GAS-LIQUID CHROMATOGRAPHY OF SUBMICROGRAM AMOUNTS OF DRUGS

II. ANALYSIS OF BARBITURATES AND RELATED DRUGS IN BIOLOGICAL MEDIA

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As early as 1960, an attempt was made by JANAK[1] to employ gas chromatography in the identification of barbiturates. His method involved heating the sample to 800° followed by gas chromatographic separation of the pyrolytic products. In 1962, a similar procedure was described by NELSON AND KIRK[2] who presented unique patterns for 22 barbiturates. These methods are probably satisfactory for analyses in which only one unknown compound is involved. In the resolution of mixtures, however, it would appear that correct interpretation of the complicated pattern so obtained would be difficult if not impossible.

Separation of barbiturates by gas chromatography has been described by COOK *et al.*[3,4]. These workers found that they could not separate the barbiturates as such, but by reaction with diazomethane overnight they were able to obtain good separation of the resulting dimethyl derivatives. Such a method will not, of course, resolve compounds which are already N-methylated from their lower non-methylated homologue. Furthermore, using a non-polar stationary phase, it was found that compounds could not be resolved which differed only in the possession of either a hexenyl or a phenyl side-chain, although COOK[4] *was* able to resolve such mixtures using the polar compound polyethylene glycol adipate, as liquid phase.

An attempt to apply gas chromatography to the separation of barbiturates as the free acids was made by PARKER AND KIRK[5] in 1961. It would seem that their solid support of acid-washed firebrick was not silanised and, as a copy of the chromatogram they obtained is not shown, it is not clear whether they experienced tailing and, if so, to what extent. Although they list the retention times for 23 barbiturates, it is not stated whether *any mixture* of these would be resolved. Indeed, they say that mixed barbiturates caused "overlapping peaks", which we interpret as meaning that resolution of certain mixtures was not achieved. A further drawback to their procedure was that they found variation of retention time with sample size; this was probably due to overloading of the column.

In a later article, PARKER, FONTAN AND KIRK[6] used acid-washed Chromosorb W coated with either 5 % SE-30 or 1 % Carbowax 20M in an attempt to provide a gas chromatographic screening procedure for barbiturates, alkaloids, sympatho-

mimetic amines and tranquillisers. Their conditions probably represented a compromise, with the result that their column was not necessarily the best that could have been prepared for the resolution of mixtures containing only barbiturates. These workers did not report any retention times for barbiturates except those included in their Table, which is inadequate. The same workers, in a subsequent article[7], refer, in their brief introduction, to the use of hexamethyldisilazane (HMDS) for silanising the solid support but they do not themselves use a silanising procedure. They use a mixed liquid phase of 1.5 % SE-30 and 2 % Carbowax 20M in, what appears to be, the mistaken belief that incorporation of a more polar compound in the liquid phase will obviate the necessity for silanising the support material. Comparison of results with and without the Carbowax was not reported, and the resolution of 7 barbiturates was not satisfactory.

Interesting results showing good resolution were obtained by CIEPLINSKI[8] who attempted to prevent tailing of barbiturates by the incorporation of dimer acid (0.75 %) with SE-30 (1.5 %) as the liquid phase. In this paper, he also reported results with a liquid phase of 0.75 % trimer acid and 3 % neopentyl glycol adipate (NPGA). In each case, the solid support material was Chromosorb W which had been acid-washed and treated with HMDS. We have repeated his work and found that the column functions satisfactorily at 180°, but when it is raised to, and held at 250° for an hour, dropping the temperature back to 180° gives results for the barbiturates which are not nearly as good as those obtained before the temperature was raised. This means that one would be precluded from carrying out temperature-programming up to 250° or so. We felt that the change we observed might be due to loss of the dimer acid at the higher temperature and wondered whether CIEPLINSKI[8] had tried the trimer acid with SE-30 and, if so, why the results were not reported.

Gas chromatographic detection of barbiturates is briefly discussed by VANDENHEUVEL, HAAHTI AND HORNING[9] in their more general article dealing with drugs and drug metabolites. They found that the barbiturates showed some tailing with a liquid phase of QF-1 and concluded that more than one column was necessary for satisfactory resolution of mixtures.

