These details concern, for instance, drying procedures, handling of small amounts of liquids, etc., and are to be published separately [7].

(D). Barbiturates can be determined below 100—200 ng per injection only after modification of the -NH groups. The reaction conditions of the derivatisation using methyl iodide in acetone lead to the quantitative conversion to N,N'-dimethyl barbiturates without any by-products [1, 4].

(E). The derivatisation mixture can be injected directly into the gas chromatograph [1]. Therefore it is desirable to carry out the reaction in as small a volume as can be injected almost totally. With the aid of a microrefluxer [3], derivatisation can be performed reproducibly in volumes of 5—7 μl. These principles lead to a method which is summarised in Fig. 1; but even after strictly adhering to these rules many interfering peaks did appear in the chromatograms of the first extraction experiments (see Fig. 4). In comprehensive test series the causes for these disturbances had to be liminated. Finally, detailed working instructions (see below) were obtained.

EXPERIMENTAL CONDITIONS AND STANDARD PROCEDURES

Chemicals

Acetone (pro analysi quality) from Merck (Darmstadt, G.F.R.) was used. If impurities were observed in control chromatograms, and for the experiments at 1 ppm, the acetone was purified over a column of aluminiumoxyd 90, aktiv, neutral (Aktivitätsstufe 1, Merck). In order to remove products of aldol condensation the eluate was distilled over a Vigreux-type column. Diethyl ether was purified over a similar alumina column. Small quantities of ether were purified every day. Methyl iodide and potassium carbonate (both pro analysi grade from Merck) were used directly. The molecular sieve, 3A, from Merck (2 mm pellets) was dried at 170° at 15 torr over P_2O_5 for 24 h.

Glassware

All glass vessels were specially made for the microlitre procedures. Glass of Duran or Solidex quality was used throughout. It is important that the shapes and the inner and outer diameters of the vessels correspond the specifications (see Fig. 2). To ensure absolute water-tightness the glass joints (N S 5)* of the two-necked vessels were polished.

Cleaning of the glassware. All glassware was cleaned with a 10% solution of a suitable detergent (i.e. R.B.S. from C. Roth, Karlsruhe, G.F.R.). The two-necked flasks and the 5-μl vessels were filled with cleaning solution with the aid of capillary glass tubes. Air bubbles in the finer parts were carefully avoided. The filled vessels were transferred to a beaker which contained a similar cleaning solution so that the vessels were completely immersed. The solution was heated to about 95° (boiling should be avoided). Boiling or incomplete filling of the vessels leads to the deposition of solid detergent on the glass walls. These deposits are quite difficult to remove and cause erroneous values. The vessels were then rinsed carefully with running tap water and bidistilled water using capillaries which point into their farthest tips. After predrying at 60° all glass vessels were treated in a Bunsen-burner flame until the flame

was lighty coloured from the glass. The vessels were heated slowly beginning from their lower tips and proceeding to their upper end, thus water and volatile impurities were removed in a kind of a steam distillation. The heating procedure was carried out under close observation. Only after the removal of the inner water layers did nonvolatile impurities become visible.

PTFE stoppers and caps

Stoppers and caps were prepared from a soft type of PTFE. All stoppers were fabricated so that they fitted tightly over the whole length of the ground-glass joints. Stoppers which showed the slightest irregularities were eliminated. When the reflux reaction was complete the 5 µl reaction vessels were closed with PTFE caps and stored at —20°. At this temperature the caps shrink and ensure absolutely tight closure. 5 µl of acetone could thus be stored for several months without noticeable losses.

The PTFE stirrers as shown in Fig. 2 were made from a 2-mm diameter steel wire which was partially coated with PTFE shrinking tubes.

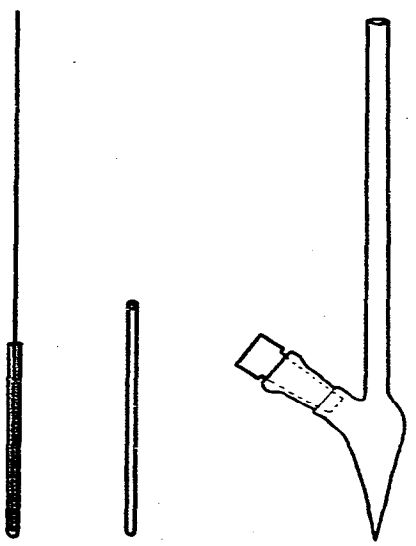

Fig.2. Glassware and microstirrer. Two-necked concentration vessel: length of reflux tube, 10 cm; I.D., 0.3 cm; ground-glass joint closed with PTFE stopper. Microvessel: length, 5.6 cm; I.D. 0.16—0.20 cm.

