(8) H. B. Bull, K. Bresse, G. L. Ferguson, and C. A. Swenson, *Arch. Biochem. Biophys.*, **104**, 297(1964).
(9) W. H. R. Shaw and J. J. Bordeaux, *J. Amer. Chem. Soc.*, **77**, 4729(1955).
(10) M. A. Schwartz and E. Nelson, in "Husa's Pharmaceutical Dispensing," 6th ed., E. W. Martin, Ed., Mack Publishing Co., Easton, Pa., 1966.
(11) R. H. Stokes, *Aust. J. Chem.*, **20**, 2087(1967).
(12) "International Critical Tables," vol. V, McGraw-Hill, New York, N. Y., 1928, p. 22.
(13) V. K. Venkatesan and C. V. Suryanarayana, *J. Phys. Chem.*, **60**, 775(1956).
(14) S. Glasstone, "Introduction to Electrochemistry," D. Van Nostrand, Princeton, N. J., 1964, p. 64.
(15) G. Milazzo, "Electrochemistry," Elsevier Publishing Co., Amsterdam, The Netherlands, 1963, p. 60.
(16) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," 3rd ed., Reinhold, New York, N. Y., 1959, pp. 111–114.
(17) J. T. Edsall and J. Wyman, "Biophysical Chemistry," vol. I, Academic, New York, N. Y., 1958, pp. 371, 372.
(18) W. J. Svirbely and J. C. Warner, *J. Amer. Chem. Soc.*, **57**, 1883(1935).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 18, 1971, from the *School of Pharmacy, University of Connecticut, Storrs, CT 06268*

Accepted for publication March 25, 1971.

This report is based upon work supported in part by the Office of Water Resources, U. S. Department of the Interior, as authorized under the Water Resources Research Act of 1964. Computer time was provided under Grant GJ-9, National Science Foundation

* To whom reprint requests should be addressed.

Combined GLC and High-Resolution Mass Spectroscopic Analysis of Diphenylhydantoin

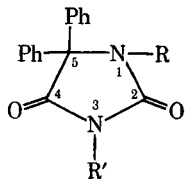
KHALID SABIH* and KHAWLA SABIH*

Abstract □ A sensitive GLC method for the analysis of diphenylhydantoin in biological material is described. This method involves the conversion of diphenylhydantoin to its methyl derivative. The identity of the methyl derivative was determined by combined GLC–mass spectroscopy. The high-resolution mass spectra of diphenylhydantoin methyl derivatives indicate that the methyl groups were introduced at the nitrogen atoms of the hydantoin ring to produce 3-methyl- and 1,3-dimethyldiphenylhydantoin.

Keyphrases □ Diphenylhydantoin and methyl derivatives, analysis in biological material—GLC and mass spectroscopy □ GLC—analysis, diphenylhydantoin and methyl derivatives □ Mass spectroscopy—analysis, diphenylhydantoin and methyl derivatives

Several spectrophotometric, colorimetric, and UV methods to determine levels of diphenylhydantoin (I) in biological material were published (1–5). However, most of these methods are nonspecific and time consuming, and they require many selective extractions to avoid interference of other drugs, *e.g.*, barbiturates. Recently, a GLC method was reported (6) which involved the conversion of diphenylhydantoin to its methoxy derivative by treatment with diazomethane. However, the structure of the methylated derivative has not been determined. In a recent report (7), a direct GLC method was described for the determination of the drug at therapeutic levels in blood. Another recent report (8) described a GLC method for the determination of diphenylhydantoin in which the drug was methylated with tetramethylammonium hydroxide. The corresponding peak of the methylated drug was identified by NMR and mass spectroscopy. However, no discussion of the mass spectra was included.

This report describes a more sensitive GLC method which measures even subtherapeutic levels of the drug in biological material. However, when using GLC for qualitative determination of drugs in biological material, it is necessary to have parameters other than the retention time of the drug in order to make a more certain identification of the drug. This is especially true if derivatives of the drugs are being analyzed when more than one reactive center is available in the drug molecule. Mass spectroscopy, therefore, was utilized as a tool for identification of diphenylhydantoin derivatives.