It seems to us that one of the most outstanding articles dealing with the gas chromatographic separation of barbituric acid derivatives which has so far appeared in the literature is that of BROCHMANN-HANSSEN AND SVENDSEN[10]. It is noteworthy that these workers were able to run the barbiturates at a temperature of about 140° (some 40° lower than that found necessary by other workers), which means that they were using a column showing much less adsorption of barbiturates. Although they show chromatograms of the resolution of some 9 to 12 barbiturates on columns using four different stationary phases, it is clear from their list of 22 barbituric acid derivatives that any *one* of their five columns would not be capable of resolving *any* mixture of barbiturates. The amounts of barbiturates which they injected varied from 5 to 10 μg. Their solid support was 60–80 mesh Chromosorb W which had been washed with concentrated hydrochloric acid and methanolic potassium hydroxide and then treated with HMDS prior to application of the stationary phase.

In a recent article, MCMARTIN AND STREET[11] have described their investigations into the treatment of solid support material, the preparation of columns showing considerably reduced adsorption, the effect of tristearin on such columns, and the scope and limitations of these columns. It is the purpose of the present paper to de-

scribe the application of these investigations to the analysis of barbituric acid derivatives both in pure solution and in extracts of biological material. With our procedure, most of the barbiturates can be detected down to the 0.04 μg level; in some cases, 0.01 μg can be detected. This description also includes analysis of certain other compounds related to the barbiturates either structurally, *e.g.*, the glutarimides Doriden and Megimide, or because they may appear in the same analytical fraction, *e.g.* salicylic acid.

METHODS, APPARATUS AND MATERIALS

Gas–liquid chromatography details

All the results were obtained using a Perkin-Elmer Model 800 gas chromatograph fitted with a flame-ionisation detector and equipped for temperature-programming. Nitrogen was used as the carrier gas at a flow rate of about 30 ml per min. The flow rates of hydrogen and air were optimised. The temperature of the injector block was usually between 50° to 100° above column temperature. Detector temperature was approximately the same as column temperature. The signal was recorded on a Honeywell −0.05 to +2.5 mV recorder. The recorder chart has 10 small squares to the inch.

Stainless steel columns, 6 ft. × 0.085 in. I.D., 1/8 in. O.D., were packed with Chromosorb W (100–120 mesh) which had been treated and coated with either a mixture of silicone gum rubber (SE-30) 2 %, and tristearin, 0.1 %, as described by MCMARTIN AND STREET[11], or by a similar procedure in which the SE-30 was replaced by a fluorosilicone polymer (QF-1). Samples were injected with a 10 μl Hamilton glass syringe, graduated in divisions of 0.2 μl, fitted with a fixed 2 in. stainless steel needle.

Preparation of samples

Blood. Extracts were prepared either by the method of STREET AND MCMARTIN[12] or by a scaled-down version of this method starting with 1 ml of blood and reducing the volumes of reagents by a factor of 5. Residues were dissolved in 50, 100 or 200 μl of ethanol and from 1 to 5 μl of these solutions were injected into the gas chromatograph.

Liver. Extracts were prepared by the procedure described by STREET[13] but starting with 25 g of liver in place of 100 g, and using proportionately less volumes of reagents. One tenth of the dried ether extract from such a preparation was taken carefully to dryness and the residue dissolved in 200 μl of ethanol. From 1 to 5 μl of this solution were injected into the gas chromatograph.

Standards. Solutions were prepared from the free acids of the barbituric acid derivatives kindly supplied by Messrs. May and Baker, Ltd., England. These barbiturates were made up in either ethanol or methanol as 0.1, 0.02, 0.004 and 0.001 % solutions containing respectively, 1, 0.2, 0.04 and 0.01 μg of the drug per μl. For nomenclature of the drugs, that given in the Merck Index (1960) has been adopted.