*Extraction procedure**

20 µl of blood (either bovine or human blood) were measured into glass vessels (see Fig. 2) using Blaubrand micropipettes, (Brand, Wertheim, G.F.R.). The blood contained 1/5 of citrate buffer. Traces of blood should be avoided at the upper inner walls while adding the sample. The barbituric acids (or the internal standards) were added dissolved in citrate buffer; 5 µl of such a solution were mixed with blood. For extraction 50 µl of a mixture of ace-

*All glass vessels with or without liquid were kept in ice baths.

tone—ether (1:1, v/v) were added. A PTFE stirrer (see Fig. 2) was introduced into the tip of the vessel. It was driven by a lab motor at about 10 rpm. After 1 min the organic layer was removed with a pasteur pipette. Suction of small droplets from the aqueous layer should be avoided. The extraction procedure was repeated twice. The combined extracts were dried with activated molecular sieve for several minutes.

*Concentration procedures**

The dried extract was transferred to a two-necked vessel (see Fig. 2). The side-arm of the vessel was closed with a PTFE stopper without use of grease. The glass vessels were immersed into water at 48°. The water should just cover the upper edge of the stoppers. After 10 min, ether was evaporated completely (under partial reflux). The vessels were then heated in a water-bath at 59° until the volume of the acetone solution was reduced to about 5 μl (after 10—15 min). The vessels were then removed quickly and cooled in ice. The immersion in ice caused condensation of the vaporised acetone still present. The condensing liquid rinsed solutes from the walls of the vessel. The remaining acetone (about 20 μl) was transferred to the small flasks and reduced to a volume of about 3 μl in the water-bath of 59°. After immersion in an ice-bath the volume increased to about 6—7 μl.

Equipment for derivatisation

With the aid of the microrefluxer [3] reflux reactions are possible in the range below 50 μl. This device is available in Europe from Berghof GmbH (Tübingen, G.F.R.), and in U.S.A. from Regis Chemical Co. (Morton Grove, Ill.). With the millilitre attachment [4] reflux experiments in the range 0.2—5 ml can be performed in series of 6—9 experiments per run. This equipment has been useful for the preparation of small samples of internal standards, for example.

Derivatisation procedure

To the concentrated acetone extract (5 μl) the following additions were made: boiling chips (diameter 0.5—0.75 mm), about 1 mg freshly powdered potassium carbonate and about 2 μl of acetone containing methyl iodide in a 5—20 *M* excess to the barbiturates. For ultratrace analysis with N-sensitive detection the boiling chips were purified with acetone. The vessels were fixed in the appropriate holes in the cooler of the micro-refluxer. The lower parts of the vessels were immersed in the heated air-bath at about 120°. After a reaction time of 30 min the vessels were taken out of the micro-refluxer. Then they were closed with PTFE caps and cooled in ice.

Gas chromatography

All experiments in the ppm range were carried out with conventional flame ionisation detection (FID). A Perkin Elmer gas chromatograph Model 900 with FID was used. Conditions used were: nitrogen (as carrier gas) flow-rate, 30 ml/min; hydrogen flow-rate, 25 ml/min; air flow-rate, 250 ml/min; glass

*All glass vessels with or without liquid were kept in ice baths.

columns 65 cm × 0.2 cm I.D., filled with 3% OV 225 on Chromosorb W-HP, 120—140 mesh. Temperature programme: 100°, 2-min hold, then 8°/min to 240° (chromatograms in Figs. 3—5) or 100°, 4-min hold, then 4°/min to 240° (chromatograms in Figs. 6 and 7).

For the analysis of barbiturates in the ppb range an L 350 gas chromatograph from Siemens (Karlsruhe, G.F.R.) was used. It was equipped with an alkali flame-ionisation detector (A-FID, "N-FID, system R.E.K."*) [8] and with column switching modules as described by Deans [9, 10]. Column: 3% OV 17 on Chromosorb W-HP, 100—120 mesh; 0.2 cm I.D.; precolumn, 50 cm; separation column, 80 cm. Carrier gas: helium 25 ml/min; hydrogen 40 ml/min; air 110 ml/min. Temperature of detector: 270°. Temperature programme: 170 to 240°, 25°/min, then hold. Heart-cutting analyses were performed with a programme of type IV (see [11]) in the following way: (a) The solvent peak was cut up to 35 sec. During this time acetone and methyl iodide were vented before reaching the separation column. (b) Part of the main fraction was then transferred to the separation column for 15 sec. (c) The rest of the main fraction was then cut off and vented. The separation was performed as usual with this "heart-cut fraction" on the main column.

