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GAS CHROMATOGRAPHIC ANALYSIS OF THE COMMONLY PRESCRIBED BARBITURATES AT THERAPEUTIC AND OVERDOSE LEVELS IN PLASMA AND URINE

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SUMMARY

A critical examination has been conducted of some gas chromatographic columns, many of which have been previously recommended for barbiturate analysis, together with a tested extraction procedure which is sensitive enough to analyse therapeutic drug levels, yet is equally rapid and suitable for emergency toxicological purposes.

INTRODUCTION

The barbiturates were introduced to medical practice about seventy years ago and over the ensuing years they have been prescribed throughout the world on an enormous scale. Today they figure as the second commonest cause of fatal poisoning in the United Kingdom and requests for their analysis in blood and urine are increasing in number. It is desirable, therefore, to have some reliable, accurate and specific means for their measurement in body fluids at both therapeutic and overdose levels. Without knowing the identity of the particular drug involved, a plasma concentration in terms of "barbiturate" will, however, be relatively meaningless. Moreover, one is often asked to undertake serial analyses on the same patient with samples taken at timed intervals in order to monitor the changing plasma drug status and hence the patient's progress.

This increasing work load, coupled with the more stringent requirements arising from the need to identify the barbiturate present, must lead every laboratory performing drug level studies to re-appraise their method. Often a spectrophotometric procedure based on that of Broughton[1] is adopted or, alternatively, a colorimetric method based on that of Curry[2]. Although both methods can provide a level neither will identify the drug, so they are sometimes used in conjunction with either a hydrolysis procedure[3] or a thin-layer chromatographic technique for this purpose[4–6]. We consider that the method of choice is gas chromatography, since this will simultaneously provide both a rapid qualitative and a reliable quantitative result. Several gas–liquid chromatographic (GLC) procedures have been described previously[7–24],

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although the plethora of methods only serves to illustrate the lack of any particular one to meet the various needs.

Accordingly, in this laboratory a prolonged, exhaustive and critical study has been made of the main aspects of barbiturate analysis by examining columns and testing extraction methods suitable for both therapeutic levels and toxicological use. This present paper embodies a description of the work thus undertaken and the lessons and recommendations that have been derived from it.

EXPERIMENTAL

Instrumentation

A Pye 104 Model 24, dual-column gas chromatograph equipped with flame ionisation detectors was used throughout in conjunction with a Hitachi 159 recorder which had been converted to 1 mV f.s.d. The column temperature in each case was that specified in Table I. The injection port was held 30° above the column temperature and nitrogen carrier gas flowing at 50–60 ml/min was used in all cases. The hydrogen and air flow-rates were 50 and 500 ml/min, respectively, and amplifier sensitivity was $2 \cdot 10^{-10}$ A.

TABLE I

SUMMARY OF COLUMNS PREPARED AND TESTED

Stationary phase	*Loading* (%)	*Conditioning temperature* (°C)	*Operating temperature* (°C)	*Solvent*
Carbowax 20M*	1	225	205	chloroform
Poly A-103*	3	275	215	chloroform
NGA+Trimer acid**	3+0.7	220	230	methanol
SP-1000*	1	250	230	chloroform
PPE-20*	3	250	200	chloroform
Apiezon L***	10	250	190	toluene
CDMS***	4	250	220 or 240	dichloromethane
OV-1*	3	300	155	chloroform
OV-17*	5	275	200	acetone
OV-25*	3	275	165	chloroform
OV-210*	4	250	160	acetone
OV-225*	4	250	205	chloroform

* Field Instruments Ltd., Richmond, Great Britain.
** Phase Separations Ltd. Queensferry, Great Britain.
*** Perkin-Elmer Ltd., Beaconsfield, Great Britain.

Columns

Each column was a coiled glass tube 5 ft. long × ¼ in. I.D. This was silanised by filling with 5% dimethyldichlorosilane in toluene for 24 h. Glass wool was silanised in the same solution. After drying the column at 110° it was filled by applying a vacuum to one end and gently tapping the tube whilst the packing material was introduced. The end was then closed with glass wool and the column conditioned with the carrier gas flowing for 24 h at the temperature indicated.

The twelve packings studied are summarised in Table I, their preparation being as follows. The amount of stationary phase calculated to give the correct loading was weighed into a 1-litre round-bottomed flask and dissolved in 200 ml of solvent (AnalaR grade) as indicated in the table. Sufficient Chromosorb W, HP, 80–100 mesh (Perkin-Elmer Ltd.) to give a total of 25 g of coated support was added to the solution (*i.e.* 1 g of stationary phase + 24 g of support = 4% loading). The flask was left to stand with occasional swirling for 2 h and the solvent was then removed under vacuum in a rotary evaporator, the final stages of evaporation being completed in a water-bath at 90° for 30 min.

Qualitative recognition

Chloroform solutions each containing 10 mg/100 ml of free barbituric acid were prepared for most of the barbiturate drugs available on the British pharmaceutical market and listed in Table II.

TABLE II

BARBITURATE DRUGS

Approved name	*Chemical name*
Allylbarbitone	5-allyl-5-isobutylbarbituric acid
Amylobarbitone	5-ethyl-5-isopentylbarbituric acid
Barbitone	5,5-diethylbarbituric acid
Butobarbitone	5-butyl-5-ethylbarbituric acid
Butylallylbarbitone	5-allyl-5-*n*-butylbarbituric acid
Cyclobarbitone	5-cyclohex-1-enyl-5-ethylbarbituric acid
Heptabarbitone	5-cyclohept-1-enyl-5-ethylbarbituric acid
Hexobarbitone	5-cyclohex-1-enyl-1,5-dimethylbarbituric acid
Methohexitone	α-(±)-5-allyl-1-methyl-5-(1-methylpent-2-ynyl)barbituric acid
Nealbarbitone	5-allyl-5-neopentylbarbituric acid
Pentobarbitone	5-ethyl-5-(1-methylbutyl)barbituric acid
Phenobarbitone	5-ethyl-5-phenylbarbituric acid
Quinalbarbitone	5-allyl-5-(1-methylbutyl)barbituric acid
Thiopentone	5-ethyl-5-(1-methylbutyl)-2-thiobarbituric acid

Where the acid itself was not available, it was prepared by liberation from the salt, extraction and drying to constant weight before use.

