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SOLVENT SUPPRESSION OF THE DECOMPOSITION OF PHENOBARBITAL DURING ON-COLUMN METHYLATION WITH TRIMETHYLANILINIUM HYDROXIDE

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SUMMARY

Gas–liquid chromatographic (GLC) analysis of phenobarbital by on-column methylation with trimethylanilinium hydroxide gives rise to a major decomposition product, N-methyl-2-phenylbutyramide, in addition to the methylated barbiturate, N,N′-dimethylphenobarbital. This reaction occurs nearly exclusively in the solution phase in the injection port of the gas chromatograph. A mechanism for the decomposition reaction consistent with the available information is presented. This decomposition is shown to be inhibited by certain solvents and this effect forms the basis of a new analytical technique for the simultaneous GLC analysis of phenobarbital, primidone, and diphenylhydantoin.

INTRODUCTION

Several published gas–liquid chromatographic (GLC) procedures for the analysis of phenobarbital employ on-column methylation with trimethylanilinium hydroxide (TMAnH)[1–5]. In these techniques, the final step is the injection of the anilinium salt of the drug, which undergoes thermolytic degradation in the injection port of the chromatograph to yield neutral and volatile products (dimethylaniline and N,N′-dimethylphenobarbital).

Some workers have reported a major decomposition product of phenobarbital with TMAnH, emerging from the GLC column much earlier than N,N′-dimethylphenobarbital[4–8]. Initially, this compound was thought to be a methylated derivative of phenylethylmalonyldiamide[7,8]; however, characterization by gas chromatography–mass spectrometry revealed that it was N-methyl-2-phenylbutyramide[5–9] which was termed "early phenobarbital" (EP)[4]. The analytical procedure of Osiewicz *et al.*[5] employed conditions which favored the decomposition reaction, utilizing the chromatographic peak produced by EP for quantitation of phenobarbital.

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Recently, Osiewicz and Valentour published a method for the determination of diphenylhydantoin in biological materials[10]; they used a dilute solution of TMAnH (0.2 *M*) in ethylene glycol–methanol (9:1) for the methylating agent. When phenobarbital dissolved in this solvent is injected onto the column of the gas chromatograph, there is very little decomposition. Only the dimethylated derivative emerges from the column.

In this paper, we characterize the decomposition reaction, propose a mechanism for it, describe the influence of various solvents on the process, and report a new procedure for the simultaneous analysis of barbiturates, primidone and diphenylhydantoin which is rapid and sensitive and which produces a stable extract suitable for manual injection or automatic sampling devices.

EXPERIMENTAL

Apparatus

A Perkin-Elmer (Norwalk, Conn., U.S.A.) Model 900 gas chromatograph with a Model AS-41 automatic sampling system and a Hewlett-Packard (Avondale, Pa., U.S.A.) Model 7620 gas chromatograph were used. Each instrument was equipped with an identical 6 ft. × 1/8 in. O.D. stainless-steel column packed with 10% UC-W98 on 80–100 mesh Chromosorb W HP DMCS. The chromatographic data were collected by a Perkin-Elmer Model PEP-1 data processor. The GLC conditions on the Model 900 were: injection port and detector temperature, 280°; column temperature, 200°. Flow-rates were: nitrogen, 30 ml/min; hydrogen, 30 ml/min; and air, 325 ml/min. The GLC conditions on the Model 7620 were: injection port, 270°; detector temperature, 290°; column temperature, 200°. Flow-rates were: nitrogen, 35 ml/min; hydrogen, 35 ml/min; and air, 290 ml/min. Concentratubes from Laboratory Research Co. (Los Angeles, Calif., U.S.A.) were used for extracting the toluene layer with TMAnH.

Reagents

All chemicals were ACS reagent grade.

2.0 M Trimethylanilinium hydroxide (TMAnH). Trimethylanilinium iodide (trimethylphenylammonium iodide), 7.0 g (13.5 mmoles), silver oxide, 3.6 g (14 mmoles), and 10 ml of anhydrous methanol were placed in a 50-ml PTFE-lined screw-capped culture tube, shaken briefly, and rotated for 6–24 h. The mixture was centrifuged at 1000 *g* for 10 min and the liquid phase was removed into a brown glass bottle. This solution was stable at room temperature for three weeks.

