Journal of Chromatography, 157 (1978) 311–320

CHROM. 11,090

RAPID MICRO-METHOD FOR THE MEASUREMENT OF PHENOBARBITONE, PRIMIDONE AND PHENYTOIN IN BLOOD PLASMA OR SERUM BY GAS–LIQUID CHROMATOGRAPHY

D. M. RUTHERFORD* and R. J. FLANAGAN
Poisons Unit, Guy's Hospital, St. Thomas' Street, London SE1 9RT (Great Britain)
(Received March 23rd, 1978)

SUMMARY

A rapid gas–liquid chromatographic method has been developed for the analysis of phenobarbitone, primidone and phenytoin in small (50 μl) volumes of either blood plasma or serum. Neither solvent transfer nor evaporation are required in the extraction, which takes less than 3 min to complete, and a quantitative analysis may be performed, in duplicate, within 20 min. Sources of interference in the assay are minimal, and prior treatment of the column with γ-glycidoxypropyltrimethoxysilane facilitates the measurement of as little as 10 ng of underivatized drug "on-column" using a flame-ionisation detector. The method has proved valuable when used for the detection and measurement of these three compounds at concentrations of 2 mg/l or greater.

INTRODUCTION

The measurement of the plasma or serum concentrations of phenobarbitone, primidone and phenytoin may be of importance both in the treatment of epilepsy and in the diagnosis of acute poisoning. Where samples which may contain more than one of these drugs are to be analysed, either a gas–liquid (GLC) or a liquid chromatographic procedure is preferable since both qualitative and quantitative information can be obtained from a single step. However, many of the methods currently available require relatively large (1 ml) sample volumes[1–3] and involve tedious extract purification and concentration procedures[1–6]. Moreover, derivatization prior to GLC has been advocated[2,3,6] while, in addition to flame-ionisation (FID), nitrogen-selective detection has been employed[4,6].

The method described here is an extension of that described for the analysis of barbiturates and some other drugs in plasma specimens obtained from poisoned patients[7]. A small (50 μl) volume of sample was extracted under acidic conditions with an equal volume of chloroform containing an internal standard, and 3–5-μl portions

* Present address: Biochemistry Department, Alfred Hospital, Commercial Road, Prahran, Victoria 3181, Australia.

of this extract were analysed by GLC–FID using two independent column systems. However, the analysis, again by GLC–FID, of a portion of this same extract on a third column has been found to facilitate the simultaneous identification and measurement of phenobarbitone, primidone and phenytoin at the concentrations attained not only after overdosage but also during therapy.

EXPERIMENTAL

Chemicals and reagents

Phenobarbitone, primidone and phenytoin (micronised) were obtained from May and Baker (Dagenham, Great Britain), I.C.I. (Macclesfield, Great Britain) and Parke Davis & Co. (Pontypool, Great Britain), respectively. γ-Glycidoxypropyltrimethoxysilane (A-187) was supplied by Union Carbide U.K. (Southampton, Great Britain). The internal standard, tetraphenylethylene (TPE), was obtained from Koch-Light Labs. (Colnbrook, Great Britain) and was used as a 10 mg/l solution in chloroform (analytical-reagent grade). Sodium dihydrogen orthophosphate (analytical-reagent grade) was used as a 4 mole/l aqueous solution ("phosphate buffer").

Gas–liquid chromatography

A Pye 104 Model 24 dual-column gas chromatograph fitted with FIDs was used, and integration of peak areas was performed by a Hewlett-Packard 3352 data system. The column and detector oven temperatures were 260 and 300°, respectively; injection port heaters were not employed. The carrier gas (argon) flow-rate was 60 ml/min and the hydrogen and oxygen[7] inlet pressures were 15 and 10 p.s.i., respectively, giving flow-rates of approximately 45 and 200 ml/min.

