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THE QUANTITATIVE ANALYSIS OF PHENOBARBITAL WITH TRIMETHYLANILINIUM HYDROXIDE

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SUMMARY

The gas–liquid chromatographic analysis of phenobarbital which uses trimethylanilinium hydroxide (TMAH) as a methylating agent results in the formation of two products: previously identified N,N-dimethylphenobarbital and a heretofore unidentified decomposition product. By mass spectrometry and synthesis, this compound was identified as N-methyl-α-phenylbutyramide. Two parameters affecting its formation are the concentration of TMAH and the time phenobarbital is in TMAH prior to GLC injection. Using a high TMAH concentration (1.8 *M*), the amount of N-methyl-α-phenylbutyramide formed is reproducible, a linear function of the phenobarbital concentration and a better basis for the quantitative determination of phenobarbital than N,N-dimethylphenobarbital. Application to the simultaneous determination of phenobarbital, primidone and diphenylhydantoin in 12 min is given.

INTRODUCTION

There has been much discussion[1] and many publications[2–4] dealing with gas–liquid chromatographic (GLC) methods for the determination of phenobarbital. These involve the free acid or derivatized phenobarbital. To achieve derivatization, diazomethane, dimethylsulfoxide, bis(trimethylsilyl)acetamide, tetramethylammonium hydroxide (TMH) or trimethylanilinium hydroxide (TMAH) have been used. Of these methods, derivatization employing an alkylammonium salt is now accepted as a preferred analytical technique. Several reasons account for this: use of TMAH permits a simple extraction procedure which results in a clean, concentrated solution suitable for GLC analysis; the methylated derivative has GLC characteristics (better separation, less tailing and improved sensitivity) superior to the corresponding free acid.

When TMAH reacts with phenobarbital, a second product (EP) is formed[5] along with the expected N,N-dimethylphenobarbital. This product (EP) either was not observed[6] or disregarded by many observers. Others realized its significance but failed to identify it correctly[7].

This paper reports the identity of EP along with the parameters affecting its formation. EP can be used for the quantitative determination of phenobarbital.

Other drugs such as doriden, anticonvulsants (*e.g.*, dilantin, primidone), antidiabetics (*e.g.*, chlorpropamide, tolbutamide) are also detectable by the procedure developed for the determination of phenobarbital and other barbiturates.

EXPERIMENTAL

Apparatus

A Hewlett-Packard (Avondale, Pa., U.S.A.) Model 7620 gas chromatograph equipped with a 6 ft. × 1/8 in. O.D. stainless-steel column packed with 10% U C-W98 on 80–100 mesh Chromosorb W HP was used. The GLC conditions were: injection port and detector temperature, 260°; column temperature, hold for 10 min at 175°, then program from 175° to 290° at 20°/min and hold at 290° for 1 min. Concentratubes, obtainable from Laboratory Research Co. (Los Angeles, Calif., U.S.A.), were used for extracting toluene with TMAH.

Reagents

All chemicals were ACS reagent grade.

Trimethylanilinium hydroxide (TMAH). Trimethylphenylammonium iodide, 20.7 g (40 mmoles), silver oxide, 5.1 g (20 mmoles), and 30 ml of anhydrous methanol were placed in a 50-ml PTFE-lined screw-cap culture tube and the mixture was rotated for 4–5 h. The mixture was centrifuged for 5 min and the liquid phase decanted into a brown glass bottle. When stored at 4–5°, this solution is stable for at least three weeks. The concentration of this TMAH solution ranges between 1.8 *M* and 2.0 *M*. The exact concentration may be determined by titrating 1 ml of this solution with 0.1 *N* hydrochloric acid using phenolphthalein as the end-point indicator. Lower concentrations of TMAH were obtained by suitably diluting the stock TMAH solution with anhydrous methanol.

Phosphoric acid, 0.2 M. Dilute 1.15 ml of 85% phosphoric acid to 100 ml with distilled water.

Phenobarbital stock solution, 10 mg/dl. Transfer 27.5 mg of sodium phenobarbital to each of two 250-ml volumetric flasks and dilute to volume with distilled water and anhydrous methanol, respectively.

Aqueous phenobarbital reference solutions. Dilute a suitable volume of the aqueous stock solution to 100 ml with distilled water to obtain final concentrations of 0.5, 1.0, 2.0, 3.0, 5.0 and 8.0 mg of phenobarbital per dl.

