A rapid micro-method for the screening and measurement of barbiturates and related compounds in plasma by gas-liquid chromatography

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SYNOPSIS A rapid gas-chromatographic method has been developed for the determination of butobarbitone, amylbarbitone, hexobarbitone, pentobarbitone, quinalbarbitone, phenobarbitone, glutethimide, and methaqualone in 'finger-prick' samples of plasma. This has been applied to the analysis of some of these drugs in plasma taken from patients after therapeutic dosage and over-dosage.

Barbiturates and related hypnotics are commonly taken in overdosage and their rapid identification and measurement in plasma may be of assistance to the clinician. At present the method of choice is solvent extraction followed by gas-liquid chromatography, since this will give both a tentative identification and an accurate measurement in one step. Several such procedures have been reported (Brad-dock and Marec, 1965; Leach and Toseland, 1968; McMartin and Street, 1966; Reith, van der Heide, and Zwaal, 1965; Street and McMartin, 1963; Sunshine and Maes, 1968) but only those of Jain, Fontan, and Kirk (1964), Anders (1966), MacGee (1971), Scott (1971), and Källberg, Agureel, Jalling, and Boréus (1971) can be quickly performed on less than the 1 to 5 ml of whole blood or plasma required by other methods. Moreover, these volumes are not always readily obtainable, especially from young children.

Jain, Fontan, and Kirk (1964) took 0.1 ml of whole blood, extracted this four times with 0.5 ml of solvent and chromatographed the combined extract. The accuracy of the method was indeterminate since no internal standard was incorporated and no recovery studies were described.

Anders (1966) carried out solvent extraction of 0.1 ml of acidified whole blood with 0.1 ml of chloroform in 5 ml centrifugal separators. Aliquots of the chloroform layer were withdrawn into a micro-syringe, via a rubber septum situated at the base of

the separator, and these were injected directly onto the gas chromatograph. The separators are expensive, difficult to obtain, and not easily cleaned. Furthermore, the extracts may become contaminated by compounds dissolved out of the septum, although this possibility has been minimized by the insertion of an aluminium disc directly above the septum.

In the methods of both MacGee (1971) and Scott (1971), extraction was performed using a solvent containing a standard mixture of barbiturates. The chromatogram obtained from this extract was compared to that obtained from the standard mixture alone. Measurement of barbiturate in the sample was achieved using an adjacent barbiturate peak as an internal standard. Although a novel approach, the wisdom of deliberately introducing a multitude of peaks onto the chromatogram is questionable. Gas-chromatographic analysis of blood and plasma samples is notoriously susceptible to interference from contaminants of solvents and glassware and from other blood components. The degree of purity of individual extracts would be indeterminate using these procedures.

Källberg *et al* (1971) recently reported a method for the estimation of phenobarbitone in 100 μ l plasma samples, but the behaviour of other barbiturates in this system was not examined.

The method now described is a modification of that of Anders (1966). The use of Dreyer tubes (to BS 625), which are cheap and therefore disposable, avoids any cross-contamination of samples. An additional refinement is the inclusion of an internal standard to eliminate the possibility of injection errors.

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Gas Chromatography

A Pye series 104 dual-column gas chromatograph equipped with a flame ionization detector and a 1 mv Perkin-Elmer 159 recorder was used. The column was a 5 ft $\times$ $\frac{1}{8}$ in. OD coiled glass tube which had been silanized with a 5% solution of dimethyldichlorosilane in benzene over a period of 24 hours. Glass-wool was silanized in the same solution. After drying overnight at 100° the column was packed with 4% cyclohexanedimethanol succinate (CDMS) (Perkin-Elmer Limited, Beaconsfield, Buckinghamshire) on 85-100 mesh Diatomite C.Q. (J.J.'s Chromatography Limited, King's Lynn, Norfolk) according to the method of Berry (1969). The column was conditioned for 48 hours at 240° with a nitrogen flow rate of 60 ml/minute. Thereafter, the column was tapped down, more packing material added, and reconditioned for a further 48 hours before use.

The instrument settings were as follows: column oven temperature 225°; injection port setting 2; detector oven temperature 250°; nitrogen (carrier gas) flow rate 60 ml/min; attenuation $\times$ 50; hydrogen flow rate 60 ml/min; air flow rate 600 ml/min. If the presence of phenobarbitone was suspected in the sample, a column oven temperature of 235°C was selected.

The retention times of the drugs under study are given in Table I.

Column Oven Temperature 225°		Column Oven Temperature 235°	
Drug	Retention Times in Minutes	Drug	Retention Times in Minutes
Butobarbitone	3.5	Tetraphenylethylene	4.3
Amylobarbitone	3.8	Phenobarbitone	12.7
Hexobarbitone	3.8		
Glutethimide	4.1		
Pentobarbitone	4.2		
Quinalbarbitone	5.1		
Tetraphenylethylene	6.9		
Methaqualone	8.1		

Table I Retention times

EXTRACTION PROCEDURES

The following reagents were employed: 4.0 M sodium dihydrogen orthophosphate (AR grade) in distilled water (phosphate buffer); 10 μ g/ml tetraphenylethylene (TPE) (available from Koch-Light Laboratories, Colnbrook, Buckinghamshire) in chloroform (AR grade, redistilled).