The column, a coiled glass tube (1.5 m × 4 mm I.D.) was immersed in a solution of 5% dichlorodimethylsilane in toluene for 1 h. It was then washed with methanol, dried at 100° and subsequently packed with 2% (w/w) SP-525 (Chromatography Services, Merseyside, Great Britain) on Varaport 30 (80–100 mesh). The column packing was prepared using the rotating evaporator technique, and phosphoric acid-treated glass wool (Chromatography Services) was used to plug the injection-end of the column. The packed column was conditioned at 275° with an argon flow-rate of 60 ml/min for 16 h prior to the injection of from 10 to 20 μl of A-187. Thereafter, occasional injections of 5 to 10 μl of this compound were performed to maintain the column in the deactivated form.

The chromatography on this system of a solution containing phenobarbitone, primidone and phenytoin together with TPE is illustrated in Fig. 1. The retention times of these compounds and some additional drugs on this same system are given in Table I.

Extraction procedure

Sample (50 μl), phosphate buffer (5 μl) and internal standard solution (50 μl) were added[7] to a clean Dreyer tube (Poulten, Selfe and Lee, Wickford, Great Britain). The contents of the tube were mixed thoroughly on a vortex mixer for 30 sec and the tube was centrifuged for 2 min at 9950 *g* in an Eppendorf centrifuge 5412 (Anderman & Co., East Molesey, Great Britain) which was modified to accept Dreyer tubes by slight drilling-out of the 0.4-ml test tube centrifuge adaptors. Subsequently, a 3–5-μl

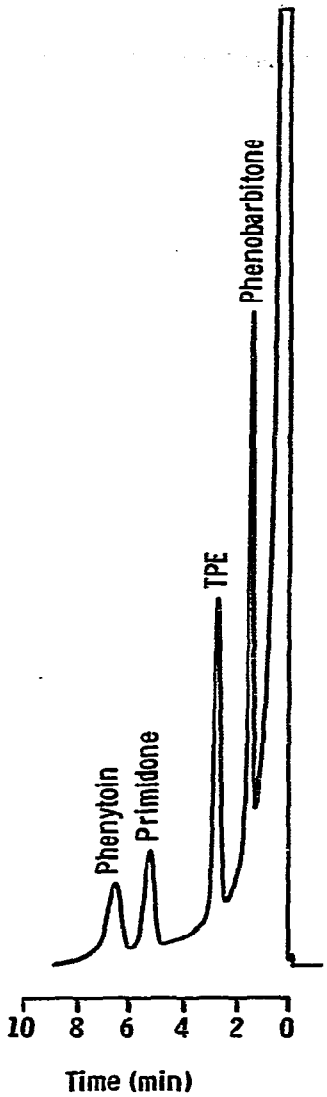


Fig. 1. Analysis of a chloroform solution containing phenobarbitone (20 mg/l), primidone, phenytoin and TPE (all 10 mg/l) on the SP-525 column system; 4-μl injection.

portion of the chloroform phase was obtained[7] and injected onto the column of the gas chromatograph using a syringe fitted with an 11.5-cm needle. The extraction was performed in duplicate and the mean result taken. If the difference between the duplicates was greater than approximately 10%, both the extractions and the analysis were repeated.

Instrument calibration and calculation of results

Standard solutions containing each of the drugs under study at a range of concentrations were prepared in chloroform by dilution of an ethanolic solution of

TABLE I

RETENTION TIMES OF PHENOBARBITONE, PRIMIDONE, PHENYTOIN AND SOME OTHER COMPOUNDS ON THE SP-525 SYSTEM

Compound	*Retention time*	
	min	*Relative to TPE*
Pheneturide	0.48	0.17
Barbitone	0.51	0.18
Allobarbitone	0.60	0.21
Allylbarbitone	0.60	0.21
Amylobarbitone	0.60	0.21
Butobarbitone	0.60	0.21
Pentobarbitone	0.63	0.22
Phenacetin	0.66	0.23
Quinalbarbitone	0.71	0.25
Meprobamate	0.80	0.28
Hexobarbitone	0.88	0.31
Methoin	0.94	0.33
Glutethimide	1.03	0.36
Propylphenazone	1.08	0.38
Ethotoin	1.11	0.39
Caffeine	1.31	0.46
Cyclobarbitone	1.40	0.49
Phenazone	1.45	0.51
Phenobarbitone	1.58	0.55
Heptabarbitone	1.65	0.58
Iminostilbene	1.71	0.60
Methaqualone	2.34	0.82
TPE	2.85	1.00
Phenylbutazone	3.16	1.11
Feprazone	3.62	1.27
Azapropazone	4.53	1.59
Primidone	5.40	1.89
Carbamazepine	5.87	2.06
Phenytoin	6.71	2.35
Chlordiazepoxide	8.21	2.88
Cholesterol*	(13.4)	(4.70)

