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SIMULTANEOUS DETERMINATION OF METHYLATED BARBITURATES AND OTHER ANTICONVULSANT DRUGS BY HIGH-RESOLUTION GAS CHROMATOGRAPHY

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SUMMARY

Several barbiturates and other anticonvulsant drugs can be analyzed simultaneously as dimethylated derivatives by high-resolution gas chromatography with a solid flash methylation injector.

Quantitative determinations were performed on test serum during a quality control scheme. The sensitivity ($\pm$ 0.1 μg/ml) and the accuracy are compatible with the measurement of therapeutic levels of barbiturates. The advantages of the method are discussed.

INTRODUCTION

The widespread availability of barbiturates, obtained by both prescription and illicit means, in addition to the increasing use of biochemical monitoring of anticonvulsant therapy in epileptic patients, has necessitated the simultaneous determination of several anticonvulsant drugs in serum. It is therefore imperative to have some reliable, accurate and specific methods for their measurement in body fluids at both the therapeutic and overdose levels.

Spectrophotometric techniques[1–4] are generally time consuming, have a low specificity and require solvent partitioning or thin-layer chromatographic (TLC) separation in order to isolate and identify the drugs prior to their quantitative assay. TLC methods[5,6] are applicable only with clean extracts. When the chromatogram indicates the presence of several drugs, the quantitative data may be unreliable. Gas–liquid chromatographic (GLC) techniques on packed columns have been widely applied to the simultaneous analysis of barbiturates. Some workers chromatograph the unchanged drugs[7–12], while others derivatize the acidic hydrogens of the molecules in order to reduce their polarity and subsequently their adsorption on the column and peak tailing.

Several derivatives have been prepared: the N-trimethylsilyl (TMS)[13–15], the N-pentafluorobenzyl (PFB)[16] and the most widely used N-1,3-dimethyl derivatives. Methylation with diazomethane or dimethyl sulphate prior to gas chromatography[17–22] increases the manipulation steps. On-column and flash methylation with tetra-

methylammonium hydroxide[23–26] or trimethylanilinium hydroxide[27–31] are simpler techniques.

On a smaller scale, the more expensive and sophisticated gas chromatography–mass spectrometry[32–34] and the high-performance liquid chromatographic techniques[35,36] are also used for determination of anticonvulsant drugs: however, they are only feasible in large laboratories.

The quality control scheme developed in the last 3 years by Richens[37] to validate the methods for the measurement of these drugs concluded that spectrophotometric methods give highly unreliable results compared with TLC and particularly GLC, which appeared to be the most appropriate for routine applications. Nevertheless, a large number of endogenous or exogenous compounds present in biological fluids and co-extracted with the drugs necessitate frequent column reconditioning and detector cleaning as well as identification of the barbiturates. This problem can be overcome by the use of a rapid clean-up procedure following the initial extraction and by the improvement of the chromatographic conditions. This paper describes a method for the simultaneous determination of several barbiturates and anticonvulsant drugs. After extraction, the drugs are methylated by a solid flash procedure and their concentrations determined by high-resolution glass capillary chromatography on a support-coated open-tubular column.

EXPERIMENTAL

Apparatus

A Model IGC 12 DFL Intersmat gas chromatograph was used with a hydrogen flame-ionization detector (FID). The recorder was a 1-mV strip-chart Kipp and Zonen Model BD 7. A 20-m SE-30 coated open-tubular glass capillary column (I.D. 0.5 mm) was used for the chromatographic separations. Hydrogen, supplied from a General Electric Model 225 generator, served as the carrier gas. The inlet pressure, measured at ambient temperature, was set at 16 p.s.i. Nitrogen was used as the make-up gas with a flow-rate of 35 ml/min. The following conditions were applied to all recordings: injection port temperature, 260°; oven temperature, programmed between 135° and 245° at 8°/min; gas flame supply, air 250 ml/min and hydrogen 25 ml/min. The all-glass solid injection device was described by Van den Berg and Cox[38].

Reagents and chemicals

All chemicals were of ACS reagent grade, purchased from E. Merck (Darmstadt, G.F.R.) and used without further purification. Sodium hydroxide (0.45 *N*) and sulphuric acid (1 *N*) solutions were prepared weekly with distilled water. Trimethylanilinium hydroxide (TMAH) was purchased from Pierce (Rockford, Ill., U.S.A.). Barbiturates, diphenylhydantoin and primidone standards in the acidic form were obtained from different manufacturers. The different drug stock solutions (each a 5 mg/ml solution in chloroform) were stored at 4°. The working standards were prepared daily at a concentration of 0.1 mg/ml by diluting 1 ml of stock solution to 50 ml with chloroform. Secobarbital (25 μg) was used as the quantitative internal standard and was introduced into all the extraction tubes. *p*-Toluyldiphenylhydantoin (50 μg) served as the qualitative internal standard for checking the identification of the peaks.