I: R = R′ = H; diphenylhydantoin
II: R = H, R′ = CH_3; 3-methyldiphenylhydantoin
III: R = R′ = CH_3; 1,3-dimethyldiphenylhydantoin

EXPERIMENTAL

Reagents—Diphenylhydantoin[1] and dimethyl sulfate[2] were used. Heptane and chloroform were redistilled before use. Acetate buffer (0.2 *M*, pH 5.6) was made by mixing 4.8 ml. of acetic acid solution (0.2 *M*, 11.55 ml. in 1000 ml. water) and 45.2 ml. of sodium acetate solution (0.2 *M*, 16.4 g. in 1000 ml. water), and the mixture was diluted with water to 100 ml. Methanolic potassium carbonate solution was made by mixing 1 ml. of 2–5% aqueous solution of potassium carbonate and 9 ml. of methanol (analytical reagent).

Preparation of Methyl Derivatives of Diphenylhydantoin—*Monomethyl Derivative*—A solution of 1.0 g. diphenylhydantoin in 30 ml. methanolic potassium carbonate was placed in a three-necked, 150-ml. flask fitted with a reflux condenser and a magnetic stirrer. Twenty milliliters of dimethyl sulfate was added, and the reaction was allowed to proceed for 15 min. at 70°. The reaction mixture was then cooled, and methanol was removed under reduced pres-

[1] Parke-Davis and Co., Detroit, Mich.
[2] Matheson Coleman and Bell, Norwood, Ohio.

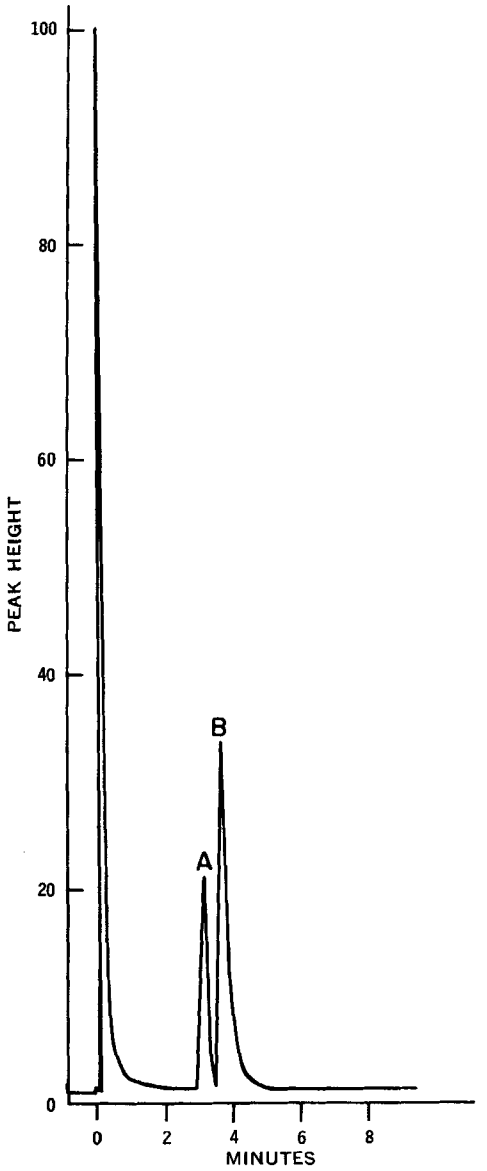


Figure 1—*Gas chromatogram of: (A) dimethyldiphenylhydantoin and (B) monomethyldiphenylhydantoin. The curve represents 5 μl. of a solution of 40 mcg. diphenylhydantoin products in 100 μl. chloroform.*

sure. The resulting mixture was extracted three times with 150-ml. portions of heptane and once with chloroform (100 ml.). The organic layers were mixed and dried over sodium sulfate, and the solvents were removed under reduced pressure to give a white solid material. This material was recrystallized from benzene, m.p. 223°.