Internal GLC standard solutions. Secobarbital, 10 mg/dl — dissolve 27.5 mg of sodium secobarbital in 250 ml of anhydrous methanol. Aprobarbital, 0.5 mg/dl — Dissolve 25 mg of aprobarbital in 500 ml of toluene. Dilute 10 ml of this stock solution to 100 ml with toluene.

Absolute GLC reference standard, 1 μg/μl. Mix 1 ml each of phenobarbital stock solution (methanol) and secobarbital internal standard. Evaporate the mixture to dryness and redissolve the residue in 100 μl of TMAH. Repeat for each desired concentration of TMAH.

Preparation of α-phenylbutyramide, N-methyl-α-phenylbutyramide (EP) and N,N-dimethyl-α-phenylbutyramide. To a 250-ml round-bottom flask equipped with

a magnetic stirrer and reflux condenser were added 25 g (0.15 moles) of α-phenylbutyric acid and 22.3 ml (0.3 moles) of thionyl chloride. After refluxing for 0.5 h, the mixture was cooled and divided into three equal portions. One portion was slowly added with stirring to 100 ml of concentrated ammonium hydroxide. Similarly, a second portion was reacted with 100 ml of aqueous monomethylamine (40%, w/v) and the third with 100 ml of aqueous dimethylamine hydrochloride (20%, w/v). The aqueous dimethylamine solution (130 ml) was extracted twice with 100 ml of ether. The ether extract was dried over $MgSO_4$ and the ether was removed on a rotoevaporator to yield a liquid, N,N-dimethyl-α-phenylbutyramide [NMR (CS_2): δ0.8 (T, 3, $-CH_3$), δ1.65 (m, 2, $-CH_2-$), δ2.8 (S, 6, $-N(CH_3)_2$), δ3.45 (T, 1, CH) and δ7.13 (S, 5, phenyl)].

The precipitates formed in the ammonium hydroxide and monomethylamine solutions were filtered by suction, washed with cold water and allowed to air dry. They were identified, respectively, as: α-phenylbutyramide [NMR (CS_2): δ0.86 (T, 3, $-CH_3$), δ1.92 (m, 2, $-CH_2-$), δ3.27 (T, 1, CH), δ5,65, 6.2 (broad singlets, 2, $-NH_2$) and δ7.3 (S, 5, phenyl)] and N-methyl-α-phenylbutyramide [NMR (CS_2): δ0.86 (T, 3, $-CH_3$), δ1.97 (m, 2, $-CH_2-$) δ2.68 (D, 3, $-NCH_3$), δ3.3 (T, 1, CH), δ6.33 (broad singlet, 1, $-NH$) and δ7.27 (S, 5, phenyl)].

Procedure

To a PTFE-lined screw-cap culture tube was added 1 ml of the aqueous phenobarbital reference solution, 0.5 ml of 0.2 *M* phosphoric acid and 5 ml of the aprobarbital standard solution. The tube was vigorously shaken for 2 min and then centrifuged for 5 min at 2000 r.p.m.

Approximately 4 ml of the toluene layer were transferred to a concentratube using a Pasteur pipette. While mixing this solution with a vortex mixer, 25 μl of 1.8 *M* TMAH were added dropwise. After completing the addition and "vortexing" for an additional 10 sec, the tube was centrifuged for 10 min at 2000 r.p.m. Three microliters of the TMAH layer were then slowly injected into the gas chromatograph (over a 10-sec interval).

In order to study the effect of the TMAH concentration, 100 μl of the appropriate TMAH solution (0.05, 0.1, 0.22, 0.45, 0.90, 1.35, and 1.8 *M*) was added to an absolute GLC reference standard. After vortexing for 10 sec, 3 μl of the solution were injected slowly.

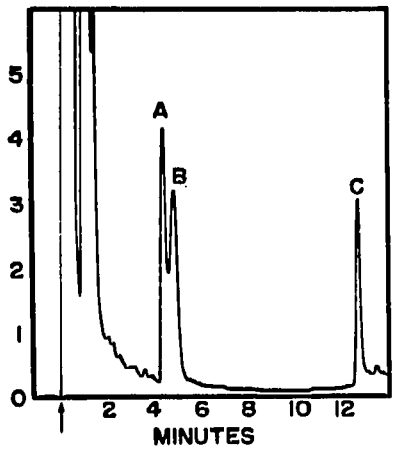
For studying the precision of the analysis, ten 3 mg/dl aqueous phenobarbital reference solutions were extracted with toluene according to the above procedure. Three microliters of each resulting TMAH solution were then analyzed.

