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Rapid, Simultaneous GLC Determination of Phenobarbital, Primidone, and Diphenylhydantoin

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Abstract □ A rapid method is described for the simultaneous determination of phenobarbital, primidone, and diphenylhydantoin. The method gives greater sensitivity and reproducibility for phenobarbital and primidone than the short methods now in use, maintains the efficiency of much longer techniques, and takes only 45 min. from the time the sample is received until the results are reported.

Keyphrases □ Phenobarbital—rapid, simultaneous GLC determination with primidone and diphenylhydantoin □ Primidone—rapid, simultaneous GLC determination with phenobarbital and diphenylhydantoin □ Diphenylhydantoin—rapid, simultaneous GLC determination with phenobarbital and primidone □ GLC—rapid, simultaneous determination of phenobarbital, primidone, and diphenylhydantoin

Since the introduction of the on-column methylating reagents, tetramethylammonium hydroxide (1, 2) and trimethylphenylammonium hydroxide (3), the extraction and GLC analysis of anticonvulsant drugs have been greatly simplified. These reagents are normally used as either a dilute (0.1–0.2 *M*) solution (4, 5) or a concentrated (24%) solution (6, 7) in methanol. The methods published to date concerning two of the three primary anticonvulsants, phenobarbital and primidone, are lacking in sensitivity or reproducibility or the extraction time required is unnecessarily long.

This paper describes a combination of two methods (5, 6) which increases the sensitivity and reproducibility of the analysis of phenobarbital and primidone and also decreases the time necessary to give a clean, efficient extraction.

EXPERIMENTAL

Apparatus—A gas chromatograph[1] with four flame-ionization detectors was used for the determinations. The column was a 1.82-m. (6-ft.) by 2-mm. i.d. glass U-tube packed with 3% OV-17 (phenyl methyl silicone oil) on 80–100-mesh Chromosorb W-HP. The injection port was heated to 360° and the detector to 260°. The column temperature was programmed from 140 to 220° at 8°/min. The carrier gas flow rate was adjusted to around 60 ml./min. to give a retention time of 5.0 ± 0.2 min. for methylated diphenylhydantoin at 220°.

Reagents—Trimethylphenylammonium hydroxide[2] was available as a 0.1 *M* solution in methanol; it was concentrated to 1.5 *M* under dry nitrogen over low heat. 5-(*p*-Methylphenyl)-5-phenylhydantoin[3] (I) and phenylethylmalonamide[4] were also used.

Procedure—One milliliter of plasma, 0.50 ml. of the internal standard (20 mg. I diluted to 500 ml. with 0.1 *N* NaOH), 0.5 ml. 0.1 *N* NaOH, 0.5 ml. of a 1.0 *M* H_3PO_4 buffer, pH 2.7, and 13 ml. of ether were combined in a 50-ml. centrifuge tube. The tube was sealed by wetting the ground-glass portion of the stopper with distilled water before insertion, shaken mechanically for 10 min., and centrifuged at 2000 r.p.m. for 1 min. The ether was transferred to a 15-ml. conical centrifuge tube and evaporated under nitrogen at 50°. The residue was taken up in 5 ml. of toluene, and 50 μl. of 1.5 *M* trimethylphenylammonium hydroxide was added. The tube was shaken for 1 min. and then centrifuged for 2 min.; 1 μl. of the lower phase was injected into the chromatograph.

Drug levels were determined from a standard curve of the peak height ratio of the drug to the internal standard *versus* concentration. Phenobarbital levels were determined by summing the heights of the three peaks shown in Fig. 1 after adjusting to constant peak width. The total phenobarbital (Pb) peak height is given by Eq. 1:

$$h_{Pb} = \frac{w_1}{w_3}h_1 + \frac{w_2}{w_3}h_2 + h_3 \qquad \text{(Eq. 1)}$$

where h is the height and w is the width at half height of the peaks shown by the subscripts. The peak width ratios are constant for any given set of chromatographic conditions.

RESULTS AND DISCUSSION

The chromatogram of an extracted sample containing therapeutic levels of phenobarbital, primidone, and diphenylhydantoin, along with an extraction of standard plasma, is shown in Fig. 1. The first two phenobarbital peaks shown in this figure are produced in the chromatograph during on-column methylation with 1.5 *M* trimethylphenylammonium hydroxide. They are also the only peaks produced by methylation of the total product of the aqueous alkaline hydrolysis of phenobarbital. GLC and NMR studies of these two products indicate that they are methylated derivatives of phenyl-

[1] Varian 2100.

[2] Eastman.

[3] Aldrich Chemical Co.

[4] Supplied by Dr. C. E. Pippenger and Dr. B. B. Gallagher.