The compounds studied were:
Barbital (Barbitone), 5,5-diethylbarbituric acid;
Di-allylbarbituric acid (Allobarbitone), 5,5-di-allylbarbituric acid;

Butethal (Butobarbitone), 5-butyl-5-ethylbarbituric acid;
Pentobarbital (Pentobarbitone), 5-(1-methylbutyl)-5-ethylbarbituric acid;
Amobarbital (Amylobarbitone), 5-(3-methylbutyl)-5-ethylbarbituric acid;
Aprobarbital, 5-allyl-5-isopropylbarbituric acid;
Rutonal, 5-phenyl-5-methylbarbituric acid;
Secobarbital (Quinalbarbitone), 5-allyl-5-(1-methylbutyl)-barbituric acid;
Cyclobarbital (Cyclobarbitone), 5-(1-cyclohexen-1-yl)-5-ethylbarbituric acid;
Heptabarbital (Medomin), 5-(1-cyclohepten-1-yl)-5-ethylbarbituric acid;
Hexobarbital (Hexobarbitone), 3,5-dimethyl-5-(1-cyclohexen-1-yl)-barbituric acid;
Salicylic acid, *o*-hydroxybenzoic acid;
Acetylsalicylic acid (Aspirin), 2-acetoxybenzoic acid;
Doriden (Glutethimide), α-ethyl-α-phenyl glutarimide;
Bemigride (Megimide), β-methyl-β-ethyl glutarimide;
Caffeine, 1,3,7-trimethylxanthine.

RESULTS AND DISCUSSION

Analysis of pure solutions of drugs

Figs. 1 and 2 show the results obtained by injection of 0.2 μg and 0.01 μg, respectively, of mixtures containing each of the five barbiturates, di-allylbarbituric acid, butethal, amobarbital, pentobarbital and secobarbital. Even at the level of 10 nanograms, partial resolution of the two isomers amo- and pentobarbital is effected. Sensitivity around the 10 nanogram region means that it should be possible to determine the "free" (as distinct from protein-bound) plasma levels of certain drugs which could lead to an attempt to correlate such levels with toxicity and therapeutic effectiveness. Using only a single compound, *e.g.* in clinical trials, gas-chromatographic conditions could be chosen to suit that particular compound rather than those to suit resolution of a mixture. In this connexion too, solid sampling (which we have not investigated) would seem to be the answer to possible interference by solvent, and would also allow smaller initial samples of biological material to be used.

The results obtained by injection of 4 μl of a solution containing 2 μg each of a mixture of 10 barbiturates run at 160° at an attenuation of × 100 are shown in Fig. 3. It will be noted that, at this temperature, 34 min are required before the last peak (heptabarbital) emerges. The same mixture of barbiturates can be separated in 12 min by temperature-programming from 150° at 5° per min, when the peaks are actually sharpened. These results are shown in Fig. 4 where 0.5 μg of each of 10 barbiturates was injected at an attenuation of × 50. Fig. 5 shows the results obtained by temperature-programming a mixture of 0.05 μg of each of the barbiturates, secobarbital (1), hexobarbital (2), rutonal (no peak), cyclobarbital (4) and heptabarbital (5), at attenuation × 5. The tailing of rutonal which is apparent in both Figs. 3 and 4 and its disappearance as shown in Fig. 5, is difficult to account for in view of the fact that cyclobarbital shows less tailing. We have noticed that certain other compounds display anomalous results. For example, 4-hydroxyacetanilide shows considerably more tailing even at 200° than either *p*-aminophenol or salicylic acid which run well at 150°.