For analysis by gas chromatography–mass spectrometry (GC–MS), 100 μl of 1.8 *M* TMAH were added to an absolute GLC reference standard. After vortexing, 3 μl of this solution was injected slowly into the GC–MS apparatus*.

RESULTS AND DISCUSSION

In the GLC analysis of phenobarbital in 1.8 *M* TMAH, one other product was formed besides the expected N,N-dimethylphenobarbital (I) (Fig. 1). This other peak was initially labelled "Early Pheno" (EP)[8]. MS analysis indicated that in addition to EP, another minor reaction product was formed.

* See footnote on p. 160.



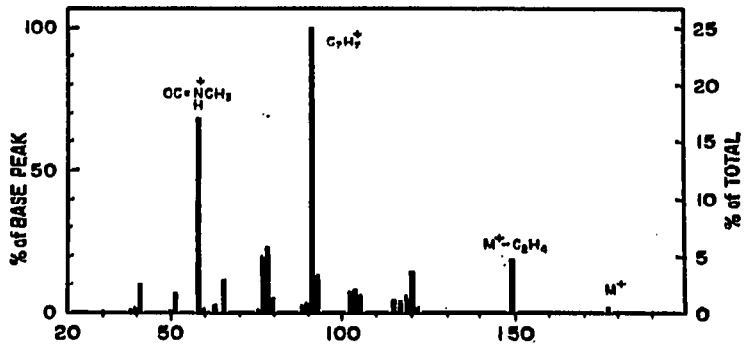
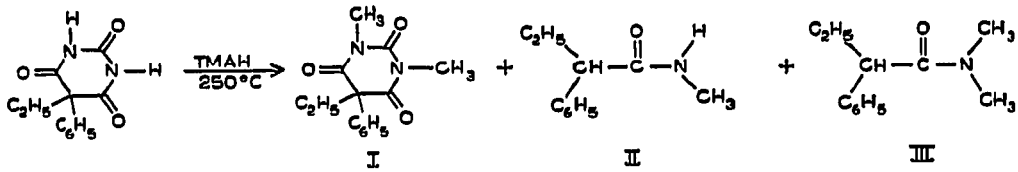


Fig. 1. Chromatogram of a 2 mg% aqueous phenobarbital standard. (A) N-Methyl-α-phenylbutyramide (EP); (B) aprobarbital; (C) phenobarbital.

Fig. 2. Mass fragmentation pattern of N-methyl-α-phenylbutyramide (EP).

Chemical ionization studies* indicated that the molecular weight of the major peak was 177 while that of the minor peak was 191. Coupling this information with the electron fragmentation pattern (Fig. 2) of the major peak and a knowledge of the products formed when phenobarbital is decomposed in a basic medium[9], the two peaks were tentatively identified as N-methyl-α-phenylbutyramide (II) (EP) and N,N-dimethyl-α-phenylbutyramide (III). To confirm this, these compounds were synthesized.



I II III

The electron fragmentation patterns and GLC retention times of these synthetic compounds compared with those of the compounds obtained via the TMAH reaction. These data were identical and thus the identities of the reaction products were firmly established.

By GLC alone, only the major decomposition product, EP (II), was detected together with N,N-dimethylphenobarbital (I) and thus only these two compounds are of concern in this analysis procedure. The second decomposition product (III) was formed in such minor amounts that it was not observed under the GLC operating conditions.

To study the parameters affecting the formation of EP during methylation of phenobarbital, the concentration of TMAH was varied from 0.05 *M* to 1.8 *M*.

* The analysis and the interpretation of the mass spectrometry data were performed by Roger Foltz, Batelle Memorial Laboratories, Columbus, Ohio, U.S.A.

