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Quantitative Flash-Methylation Analysis of Phenobarbital

KOUICHI KURATA ˣ, MASASHI TAKEUCHI, and KEIKO YOSHIDA

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Abstract □ In phenobarbital measurement by GLC with the flash-methylation technique, using trimethylanilinium hydroxide as a methylating reagent, a small amount of water decomposed phenobarbital and interfered with the quantitative analysis. Thus, both the sample and the methylating reagent must be sufficiently dehydrated to attain quantitative analyses. The hydrolysis decomposition product of phenobarbital was *N*-methyl-2-phenylbutyramide, as shown by its mass spectrum. The sum of methylated phenobarbital and *N*-methyl-2-phenylbutyramide (if observed in the spectrum) can be used for an accurate phenobarbital assay in the present flash-methylation technique.

Keyphrases □ Phenobarbital—analysis, flash-methylation GLC, degradates □ GLC, flash methylation—analysis, phenobarbital in biological fluids, degradates □ Mass spectrometry—analysis, phenobarbital in biological fluids, degradates

GLC determination of anticonvulsant drugs in biological fluids has been widely adopted in clinical laboratories. Phenytoin and phenobarbital have usually been assayed with the flash-methylation technique, using trimethylanilinium hydroxide as a methylating reagent. Some authors (1, 2) described the common errors in the assay of phenobarbital due to its decomposition by hydrolysis, and the GLC peak derived from the decomposition product has been called "early phenobarbital" (3); its structure has been speculated to be *N*-methyl-2-phenylbutyramide (III) (4, 5).

In the present work, the authors identified the decomposition product as III by mass spectrometry and found that its formation was due to the basic phenobarbital hydrolysis caused in pretreating the sample with basic methylation reagent and the subsequent methylation of the resulting 2-phenylbutyramide (I) in the GLC unit. Several problems in the assay of the anticonvulsant drugs by flash methylation are discussed here also.

EXPERIMENTAL

Methylation—Various concentrations of trimethylanilinium hydroxide in methanol were prepared as described previously (6) and stored at 4°. A 10-μg sample of phenobarbital was dissolved in 30 μl of trimethylanilinium hydroxide–methanol solution (the reagent must be used within 2 weeks of preparation), and the mixture was kept standing for 5 min. Each 3-μl sample obtained was then subjected to GLC.

GLC—The gas–liquid chromatograph[1] was equipped with a dual flame-ionization detector and columns for linear temperature programming. The glass columns, 200 cm long × 4.0 mm i.d., were packed with an equal amount of either 1.5% QF-1 on 60–80-mesh Chromosorb W or 3% OV-17 on 80–100-mesh Shimalite[2]. The analysis was performed under the following conditions for 1.5% QF-1: injection temperature, 245°; detector temperature, 245°; and column temperature, initial 150° and programmed to 215° at 6°/min. For 3% OV-17, the injection temperature