0.2 M Phosphoric acid. Phosphoric acid, 85% (1.15 ml), was diluted to 100 ml with distilled water.

Anticonvulsant drug stock solutions (50 mg/dl). Methanolic solutions of phenobarbital, primidone, and diphenylhydantoin (phenytoin) were prepared by dissolving 25 mg of each drug (free acid) in methanol and diluting each solution to 50 ml with methanol.

Plasma working standard (2.0 mg/dl). Two milliliters of the stock solutions of phenobarbital, primidone, and diphenylhydantoin were transferred to a 50-ml volumetric flask and evaporated to dryness in a 40° water bath using a stream of filtered

air. The residue was dissolved in drug-free plasma and diluted to volume with the same medium.

Internal GLC standard solution. Cyclobarbital was dissolved in ether to a concentration of 0.25 mg/dl.

N^1-Methyl-2-phenylbutyrylurea (VIII). This material was prepared by a modification of the method of Maulding *et al.*[11]. N-Methylphenobarbital (mephobarbital), 10 mg, was dissolved in 2.0 ml of 3 *M* NaOH and incubated overnight at ambient temperature. The solution was neutralized with 0.5 *M* sulfuric acid and extracted with 10 ml of ether. The ether phase was removed, washed with distilled water, dried with 1.0 g of anhydrous sodium sulfate, filtered, and evaporated to dryness. The procedure yielded *ca.* 1 mg of crystalline product [nuclear magnetic resonance (NMR) ($CDCl_3$): δ 0.86 (T,3,–CH_3), δ 1.89 (m, 2, –CH_2–), δ 2.83 (D,3,–NCH_3), δ 3.3 (T,1,CH), δ 6.78 (broad singlet, 1,–NH), δ 7.25 (S,5, phenyl), δ 8.34 (broad singlet, 1, CO–NH–CO)]. The NMR spectra were obtained by Lou Schnierer, Chemistry Department, Case Western Reserve University, Cleveland, Ohio, U.S.A.

N,N'-Dimethylphenobarbital. This was prepared by a modification of the method of Greeley[12]. Stock phenobarbital standard, 0.1 ml (50 μg), was placed in a 16 ml PTFE-lined screw-capped tube and evaporated to dryness. Fifty microlitres of 2.0 *M* methanolic tetramethylammonium hydroxide (Southwestern Analytical Chemicals, Austin, Tex., U.S.A.), 250 μl of N,N-dimethylacetamide, and 20 μl of methyl iodide were added to the residue. This was vortex-mixed briefly and allowed to stand for 15 min. One milliliter of distilled water and 4.0 ml of toluene were added and the solution was again mixed. The solution was centrifuged and the toluene layer removed.

N-Methyl-2-phenylbutyramide (EP). This material, prepared by the method of Osiewicz *et al.*[5], was a gift of R. Osiewicz.

Extraction procedure

Plasma (1.0 ml) is pipetted into a 16-ml PTFE-lined screw-capped culture tube. 0.2 *M* Phosphoric acid (0.5 ml) and 5 ml of ether containing the internal standard (0.25 mg/dl cyclobarbital) are added and the tube is capped and shaken vigorously for 2 min. The mixture is then centrifuged for 2 min at 750 *g* and approximately 4 ml of the ether layer transferred to a Concentratube with a Pasteur pipet. The ether solution is evaporated to dryness in a 40° water bath under a stream of filtered air. The residue is dissolved in 2.0 ml of toluene, washing down the sides of the tube in the process. While mixing this solution in a vortex mixer, 35 μl of 2.0 *M* TMAnH are added with a 50-μl syringe. The mixture is vortexed for an additional 10 sec, then centrifuged for 2 min at 2000 rpm. Four microliters of the TMAnH layer are removed and added to 4.0 μl of 50% glycerol in methanol previously placed in the tip of another Concentratube. The solutions are mixed quickly by repeated aspiration into the 10-μl syringe. (The syringe must be rinsed with methanol before proceeding to the next sample.) Three microliters of the final solution are injected into the gas chromatograph over an interval of 10 sec or prepared for automatic sampling. Phenobarbital, primidone and diphenylhydantoin are quantitated in relation to the cyclobarbital internal standard.

The precision of the analysis was evaluated by extracting multiple aliquots of the plasma working standard and analyzing them isothermally on the Model 900 at 220° with the automatic sampling device.