Secobarbital was used as the internal standard to insure that aprobarbital, which is incompletely resolved from EP, did not interfere with the quantitation of EP. These studies showed that formation of EP was dependent on both the TMAH (base) concentration and the length of time phenobarbital was in contact with the TMAH prior to injection into the gas chromatograph (Fig. 3). At low TMAH concentrations, the amount of EP formed was small when phenobarbital was analyzed immediately after being dissolved in TMAH. Increasing the TMAH concentration increased the amount of EP, until, at a base concentration of 1.8 *M*, the amount formed remained constant. At this concentration, the EP to N,N-dimethylphenobarbital ratio was 65:35.

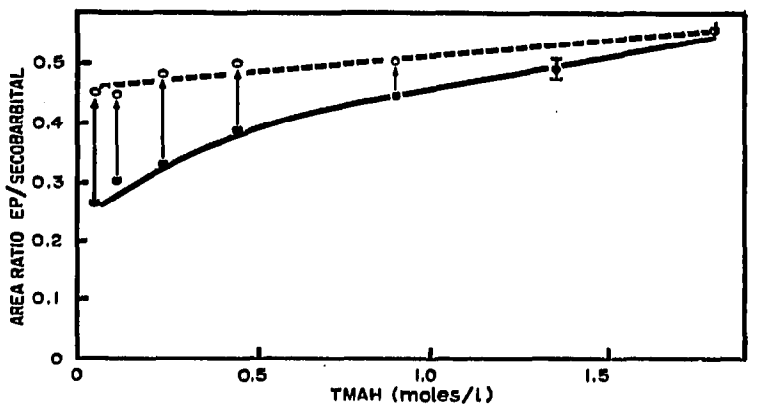


Fig. 3. Graph of the effect of time and TMAH concentration on formation of N-methyl-α-phenylbutyramide (EP). Solid black circles indicate initial extent of formation of EP when analyzed directly after mixing reagents; arrows indicate the extent of increase of EP if the samples are periodically re-analyzed over several hours; open circles indicate the final ratio where the amount of EP formed remained constant.

When the above phenobarbital solutions were re-analyzed at 30-min intervals after the addition of TMAH, the amount of EP in each sample increased with time until each had reached a value comparable to that formed in the sample containing 1.8 *M* TMAH. At this concentration of TMAH, the amount of EP formed remained constant over a period of several hours.

Thus this GLC analysis method for phenobarbital is based on the use of a high TMAH concentration in order to insure a reproducible formation of EP. In the actual extraction of toluene with TMAH, the concentration of TMAH increases from 1.8 *M* before extraction to 2.2 *M* after extraction. This increase in concentration, probably due to a loss of methanol in toluene, is desirable since, in view of the data presented, the increased concentration would tend to insure the reproducibility of the procedure.

For quantitative purposes, the concentration of EP is the better measure of the phenobarbital concentration and is a linear function of the phenobarbital concentration from 0.5 to 8.0 mg/dl (Fig. 4).

Replicate analysis of a 3 mg/dl aqueous phenobarbital standard (aprobarbital internal standard) showed less than 3% variation in the EP to N,N-dimethylaprobarbital (internal standard) peak ratio. For N,N-dimethylphenobarbital, the

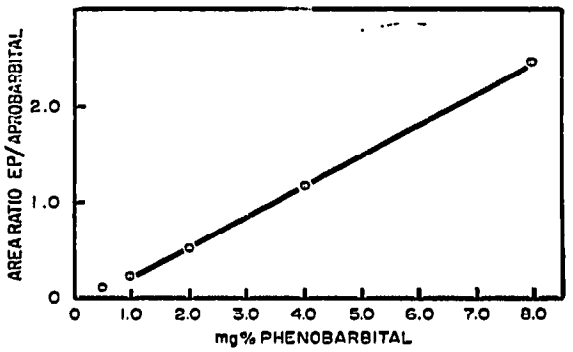


Fig. 4. Graph showing linearity of phenobarbital analysis method over the range of 0.5–8.0 mg/dl.

variation of a similar peak ratio with the internal standard was greater, possibly because the small size of the peak made accurate quantitation more difficult. Thus, while the results of quantitation based on either of these peaks should be of the same order of magnitude, quantitation of EP will be more precise and reproducible (see Table I).

TABLE I

PRECISION OF ANALYSIS

Three mg/dl aqueous phenobarbital standards were used for these determinations. CV= Coefficient of variation.