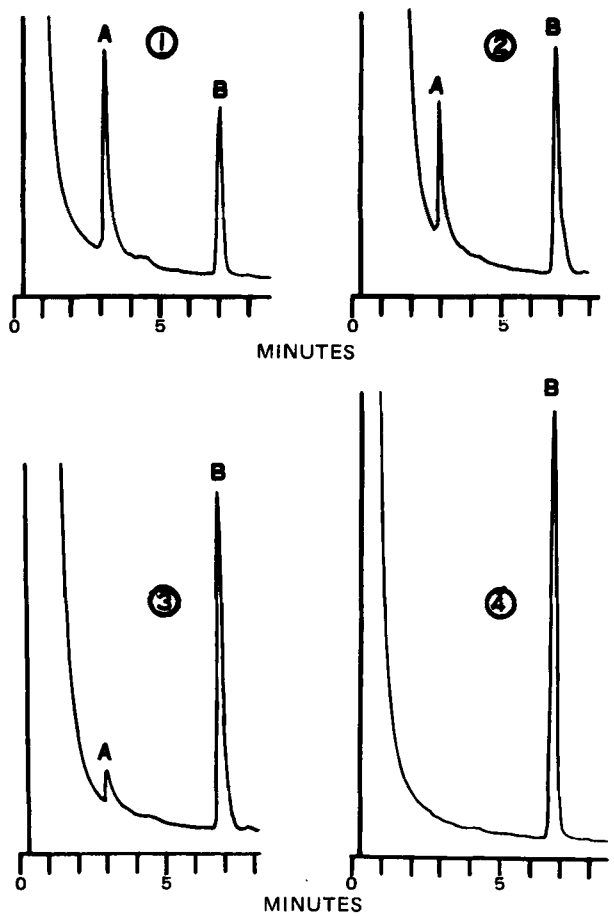

Figure 1—*Gas chromatograms of phenobarbital treated with various trimethylanilinium hydroxide concentrations. Phenobarbital was analyzed with 1.5% QF-1 in 0.20 (①), 0.10 (②), 0.05 (③), and 0.025 (④) M trimethylanilinium hydroxide solution. A 10-μg sample of phenobarbital was dissolved in 30 μl of trimethylanilinium hydroxide solution; after 5 min, a 3-μl sample was injected into the chromatograph.*

[1] Shimadzu GC-4CMPF, Shimadzu Ltd., Japan.

[2] Wako Chemical Industries Ltd., Japan.

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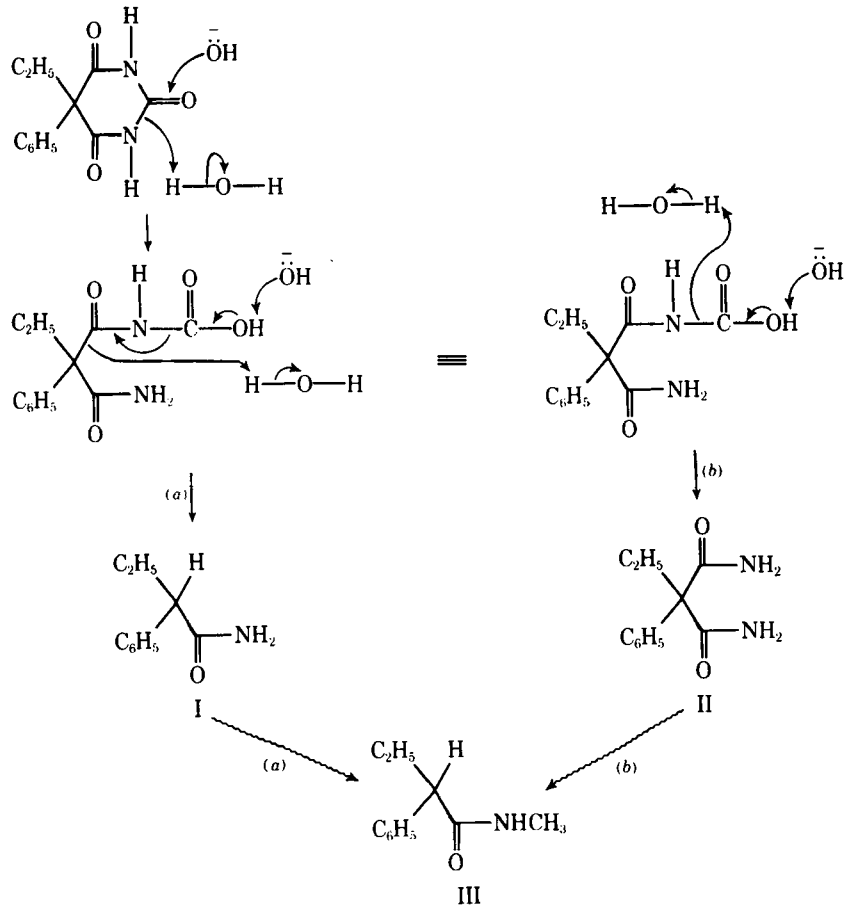

Scheme I—Mechanistic consideration through which N-*methyl-2-phenylbutyramide (III) should be derived from phenobarbital by basic hydrolysis and methylation. The hydrolyzed product should be considered as 2-phenylbutyramide [path* a *(I)] or 2-ethyl-2-phenylmalondiamide [path* b *(II)].*

was 300°, the detector temperature was 300°, and the column temperature was 150–275°, programmed to rise at 10°/min. The gas flows were: hydrogen, 50 ml/min; air, 800 ml/min; and nitrogen (as a carrier), 50 ml/min.