Table I—Two-Hour Time Study of Phenobarbital Peak Heights with Diphenylhydantoin as Internal Standard

Injection Time, min.	Peak 1		Peak 2		Peak 3		Total	
	Column 1	Column 2	Column 1	Column 2	Column 1	Column 2	Column 1	Column 2
0	0.391	0.355	0.620	0.650	1.32	1.27	2.23	2.18
25	0.288	0.326	0.900	0.933	1.21	0.979	2.29	2.12
50	0.233	0.355	0.520	0.931	1.57	1.05	2.25	2.22
75	0.393	0.369	0.704	0.796	1.23	1.06	2.22	2.12
100	0.590	0.396	0.864	1.22	1.07	0.764	2.38	2.23
σ	0.136	0.0254	0.161	0.211	0.185	0.182	0.0650	0.0527
$\bar{x}$	0.379	0.360	0.722	0.906	1.28	1.02	2.27	2.17
RSD, %	36	7	23	23	15	18	2.9	2.5
σ_T	0.0930		0.202		0.219		0.0768	
$\bar{x}_T$	0.370		0.814		1.15		2.22	
RSD_T, %	25		25		19		3.5	
Variation	10.5–26.6%		23.4–55.0%		34.4–70.7%			

ethylmalonamide, a metabolite of primidone. However, the recovery of phenylethylmalonamide from plasma using this extraction technique is 9.9% at a concentration of 20 mcg./ml. of plasma and 8.5% at a concentration of 5 mcg./ml. of plasma. Gallagher and Baumel (8) reported levels of phenylethylmalonamide in serum of 10 and 12 mcg./ml. in two patients who had been taking primidone (750 mg./day) for more than 2 years. Therefore, for practical drug level determinations, it is safe to assume that these peaks are produced on the column by phenobarbital and are not the result of the metabolism of primidone. The third peak has been shown by NMR to be 1,3-dimethylphenobarbital. A study is underway to determine the structures of the two methylated derivatives of phenylethylmalonamide.

Table I shows the variation of the peak height ratios of the three phenobarbital peaks over 2 hr. for one sample. Each peak has been found to vary continuously with time in an unpredictable manner, supporting the necessity of using all three peaks for quantitation. Data are shown for the three separate peaks and their combined total using two identically prepared columns. The range of variation of the ratios for each peak as a percentage of the total ratio is also shown. The same values were calculated for seven replicate samples analyzed on one column, and these showed relative standard deviations of 28, 12, 18, and 5% for peaks one, two, three, and the total, respectively.

To show that the response-mole ratios of phenobarbital peaks one and two are equivalent to peak three after correction, standard samples containing phenobarbital, primidone, diphenylhydantoin, and the internal standard were extracted and chromatographed. The samples were four each of six levels containing from 100.4 to 4.02 mcg. of phenobarbital/ml. of plasma. Each sample was run on two columns simultaneously and averages of the measured values were used. The ratio of the concentration of phenobarbital to that of diphenylhydantoin was constant for all samples. Peak height ratios were calculated for phenobarbital, using the total height from Eq. 1, and for diphenylhydantoin. The ratio for phenobarbital was divided by that for diphenylhydantoin for each sample, giving a mean of 2.04 with a relative standard deviation of 8%.

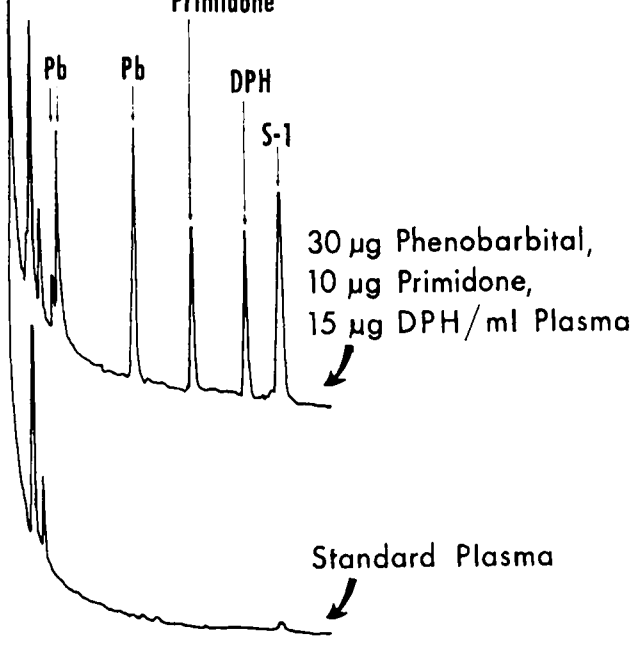

Figure 1—*Typical chromatogram. Upper trace shows extraction of plasma containing therapeutic levels of phenobarbital (Pb), primidone, and diphenylhydantoin (DPH); lower trace shows extraction of standard plasma. S-1 is the internal standard.*

The recoveries of phenobarbital, primidone, and diphenylhydantoin were 100, 72, and 100%, respectively, and the calibration curve for each drug was linear over the concentration range normally found in plasma. The relative standard deviations of six replicate extractions of a patient sample containing all three drugs were 3.2% for 32.7 mcg. of phenobarbital, 3.3% for 7.66 mcg. of primidone, and 5% for 12.2 mcg. of diphenylhydantoin/ml. of plasma. The limit of detection for each drug is 0.5 mcg./ml. of plasma.

The extraction procedure described is as clean and efficient as longer extractions now in use, yet it takes only 25 min. The method is applicable to the other hydantoin and succinimide anticonvulsants, except methsuximide and mephenytoin, in which case the derivatizing reagent is added directly to the ether residue. We are presently adapting the method to analyze small volumes of plasma and whole blood so that accurate multiple-drug determinations can be made from finger-stick samples.

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