To assess column performance, an aliquot of each solution was injected in turn and the retention times recorded (see Table III). The two non-barbiturate hypnotics, methaqualone and glutethimide, and the compound chosen as internal standard, tetraphenylethylene (TPE), were similarly tested.

For routine work, however, it was more convenient to prepare two mixtures consisting of the most frequently encountered drugs. The first contained the free acids of barbitone, butobarbitone, amylobarbitone, pentobarbitone and quinalbarbitone, each at a concentration of 10 mg/100 ml in chloroform. The second contained heptabarbitone, cyclobarbitone and phenobarbitone in addition to the other five and at the same concentration. Injection of one of these mixtures at the beginning of an analysis provided a basis to which any unknown could be related.

TABLE III

RETENTION TIMES OF COMPOUNDS TESTED ON EACH COLUMN

Compound	*Retention time (min)*											
	1% Carbowax 20M	*3% Poly A-103*	*3% NGA+ Trimer acid*	*1% SP-1000*	*3% PPE-20*	*10% Apiezon L*	*4% CDMS*	*3% OV-1*	*5% OV-17*	*3% OV-25*	*4% OV-210*	*4% OV-225*
Allylbarbituric acid	4.4	3.2	4.8	2.8	2.4	3.7	4.0	2.5	2.9	2.7	2.8	4.7
Amylobarbitone	4.2	3.4	5.0	2.7	2.2	4.4	4.1	3.2	3.4	3.2	3.8	5.3
Barbitone	2.6	1.8	2.6	1.8	1.8	1.8	2.5	1.2	1.7	1.3	1.7	3.1
Butobarbitone	4.0	3.0	4.5	2.6	2.6	3.7	3.8	2.6	3.0	2.7	3.2	4.8
n-Butylallylbarbitone	5.2	3.7	5.4	3.1	2.6	4.4	4.7	3.1	3.6	3.3	3.5	5.8
Cyclobarbitone	16.5	10.7	13.9	9.3	9.2	14.4	14.3	9.3	12.0	14.6	10.5	18.8
Glutethimide	2.5	2.4	4.3	1.8	3.2	10.7	4.2	5.2	7.7	8.6	7.1	8.2
Heptabarbitone	22.4	15.7	20.1	12.4	13.6	21.6	19.4	13.6	16.7	20.6	14.4	25.3
Hexobarbitone	2.9	2.9	4.6	1.9	3.0	9.4	4.1	5.6	7.4	8.5	6.6	8.0
Methaqualone	4.4	5.7	8.6	3.1	9.4	—	8.6	—	27.0	—	14.1	17.2
Methohexitone	1.5	1.4	2.2	1.0	1.1	5.5	1.8	4.0	4.4	4.5	2.9	3.6
Nealbarbitone	4.9	3.8	5.3	3.0	2.2	4.8	4.5	3.0	3.6	3.3	3.3	5.5
Pentobarbitone	4.6	3.8	5.2	2.9	2.3	5.2	4.5	3.5	3.8	3.7	4.0	6.1
Phenobarbitone	29.4	16.2	20.6	15.8	Poor response	14.2	23.4	9.8	13.2	16.6	11.1	25.6
Quinalbarbitone	6.0	4.6	6.2	3.6	2.8	6.3	5.6	4.3	4.6	4.5	4.3	7.1
Tetraphenylethylene	5.3	8.0	10.4	3.4	9.8	>60	7.8	>60	66.4	>60	8.5	17.8
Thipentone	5.0	6.1	7.0	3.2	No response	11.1	6.2	5.5	6.1	5.8	4.0	—

Quantitative measurement

A range of standard solutions each containing 50 μg/ml of TPE as internal standard and from 20–200 μg/ml of the barbituric acid were made up in chloroform for the eight most commonly prescribed barbiturates already mentioned. Stock solutions of TPE and barbituric acid were made and aliquots of each mixed and diluted to 50 ml with chloroform as indicated in Table IV.

TABLE IV

THE COMPOSITION OF STANDARD SOLUTIONS OBTAINED BY DILUTING STOCK SOLUTIONS OF TETRAPHENYLETHYLENE (100 mg/100 ml) AND BARBITURIC ACID (100 mg/100 ml) TO 50 ml WITH CHLOROFORM

Barbituric acid stock solution (ml)	*Tetraphenylethylene stock solution (ml)*	*Barbituric acid (μgequiv. in 0.1 ml)*
1	2.5	2
2	2.5	4
3	2.5	6
4	2.5	8
5	2.5	10
6	2.5	12
7	2.5	14
8	2.5	16
9	2.5	18
10	2.5	20

These sets of standard solutions were stored in a dark cupboard and were thus always ready for use. Once the barbiturate involved had been identified from the qualitative mixture, a calibration curve was readily prepared by injecting 3–5-μl aliquots of the appropriate standards and plotting a graph of peak-height ratios against concentration. The ratio of drug to internal standard peak height was then measured for the unknown sample and related back to the calibration graph to give the drug content of the sample directly in terms of μg per extract. Knowing the plasma or urine volume extracted, the drug concentration can easily be calculated in its customary form of mg/100 ml, *viz.*:

If n=total number of μg in extract (from calibration graph)

N=volume of plasma analysed (ml)

then the drug concentration (mg/100 ml) = $\frac{n}{N} \cdot \frac{100}{1000}$

Reagents

The following reagents were used:

AnalaR chloroform, redistilled before use.