RESULTS AND DISCUSSION

The decomposition of phenobarbital during on-column methylation with TMAnH[4–8] should resemble the base-catalysed hydrolysis reaction occurring in aqueous solution (Fig. 1)[13–15]. The unionized molecule (I) is subject to nucleophilic attack by hydroxide ion (or water) at either carbonyl C-2 or one of the equivalent C-4 and C-6 carbonyl positions. Attack at the 2-position leads to cleavage of the 1–2 (2–3) bond, producing phenylethylmalonyldiamide (II). Attack at either of the other positions gives rise to a mixture of phenylethylmalonate, urea, and 2-phenylbutyrylurea (III). The latter is apparently a decarboxylation product of the intermediate, 2-phenyl-2-ethylmalonurate, which is not isolated[14,16]. The singly ionized species (IV) gives exclusively the hydrolysis products expected from reaction at the 4- or 6-position. The dianion (V) hydrolyses slowly, if at all[15], reflecting the difficulty of nucleophilic attack on a species which has a double negative charge. The rate of phenobarbital hydrolysis stabilizes above pH 10.8, the second pK of the molecule[15], and is virtually the same in 0.1 N and 1.0 N potassium hydroxide[17].

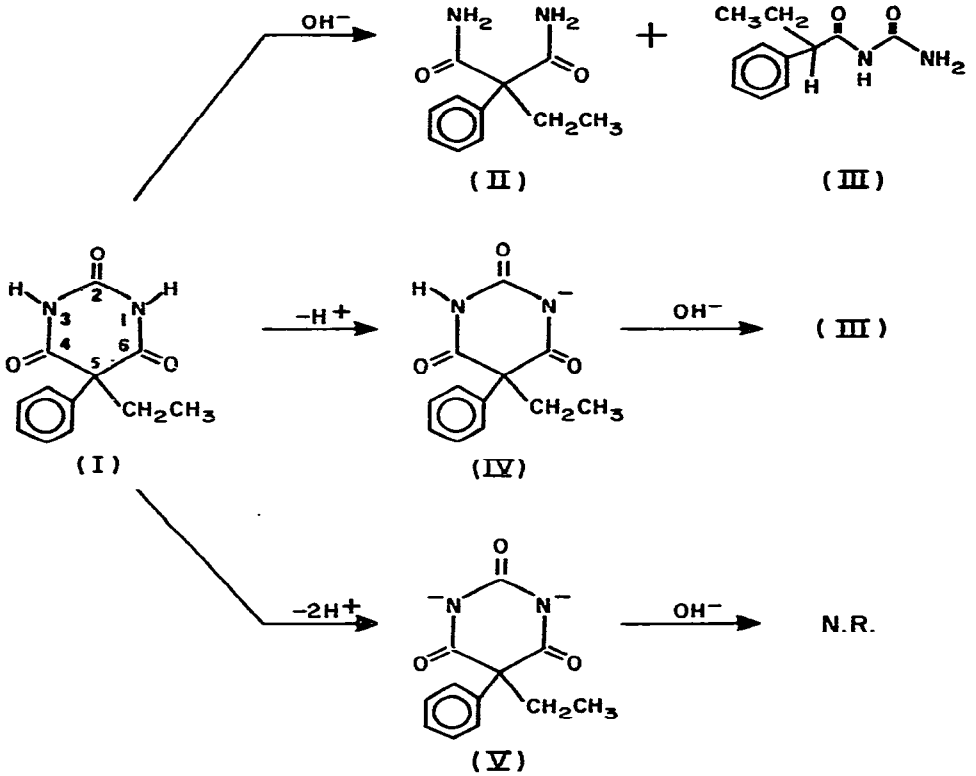

Fig. 1. Hydrolysis of phenobarbital in aqueous solution.

These facts suggest that the monoanion of phenobarbital is the primary species which experiences ring cleavage in basic solution. The delocalized forms which can be written for the ionized molecule (Fig. 2) show why this may be so, and also help to