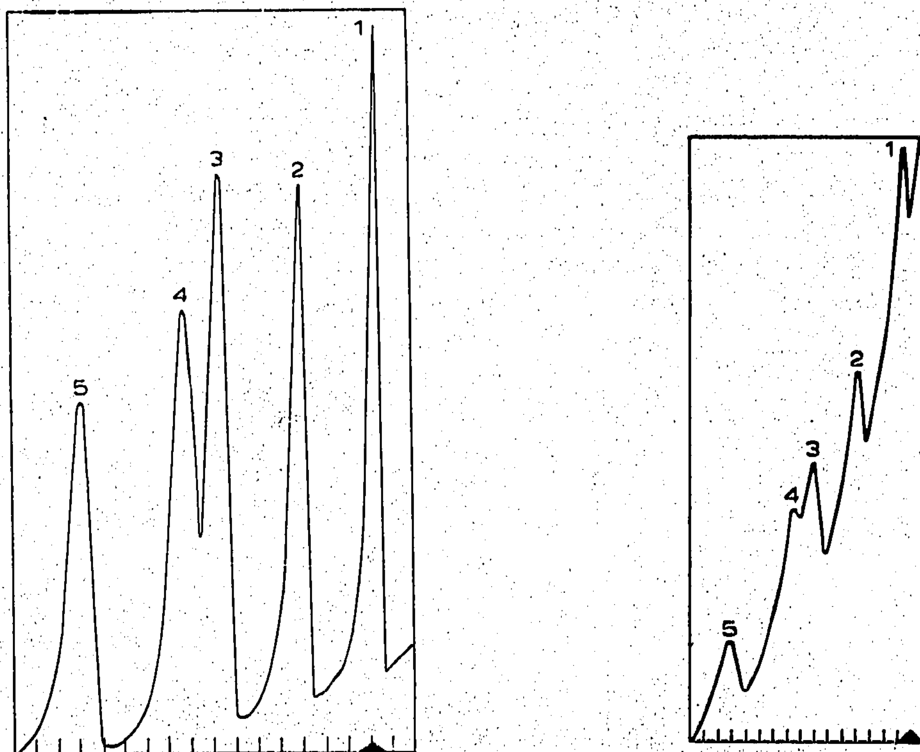


Fig. 1. Chromatogram obtained by injection of 0.2 μg each of a mixture of di-allylbarbituric acid (1), butethal (2), amobarbital (3), pentobarbital (4), and secobarbital (5) in 1 μl of ethanol. SE-30–tristearin column at 160°. Attenuation: × 10. Y-axis = detector response. X-axis = time after injection (increasing to left). 1 division ≡ 0.1 in. ≡ 24 sec. ▲ is 1 in. from point of injection.

Fig. 2. Details as in Fig. 1 but 0.01 μg of each of the 5 barbiturates injected in 1 μl of ethanol at attenuation × 1. Axes and ▲ as in Fig. 1.

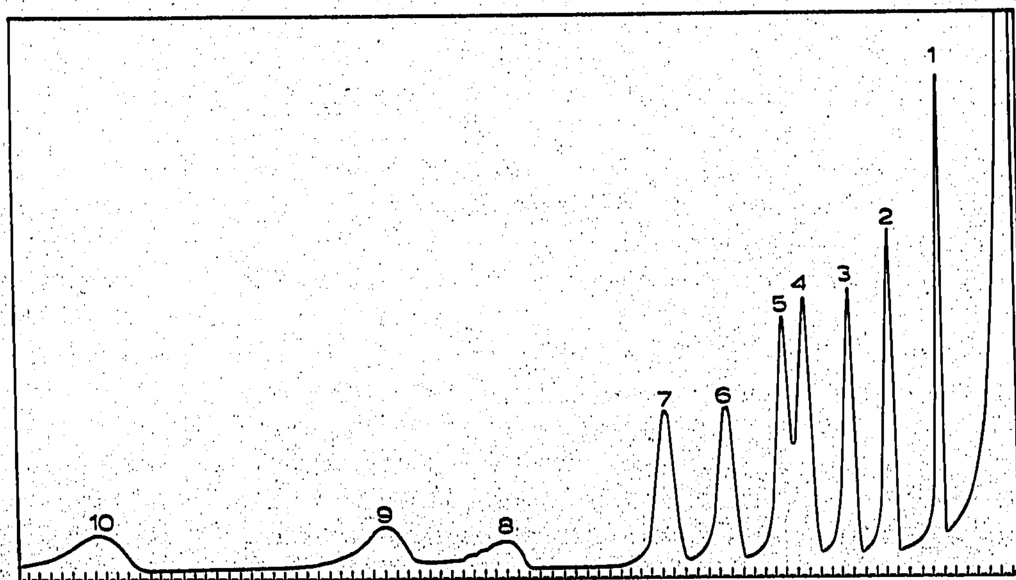
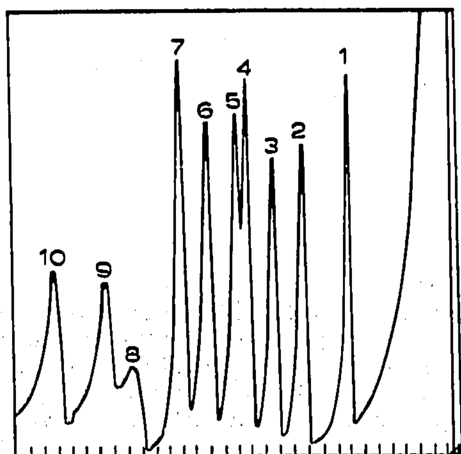


