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Note

Simultaneous assay of methylphenobarbitone and phenobarbitone using gas–liquid chromatography with on-column butylation

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The methods available for barbiturate analysis have recently been assessed by Kananen *et al.*[1], who advocated the use of a gas–liquid chromatographic (GLC) procedure involving methylation of the barbiturates with trimethylphenylammonium hydroxide[2]. While this technique is excellent for many barbiturates, it is unsuitable for the simultaneous determination of methylphenobarbitone (MPB) and phenobarbitone (PB) since these compounds yield a common 1,3-dimethyl product when methylated[3]. MPB is sometimes used as an anticonvulsant[4], and is known to be converted in the body to PB[5]. Therefore, a simultaneous quantitative determination of the two compounds is desirable. This has been achieved by GLC of the underivatized barbiturates[5–8], but such methods suffer from the widely acknowledged difficulties associated with chromatographing underivatized barbiturates[1,9]. Ethyl derivatives have been used[3], but we have not found them satisfactory for quantitation. Greeley[9] has recently described a technique for the formation of N-alkyl-barbiturates which did not involve on-column alkylation, and has demonstrated its application to the quantitative analysis of barbiturates in blood.

The present report describes a simple and convenient on-column butylation procedure for the assay of MPB and PB in plasma or serum which is based on the work of Kowblansky *et al.*[10] and does not suffer from the difficulties ascribed to on-column alkylation by Greeley[9].

EXPERIMENTAL

Materials

The barbiturates were obtained from the following pharmaceutical companies: phenobarbitone from Drug Houses of Australia, methylphenobarbitone from Sterling Pharmaceuticals (Sydney, Australia), and Alphenal from Analabs (New Haven, Conn., U.S.A.)*. Tetrabutylammonium hydroxide (TBAH) titrant (25% in methanol) was obtained from Eastman-Kodak, Rochester, N.Y., U.S.A. All other reagents were of analytical grade.

GLC was carried out on a Varian Model 2100 instrument, equipped with flame ionization detectors. Columns were 1.8 m × 2 mm I.D. Pyrex U-tubes, packed with 3% OV-101 on Chrom G HP, 100–120 mesh (Varian). The instrument parameters

* Generously donated by Warner-Lambert International, Morris Plains, N.J., U.S.A.

used were: nitrogen, 50 ml/min, hydrogen, 35 ml/min, air, 300 ml/min; the column oven temperature was 195° and the injector and detector temperatures were 275°.

Method

Plasma (1.0 ml), hydrochloric acid (1.0 ml; 0.2 *M*) and chloroform (5.0 ml, containing Alphenal, 3.0 mg/l) were added to a screw-capped Pyrex tube (150 × 20 mm), which was capped and shaken for 2 min. After centrifugation (2 min at 1000 *g*), the aqueous layer was aspirated and discarded, and the chloroform phase poured carefully into a Pyrex test tube with a ground-glass conical joint. The solvent was removed *in vacuo* using a Buchi Rotovapor R, and the tube flushed briefly with a stream of nitrogen to ensure complete removal of chloroform. The residue was taken up in TBAH (50 μl), and 1–2 μl were injected into the gas chromatograph, operated as described above.

Aliquots of MPB and PB were added to samples of Red Cross Blood Bank plasma to give single or mixed standard solutions ranging in drug concentration from 1.0–50.0 μg/ml. These were compared with aqueous standards, similarly prepared.

RESULTS

A typical chromatogram of an aqueous standard (MPB and PB, each 20 μg/ml) is shown in Fig. 1A, and the corresponding plasma standard in Fig. 1B. The retention times for the butylated barbiturates were: MPB, 4.4 min; PB, 8.3 min; Alphenal, 9.8 min. Comparison of plasma standards with aqueous standards gave recoveries for MPB and PB which were greater than 95%.

Calibration curves for plasma standards, using ratios of peak heights to concentration are given in Fig. 2. Linear regression analysis gave coefficients of determination (r^2) values for the two assays as 0.995 (MPB) and 0.996 (PB). The respective regression equations were

$$\text{MPB conc.} = 11.48 \times R_{II} \frac{\text{MPB}}{\text{Alphenal}}$$

$$\text{PB conc.} = 16.97 \times R_{II} \frac{\text{PB}}{\text{Alphenal}} + 0.39$$

Table I gives the results of applying this technique to determining both MPB and PB in the plasma of patients taking the doses of MPB indicated. These assays were carried out when each patient had been taking the indicated dosage for at least one month, so that steady-state plasma concentrations had had time to develop.

DISCUSSION

Kowblansky *et al.*[10] showed that on-column N-butylation of xanthines and barbiturates could be effected using tetrabutylammonium hydroxide. They noted that the method offered some advantages over N-methylation, particularly with respect to selectivity. The present report describes one such application, in which simple, rapid quantitative simultaneous determination of MPB and PB is achieved.