Extraction procedure

A 1-ml volume of plasma, diluted with an equal volume of water containing the internal standards, was introduced into a 25-ml centrifuge tube. After acidification with 1 ml of 1 *N* sulphuric acid, the sample was extracted with 25 ml of chloroform by gentle shaking for 5 min. After spinning at 3000 rpm for 3 min, the organic layer was withdrawn and, after filtration through paper, was transferred into another conical tube. This procedure prevents the transfer of any precipitates from the interface. An aliquot of 20 ml of the chloroform layer was back-extracted in 5 ml of 0.45 *N* sodium hydroxide solution. After centrifugation, the solvent was discarded and the aqueous phase re-acidified with 3 ml of 1 *N* sulphuric acid. Finally, it was shaken with 20 ml of chloroform for 3 min. The organic layer, transferred into a conical tube, was evaporated to dryness under a stream of nitrogen and the residue was carefully dissolved in 0.1 ml of chloroform. A 1-μl volume of this solution was placed on the tip of the injector needle as a thin drop with a syringe containing 2 μl of TMAH. The needle was at the loading position. The hydrogen entered beneath the injection device and split into one flow entering the column and another flow along the needle, blowing off the volatile derivatization agent and solvent via the restrictor. When evaporation was completed, the needle was pushed inside the injection block and flash methylation of the barbiturates and similar drugs occurred. Under these experimental conditions, TMAH gave complete derivatization for up to at least 2 μg of the drug; this was confirmed by GC–MS.

Gas chromatographic column preparation

The support-coated open-tubular column was prepared according to the method developed by Rutten and co-workers[39,40]. The drawn Pyrex column was deactivated with a 2% solution of benzyltriphenylphosphonium chloride in *n*-hexane. Chromosorb R 6470-1 (Supelco, Bellefonte, Pa., U.S.A.) was used as support and was dynamically coated with SE-30.

RESULTS

Qualitative

To assess the column performance, an aliquot of each drug was injected. On the basis of the recorded retention times, the temperature conditions were selected. Fig. 1 shows a chromatogram obtained after the extraction of a serum sample spiked with the most frequently used barbiturates plus diphenylhydantoin (Table I). The confirmation of the identity of the peaks is based on their retention times relative to that of the qualitative internal standard, *p*-toluylphenylhydantoin.

Except for mephobarbital and phenobarbital, which gave the same 1,3-dimethylated compound after derivatization with TMAH, the separation of the drugs was satisfactory. For the ready separation of mephobarbital and phenobarbital, one must derivatize the sample with TMS or, better, with PFB in order to obtain a mono- and a di-substituted derivative, respectively.

On the other hand, as phenobarbital is the active metabolite of mephobarbital, the total amount of the two compounds is a useful measure. The recording time for the completion of the chromatogram was 15 min if screening for 13–15 drugs was required. The dimethylated barbiturates gave single symmetrical peaks when the flash

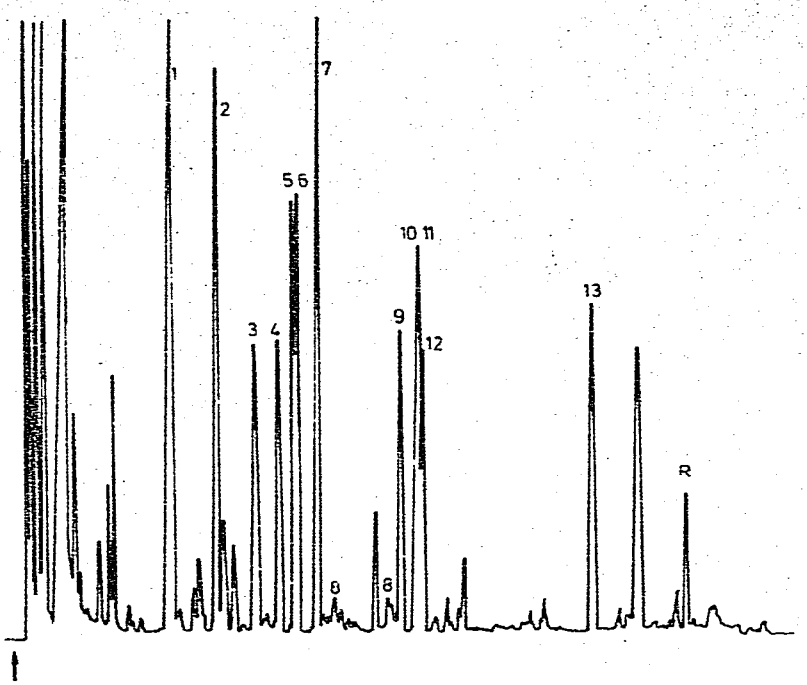

Fig. 1. Gas chromatogram of a standard mixture of barbiturates and diphenylhydantoin. For conditions, see text.