0.5 N Sodium hydroxide and 1 N hydrochloric acid, prepared from concentrated volumetric solutions (Hopkin & Williams, Chadwell Heath, Essex) and washed three times with chloroform before use.

Internal standard: a 1.0 mg/100 ml solution of TPE (Koch-Light, Colnbrook, Bucks.) in chloroform.

The commonly prescribed barbiturates were obtained generally as their free acids, in a standard pack from May & Baker Ltd., Dagenham, Essex. Others which were not available from this source were kindly supplied by their respective distributing companies.

Glass wool: The quality was variable. Sometimes it could be used directly, but often it was necessary to wash out impurities with chloroform. This was conveniently achieved by treating large batches in a Soxhlet extraction apparatus.

Extraction procedures

(1) Therapeutic levels. 5.0 ml of plasma or urine were extracted with 15 ml of chloroform by gently shaking for 10 min in a 30-ml centrifuge tube. After spinning at 3,000 rpm (8-in. rotor) the organic layer was withdrawn by means of a Pasteur pipette and transferred into a conical tube (Q & Q) via a glass-wool plug fixed into a small funnel. This prevented the transfer of any precipitate present at the liquid interface. The sample was extracted with another 10 ml of chloroform and the second organic layer pooled with the first.

To the chloroform, 5.0 ml of 0.5 *N* sodium hydroxide were added and shaken gently for 10 min. After centrifuging the solvent was discarded. The sodium hydroxide was washed with successive 10-ml aliquots of chloroform until no interface precipitate remained (usually two-or-three such washings were required). To the washed sodium hydroxide extract, 3.0 ml of *N* hydrochloric acid were added and the aqueous phase was shaken with 10 ml of chloroform for 10 min. The chloroform was transferred to a 10.0-ml conical tube containing 0.5 ml of the internal standard solution. With the tube immersed in a water-bath at 60°, the solvent was evaporated to dryness under a stream of air. The remaining aqueous phase was re-extracted with a further 5.0 ml of chloroform and evaporated to dryness with the rest. The residue was carefully dissolved in 0.1 ml of chloroform and a 5-μl aliquot was injected on to the gas chromatograph.

(2) Overdose levels. The extraction followed the same lines as for therapeutic levels, but with the following modifications:

The sample size was reduced to 1.0 ml and this was extracted once with 15 ml of chloroform. When back-extracting into 5 ml of 0.5 *N* sodium hydroxide the shaking time was reduced to 5 min. The number of washings of the sodium hydroxide layer could be reduced to one since a smaller sample was used initially. The final re-extraction into chloroform was done by shaking once for 5 min with 10 ml of solvent. The volume of internal standard used was increased to 1.0 ml and the volume of extract introduced into the gas chromatograph was reduced to 1–2 μl.

RESULTS

Qualitative separation

Table III shows the retention times of fourteen barbiturate drugs on all of the stationary phases under test. Figs 1–11 show the separations obtained with the various columns of a mixture containing the eight barbiturates most commonly encountered in practice. No trace is shown for the PPE-20 column since the performance was so

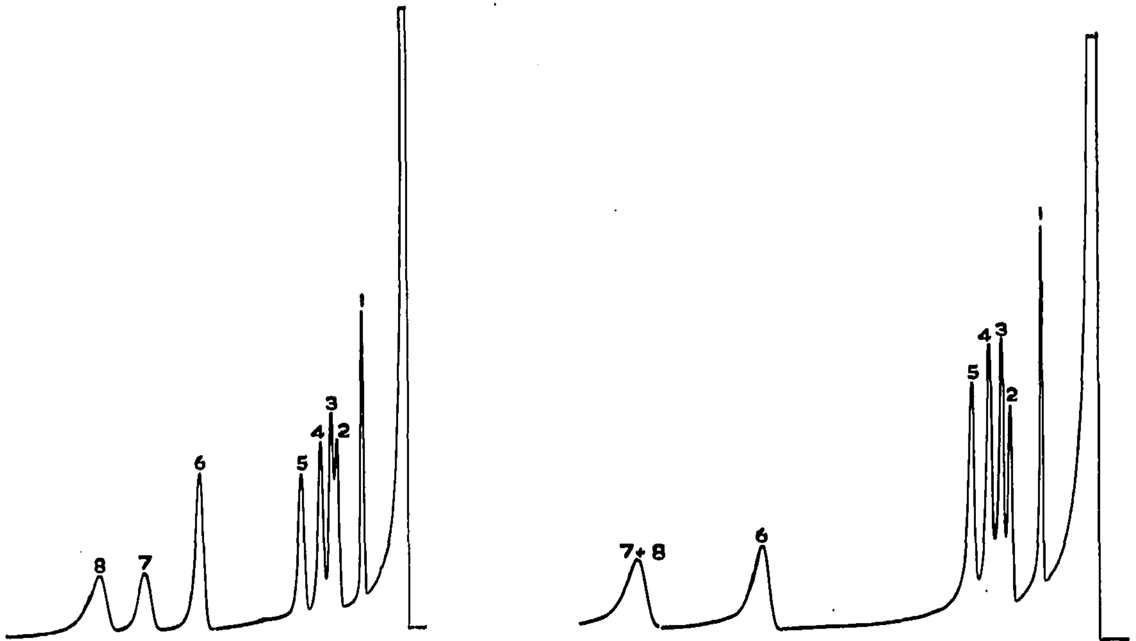

Fig. 1. Separation of a qualitative barbiturate mixture containing the eight most frequently encountered drugs. Peaks are numbered as follows: 1 = barbitone; 2 = butobartitone; 3 = amylobarbitone; 4 = pentobarbitone; 5 = quinalbarbitone; 6 = cyclobarbitone; 7 = heptabarbitone; 8 = phenobarbitone. Stationary phase, CDMS.