Anal.—Calc. for $C_{16}H_{14}N_2O_2$: C, 72.18; H, 5.26; N, 10.52. Found: C, 72.29; H, 5.29; N, 10.62.

The yield was 95% of the monomethyl derivative. GLC of this material, using the DC-200 column, showed the presence of one major component.

Dimethyl Derivative—The procedure described above was used to methylate diphenylhydantoin, except sodium hydroxide was utilized instead of potassium carbonate in concentration ranging between 2.5 and 10%. The procedure gave a mixture of two components, as shown by the presence of two spots on silica gel TLC. Attempts to separate the two components on a silica gel column were not successful. The gas chromatogram of this product, using the DC-200 column, showed the presence of two major peaks (Fig. 1).

Mass Spectra—The high-resolution mass spectra of diphenylhydantoin and its methyl derivatives were determined using an A.E.I. MS-9 double-focusing mass spectrometer. The MS-9 was

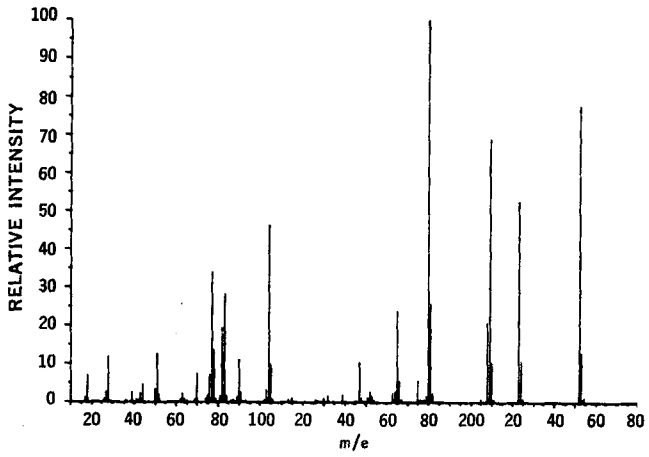


Figure 2—*Mass spectrum of diphenylhydantoin.*

coupled to a digital data system[3]. Computer processing of the digitalized data was carried out on Battelle's CDC-6400 computer. Diphenylhydantoin was introduced by a direct probe operating at 220°, while the methyl derivatives were introduced through a gas chromatograph connected to the MS-9. These derivatives were chromatographed using 0.61-cm. (0.125-in.) stainless steel tubing [3.05 m. (10 ft.) long] packed with 2% OV-17 on Gas Chrom Q. The gas chromatograph (Varian Aerograph model 1740) was connected to the mass spectrometer by means of a McCloskey-type separator (9).

Each component emerging from the column of the gas chromatograph goes directly to the ion source of the mass spectrometer where it is ionized by electron impact. Helium was used as the carrier gas at a rate of 30 ml./min. with the column temperature programmed from 210 to 280 at 2°/min. All of the mass spectra were obtained at 70 e.v. Other operating parameters were: injection port, 210°; ion source, 200°; separator temperature, 250°; and accelerating voltage, 8 kv.

Gas Chromatography—An F&M model 5755 B gas chromatograph, equipped with a flame-ionization detector, was used for routine analyses. The column was 1.4-m., 0.61-cm. (4.5-ft., 0.125-in.) stainless steel tubing packed with Gas Chrom Q 80–100 mesh coated with 3% DC-200. The operating temperatures used for analysis were: column, 200°; and injection port, 310°. Helium was used as the carrier gas with a flow rate of 40 ml./min.

Extraction Procedure—*Standards*—A stock solution of 5 mg. diphenylhydantoin in 10 ml. chloroform was prepared. Aliquots containing 0, 2.5, 5, 10, 20, 50, 80, and 100 mcg. were placed in separate glass-stoppered centrifuge tubes, and solvent was removed