TABLE I

RETENTION TIMES OF THE MAIN BARBITURATES

For experimental conditions see text.

Peak No.	*Compound*	*Substituent 1*	*Substituent 2*	*Retention time (min)*	*Relative retention time (p-toluylphenylhydantoin = 1.00)*
1	Barbital	Ethyl	Ethyl	4.2	0.25
2	Allobarbital	Allyl	Allyl	5.4	0.32
3	Butabarbital	Ethyl	*sec*-Butyl	6.4	0.38
4	Amobarbital	Ethyl	Isoamyl	7.1	0.42
5	Pentobarbital	Ethyl	1-Methylbutyl	7.5	0.44
6	Vinbarbital	Ethyl	1-Methyl-1-butenyl	7.65	0.46
7	Secobarbital	Allyl	1-Methylbutyl	8.15	0.49
8	Brallobarbital	Allyl	2-Bromoallyl	8.55–10	0.51–0.59
9	Hexobarbital	Methyl	1-Cyclohexen-1-yl	10.35	0.62
10	Mephobarbital	Ethyl	Phenyl	10.85	0.64
11	Phenobarbital	Ethyl	Phenyl	10.85	0.64
12	Cyclobarbital	Ethyl	1-Cyclohexen-1-yl	10.95	0.65
13	Diphenylhydantoin	—	—	15.5	0.92
R	*p*-Toluylphenyl-hydantoin	—	—	16.8	1.00

methylation was carried out with TMAH. The only exception was brallobarbital containing a brominated substituent; this decomposed and gave two small peaks at 8.5 and 10 min.

Quantitative

The quantitative performance of the procedure was first checked with four standard solutions containing between 2.5 and 70 μg/ml of barbituric acid (barbital, butabarbital, amobarbital, phenobarbital) and diphenylhydantoin. The peak height ratios measured for these drugs and secobarbital indicate straight line calibration graphs passing through the origin (Fig. 2). These results confirm the general reproducibility of the methylation yield and the relatively low adsorption of these dimethylated barbiturates in the chromatographic system. The overall detection limit lies between 200 and 500 pg, but good precision for a quantitative assay can be obtained for approximately 5 ng of injected drugs. We also controlled the complete analytical procedure on blank serum spiked with increasing amounts of barbiturates (Fig. 3). The mean recoveries attained with the extraction procedure described above are independent of the drug concentration. Diphenylhydantoin gave the highest recovery of 102 $\pm$3%, with phenobarbital 85 $\pm$ 5% and primidone (retention time 13.2 min), another anticonvulsant drug, 71 $\pm$ 6%. The standard deviations were satisfactory despite the difficulty of chromatographing nanogram amounts of drugs. The overall