Greeley[9] introduced an alkylation procedure which did not involve on-column derivatization. While that procedure has the significant advantage of providing a route to any N-alkyl derivative using the corresponding primary alkyl iodide, the on-

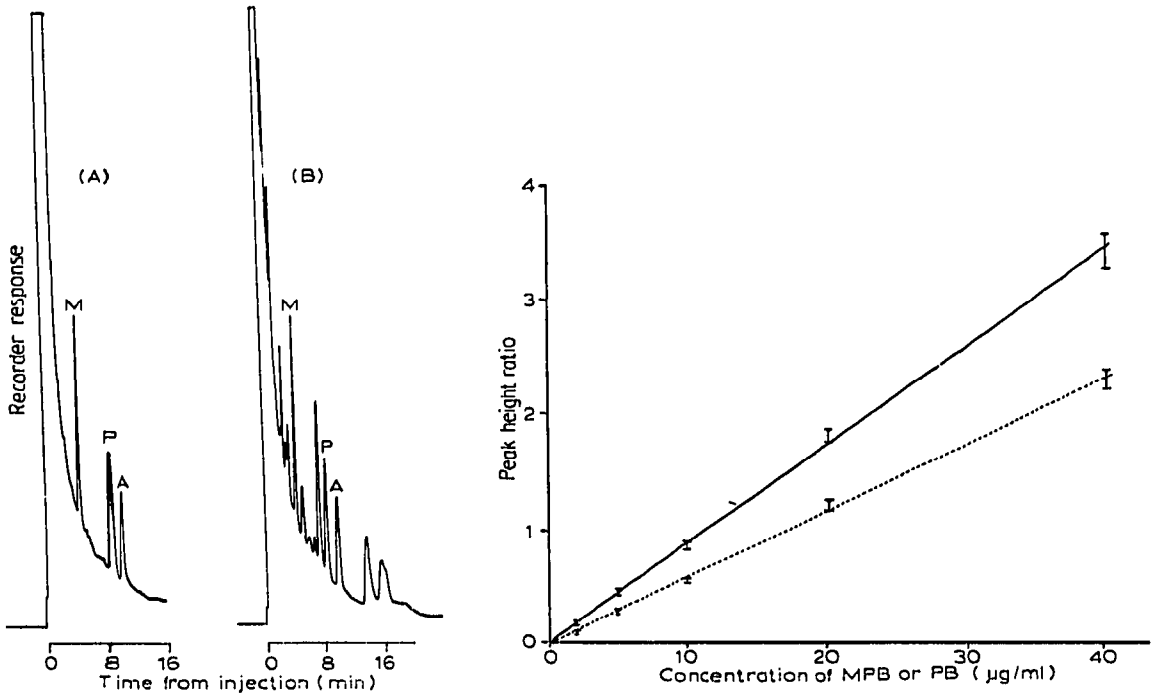

Fig. 1. Chromatograms from aqueous standard (A) and plasma standard (B), each containing MPB (peak M) and PB (peak P) at 20 μg/ml. The internal standard, Alphenal, gives peak A.

Fig. 2. Calibration curves for plasma standards of MPB (continuous line) and PB (broken line). Bars indicate one standard deviation either side of the mean of six determinations.

column procedure offers convenience and simplicity. We have not experienced problems with on-column butylation to the degree which Greeley reported. Although column life is certainly variable and unpredictable, one column has been in continuous use for nine months without loss of performance, and column conditioning with "Silyl 8" (Pierce, Rockford, Ill., U.S.A.) was needed only occasionally.

While we have applied the technique only to the determination of two barbiturates of particular interest, it is apparent that the procedure could be readily extended to include more barbiturates, possibly with the use of temperature programming, and a lower initial temperature than 195°.

Initial attempts at assaying plasma standards were made using expired blood (3–6 weeks' storage at 4°) from the Red Cross Blood Bank. However, all samples of these gave rise to a large peak which coincided with that of PB. It was subsequently shown that a freshly collected bag of blood lacked this peak, but it was present once the blood had been stored in the refrigerator for two weeks or more. Possibly the interfering peak was a plasticizer component which slowly dissolved from the bag[11], since storage for two weeks in glass of an aliquot of the blood removed from a bag immediately after collection produced no corresponding peak. This raised the possibility of false-positive values for PB if blood is collected in plastic tubes, but we have not observed this in the commonly used polypropylene tubes, even after extended storage periods.

The simplicity of the extraction technique used has been obtained at the cost of slowing the analysis due to the late peaks which arise from plasma (Fig. 1B).

TABLE I

STEADY-STATE CONCENTRATIONS OF MPB AND PB IN PLASMA OF PATIENTS TAKING MPB

Patient	*MPB dose (mg/day)*	*MPB (μg/ml)*	*PB (μg/ml)*	*DPH (μg/ml)**
1	90	1.0	18.6	16.8
2	180	1.4	22.6	20.9
3	90	0.7	11.1	20.8
4	120	0	6.0	6.2
5	240	2.2	16.9	27.9
6	600	4.3	35.1	8.5
7	180	0	7.8	8.6
8	120	4.4	6.5	5.4
9	90	0.7	7.2	13.2
10	45	0	1.2	2.4

* DPH = diphenylhydantoin; assayed separately by a minor modification of the method of MacGee[12].

However, samples can be injected at intervals of 15 min, which permits complete elution of these late peaks between successive samples.

Each of the barbiturates tested gave rise to two peaks under the butylation conditions used, but with the chromatographic conditions selected the "early" peaks were eluted in the solvent front. This is similar to the documented behaviour of PB when methylated[13], but does not affect the utility of the assay. We have not noted significant variations in the relative proportions of the two peaks from either MPB or PB when these were allowed to stand at room temperature for up to 3 h, although the corresponding effect has been reported to cause errors in the methylation of PB[14].

The results of determining steady-state plasma levels of MPB and PB in patients taking MPB (Table I) illustrate the utility of the technique. The levels of PB are markedly higher than those of MPB in most cases, and there is not a good correlation between MPB dose and the plasma level of either drug. Further studies on the metabolic fate of MPB are in progress, and will be reported separately.

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