Sample No.	*N-Methyl-α-phenylbutyrimide / N,N-Dimethylaprobarbital**	*N,N-Dimethylphenobarbital / N,N-Dimethylaprobarbital*
1	0.91	0.32
2	0.84	0.41
3	0.87	0.39
4	0.89	0.44
5	0.88	0.40
6	0.84	0.43
7	0.83	0.40
8	0.84	0.41
9	—	0.39
10	0.86	0.39
	Av. 0.86±0.02	Av. 0.40±0.05
	CV 2.3%	CV 5%

* N,N-Dimethylaprobarbital= internal standard.

For quantitation of an unknown barbiturate mixture, aprobarbital is preferred as the internal standard over secobarbital since it is a rarely used barbiturate. The linearity and precision of the analysis procedure using aprobarbital indicates that it does not interfere with quantitation of EP.

During the period of time that this procedure has been used on actual samples in our laboratory, other drugs and/or their metabolites were detected (Table II). On the GLC column used, phenacetin has a retention time similar to amobarbital and thus the identity of the peak at this retention time must be checked by another

TABLE II

RELATIVE RETENTION TIMES OF SOME DRUGS

Drug	*Relative retention time (programmed)*	*Relative retention time (isothermal)*
Salicylic acid	0.48	
N-Methyl-α-phenylbutyramide (EP)	0.89	0.59
Aprobarbital	1.00*	–
Phenacetin	1.38	–
Tolbutamide	1.48	–
Chlorpropamide	1.64	–
Secobarbital	1.78	1.00**
Glutethimide	2.60	–
Mesantoin	2.28	–
Phenobarbital	2.52	1.68
Primidone	3.10	2.43
Dilantin	3.14	4.45
Phenylbutazone	3.49	–

* Aprobarbital, absolute retention time 4.5 min.
** Secobarbital, absolute retention time 2.6 min.

independent qualitative procedure. Glutethimide and its metabolites may also be detected by this procedure. In most patients exposed to glutethimide the blood sample usually will only show the presence of the parent drug, while the urine contains many metabolites whose peaks appear after glutethimide on the chromatogram and which are a qualitative confirmation of the presence of glutethimide.

By far and large the greatest potential for this procedure is its application to the analysis of phenobarbital and dilantin. Neurologists frequently desire to know the blood concentrations of these drugs in patients for whom they were prescribed.

By slightly modifying the suggested procedure it is possible to determine phenobarbital, primidone and dilantin in 12 min (Fig. 5). The modifications of the described procedure needed to achieve this analysis are: isothermal analysis of the extracted sample at 225°; and substitution of secobarbital for aprobarbital as the internal standard since at this temperature, EP and aprobarbital are not adequately resolved.

Preliminary investigations indicate that when analyzing samples obtained from patients who were given primidone there may be a slight interference from phenylethylmalonamide (PEMA), a metabolic product of primidone. VanMeter and Gillen[7] have reported the formation of a characteristic peak following the injection of a mixture of PEMA and TMAH. We found that this mixture gives a peak which has the same retention time as EP (4.01 min) and is significantly lower than the retention time of PEMA in the absence of TMAH (12.9 min).

We presume that the early peak from PEMA and TMAH is EP. Using the procedure described above, less than 5% of any PEMA present is extracted and therefore its contribution to any EP peak will be no more than 5% of the PEMA concentration. This will not be a significant effect, unless large amounts of primidone

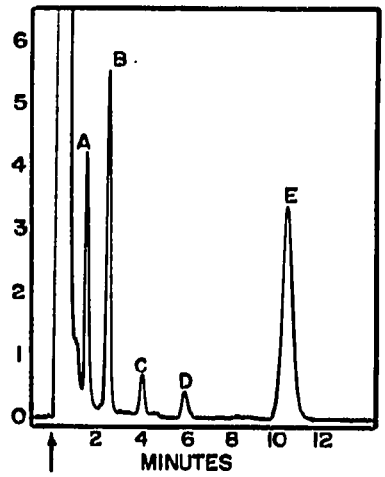


Fig. 5. Chromatogram of a mixture of anticonvulsants. (A) N-Methyl-α-phenylbutyramide (EP); (B) secobarbital; (C) phenobarbital; (D) primidone; (E) diphenylhydantoin.

are present. This problem can then be resolved by using the dimethylphenobarbital peak to quantitate the phenobarbital concentrations.

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