Fig. 2. Separation as in Fig. 1. Stationary phase, OV-225.

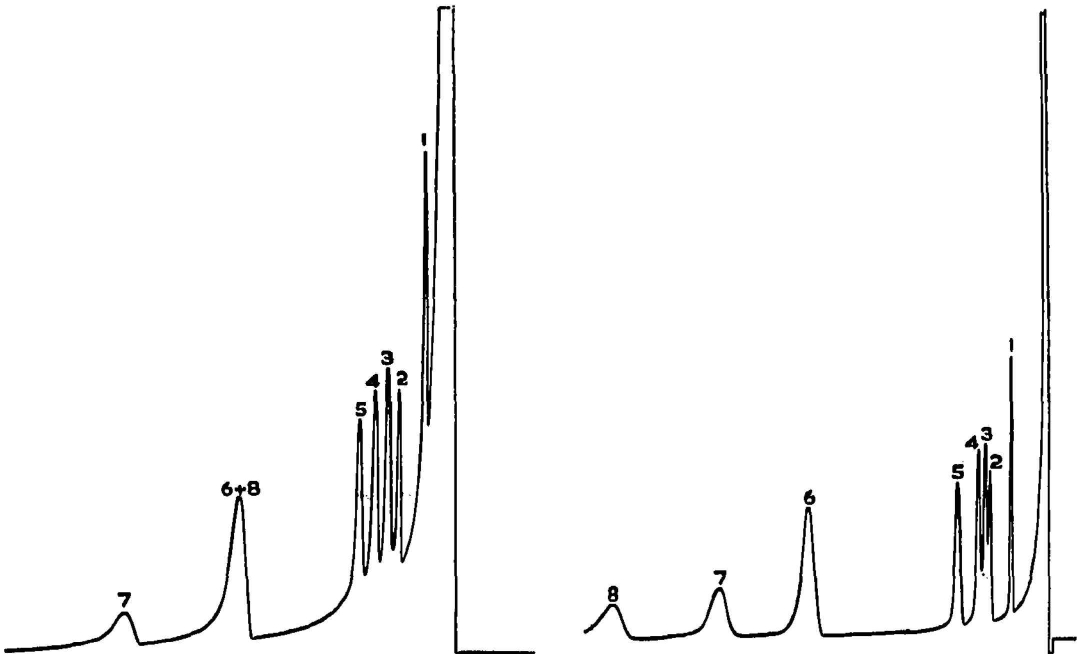

Fig. 3. Separation as in Fig. 1. Stationary phase, Apiezon L.

Fig. 4. Separation as in Fig. 1. Stationary phase, Carbowax 20M.

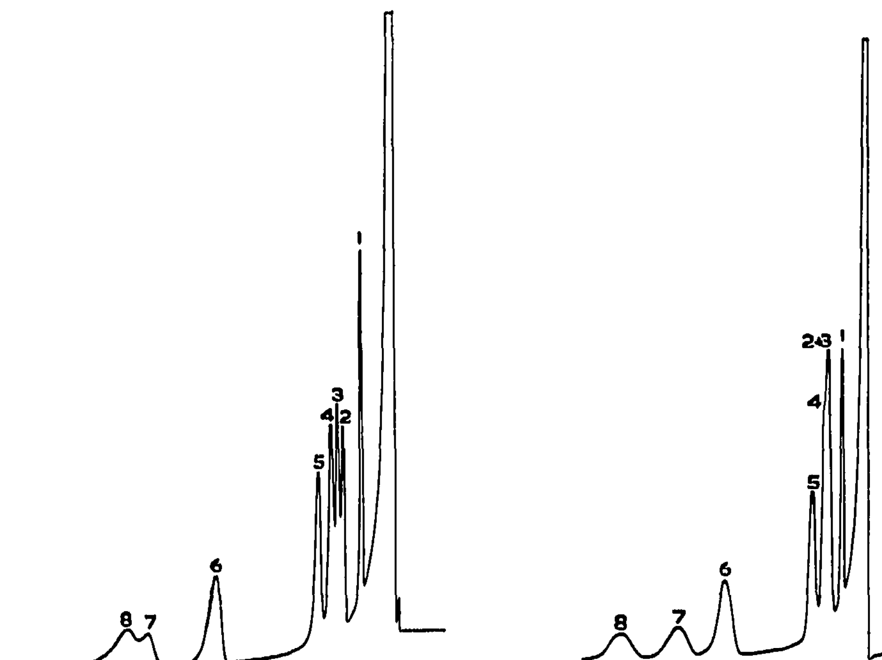

Fig. 5. Separation as in Fig. 1. Stationary phase, NGA+Trimer acid.
Fig. 6. Separation as in Fig. 1. Stationary phase, SP-1000.