Five μ l of buffer solution was introduced into a clean Dreyer tube using a Hamilton repeating dispenser

fitted to a 250 μ l gas-tight syringe (available from Field Instruments Limited, Richmond, Surrey). A 50 μ l aliquot of the plasma sample was added via an Eppendorf semi-automatic pipette (available in the UK from V. A. Howe and Company, London, W.11) and finally 50 μ l of 10 μ g/ml TPE solution was dispensed into the tube using a Hamilton repeating mechanism fitted to a 2500 μ l gas-tight syringe (5 μ g/ml TPE solution was used if the presence of phenobarbitone in the sample was suspected).

The contents of the tube were mixed thoroughly for 30 seconds on a Whirlmixer and centrifuged for two to three min at 2000 to 3000 rpm.

The presence of a lipoprotein interface between the aqueous and organic layers made careful withdrawal of the extract essential. To prevent contamination or blockage of the syringe needle, a small quantity of air was taken into the syringe and, after passing the needle through the protein layer, was slowly expelled.

A suitable quantity of extract was taken up and, on withdrawing the sample, the outside of the needle was wiped with a tissue before injection onto the column of the gas chromatograph.

QUANTITATION

For each of the drugs under study, a range of standard solutions in redistilled chloroform was prepared. These standards contained from 5 to 50 μ g/ml of drug and 10 μ g/ml of TPE, with the exception of phenobarbitone standards which contained 5-100 μ g/ml of the drug and 5 μ g/ml of TPE. The ratio of the peak height of drug to the peak height of the internal standard was linear in each case over the stated concentration range. Calibration graphs were prepared by injecting 3-5 μ l aliquots of these solutions after injection of unknown samples.

Figures 1 to 4 illustrate the responses and separations obtained with the standard chloroform solutions of the drugs under study.

Results and Discussion

RECOVERY STUDIES

Standards containing phenobarbitone at a concentration of 10, 20, and 50 μ g/ml were prepared in heparinized bovine plasma. Concentrations of 5, 10, and 20 μ g/ml were used for the other drugs under study.

These standards were taken through the extraction procedure described above and the results obtained are shown in Table II. It can be seen that recoveries were of the order of 90%, with a standard deviation of 8% or less for all drugs, and were uniform over the concentration range studied.

Fig. 1.

Fig. 1 Two μ l injection of standard barbiturate mixture containing 10 μ g/ml of (1) butobarbitone, (2) amylobarbitone, (3) hexabarbitone, (5) pentobarbitone, (6) quinalbarbitone, and (S) tetraphenylethylene.

Fig. 2.

Fig. 2 Three μ l injection of standard methaqualone solution, containing 10 μ g/ml methaqualone (7) and 10 μ g/ml tetraphenylethylene (S).

Fig. 3.

Fig. 3 Three μ l injection of 10 μ g/ml glutethimide standard (4) with 10 μ g/ml tetraphenylethylene (S).

Fig. 4.

Fig. 4 Three μ l injection of 5 μ g/ml standard phenobarbitone (8) solution. Internal standard = 5 μ g/ml tetraphenylethylene (S).

Drug	% Recovery	Standard Deviation (%)
Butobarbitone	83	5
Amylobarbitone	91	8
Pentobarbitone	94	5
Hexabarbitone	94	4
Quinalbarbitone	85	8
Phenobarbitone	78	6
Methaqualone	88	5
Glutethimide	100	7

Table II Recoveries of added drug from heparinized bovine plasma over the concentration range 5-20 µg/ml (phenobarbitone 10-50 µg/ml)¹

¹Each result represents the mean of 15 samples.

COMPARISON WITH THE STANDARD MACRO-SCALE EXTRACTION PROCESS

The micro-method has been compared—with respect to results obtained, contamination in extract, and time taken to perform in duplicate—with a more elaborate procedure (Berry, to be published). In this method 1 ml of acidified plasma was extracted with 25 ml of chloroform; barbiturates present were back-extracted into 5 ml of 0.5N aqueous sodium hydroxide solution, and this was acidified and re-extracted with a further 15 ml of chloroform. One ml of TPE solution (internal standard) was added and the extract evaporated to dryness. Finally, the residue was taken up in 0.1 ml of chloroform and chromatographed on the system described previously.

Drug	Sample No.	Method A		Method B
		No. of Determinations	Result	Result (mean of triplicate)
Pentobarbitone	1	3	13.1	14.9
	2	3	17.1	16.6
	3	3	16.9	20.2
	4	2	38.1	34.5
	5	2	33.6	37.2
	6	2	29.2	32.0
Butobarbitone	1	1	34.7	36.5
	2	1	27.4	30.6
	3	1	22.1	25.9
	4	2	15.9	19.4
	5	2	11.6	13.1
	6	3	48.3	52.5
	7	3	19.7	24.5
Phenobarbitone	1	3	98.0	125.0
	2	3	118.0	122.0
Amylobarbitone (from Tuinal)	1	2	4.3	6.7
	2	3	4.0	3.6
	3	2	18.7	24.1
	4	3	6.5	10.0
	5	2	23.2	31.2
	6	2	21.9	17.3
Quinalbarbitone (from Tuinal)	1	2	2.3	4.1
	2	3	3.1	2.6
	3	2	16.0	20.7
	4	3	4.7	6.4
	5	2	18.0	19.3
	6	3	7.2	7.8

Table III A comparison of results obtained using the method of Berry (A) and the micro-method (B)¹

¹All results in µg/ml, corrected for extraction losses.