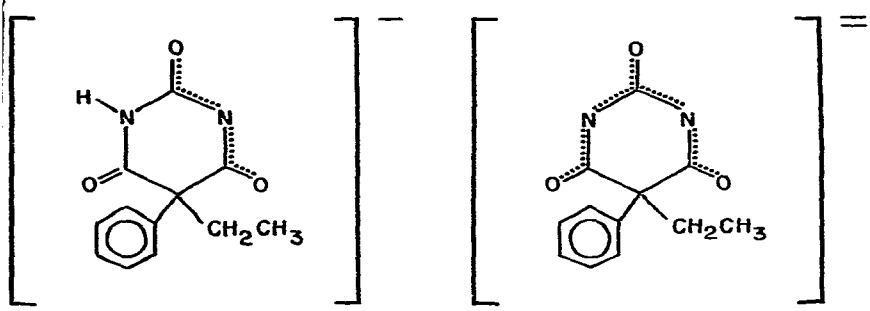

Fig. 2. Charge delocalization of phenobarbital ions.

explain the observed reactivities and products. For the monoanion, the contribution of three resonance structures results in a delocalization of the negative charge over five atoms, and significantly reduces the positive charge density on C-2 and C-6. Thus, C-4 is expected to be preferentially attacked by hydroxide ion. Because of the plane of symmetry passing through C-2 and C-5 ,removal of either acidic proton gives rise to the same ion. Considerations similar to the above apply to the dianion, except that for this form one can visualize six resonance forms. All three carbonyl carbon atoms are unlikely sites of nucleophilic attack due to delocalization of the double negative charge. The rate of hydrolysis depends both upon the concentration (activity) of the monoanion, and upon that of hydroxide ion. At pH's above the second pK of the molecule, an essentially constant rate of hydrolysis is achieved[17].

Factors favoring the decomposition of phenobarbital during TMAnH methylation

Previous workers have shown that the decomposition of phenobarbital during on-column methylation with TMAnH is favored by high TMAnH concentrations and by continued incubation of the phenobarbital-TMAnH solution before injection into the gas chromatograph[5]. Two additional factors affecting the process are injection port temperature and injection time. Other factors being equal, a greater amount of decomposition occurs at higher injection port temperatures and at longer injection times. The maximum percentage of EP generated under these conditions[5] is about 80%.

These observations suggest that the decomposition reaction occurs primarily in solution in the injection port, that is, "on-needle". This fact is documented in Fig. 3. Figs. 3A and 3B show chromatograms produced by EP and by N,N'-dimethylphenobarbital. Fig. 3C shows an injection of phenobarbital and 2 M TMAnH, dissolved in methanol. Both the decomposition product and N,N'-dimethylphenobarbital peaks appear, the former accounting for 70% of the combined area. Fig. 3D is the result of simultaneous injection of phenobarbital and TMAnH using different syringes. The early peak is less than 2% of its previous area, indicating that the decomposition reaction occurs nearly exclusively in the solution phase. It is also noteworthy that the dimethylated product is produced much less efficiently when the reactants mix only in the gas phase.