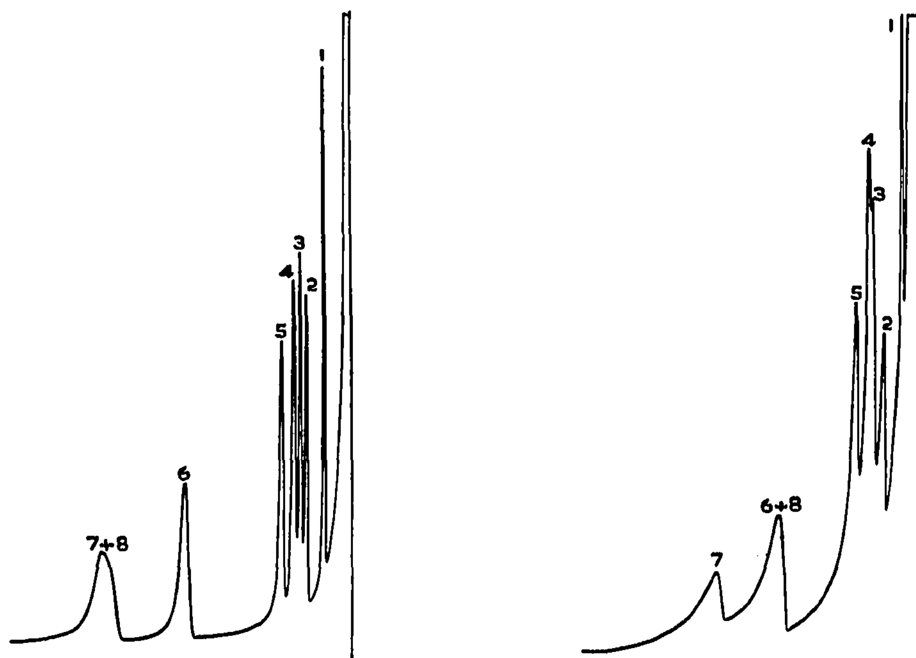

Fig. 7. Separation as in Fig. 1. Stationary phase, Poly A-103.
Fig. 8. Separation as in Fig. 1. Stationary phase, OV-1.

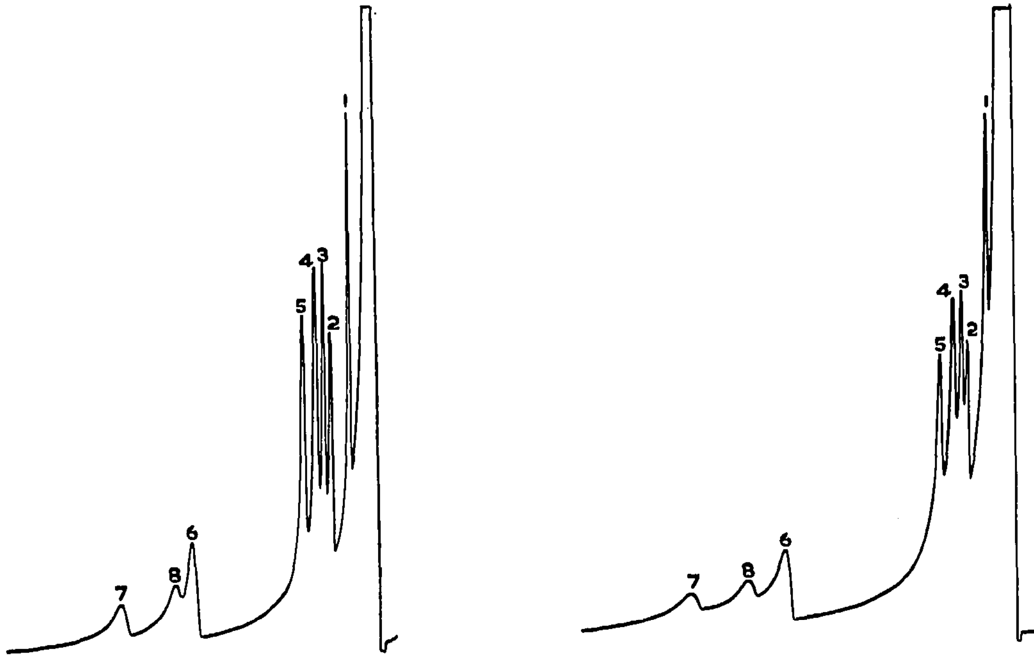

Fig. 9. Separation as in Fig. 1. Stationary phase, OV-17.

Fig. 10. Separation as in Fig. 1. Stationary phase, OV-25.

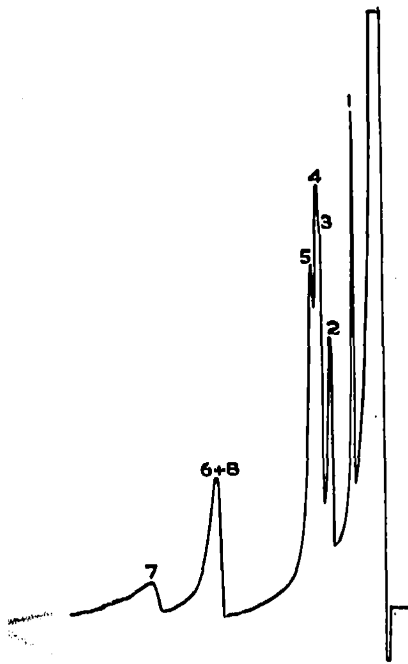

Fig. 11. Separation as in Fig. 1. Stationary phase, OV-210.

poor that it was not considered worthwhile to reproduce it. It can be seen that several of the columns could be regarded as satisfactory with respect to separation of the mixture, but there are other important factors which influence the final choice and these will be discussed later.

The non-barbiturate hypnotics, glutethimide and methaqualone, were also chromatographed on these same columns, although a different extraction procedure had to be employed for them[25,26].