Fig. 5.

Fig. 6.

Fig. 5 One µl injection of extract of butobarbitone overdose sample. Internal standard (S) 10 µg/ml tetraphenylethylene.

Fig. 6 Three µl injection of sample from case of suspected methaqualone ingestion. Internal standard 10 µg/ml tetraphenylethylene.

The comparison of results obtained by the two procedures for barbiturate estimation is shown in Table III.

Both methods yielded spurious peaks on the chromatograms, but the micro-method gave a considerably cleaner extract from many samples (Figs. 5 and 6). In neither case were peaks encountered that interfered with the analysis, except sometimes at levels below $5 \mu\text{g/ml}$ (Fig. 7 could indicate a trace of pentobarbitone). (The micro-method involves no concentration of organic phase—this could contribute towards extract cleanliness.)

At the higher column oven temperature used for the measurement of phenobarbitone a contaminant peak with twice the retention time of the drug was consistently found when using the rapid method (Fig. 8). This difficulty was overcome by carefully timing the sample injections. At 225°C this peak lost much definition and could be injected over quite easily.

Finally, the micro-method took five minutes to extract a sample in duplicate; the long-extraction procedure took upwards of one hour.

EXTRACTION TIME

Experiments were performed to examine the effect

of extraction time on recovery and clarity of extract. It was found that 30 seconds was the optimum time period. Mixing for 60 seconds, or 120 seconds, increased the amount of contamination in the extract, but did not significantly effect the recovery of the drug.

REPRODUCIBILITY

The simplicity of the method allows highly reproducible results to be obtained (Table IV). Since the whole process takes place in a single tube, no losses of drug occur by multiple transfers of extracts between tubes. Furthermore, as there is no evaporation stage in the method, no losses can occur through solvent splashing out of the tube.

Sources of error can arise when measuring out the plasma and extracting the solvent. These are minimized by correct use of the Hamilton dispenser and the Eppendorf pipette, which in our hands have been shown to deliver volumes reproducible to within $\pm 2\%$.

POSSIBLE INTERFERENCE FROM OTHER DRUGS

Here, consideration is limited to those drugs commonly occurring in overdose at concentrations of, say, $5 \mu\text{g/ml}$ or greater. Acetylsalicylic acid, sali-

Fig. 7.

Fig. 7 Three μl injection of extract of plasma sample screened for presence/absence of drugs under study. S = $10 \mu\text{g/ml}$ tetraphenylethylene.

Fig. 8.

Fig. 8 Two μl of extract of plasma sample from phenobarbitone overdose case. Internal standard $5 \mu\text{g/ml}$ tetraphenylethylene.

	Sample	Peak Ratio	% Recovery
5 µg/ml	A	0.30	92
	B	0.28	87
	C	0.27	91
	D	0.28	93
	E	0.25	83
10 µg/ml	A	0.54	85
	B	0.56	87
	C	0.58	90
	D	0.59	93
	E	0.56	88
20 µg/ml	A	1.13	85
	B	0.97	74
	C	1.18	91
	D	1.14	87
	E	1.14	87

Table IV Results obtained from analysis of methaqualone bovine plasma standards of 5, 10, and 20 µg/ml concentration

Drug	Retention Time	Response
Phenacetin	0.42	Poor
Butobarbitone	0.49	Good
Quinalbarbitone	0.72	Good
Ethotoin	0.84	Good
Phenazone	0.89	Poor
Meprobamate	0.97	Very poor

Table V Drugs extracted and chromatographed by the system under study¹

¹Retention times relative to tetraphenylethylene at column oven temperature 225°C.

cyclic acid, paracetamol, phenytoin, methyprylone, and ethchlorvynol have been found not to interfere.

The four drugs which have been demonstrated to be both extracted and chromatographed are shown in Table V.

APPLICATION

The method described here offers a rapid, sensitive procedure for the detection and estimation of barbiturates, methaqualone, and glutethimide in small quantities of plasma. As a screening device it can eliminate, or suggest, the presence of a barbiturate or related drug, in 10 minutes. A complete quantitative analysis in duplicate takes less than 30 minutes.

Since a single retention time on a gas-chromatographic system is not necessarily an adequate criterion of identification it is recommended that, in

cases of drug overdose, the analysis should be accompanied by more specific qualitative procedures such as thin-layer chromatography. Here, also, the extraction can be speeded up by mixing the required amount of plasma with proportionate volumes of chloroform and buffer.

The adaptability of the extraction is further demonstrated by its use in estimating barbiturate concentrations in 20 µl plasma samples as, for example, from rodents.

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