Fig. 3. Chromatogram obtained by injection of 2 μg each of a mixture of barbitone (1), di-allylbarbituric acid (2), butethal (3), amobarbital (4), pentobarbital (5), secobarbital (6) hexobarbital (7), rutonal (8), cyclobarbital (9), and heptabarbital (10) in 4 μl of ethanol. SE-30–tristearin column at 155°. Attenuation: × 100. Axes as in Fig. 1. ▲ is at point of injection.



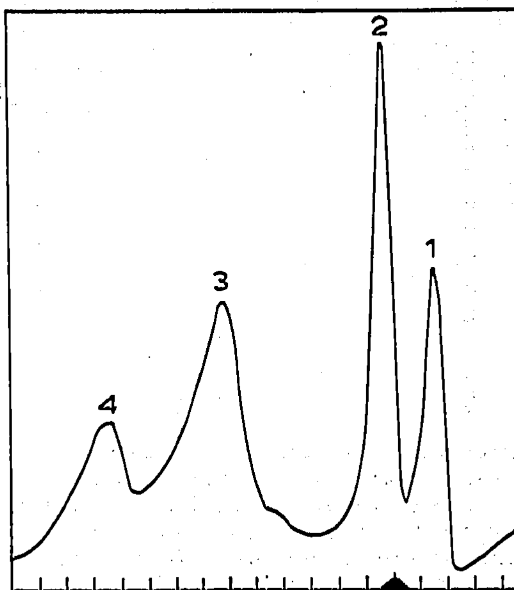


Fig. 4. Chromatogram obtained by injection of 0.5 μg each of a mixture of those barbiturates referred to in Fig. 3, in 2 μl of ethanol. SE-30–tristearin column temperature-programmed from 150° at 5° per min. Attenuation: × 50. Axes as in Fig. 1. ▲ is at point of injection.

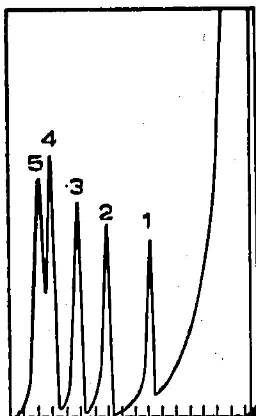
Fig. 5. Chromatogram obtained by injection of 0.05 μg each of a mixture of secobarbital (1), hexobarbital (2), rutonal (no peak), cyclobarbital (3), and heptabarbital (4), in 1 μl of ethanol. SE-30–tristearin column temperature-programmed from 150° at 5° per min to 200° then isothermal. Attenuation: × 5. Axes as in Fig. 1. ▲ is 2 in. from point of injection.

Separation of the mixture of 10 barbiturates referred to above can be effected in 6 min by running at 200° but in this case the resolution of amobarbital and pentobarbital at 200° is not as good as either the 160° or the temperature-programmed run.

On the SE-30–tristearin column, it is not possible to achieve good resolution of a mixture of aprobarbital and di-allylbarbituric acid but, by using the more polar QF-1 phase, (also containing tristearin), these two barbiturates may be satisfactorily resolved. On the other hand, a mixture of rutonal and cyclobarbital is not resolved at all on QF-1–tristearin but, on SE-30–tristearin, the two drugs are separated quite cleanly.