* This compound was not eluted from some of the column systems used (see text).

phenobarbitone (2.0 g/l), primidone and phenytoin (both 1.0 g/l). The range of concentrations obtained is given in Table II. Each standard also contained TPE (10 mg/l), obtained by dilution of a solution (1.0 g/l) of this compound in chloroform. These standard solutions were stable for at least one year if stored at ambient temperature and in the absence of light.

The ratio of the peak area of drug to the peak area of TPE, when plotted against drug concentration, was linear for all three drugs over the stated concentration ranges. Details of the calibration graphs normally obtained are given in Table II. The drug concentration in the extract was ascertained from the appropriate graph, and the drug concentration in the sample was calculated by use of the recovery data presented in Table III.

TABLE II

DRUG STANDARD SOLUTIONS AND CALIBRATION DETAILS

Drug	*Regression coefficient (l/mg)*	*Standard error of coefficient*	*Intercept on y-axis*	*Standard solutions of drug available (mg/l)* *											
				5	*10*	*20*	*30*	*40*	*50*	*60*	*75*	*80*	*100*	*150*	*200*
Phenobarbitone	0.053	0.001	−0.040		×	×		×		×		×	×	×	×
Primidone	0.058	0.001	−0.033	×	×	×	×	×	×		×		×		
Phenytoin	0.065	0.001	−0.098	×	×	×	×	×	×		×		×		

* Each standard also contained TPE (10 mg/l).

TABLE III

RECOVERY OF ADDED DRUG FROM HEPARINISED BOVINE PLASMA ($n = 25$ IN EACH CASE)

Compound	*Percentage recovery (mean ± S.D.)*	*Recovery factor*
Phenobarbitone	75.8 ± 3.0	1.32
Primidone	42.2 ± 1.8	2.37
Phenytoin	86.2 ± 2.5	1.16

RESULTS AND DISCUSSION

Column deactivation and stability

Both phenobarbitone and phenytoin gave rise to positively skewed peaks if the column was not deactivated with A-187[8,9]. Such treatment was only required infrequently (at most, weekly with a relatively new column, and monthly thereafter), although if the column was maintained at approximately 160° with argon flow when not in use, this minimised the need for further treatment. However, disturbance of the column packing (*e.g.* by too-rapid changing of septa) necessitated additional deactivation especially with a relatively new column. These SP-525 columns were useable for at least six months, a stability similar to that claimed[4] for the relatively polar phase WG-11. Argon was here preferred to nitrogen as the carrier gas since it was available in an oxygen-free form, and thus oxidation of the stationary phase was minimised.

Recovery studies

Standard solutions containing phenobarbitone (20–100 mg/l, in increments of 20 mg/l), primidone and phenytoin (both 10–50 mg/l, in increments of 10 mg/l) were prepared in heparinised bovine plasma[7]. The mean percentage recoveries calculated from quintuplicate analyses of each solution are given in Table III. Each recovery was uniform over the range studied, and confirmation of the validity of these recovery factors when applied to specimens from other sources was obtained. Analyses of commercially available "quality control" specimens: "Wellcomtrol" (Wellcome Reagents, Beckenham, Great Britain) and "Biotrol-Therapeutique I and II" (Laboratories