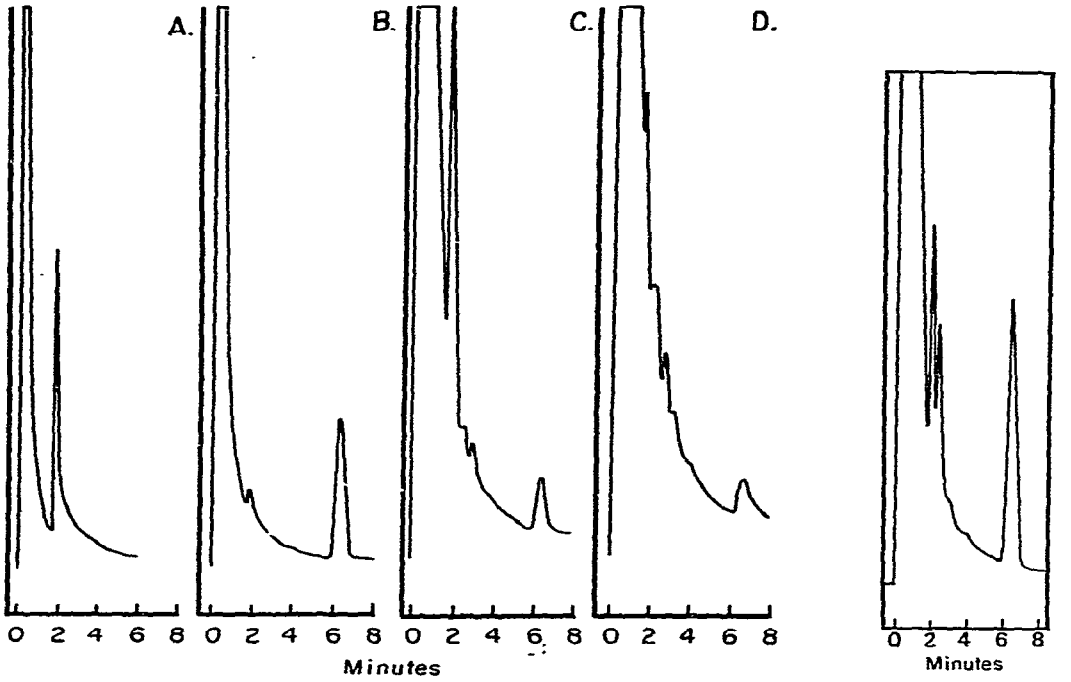

Fig. 3. GLC of Phenobarbital Methylation Products. A, N-methyl-2-phenylbutyramide (EP); B, N,N'-dimethylphenobarbital; C, Phenobarbital (5 μg) in 2.0 *M* TMAnH; D, Phenobarbital (10 μg) co-injected with 2.0 *M* TMAnH using different syringes.

Fig. 4. GLC of N,N'-Dimethylphenobarbital (5 μg) in 2.0 *M* TMAnH.

The mechanism of phenobarbital decomposition during on-column methylation with TMAnH

The experiments described below were undertaken in an effort to characterize further the mechanism of the decomposition reaction. The results of the co-injection experiment suggest that a reaction occurring in solution provides either EP or a necessary precursor or precursors. Is the species which is cleaved by hydroxide ion to produce EP N,N'-dimethylphenobarbital, or N-methylphenobarbital? Fig. 4 displays the chromatogram produced by the injection of N,N'-dimethylphenobarbital and TMAnH under the conditions used in Fig. 3C. In addition to peaks with the retention times of EP and the dimethyl derivative, a new third peak is present with a retention time just slightly longer than EP. This component may be N,N'-dimethylphenylethylmalonyldiamide or a mixture of methylated phenylethylmalonyldiamides. In any case, the appearance of this chromatogram implies that N,N'-dimethylphenobarbital is not the principal precursor of EP in the TMAnH methylation of phenobarbital, since the third peak is not usually observed. N-Methylphenobarbital under the same conditions produced a chromatogram identical to that of phenobarbital (*i.e.*, Fig. 3C). Furthermore, over a range of TMAnH concentrations from 0.2 to 2.0 *M* (in methanol), where phenobarbital produced from 31 to 71% EP, N-methylphenobarbital in each case produced an identical proportion. In no case was the second early peak produced by

N,N′-dimethylphenobarbital observed. These results suggest that the monomethylated product of phenobarbital lies on the pathway to EP.

When N-methylphenobarbital and N,N′-dimethylphenobarbital were injected into the GC with ammonium hydroxide (2.0 *M* in methanol), no EP peak was seen, though there was a 30% reduction in the size of the parent peak in each case. The basic hydrolysis product of N-methylphenobarbital, N¹-methyl-2-phenylbutyrylurea (VIII in Fig. 5), was prepared as described in Experimental. When this material was injected with TMAnH into the gas chromatograph, only a single peak with the retention time of EP was observed.

A suggested mechanism for the decomposition of phenobarbital during GLC analysis with TMAnH consistent with the above data is presented in Fig. 5. The following steps are proposed: (1) the dianion V, the predominant form in concentrated base (*e.g.*, TMAnH), is first methylated to N-methylphenobarbital (VI); (2) this molecule experiences nucleophilic attack by hydroxide ion at C-6; (3) the resulting intermediate cleaves at the 1–6 bond to give a substituted malonylurea (VII); (4) compound VII undergoes decarboxylation to a butyrylurea (VIII); (5) the imido nitrogen atom of VIII is methylated, giving a trisubstituted urea (IX); (6) the urea derivative IX thermally decomposes to give the observed major product, N-methyl-2-phenylbutyramide (EP, X) and methyl isocyanate. Steps 1–4 presumably occur in solution prior to vaporization in the injection port of the gas chromatograph. Steps 5 and 6 may occur during and after on-column injection of the trimethylanilinium salt of VIII, perhaps in concerted fashion.