Analysis of extracts from biological media

Initially, barbiturates were added to biological samples known not to contain any barbiturate. One result, typical of these experiments, is shown in Fig. 6. For comparison purposes under the same conditions, Fig. 7 shows the tracing from a standard barbiturate mixture. In this case, the barbiturates barbital, di-allylbarbituric acid, butethal, amobarbital and pentobarbital were added to 5 ml of blood to give a concentration for each barbiturate of 1 mg per 100 ml of blood. Three-fifths of the extract was taken to dryness and the residue dissolved in 100 μl of ethanol. The 5 μl of this solution which was injected would contain 1.5 μg of each barbiturate if there were 100 % recovery. By comparison of peak heights with the standard (Fig. 7), the recovery would appear to be about 50 % for butethal, amobarbital and pentobarbital.



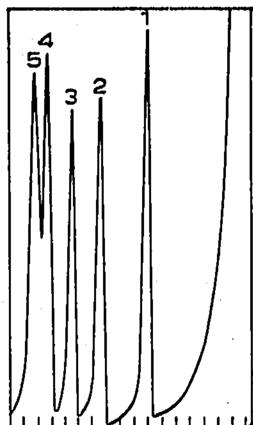


Fig. 6. Chromatogram obtained by injection of 5 μl of a solution of a residue obtained from an extract of blood (see text). In this case to 5 ml of blood had been added 50 μg each of a mixture of barbital (1), di-allylbarbituric acid (2), butethal (3), amobarbital (4), and pentobarbital (5). If recovery were 100%, 1 μl of final solution would contain 0.3 μg of each barbiturate. SE-30–tristearin column temperature-programmed from 150° at 5° per min. Attenuation: × 100. Axes as in Fig. 1. ◢ is at point of injection.

Fig. 7. Chromatogram obtained by injection of 1 μg each of a mixture of those barbiturates referred to in Fig. 6 in 1 μl of ethanol. SE-30–tristearin column temperature-programmed from 150° at 5° per min. Attenuation: × 100. Axes as in Fig. 1. ◢ is at point of injection.

One interesting point emerges by comparing *relative* peak heights, when it is seen that the recovery of barbital and of di-allylbarbituric acid is less than that of the other barbiturates. This is in keeping with previous observations regarding these two drugs and is probably related to the fact that their partition coefficients in ether–water are lower than those of most other barbiturates. The same blood sample carried through the procedure without added barbiturate gave a trace which was free from peaks in the region under observation. In each case, the runs were made at attenuation × 100 and the column was temperature-programmed from 150° at 5° per min.

Using the same batch of blood, the above experiment was repeated but with the barbiturates added to the blood this time to give a concentration of 0.1 mg per 100 ml of blood. 5 ml of blood were extracted and the residue was dissolved in 50 μl of ethanol. 2 μl of this solution were injected into the gas chromatograph and produced the results shown in Fig. 8. The column in this case was temperature-programmed from 155° at 5° per min and the attenuation was × 10. This increased sensitivity now reveals a number of peaks in the blood blank but the barbiturate peaks (corresponding to about 0.1 μg of each drug, *i.e.* again about 50% recovery) are clearly distinguished from these peaks, which we have found to be due to impurities in the ether used in the extraction procedure. Attempts to purify the ether by standard procedures did not eliminate these peaks. It is pertinent to note here that the low recoveries in each of the above two experiments may be related to the method of evaporating down the ether solutions, which may have resulted in the residue being distributed over an area too large to be covered by the small volumes of ethanol used to dissolve the residues. There is obviously room for improvement here, although the use of an internal standard, added to the sample prior to extraction, would be of value in using the procedure quantitatively. It is probable that some form of solid sampling would

obviate losses. We have not investigated this because our apparatus is not designed to permit solid sampling other than by syringe. However, the results of Fig. 8 show that it would be quite easy to follow blood barbiturate levels in patients receiving therapeutic doses of the drugs. Again, such a study would be even simpler and could be made more sensitive for a single, *known* barbiturate.