TABLE IV

RESULTS OBTAINED FROM REPLICATE ANALYSES ($n = 20$) OF "QUALITY CONTROL" SPECIMENS: WELLCOMTROL

Compound	*Results from reference laboratories (mg/l)*		*Results from the present method (mg/l)*	
	Mean	*Range*	*Mean*	*S.D.*
Phenobarbitone	21.6	12.8–33.5	22.1	0.8
Primidone	12.6	7.6–19.6	12.6	0.6
Phenytoin	15.3	10.0–21.3	15.9	0.7

TABLE V

RESULTS OBTAINED FROM REPLICATE ANALYSES ($n = 20$) OF "QUALITY CONTROL" SPECIMENS: BIOTROL-THERAPEUTIQUE

Solution No.	*Compound*	*Intended concentration (mg/l)*	*Means of results from reference laboratories (mg/l)*		*Results from the present method (mg/l)*	
			GLC	*Immunoassay*	*Mean*	*S.D.*
I	Phenobarbitone	10	10	11.5	10.0	0.5
	Phenytoin	10	10.2	11.2	10.7	0.4
II	Phenobarbitone	50	51	52	50.3	1.9
	Phenytoin	50	52.2	46	52.0	2.8

Biotrol, Paris, France) were performed, and the results of replicate analyses (n=20) of each solution are summarised, together with the reference data supplied, in Tables IV and V.

Assay reproducibility

The good reproducibility attainable is shown by the results presented in Tables III and VI. Similar coefficients of variation (5.4% or less) were obtained in the analyses summarised in Tables IV and V. These results are comparable to those given by the procedure of Soldin and Hill[10], which also employs the rapid admixture of a small volume of sample and an equal volume of an internal standard solution, followed by the chromatographic analysis of a portion of the resulting mixture. On the infrequent occasions where a difference between duplicates greater than approximately 10% was obtained with the present method, the analysis was normally repeated. Such relatively large variations were attributed to large errors in the sample volume measurement.

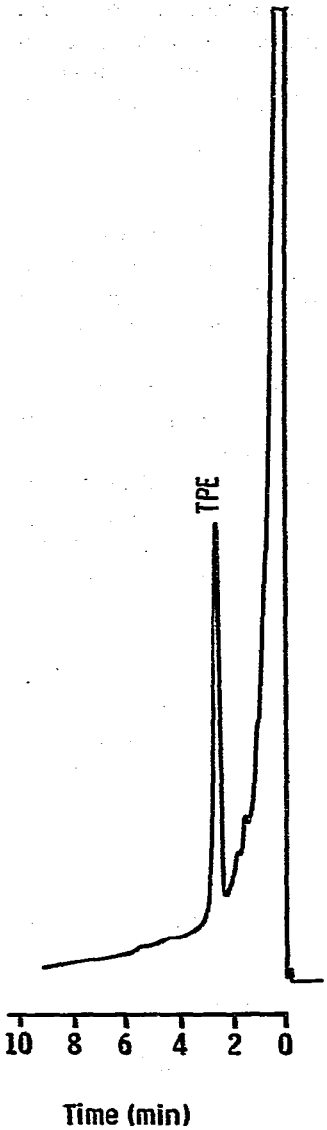
TABLE VI

COEFFICIENTS OF VARIATION (C.V.) ASSESSED FROM THE DIFFERENCE BETWEEN DUPLICATE ANALYSES

Compound	*n*	*Range (mg/l)*	*Mean (mg/l)*	*C.V. (%)*
Phenobarbitone	53	2.2–43.8	18.24	5.7
Primidone	28	2.5–11.9	7.33	4.7
Phenytoin	99	2.1–35.0	8.67	6.3

Specificity

Interference from endogenous sample constituents was minimal. An example of the chromatogram normally obtained on analysis of a drug-free human plasma specimen is given in Fig. 2; analogous extracts performed without the addition of TPE did not reveal compounds which could elute with this standard. The analysis of an extract of plasma obtained from a patient treated with primidone and phenytoin is illustrated in Fig. 3.