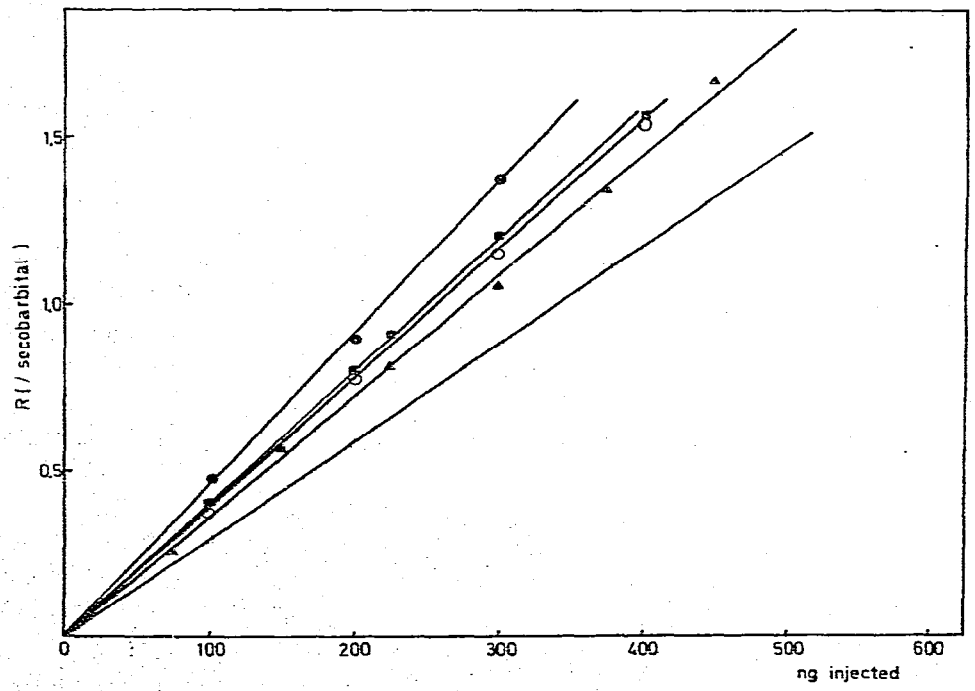

Fig. 2. Calibration graphs recorded on pure solution. Internal standard, secobarbital. ●, Barbital; ■, butabarbital; ○, amobarbital; □, phenobarbital; ▲, diphenylhydantoin.

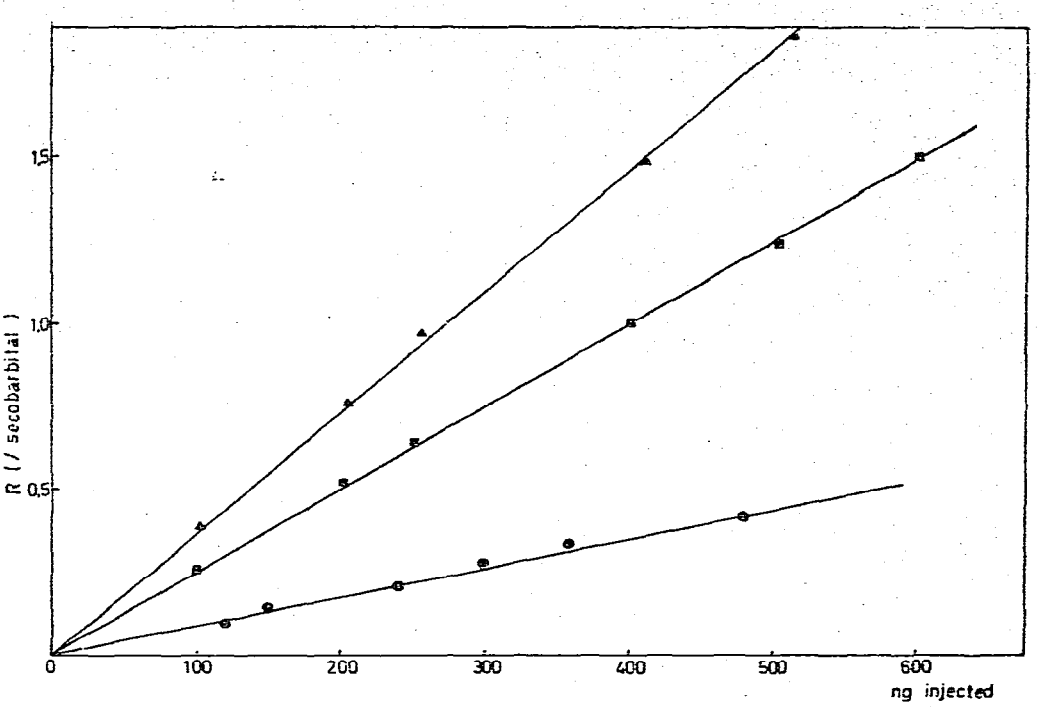

Fig. 3. Calibration graphs recorded on serum extracts. Internal standard, secobarbital. ▲, Diphenylhydantoin; ■, phenobarbital; ●, primidone.

precision of the method, determined by five replicate extractions of the same spiked serum (100 ng/ml of drugs), was 6.3% for primidone, 4.1% for phenobarbital and 3.8% for diphenylhydantoin.

Finally, the accuracy, specificity and validity of the procedure were tested on pooled specimens received as part of the quality control scheme developed by Richens[37]. The results, obtained from the first three experiments in which we participated, are reported in Table II. Except for primidone in one instance good correlations were found between our values and the mean values calculated from the data obtained in the 98 laboratories participating in this investigation. The last experiment was also

TABLE II

RESULTS OF QUALITY CONTROL EXPERIMENTS

Concentrations are given in μg/ml.