RESULTS AND DISCUSSION

Strict adherence to the experimental conditions (see above) avoided impurities which were otherwise detected in chromatograms such as shown in Fig. 3. Nearly perfect chromatograms from blanks were obtained reproducibly (see Fig. 4). It needs to be stressed that seemingly trivial deviations from the standard procedures can lead to unsatisfactory results. This implies

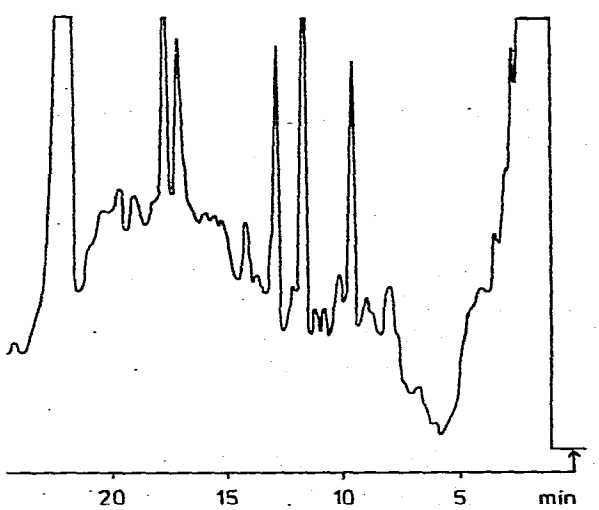

Fig.3. Gas chromatogram of an early blank experiment. 25 μl of whole blood submitted to prechromatographic techniques as described in Fig. 1 but without the use of more elaborate procedures. 1 μl injected into gas chromatograph from a total of 5 μl of the reaction mixture. Attenuation 2; range 10. For other GC details, see text.

*Manufactured by Siemens AG.

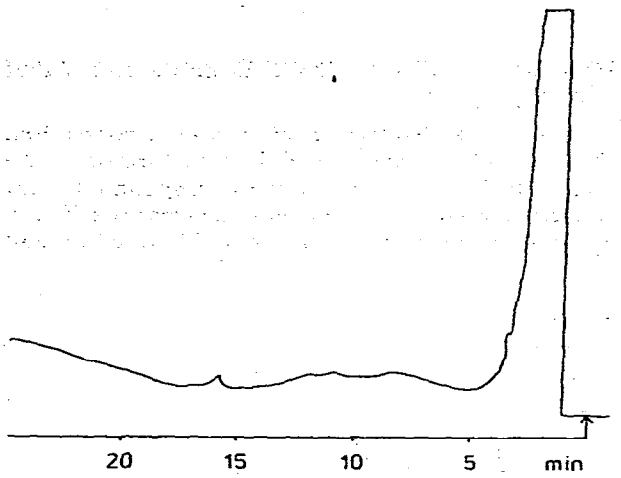

Fig.4. Gas chromatogram of a blank experiment after strict adherence to working instructions. Analytical conditions, see Fig. 2 and text.

especially to extraction or drying. Appropriate cleaning procedures and correctly shaped glassware are important as well.

Low blanks allowed quantitative experiments. In the range 1—10 ppm, recovery rates of barbiturates from 25 µl blood were about 70%. In GC, quantitative experiments are not usually carried out by analysing exactly measured aliquots but by using an internal standard. This method is much more convenient and precise (see ref. 12, for example). 200 ng each of five different barbituric acids were dissolved in 25 µl of blood. Extraction and sample preparation were performed according to the standard procedure. 1 µl of a total of 5 µl was injected into the gas chromatograph. A typical chromatogram is shown in Fig. 5. For quantitative evaluation the peak height ratios

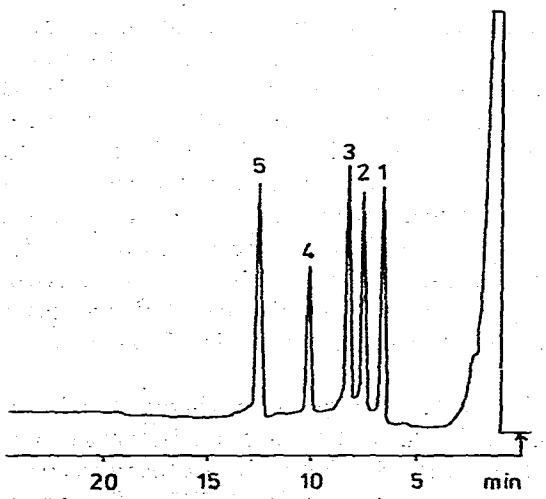

Fig.5. Gas chromatogram of a recovery experiment. 25 µl of blood contained 200 ng each of butalbital (1), amobarbital (2), secobarbital (3), propallylonal (4) and heptabarbital (5). For other conditions, see Fig. 2 and text.