Quantitative measurement

It can be seen from Fig. 12 that for all the commonly prescribed barbiturates the ratios of barbiturate to TPE peak heights were linear over the concentration range employed. The standard curves all pass through zero, with the exception of that for phenobarbitone. This indicates that either a small amount of absorption of this drug is still occurring, despite the care taken with silanisation of glassware and use of high-performance supports or, alternatively, because of the longer retention time and consequently wider peak width, the peak height relationship has become inaccurate. Peak area measurement was not used, however, since the accuracy is quite adequate for toxicological purposes.

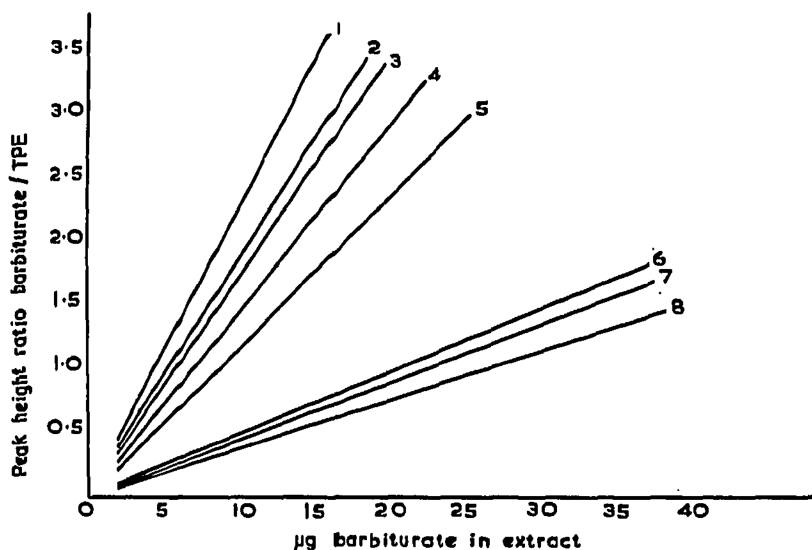

Fig. 12. Standard calibration graph relating the ratio of the peak heights of the barbiturates and TPE to the number of micrograms of barbiturate in the extract. Barbiturates are numbered as in Fig. 1.

Recovery studies

Other workers[13,21,22,24,27] have reported the barbiturate recoveries achieved with their various methods of analysis. Table V shows the recoveries attained in the present study by extraction procedure 1 in the therapautic range (0–0.4 mg/100 ml) and by procedure 2 in the overdose range (1–10 mg/100 ml).

Recovery experiments at therapeutic levels were performed by adding from

2 to 20 μg of each drug as the free acid dissolved in 1.0 ml of 0.01 N sodium hydroxide to 4.0 ml of outdated blood-bank plasma. The adequacy of the extraction procedure was then verified, first by adding sufficient acid to neutralise the sodium hydroxide and then subjecting the samples to procedure 1.

The overdose recovery experiments were performed by adding 10, 20, 40, or 80 μg of each drug dissolved in 0.2 ml of 0.01 N sodium hydroxide to 0.8 ml of outdated blood-bank plasma. These samples were then subjected to procedure 2 after first adding enough acid to neutralise the sodium hydroxide used to dissolve the drug.

TABLE V

RECOVERIES OF THE COMMONLY PRESCRIBED BARBITURATE DRUGS FROM PLASMA BY THE TWO PROCEDURES

Barbiturate	*Mean recovery (%)*	
	Procedure 1	*Procedure 2*
Barbitone	82 ± 4	55
Butobarbitone	88 ± 3	76
Amylobarbitone	95 ± 3	85
Pentobarbitone	96 ± 3	77
Quinalbarbitone	94 ± 3	93
Cyclobarbitone	85 ± 4	81
Heptabarbitone	91 ± 3	86
Phenobarbitone	85 ± 5	67

Specificity

No peaks arose from constituents of normal plasma or urine, irrespective of the state of the sample or type of preservative, provided a back-extraction was included in the work-up.

On the columns tested, glutethimide, methaqualone, methyprylon, meprobamate and phenazone (from trichloralphenazone) all have retention times similar to those of barbiturates, while the anticonvulsant drug, ethotoin, chromatographs to give a peak coincident with TPE on the CDMS column. Any of these drugs may be present in the plasma in association with barbiturates, especially in overdose cases. By performing a back-extraction with sodium hydroxide, however, these non-barbiturates, which are neutral or weakly basic, can be eliminated from the final concentrate.

There are other acidic drugs, *e.g.* acetylsalicylic acid, phenylbutazone, primidone, phenytoin, chlorpropamide, which come through the extraction and are known to cause confusion when measuring barbiturates by spectrophotometric techniques. Fortunately, on most of the gas chromatographic columns their retention times are distinct from those of the barbiturates, except perhaps for phenylbutazone, which on the CDMS column mimics cyclobarbitone, though it can be differentiated on the OV-225 column.

TABLE VI

ASSESSMENT OF COLUMNS

Amylo= Amylobarbitone; buto= butobarbitone; cyclo= cyclobarbitone; hepta= heptabarbitone; hexo= hexobarbitone; neal= nealbarbitone; pento= pentobarbitone; pheno= phenobarbitone; quinal= quinalbarbitone; TPE= tetraphenylethylene.