Compound	*Expt. 1*		*Expt. 2*				*Expt. 3*			
	Specimen 1		*Specimen 1*		*Specimen 2*		*Specimen 3*		*Specimen 4*	
	Found	*Mean**	*Found*	*Mean*	*Found*	*Mean*	*Found*	*Mean*	*Found*	*Mean*
Primidone	7.4	7.3	—	—	—	—	6.3	2.1	7.5	2.4
Phenobarbital	15.2	14.8	10.0	10.0	49.9	50.0	30.0	27.5	28.0	26.6
Diphenylhydantoin	12.2	13.3	10.0	10.0	50.0	50.0	7.9	7.6	7.2	6.8

* Mean values were calculated from the data from 80–100 laboratories.

performed on serum containing carbamazepine and sodium valproate; no interference arose from either of these drugs.

DISCUSSION

The use of support-coated open-tubular gas chromatographic columns combined with solid flash methylation injection of the barbiturates and other anticonvulsant drugs constitute a useful method for the routine analysis of these compounds.

With the high resolving power of the chromatographic column, simultaneous quantitative analysis of up to 15 different drugs can be achieved in less than 20 min. This method is applicable to the separation of amobarbital, pentobarbital and secobarbital, which are difficult to analyze[12] when simultaneously present. In combination with our extraction procedure, 20 samples per day can be analyzed by one technician. Of course, the extraction and clean-up procedures could be simplified if the monitoring of only 4–8 drugs is required, and this will enhance the recovery of some barbiturates slightly. The quality of the column also assures the specificity of the measurement and this specificity has been checked on several plasmas including that from patients treated with other drugs such as diazepam and nitrazepam. There is virtually no adsorption of the dimethylated barbiturates on the inert support-coated open-tubular column and, for this reason, the sensitivity is largely below the limit of the mean therapeutic values. This would make the analysis of smaller plasma samples (200–300 μl of capillary blood) possible, provided that the extraction is adapted for such volumes (salt–solvent pairs, such as that developed by Horning *et al.*[34] and Driessen and Emonds[10]). In addition, with this simplified extraction procedure, the complete derivatization of the drugs with TMAH eliminates the problem of an unreproducible on-column methylation of the barbiturates by plasmatic lecithin[41], which could lead to consistently low results. The thermal stability of the chromatographic column is also a useful factor.

Methylated anticonvulsant drugs have now been routinely chromatographed at the rate of 10–20 samples a day for 4 months without a significant decrease in the column resolution. The column was simply reconditioned each night at 240°, and the detector has not been cleaned during this period.

The solid flash methylation injection is also advantageous. It reduces the solvent tail considerably, thus preventing column overloading. We detected only peaks due to the decomposition of the remaining methylating agent and some extraneous peaks due to the non-volatile impurities in chloroform. The reproducibility of this mode of injection is equivalent to that of a common septum system, and it does not influence to a great extent ($<10\%$) the derivatization yield if one takes the precaution of applying the sample on to the tip of the glass needle.

The main disadvantage of the method is the double extraction procedure, which has to be performed for complete and secure screening of anticonvulsant drugs: however, we consider that accuracy is of greater importance than analysis time in the quantitative monitoring of these drugs.

In addition, any other drugs that could be present in the plasma in association with barbiturates (for instance, meprobamate, gluthetimide and methaqualone) and could interfere with the signals would be eliminated from the final solution by the back-extraction with sodium hydroxide solution. Their neutral or weakly basic char-

acter will maintain them in the chloroform layer. This back-extraction also eliminates several naturally occurring compounds that can either interfere[42,43] or accumulate on the column and be eluted during the subsequent analysis, creating secondary interferences. As a consequence, the risks of denaturating the column and contaminating the FID detector, which are particularly important after direct extract analysis[31], are eliminated.

CONCLUSION

Barbiturates and other anticonvulsant drugs such as primidone and diphenylhydantoin can be simultaneously analyzed as dimethylated derivatives by high-resolution gas chromatography with a solid flash methylation injector. The determinations were carried out using an internal standard, which could be either another barbiturate (secobarbital) or a compound similar to an anticonvulsant drug (*p*-toluyldiphenylhydantoin). A clean-up procedure is necessary if qualitative and quantitative screening of the drugs are required. The extraction could be simplified if one only needs a doseage for one or two compounds.

The sensitivity ($\pm$ 0.1 μg/ml) and the accuracy ($\pm$ 5%) are largely compatible with routine measurements of therapeutic levels of barbiturates and the specificity is controlled by the high resolving power of the chromatographic column. The specificity of the complete procedure makes it competitive and at least of comparable efficiency with others currently in use, which are generally more expensive (GC–MS, specific nitrogen detector, etc.) or more complicated (*e.g.*, mixing of the carrier gas with formic acid vapour).

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