TABLE I

COMPARISON OF GC RESULTS FROM EXTRACTION EXPERIMENTS (A) WITH THOSE FROM CALIBRATION EXPERIMENTS (B)

(A). 200 ng of barbituric acids 1—5 (see Fig. 4) were dissolved in 25 μl blood. Extraction, concentration of extract, methylation in 7-μl end volume, and GC as performed as described in the text. Peak-height ratios of barbituric acids in relation to compound 4 from four experiments are given, together with average values ($\bar{x}$) and standard deviations (S.D.). (B). 200 ng each of barbituric acids 1—5 were dissolved in 7 μl acetone. Methylation and GC were carried out as described.

	Peak-height ratios			
	1:4	2:4	3:4	5:4
Calibration experiments (A)				
1	1.57	1.53	1.60	1.45
2	1.66	1.73	1.83	1.47
3	1.71	1.72	1.94	1.57
4	1.74	1.69	1.84	1.54
$\bar{x}$	1.670	1.665	1.803	1.508
S.D. (absolute)	± 0.074	± 0.091	± 0.144	± 0.058
S.D. (relative)	± 4.4%	± 5.5%	± 8.1%	± 3.9%
Calibration experiments (B)				
1	1.62	1.81	1.87	1.47
2	1.70	1.58	1.70	1.52
3	1.54	1.36	1.61	1.54
4	1.60	1.56	1.70	1.57
$\bar{x}$	1.615	1.578	1.720	1.525
S.D. (absolute)	± 0.066	± 0.184	± 0.109	± 0.042
S.D. (relative)	± 4.1%	± 11.7%	± 6.3%	± 2.8%

of four barbituric acids to the fifth barbituric acid were calculated. Thus, propallylonal (peak 4 in Fig. 5) was used in place of an internal standard. Single values, average values and standard deviations of four series are listed in part A of Table I. They are in good accordance with the values of calibration experiments, which results are shown in part B of Table I. The data of Table I demonstrate the suitability of the method for the determination of barbituric acids in therapeutic concentrations from 25-μl blood samples. Some additional experiments were performed with 25-μl whole-blood samples containing 1-ppm amounts of barbituric acids. Small interfering peaks from impurities were seen in these chromatograms (see Figs. 6 and 7). Recoveries of about 70% and correct peak-area ratios, similar to those in Table I, were obtained.

Analyses with A-FID and column-switching techniques. With conventional GC techniques the detection limits for N,N'-dimethylated barbiturates are about 2 ng per injection (see, for example, Fig. 7). The GC detection limit for nitrogen-containing compounds can be improved with A-FID [8], especially if it is used in conjunction with column-swtiching techniques as described by Deans [9, 10]. Using the heart-cutting mode of operation [12],

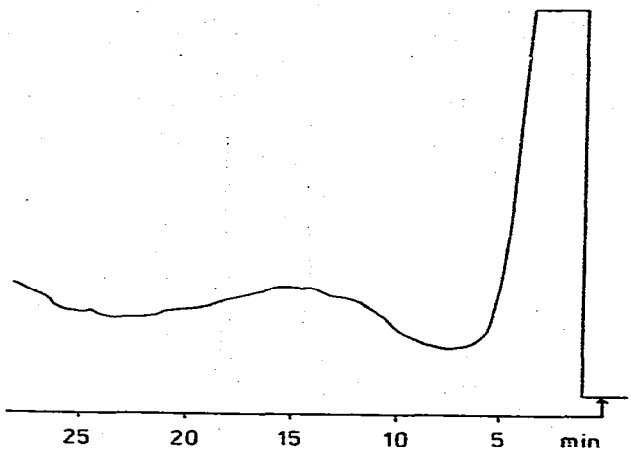

Fig.6. Gas chromatogram of a blank experiment, obtained with maximal GC sensitivity (attenuation 1, range 1) with conventional FID. Injection of 1.5 μl from 6-μl end volume. Other conditions, see text.