Mass Spectrometry—For detailed analysis of each methylation product, a GLC mass spectrometer[3] was used with the flash heater temperature at 245°. The glass column, 200 cm × 4.0 mm, was packed with 1.5% QF-1. The column temperature was 130–190°, programmed to rise at 5°/min. Helium flow was 27 ml/min. For the mass fragmentometry, a total ion detector was used. The ionization voltage was 70 ev.

RESULTS

Figure 1 shows the chromatograms analyzed with 1.5% QF-1 resulting from phenobarbital methylation using various concentrations of trimethylanilinium hydroxide. At low trimethylanilinium concentrations, only the B peak was observed; at higher concentrations, the A peak appeared and the B peak decreased. In the analysis using the two columns (1.5% QF-1 and 3% OV-17), two peaks were generally observed. The A peak was the one previously called "early phenobarbital." The sum of the A and B amplitudes was almost constant (Fig. 1) in each analysis.

The influence of water on the methylation was investigated. In Fig. 2, Chart 1 corresponds to the chromatogram of phenobarbital treated with 30 μl of 0.10 *M* trimethylanilinium hydroxide dehydrated with anhydrous sodium sulfate; Chart 2 shows the chromatogram resulting from the methylation of phenobarbital treated with 30 μl of trimethylanilinium hydroxide containing 3 μl of water. The A′ and B′ peaks showed the same retention times as A and B (Fig. 1) under identical GLC and, therefore, were identified with A and B. Only the B′ peak is observed in Chart 1, while two peaks, A′ and B′, appeared in Chart 2. This result suggests clearly that phenobarbital was decomposed through the hydrolysis in the latter case (Chart 2). However, as already described, the sum of the amplitudes of the A′ and B′ peaks is almost the same in Charts 1 and 2.

Both A and B peaks were characterized by GLC–mass spectrometry. Each molecular weight of the compounds corresponding to the A and B peaks indicated *m/e* 177 and 260. The B peak was identified as methylated phenobarbital by comparison with the mass spectrum of the authentic sample The structure of the A peak was determined as *N*-methyl-2-phenylbutyramide by reasonable fragmentation of its spectrum, and this conclusion was supported by the mechanism of its formation from phenobarbital under the basic condition (*vide infra*).

DISCUSSION

The simultaneous measurement of many anticonvulsant drugs by GLC has been adopted in many clinical laboratories. Drugs such as phenytoin and phenobarbital usually have been measured with the flash methylation technique. However, in the measurement of phenobarbital with this technique, some authors (1, 2, 7) have suggested that phenobarbital has not quantitatively changed into its methylated derivatives and have demonstrated that the longer the time spent in the pretreatment stage (before injection), the more phenobarbital decomposed (2). In accordance with this conclusion, it was demonstrated (Fig. 2) that a small amount of water contaminant decomposed phenobarbital. Hence, the decomposition (Fig. 1) might reasonably be caused by moisture contaminating the trimethylanilinium hydroxide solution.

The more trimethylanilinium hydroxide used, the more phenobarbital was decomposed. Hence, it is very important that the sample and trimethylanilinium hydroxide solution should be sufficiently dehydrated for quantitative phenobarbital analysis. However, moisture, which inevitably contaminates during operation, sufficiently decomposes phenobarbital at high trimethylanilinium hydroxide concentrations. Therefore, it is desirable that phenobarbital might be treated with a low trimethylanilinium hydroxide concentration (*e.g.*, 0.025 *M*). However,

[3] Shimadzu GCMS-7000, Shimadzu Ltd., Japan.