Stationary phase	*Ref.*	*Separation of mixture*	*Common drugs not resolved*	*Special separations*	*Peak symmetry*	*Analysis time (min)*	*TPE position*	*Barbitone position relative to solvent front*	*Phase stability*	*Sensitivity*
Carbowax 20M	34	v-good	none	none	v-good	29.4	pento/quinal	well removed	fair	v-good
Poly A-103	37	good	pheno/hepta	none	v-good	16.0	quinal/cyclo	quite well removed	v-good	v-good
NGA + Trimeracid	16	v-good	none	none	v-good	20.6	quinal/cyclo	quite well removed	poor	good
SP-1000	35	fair	buto/amylo/ pento	anticonvulsant drugs	v-good	15.8	pento/quinal	quite well removed	fair	good
PPE-20	35	poor	buto/amylo/ pento	none	poor	–	cyclo/hepta	quite well removed	poor	poor
Apiezon L	17	good	pheno/cyclo	buto/amylo/ hexo	good	21.6	very long retention time	well removed	v-good	v-good
CDMS	36	v-good	none	none	v-good	23.4	quinal/cyclo	well removed	v-good	v-good
OV-1	38	poor	amylo/pento pheno/cyclo	none	poor	13.6	very long retention time	very close	v-good	good
OV-17	39	v-good	none	none	good	16.7	very long retention time	close	v-good	v-good
OV-25	–	v-good	none	none	poor	20.6	very long retention time	close	good	fair
OV-210	38	poor	amylo/pento pheno/cyclo	none	poor	14.4	quinal/cyclo	close	fair	good
OV-225	–	good	pheno/hepta	neal/pento amylo/hexo	good	25.6	quinal/cyclo	well removed	v-good	good

Columns

The performance of each column was assessed and the criteria, considered together with the comparative results, are shown in Table VI. The table headings are self-explanatory. Peak symmetry describes the degree of tailing, and the analysis time is the time taken for the qualitative mixture of eight drugs to elute. TPE position indicates the two barbiturate drugs between which the internal standard elutes and the reference is the literature reference to earlier work which prompted us to test that particular stationary phase.

The following comments give a guide to the peculiarities of each column:

Carbowax 20M. Generally this is a good column (Fig. 4) but TPE elutes at an inconvenient point and the analysis time is long.

Poly A-103. This is a very good column (Fig. 7), its only disadvantage being the inability to separate phenobarbitone and heptabarbitone. The separation of butobarbitone and amylobarbitone is good enough to determine one in the presence of the other, using TPE as internal standard, which is difficult with the CDMS column.

NGA+Trimer acid. Being a two-component phase this is more difficult to prepare. Also stability is poor since it is operated above the maximum temperature recommended for the two components. Separation (Fig. 5) is similar to CDMS.

SP-1000. Separation is unacceptable for routine work (Fig. 6) and TPE elutes at an inconvenient point. This phase has been found useful at 2% loading for anticonvulsant drug analysis[28] using argon or helium as carrier gas to exclude traces of oxygen, since in the presence of the latter SP-1000 seemed unstable.

PPE-20. This phase has only recently been recommended for barbiturate analysis, but in our hands produced disappointing results. The general performance was so poor that we did not consider it worthwhile to reproduce the trace of the mixture chromatogram among the figures. A 1% loading was also tested since this was quoted in the original work, but the performance was no better than with the 3% loading.

Apiezon L. Hexobarbitone is not often prescribed, but the column separates it from amylobarbitone, whereas CDMS does not. General separation is good (Fig. 3), but TPE has an unacceptably long retention time. This is another useful column for confirming identity and, in particular, analysing the awkward mixture of hexobarbitone, amylobarbitone and butobarbitone, if more than one of these has been ingested. TPE, however, cannot be used as the internal standard.

CDMS. This is the best all-round column (Fig. 1). The separation of butobarbitone and amylobarbitone is not complete so it is difficult to analyse one in the presence of the other. Phenobarbitone has a long retention time at 220°, but the phase is very stable and can be operated at 240° to analyse more quickly the drugs which are retained for a longer time. It is an easy column to reproduce and is also the liquid phase of choice for the non-barbiturate hypnotic, methaqualone[26].

OV-1. This phase was tested as an improved SE-30 which had been used successfully by several workers, but in our hands its overall performance was unacceptable for this work (see Fig. 8).

OV-17. A generally good column which separated all eight barbiturates in the test mixture, although one can see from Fig. 9 that phenobarbitone and cyclobarbitone were not completely resolved. The TPE retention time was too long for

it to be used as an internal standard so, although this is a good general purpose column, it has not been recommended in this case.

OV-25. Despite complete separation (Fig. 10), this phase cannot be recommended since peak shape is poor and retention time of TPE is too long for use as internal standard.

OV-210. This was tested as an improved QF-1, but was a generally poor column (Fig. 11). Since TPE elutes in a convenient position it could be used to differentiate and quantitate some of the less commonly prescribed barbiturates that CDMS will not resolve.

OV-225. After CDMS this is the most useful column. It gives a generally good separation (Fig. 2), but will not separate phenobarbitone from heptabarbitone. It will resolve some drug pairs which CDMS will not, *viz.* nealbarbitone/pentobarbitone, hexobarbitone/amylobarbitone, and these can be quantitated against TPE, which chromatographs in a convenient place. It is an easily prepared stable phase which we have also found useful for analysis of the anticonvulsant drugs, methoin, ethotoin and ethosuximide[29] and also the non-barbiturate hypnotic, meprobamate.

DISCUSSION

Extraction procedure

Table V indicates that in practice there is very little difference in efficiency between the two extraction procedures described. With the exception of barbitone, which has an unfavourable partition coefficient, the variations which do occur may be attributed mainly to the small quantities of solvent which a single extraction leaves behind.

One difficulty when analysing large plasma volumes containing only therapeutic drug levels is the protein precipitate which forms between the chloroform and the aqueous layers. Initially this is overcome by pushing the Pasteur pipette through the pad of protein and into the chloroform to withdraw it and, further, preventing the transfer of any traces of solid material by the use of a glass-wool plug in a funnel.