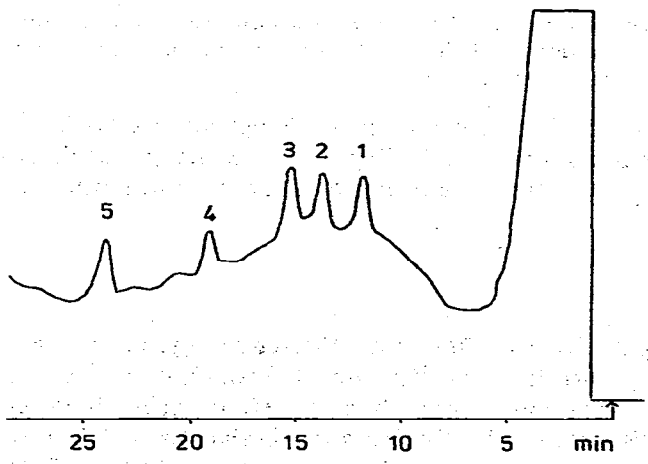

Fig.7. Gas chromatogram of a recovery experiment. Same compounds as in Fig. 4, but 20 ng of each in 25 μl blood. Other conditions, see Fig. 5 and text.

disturbing influences from methyl iodide and acetone on the A-FID are excluded. Thus, up to 10 μl of a reaction mixture can be injected into the gas chromatograph while the A-FID is operated with good sensitivity. With such a special equipment (see Experimental) 16 pg of N,N'-dimethylbarbital could be measured. For the same compound the linearity of detection was found to be between 2×10^{-10} and 1×10^{-4} g.

If 25 μl of human blood are extracted under standard conditions good chromatograms from blanks are obtained (see Fig. 8). Only one disturbing peak was found. It is only present in extracts from human blood and after methylation. It shows the same retention behavior as caffeine.

To 25 μl blood two barbiturates were added (1 ng each). After the application of the described prechromatographic techniques, the total amount of the

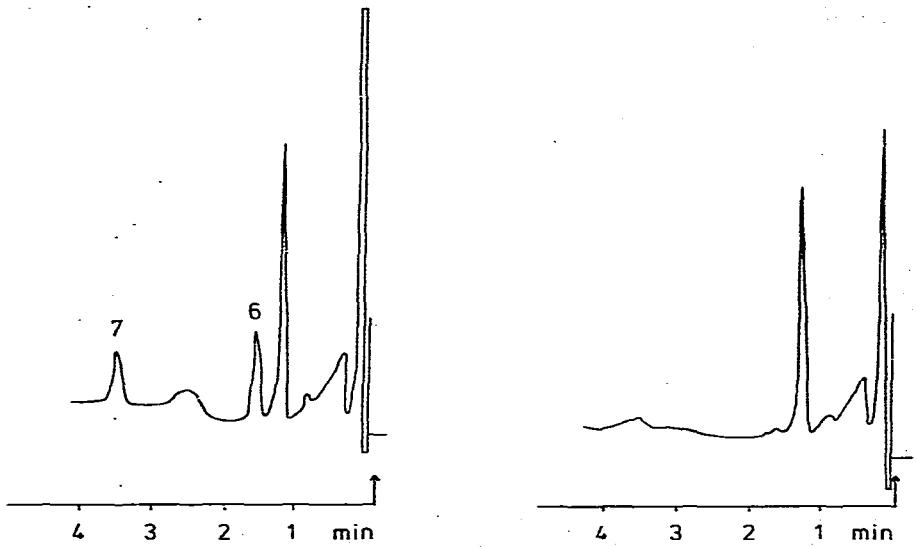

Fig.8. Gas chromatogram of a blank experiment with A-FID and heart-cutting technique. 4.0 μl (i.e. 100%) of the end volume injected. Sensitivity, 8 x 10^{-12} A. Other conditions, see text.

Fig.9. Gas chromatogram of a recovery experiment with A-FID and heart-cutting technique. Dial (7) and cyclobarbital (8) in 1 ng amounts each in 25 μl blood. Same conditions as in Fig. 7 (i.e. total injection of the end volume, here 4.8 μl).

reaction mixtures could be injected into the gas chromatograph (4 to 6 μl). The chromatogram in Fig. 9 shows the possibility of measuring subtherapeutic levels of barbiturates in minute blood samples, as subnanogram amounts can be detected as sharp peaks.

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