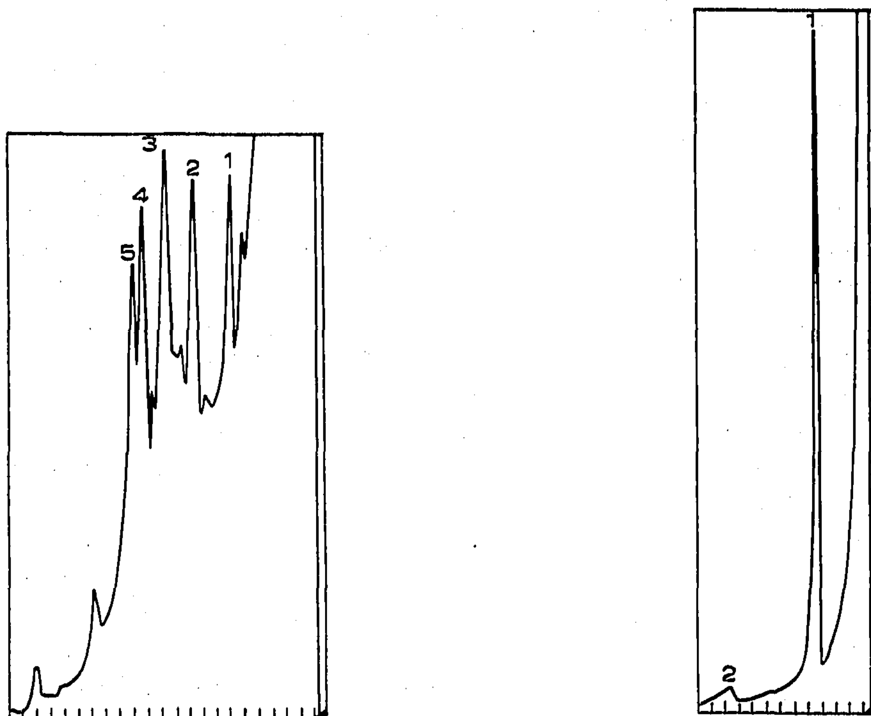


Fig. 8. Chromatogram obtained by injection of 2 μl of an ethanolic solution of a residue obtained from an extract of blood (see text). In this case, to 5 ml of blood had been added 5 μg each of a mixture of barbital (1), di-allylbarbituric acid (2), butethal (3), amobarbital (4), and pentobarbital (5). If recovery were 100 %, 1 μl of final solution would contain 0.1 μg of each barbiturate. SE-30–tristearin column temperature-programmed from 155° at 5° per min. Attenuation: × 10. Axes as in Fig. 1. ◢ is at point of injection.

Fig. 9. Chromatogram obtained by injection of an ethanolic solution of a residue from an extract of liver from a case where death was due to an overdose of amobarbital (see text). (1) amobarbital, (2) phenobarbital. QF-1–tristearin column at 200°. Attenuation: × 100. Axes as in Fig. 1. ◢ is at point of injection.

Fig. 9 shows the results obtained from an actual routine case in our department. By ultraviolet spectroscopy and elevated temperature paper-chromatography (see STREET[14, 15]) it had previously been established that death was due to ingestion of an overdose of barbiturates. The liver was found to contain 26.7 mg of barbiturates per 100 g and paper chromatography and treatment with hot sulphuric acid (see STREET AND MCMARTIN[16]) suggested that amobarbital was present. The paper chromatogram also showed a very faint absorbing spot (in 254 mμ light) in the region of phenobarbital. Part of this extract was subjected to gas–liquid chromatography (QF-1–tristearin) at 200°, and gave the results shown in Fig. 9, the retention times of which peaks agree with those of amobarbital and phenobarbital. It was concluded that death was due to an overdose of amobarbital and that the phenobarbital was probably present in therapeutic amount.

Interfering compounds

In the analysis of blood for barbiturates in poisoning cases it is important to remember that there are a few compounds which may be present in the sample which are not barbiturates and yet may appear in the same extract. Precisely which compounds will be present in any given extract will, of course, depend upon the extraction procedure used. In any event, it is well to be aware that Doriden, aspirin, salicylic acid, and caffeine may be present in the extract and may confuse the analysis. Furthermore, Bemegride is often used in the treatment of barbiturate poisoning and this drug, being a weak acid, will be found in the same extract as the barbiturates.