A second precipitate tends to appear on back extraction and this remains behind when the organic layer is discarded. It can be removed by repeat washing of the sodium hydroxide with 10-ml aliquots of redistilled chloroform, sometimes as many as four times. By performing the back extraction in conical tubes complete removal of the bottom phase is made easy.

We have found it unnecessary to dry the solvent with sodium sulphate prior to evaporation so long as care is taken with its withdrawal in the final stages. For reconstitution of the residue prior to injection, acetone or ethanol have been recommended. In our hands chloroform is superior since it gives narrower and less-tailing solvent fronts.

Because one should not rely on the resolving power of the gas chromatograph to separate the drugs of interest from co-extractable, endogenous material, both procedures involve a back extraction to minimise the possibility of interference. Several quicker methods of extraction have been reported[9,13,22,24] and very often these are quite adequate so long as drug levels are high. The limitations of such procedures have been pointed out, however, by describing a number of naturally occurring compounds that can be mistaken for barbiturates[30,31]. There are also a

number of neutral and basic drugs which extract into chloroform at physiological pH and chromatograph on most of the columns investigated. Thus, back extraction was considered to be a useful step since it reduced the drugs appearing in the final extract to those with acidic character and produced clean backgrounds from any type of sample irrespective of its age. Furthermore, certain components of blood which are carried over in the absence of back extraction have long retention times and will accumulate on the column, so shortening its life, or may elute during a subsequent analysis and create secondary interference. Time, then, may be wasted in prolonging the interval between injections thus destroying any advantage gained by quick extraction.

Another incidental advantage claimed for this back extraction is that the caustic layer before and after acidification can be subjected to ultraviolet spectrophotometry, thus getting corroborative analytical information[27].

Finally, some authors have introduced a protein precipitation when analysing plasma[23]. This, undoubtedly, prolongs and complicates the manipulations and is not conducive to good recoveries of these drugs. Our method is much more rapid and even when extracting large plasma volumes, where it may be necessary to wash the sodium hydroxide layer several times to remove interface proteins, the total time taken seldom exceeds one hour. With overdose samples and small plasma volumes, there is correspondingly less washing because less precipitate forms.

Internal standard

As an internal standard for barbiturates on the CDMS or OV-225 column TPE as recommended by Parker *et al.*[18] is excellent since it elutes in the vacant position between quinalbarbitone and cyclobarbitone. It produces symmetrical peaks, it is cheap and available in a high state of purity, though cannot be used with non-polar columns because on these its retention time is too long.

The calibration standards were quite stable when stored in the dark at room temperature, the ratios not varying by more than 2% from month-to-month. Accuracy thus remains well within the limits required for routine toxicology. TPE is, however, photodegradable and so the final evaporation should be performed away from direct sunlight.

The range of standards described is suitable for determining therapeutic plasma levels and, when quantitating the overdose procedure against them, one simply doubles the result to allow for the larger aliquot of internal standard that is used.

We found the barbiturate standard adopted by some workers offered no advantage, whilst it added to the manipulations required.

Preparation and choice of columns

The columns were all prepared by a simple, standard evaporation technique that could be carried out in any busy laboratory. More refined methods, although undoubtedly producing excellent results, tend to be too difficult and time-consuming for everyday use.

With the barbiturates, irreversible adsorption on the column readily occurs and an excellent discussion of this point is to be found in a paper by Brochmann-Hansen[32]. To overcome this complication some authors recommend initial loading of the column on beginning the day's work. Others[15,21] have resorted to forming

a methyl derivative prior to chromatography, but this leads to some loss of definition, although sensitivity is improved. Saturation of the carrier gas with formic acid has also been advocated[33].

In our experience the high performance supports now available are satisfactory for the analysis of submicrogram quantities of barbiturates without further treatment and, to maintain standard conditions, Chromosorb W, HP, was used throughout. Even so, we found that when injecting these small quantities, no drug would elute unless the glassware had been adequately silanised beforehand, as described earlier. After taking these precautions we could satisfactorily handle 1–5-ml plasma samples at overdose and therapeutic drug levels. However, when dealing with micro-samples at therapeutic levels, additional precautions against adsorption would still be required.

It could be argued that the method of preparation and loading has not been optimised for each column and some that have been dismissed here may be suitable if prepared differently. The elution order will remain the same, however, irrespective of the loading, and the aim of this communication was to compare columns similarly prepared by a simple procedure.

The most useful single column of all those tested was the 4% CDMS and it is on to this that we routinely inject our barbiturate extracts initially. It is easily prepared, gives good separation, is a very stable column with a long life and our experience with it has been most favourable. The next most useful was the OV-225, despite its inability to separate phenobarbitone and heptabarbitone. The other columns which we occasionally find helpful are the Poly A-103 and the Apiezon L, particularly if a mixture of several barbiturates has been ingested, but TPE cannot be used with the latter as internal standard.

Confirmation of identity is always useful. Even though a selective extraction procedure is used, it is possible for interferences occasionally to arise. Moreover, the drug involved may be one of the less commonly encountered barbiturates and not that suggested by the CDMS column in the first instance. Some authors prefer derivative formation to confirm their findings[18], but in our experience simple chromatography on two columns is satisfactory. This is conveniently performed by having a dual column instrument containing one CDMS and another column of choice in the second position. In our case this is usually an OV-225, because it will differentiate hexobarbitone and nealbarbitone from the more commonly prescribed amylobarbitone and pentobarbitone and still allow quantitation against the TPE internal standard. Since both of these columns can be operated over the same temperature range, it is simply a question of altering the temperature by $\pm 15°$ when moving from one column to the other.

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