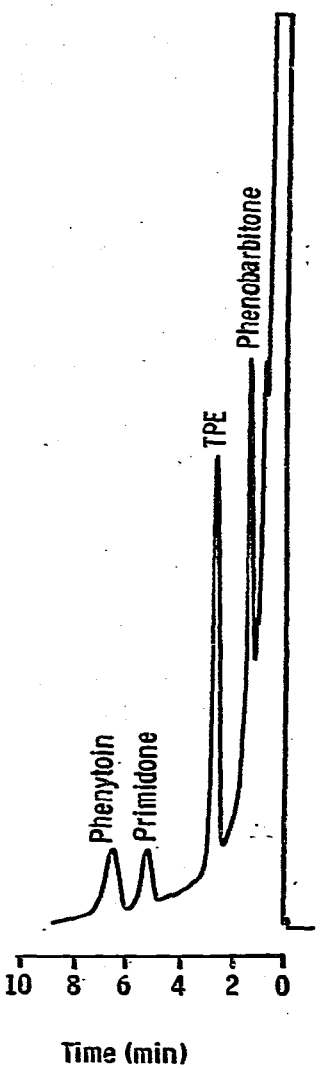


Fig. 2. Analysis of an extract of drug-free human plasma; 5-μl injection. The TPE concentration was 10 mg/l.

Fig. 3. Analysis of an extract of plasma obtained from a patient treated with primidone (750 mg/day) and phenytoin (400 mg/day); 5-μl injection. The TPE concentration was 10 mg/l, and the plasma concentrations of phenobarbitone, primidone and phenytoin were found to be 15.8, 10.0 and 8.9 mg/l, respectively.

The retention times on the SP-525 system of a number of commonly prescribed drugs are given together with those of some other compounds in Table I. Only phenylbutazone has been observed to interfere in the assay, and the presence of this compound would necessitate the choice of an alternative standard if a quantitative result was required. Cholesterol eluted from some of the columns used as a sharp, symmetrical peak, but was not observed to elute from others. This compound did not interfere in the analyses (Table I), but its presence did reduce the rate at which these analyses could be performed. The selectivity given by both the extraction procedure and the relatively high-temperature GLC column system represents an advantage of the method over liquid chromatographic procedures such as that of Soldin and Hill[10] in that interference from relatively polar compounds such as 5-(*p*-hydroxyphenyl)-5-phenylhydantoin and gentamicin did not occur.

Limits of sensitivity

The limit of sensitivity towards both phenobarbitone and phenytoin is influenced by the adsorbtion which occurs on-column, leading to intercepts on the calibration graphs corresponding to approximately 0.8 and 1.5 mg/l, respectively (Table II). On the other hand, the relatively poor recovery of primidone becomes a limiting factor even though the relevant intercept corresponded to only approximately 0.6 mg/l. Thus, the detection limit of each drug was taken to be approximately 2 mg/l, although the measurement of such low concentrations was not as accurate as those performed at higher values. However, the results presented in the following section show that these limits did not restrict the application of this method to the analysis of the drugs under study at the concentrations achieved during therapy. (Note: smaller (20 μl) or larger sample volumes may be used in the assay without affecting the limits of sensitivity provided that the other reagents are used in proportion.)

Comparison with the results obtained using a second GLC assay

The method was evaluated by comparison to the results given by a second GLC assay[1] in the analysis of 131 specimens of plasma or serum obtained from patients treated with one or more of the compounds under study. The results of the comparison are summarised in Table VII, although qualitative differences in the

TABLE VII

SUMMARY OF THE QUANTITATIVE RESULTS OF THE COMPARISON BETWEEN THE PRESENT METHOD AND A SECOND GLC ASSAY

A = Method of Toseland *et al.*[1], B = present method.