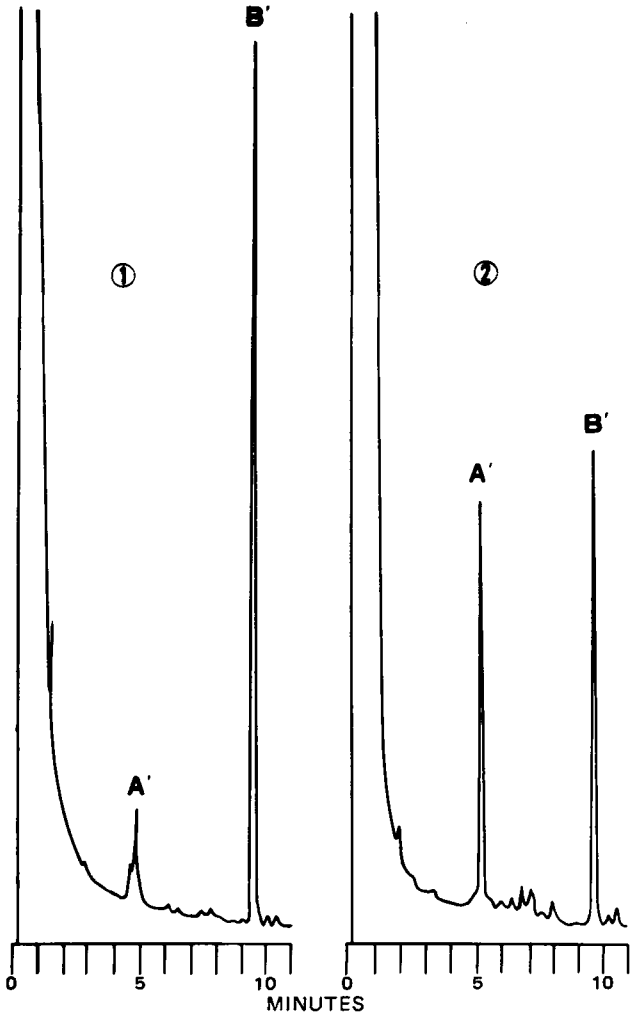

Figure 2—*Gas chromatograms of phenobarbital treated with dehydrated (Chart 1) and watered (Chart 2) trimethylanilinium hydroxide solution, analyzed with 3% OV-17. A 10-μg sample of phenobarbital was treated with 30 μl of 0.10* M *trimethylanilinium hydroxide solution dehydrated by anhydrous sodium sulfate (Chart 1) and by 0.10* M *trimethylanilinium hydroxide containing 3 μl of water (Chart 2).*

phenytoin sometimes was insufficiently methylated in 0.025 *M* because of contamination from biological fluids.

In the simultaneous measurement of phenytoin and phenobarbital, a higher concentration (0.10–0.20 *M*) of trimethylanilinium hydroxide is required for sufficient methylation. In this case, phenobarbital is frequently decomposed even if these precautions are taken. Therefore, for the quantitative analysis, operators must first check whether the A peak is present in the spectrum. When the A peak is observed, the sum of the A′ and B′ peaks should be considered as being formed from phenobarbital. Even if phenobarbital is decomposed, a peak height ratio of the sum of these two peaks to cholestane (as an internal standard) gives the accurate assay of phenobarbital.

The A peak was analyzed by GLC–mass spectrometry and showed its molecular ion at *m/e* 177, together with fragment ions at 149 ($M^+ - C_2H_4$), 120 ($C_6H_5CH_2CHO$), 91 ($C_6H_5 - CH_2^+$), and 58 ($CONHCH_3^+$). Careful mass spectrogram analysis and mechanistic considerations of its fragmentation pattern suggested this product to be *N*-methyl-2-phenylbutyramide (4, 5). Scheme I shows the reasonable mechanism of its formation from phenobarbital. Thus, phenobarbital should be first decomposed to 2-phenylbutyramide (I) through basic hydrolysis, and then this hydrolyzed product should be changed into *N*-methyl-2-phenylbutyramide (III) on column methylation (Scheme I, path *a*). Such analysis does not exclude the possibility that the A peak is *N*-formyl-*N*-methylphenylacetamide. However, from the mechanistic viewpoint, this species can be ruled out because its formation is not expected from phenobarbital by basic hydrolysis.

An alternative path of III formation also is possible (Scheme I, path *b*). Some authors (2, 8) proposed that a possible phenobarbital degradation product in alkaline solution was 2-ethyl-2-phenylmalondiamide (II). However, since the molecular ion of the A peak (*m/e* 177) fit neither that of the malondiamide (*m/e* 206) nor that of its methylated derivatives, the latter product could not be the same as the A peak. However, II could give III (A peak) by flash methylation. The study of these two paths (*a* and *b*) is now in progress.

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