The proposed mechanism is supported by several additional observations. First, the N-methylated barbiturates are known to be dramatically more prone to ring cleavage than the corresponding unmethylated molecules[15,17]; in particular, N-methylphenobarbital hydrolyses at 100 times the rate of phenobarbital (at 25° in 1.0 *M* KOH)[17]. The methylated form cannot participate in charge delocalization by resonance (as in Fig. 2); C-6 is thus more vulnerable to hydroxide ion attack. The decarboxylation step (4) was originally proposed by Gardner and Goyan[14] to account for the fact that base-catalysed cleavage of the barbiturate ring is essentially irreversible for phenobarbital, although reversible for other barbiturates[13,15]. The last step in the proposed mechanism, thermal decomposition of the butyrylurea (IX), is similar to known decomposition reactions of trisubstituted urea herbicides in GLC[18]. N,N′-dimethylphenobarbital is apparently not the principal precursor of EP, since the injection of the former material produces at least one additional major product besides EP (see Fig. 4 and ref. 19).

Suppression of phenobarbital decomposition by solvents

The decomposition of phenobarbital during derivatization with TMAnH has interfered with its quantitative estimation. Perchalski *et al.*, advocated quantitation based on the combined peak areas of N,N′-dimethylphenobarbital and two decomposition products[20]. Under the conditions used here and in previous reports from this laboratory[4,5], the only major decomposition product is EP. The conditions used by Osiewicz *et al.*[5], rendered the formation of EP from phenobarbital nearly quantitative; EP was then used to determine phenobarbital. This approach, while an improvement over others, is not entirely satisfactory because the EP peak occurs close to solvent components and thus may be subject to interference, the extract must be slowly and

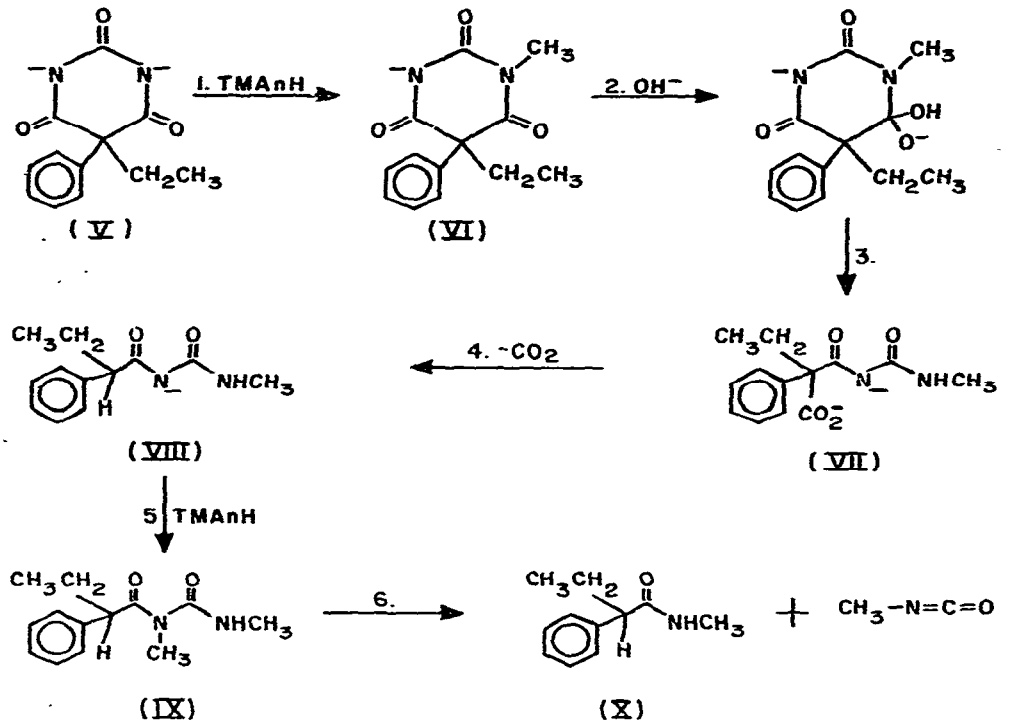

Fig. 5. Suggested mechanism for EP formation during on-column methylation of phenobarbital by TMAnH.

reproducibly injected into the GC to get reliable quantitation, and the response for primidone is relatively poor.

In an attempt to find a more suitable solvent than methanol for the analysis of phenobarbital with TMAnH, Osiewicz and Valentour used 0.2 *M* TMAnH in ethylene glycol–methanol (9:1)[10], in which the fragmentation of phenobarbital is totally suppressed. The N,N'-dimethylphenobarbital elutes from the column late enough that it is not influenced by solvent components.