Compound	*n*	*r*	*Drug concentration (mg/l)*				*Regression parameters (A on B)*
			A		*B*		
			Mean	*S.D.*	*Mean*	*S.D.*	
Phenobarbitone	65	0.87	19.68	13.30	17.21	11.14	A = 1.04 B + 1.79
Primidone	23	0.86	7.96	3.83	7.81	3.04	A = 1.08 B − 0.49
Phenytoin	99	0.92	9.54	8.25	9.20	6.56	A = 1.16 B − 1.15

results given by each method were observed in 19 cases. A compound with the retention time of phenobarbitone (4.1 and 6.2. mg/l) was detected in two specimens by the present method although neither of these patients were thought to be prescribed this drug. In contrast, phenobarbitone at concentrations of 3 mg/l or less was found in four other specimens by the second GLC assay. This drug was known to be prescribed to one of these latter patients, and was thought to be prescribed to two others. Primidone (approximately 3 mg/l) was detected by both methods in the fourth specimen, although this compound was not initially thought to be prescribed to this patient. A compound corresponding to primidone at concentrations between 2.5 and 7.6 mg/l was detected in three specimens by the present method alone; primidone (750 mg/day) was prescribed to two of these patients. Finally, phenytoin was detected at concentrations of 3.1 mg/l or less in 11 specimens by the method described here; all of these patients were either known or thought to be prescribed this drug. These differences in the detection of phenobarbitone and phenytoin occurred near the limits of sensitivity of both assays and may represent interference in those cases where the "detected" drugs were not prescribed to the patients in question. However, the failure of the second GLC assay to detect primidone in samples from patients treated with this drug may have arisen from the use of insufficient solid ammonium sulphate to ensure an acceptable recovery of primidone in this method.

Further applications of the method

The SP-525 column may be used in the rapid analysis of some other compounds (notably hexobarbitone, cyclobarbitone, heptabarbitone and methaqualone) which could be analysed previously[7] together with phenobarbitone on a Poly A 103 system at 230°. In addition, this SP-525 system is used at the same temperature as the OV-7 column employed in our laboratory for plasma benzodiazepine analyses[11] and thus both systems may be operated in the same chromatograph. The present method has also proved useful in confirming the presence of chlordiazepoxide in overdose since this compound is extracted under the conditions of this assay and elutes as a symmetrical peak (Table I). (Note: the use of the Eppendorf centrifuge 5412 instead of relatively low-speed instruments minimises both the time required for extract preparation and the frequency of emulsion formation in these direct-extract analyses).

CONCLUSIONS

The method described here has proved to be a valuable addition to the techniques available for our 24-h emergency toxicology service. The SP-525 column system is stable for a relatively long period and not only permits the detection and measurement of phenobarbitone, primidone and phenytoin by means of the same extract used for assay of other barbiturates, but also obviates the requirement for a column system for use in phenobarbitone analyses only. In addition, the technique shows advantages of minimal sample and reagent requirement, speed of assay, and good accuracy and reproducibility when used for the analysis of these compounds at the concentrations achieved during therapy.

ACKNOWLEDGEMENTS

We thank Mr. D. J. Berry and the analytical staff of the Poisons Unit for the results of the routine anticonvulsant assays, Mr. B. Soobrayen for technical assistance and Dr. B. Widdop for criticism of the manuscript.

REFERENCES

1 P. A. Toseland, J. Grove and D. J. Berry, *Clin. Chim. Acta*, 38 (1972) 321.
2 F. Dorrity and M. Linnoila, *Clin. Chem.*, 22 (1976) 860.
3 R. E. Hill and A. N. Latham, *J. Chromatogr.*, 131 (1977) 341.
4 P. A. Toseland, M. Albani and F. D. Gauchel, *Clin. Chem.*, 21 (1975) 98.
5 R. F. Adams and F. L. Vandemark, *Clin. Chem.*, 22 (1976) 25.
6 F. L. Vandemark and R. F. Adams, *Clin. Chem.*, 22 (1976) 1062.
7 R. J. Flanagan and D. J. Berry, *J. Chromatogr.*, 131 (1977) 131.
8 W. Averill, *Instrument News*, Perkin-Elmer, Norwalk, Conn., U.S.A.
9 A. M. Hayler and R. J. Flanagan, *J. Chromatogr.*, 153 (1978) 461.
10 S. J. Soldin and J. G. Hill, *Clin. Chem.*, 22 (1976) 856.
11 D. M. Rutherford, *J. Chromatogr.*, 137 (1977) 439.