0.1 µg of Bemegride is readily picked out in a mixture of 5 barbiturates (in 1 µg amounts). Barbital has the shortest retention time of the barbiturates studied; Bemegride shows a retention time of about half that of barbital when the compounds are temperature-programmed from 150° at 5° per min on an SE-30–tristearin column. Under these conditions, Doriden shows almost the same retention time as hexobarbital and a mixture of these two drugs is not resolved. This difficulty can be circumvented either by using a more polar phase or by making use of the fact that Doriden is unstable in dilute alkali at room temperature.

At 140°, aspirin and salicylic acid can be separated from each other. Their retention times are shorter than Bemegride so that they can easily be picked up and do not interfere, even in relatively large amounts, with the barbiturates.

Caffeine is such a weak base that it is generally present in the "neutral" fraction of the extraction procedure and it may, therefore, be found along with the barbiturates if a separation of "neutral" drugs has not been effected prior to chromatography. On the SE-30–tristearin column, caffeine shows the same retention time as secobarbital but resolution of the two drugs is achieved again by using QF-1 in place of SE-30.

Variation of retention time with concentration of injected solution

In a previous article (see McMartin and Street[11]) we noted that we had found identical retention times for a number of drugs for concentration ranges from 0.2 µg/µl to 0.04 µg/µl and, in cases where the drug could be detected in smaller amounts, from 0.2 µg/µl to 0.01 µg/µl. Two articles dealing with the gas chromatographic analysis of low concentrations of barbiturates have appeared very recently after our work had been completed. In the first of these articles, by Gudzinowicz and Clark[17], it was found that, for hexobarbital, their peak maxima shifted to longer retention times as the amount of sample introduced was decreased, and they state that "this effect, together with peak tailing and loss of sample when less than 0.1 µg is injected, is undoubtedly caused by adsorption on the solid support". We are inclined to agree with their statement and this is precisely why we investigated the way of reducing such adsorption (see McMartin and Street[11]). We further suggest that, as a result of our investigation, the column we have produced shows, at least for the compounds we have studied, much less adsorption than any previously reported columns, as is evidenced by the fact that the retention time is independent of concentration of injected sample over a relatively large range in the sub-microgram region.

At first sight, there might appear to be a discrepancy between the retention times of heptabarbital and cyclobarbital as shown in our Fig. 4 and those shown in our Fig. 5. However, it will be observed that in one case temperature-programming

was carried out up to 200° and then the run was isothermal, whereas in the other case temperature-programming was carried out until the last drug had emerged from the column.

BRADDOCK AND MAREC[18], working with pentobarbital and thiopental (and with barbital as "a possible internal standard") observed that their peaks all showed considerable tailing, especially at the lower concentrations. They found that the minimum levels of detection were about 0.002, 0.003 and 0.005 μg per μl for barbital, pentobarbital and thiopental, respectively. They then go on to say that "At these concentrations, the shape of the response peak and the amount of background noise interact to make the peak just barely discernible on some runs and non-existent on others." In view of this statement, we feel that their minimum levels of detection should be somewhat higher than the figures they have stated. These workers also found that at concentrations below 0.05 μg per μl, barbital, pentobarbital and thiopental all displayed a lengthening of retention time as the concentration was reduced.

It would seem, therefore, that our remarks concerning the article by GUDZINOWICZ AND CLARK[17] are also applicable to the article by BRADDOCK AND MAREC[18].

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SUMMARY

This paper describes the gas–liquid chromatographic separation of mixtures of barbiturates in pure solution and in extracts of biological material. Using a special preparation of SE-30–tristearin on acid-washed Chromosorb W in a stainless steel column it is shown that successful resolution can be obtained with submicrogram amounts of the drugs. In some cases, 10 ng of barbiturates can be detected. Interference of other drugs and methods of eliminating this interference are described.

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