The ability to inhibit this reaction is shared by several other solvents. Table I displays data obtained with 0.2 *M* TMAnH dissolved in various solvents (details given in the text to Table I). This concentration of TMAnH was chosen because it yielded an intermediate degree of fragmentation (31%) when dissolved in methanol. These experiments were carried out using the Perkin-Elmer AS-41 automatic sample injection system, which ensured that the GLC conditions for all samples were precisely identical.

The data of Table I demonstrate that the splitting of phenobarbital in these solvents varies from none to complete. The most consistent property relating to the inhibitory effect is solvent viscosity. This is not unexpected, since the rate constant for a reaction is proportional to the diffusional coefficients of the reacting species. Most

of the effective compounds are polyhydric alcohols; thus, a solvent effect on the activity of hydroxide ion is a possibility. The two aprotic solvents, dimethylformamide and dimethylsulfoxide, seem to promote the phenobarbital decomposition. The polarity of the solvent is apparently unrelated to the suppressive effect. A more detailed explanation of these results is not available.

The procedure described in Experimental employs the solvent suppressive effect of glycerol in a new analytical scheme for the simultaneous GLC determination of phenobarbital, primidone, and diphenylhydantoin (phenytoin). The technique involves extraction of a plasma sample with ether, evaporation of the ether, dissolution of the residue in toluene, and extraction of the toluene with 2.0 *M* TMAnH in methanol. An aliquot of the TMAnH layer is diluted with glycerol–methanol (1:1) and injected into the gas chromatograph.

This technique offers several advantages over other published methods. Primidone is extracted from acidified plasma much more efficiently by ether than by other solvents, such as toluene[17,20]. On the other hand, the drugs are more efficiently recovered from toluene than from ether by TMAnH extraction. Dissolving the ether residue in toluene rather than the TMAnH reduces interference from endogenous blood constituents. A solution of 2 *M* TMAnH is employed to optimize recovery of

TABLE I

EXTENT OF PHENOBARBITAL DECOMPOSITION WITH 0.2 *M* TMAnH DISSOLVED IN VARIOUS SOLVENTS, AND THEIR PHYSICAL PROPERTIES

% EP: 30 μl 0.2 *M* TMAnH solution in each solvent (a 10-fold dilution of 2.0 *M* methanolic TMAnH with the solvent) was added to 50 μg of phenobarbital in the tip of a Concentratube and mixed. 3 μl of this solution were encapsulated and injected immediately into the Model 900 gas chromatograph. Each solution was prepared and processed in duplicate and the values for % EP (peak area of EP as a percentage of the sum of the EP and N,N'-dimethylphenobarbital peak areas) agreed within ±2% of the total. The mean value is given in the table.

Solvent	*Dielectric constant*[21]	*Viscosity at 25° (cP)*[22,23]	*% EP*
DMF	36.7	0.8	100
DMSO	46.7	—	81
Water	78.5	0.9	72
Methanol	32.7	0.5	31
Ethanol	24.6	1.1	35
n-Propanol	20.3	1.9	33
iso-Propanol	19.9	2.1	32
n-Butanol	17.5	2.5	36
tert.-Butanol	1.8	3.3	37
p-Dioxane	2.2	1.2	11
1,3-Propanediol	35.0	—	8
2-Ethoxyethanol	29.6	2.0	0**
n-Octanol	10.3	—	0**
Ethylene glycol	37.7	17.4	0**
1,2-Propanediol	32.0	43.0	0**
4.5% Glycerol*	—	—	2
18% Glycerol*	—	—	0**
Glycerol	42.5	43.0	—

* In methanol.

** No peak with a retention time within ±5% of EP was observed.

primidone, which because of its weaker acidity is not well extracted at lower base concentrations. The final dilution of the TMAnH extract of the drugs into glycerol–methanol stabilizes the phenobarbital and allows the use of cyclobarbital, another base-labile barbiturate (with a desirable retention time relative to the drugs of interest) as internal standard.

Replicate analyses of the plasma working standard (2.0 mg/dl, $n = 10$) yielded the following coefficients of variation: phenobarbital, 1.2%; primidone, 3.2%; and diphenylhydantoin, 4.7%. The procedure may be applied to other anticonvulsants, barbiturates, and glutethimide, as well as other acidic drugs[5]. It may be scaled-down at least ten-fold without difficulty.

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