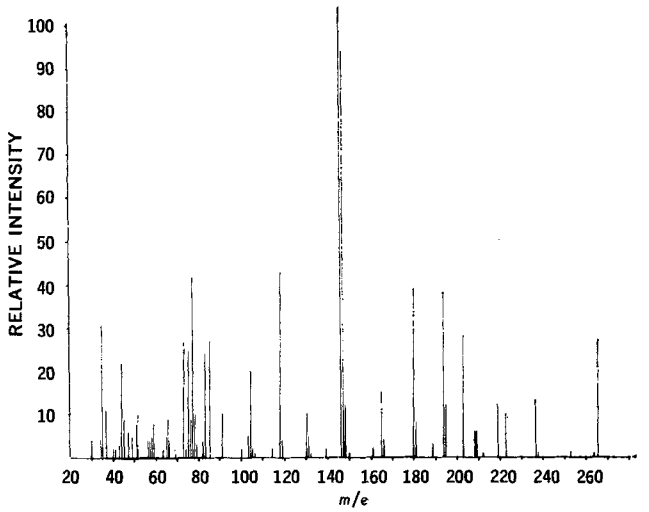


Figure 3—*Mass spectrum of monomethyldiphenylhydantoin.*

[3] Designed and built at Battelle.

Table I—High-Resolution Mass Measurement of Diphenylhydantoin

Measured Mass	Relative Intensity	Calculated Mass	Composition
103.0433	3.24	103.0422	C_7H_5N
104.0513	46.67	104.0500	C_7H_6N
104.5423	3.59	209.0814	Peak doubly ionized
105.0346	2.09	105.0340	C_7H_5O
163.0542	2.42	163.0548	$C_{13}H_7$
165.0678	24.03	165.0664	$C_{13}H_9$
180.0823	100.00	180.0813	$C_{13}H_{10}N$
209.0814	69.31	209.0841	$C_{14}H_{11}NO$
252.0922	78.00	252.0939	$C_{15}N_{12}N_2O_2$ (M^+)

under a stream of air. Each residue was dissolved in 1 ml. of methanolic potassium carbonate followed by 0.1 ml. of dimethyl sulfate. The mixtures were then heated for 5 min. at 70° on a water bath followed by removal of methanol at the same temperature by applying a stream of air. Each residue was extracted with 10 ml. of heptane by shaking on a vortex mixer. Eight milliliters of the heptane layer was taken to dryness, and each residue was dissolved in 100 μl. of chloroform. Samples (1–5 μl.) were injected onto the column under the conditions mentioned previously.

Biological System—From Plasma—A stock solution of 5 mg. diphenylhydantoin in 10 ml. of 5% sodium hydroxide was prepared. The sodium salt of diphenylhydantoin was added to plasma samples in varying amounts (0–40 mcg./ml. plasma). Two milliliters of the resulting mixtures was acidified with 1 ml. of 1 *N* HCl followed by extraction with 10 ml. of chloroform and shaking for 2 min. Layers were separated by centrifugation, and 5–8 ml. of the chloroform solution was taken to dryness. The residues were treated with dimethyl sulfate in the presence of base and chromatographed as mentioned previously.

Blood samples from patients taking the drug were treated similarly, using 1–2 ml. of plasma.

Biological System—From Tissues—Liver samples (0.5–2.0 g.) were weighed and placed in a graduated cylinder; water was added to give a final volume 10-fold greater than the weight of the tissue. The content of the cylinder was transferred to a blending tube and homogenized for 2 min. A 5–10-ml. portion was then transferred to a glass-stoppered centrifuge tube and acidified with 5 ml. of 1 *N* HCl. The mixture was extracted by shaking with 25 ml. of chloroform for 5 min. Layers were separated by centrifugation, and the water layer was aspirated. Chloroform was removed under a stream of air, and the residue was treated with dimethyl sulfate and analyzed using the procedure described previously.

RESULTS AND DISCUSSION

Methylation—The product obtained from the methylation of diphenylhydantoin was dependent on the strength and type of the base used in the reaction. Lower concentrations of methanolic potassium carbonate (2–5%) gave solely the monomethyl derivative of the drug. The gas chromatogram obtained on this product showed one major peak. Microanalysis of this product showed that it was the monomethyl derivative. When higher concentrations of potassium carbonate were used, two products were obtained,

Table II—High-Resolution Mass Measurement of 3-Methyldiphenylhydantoin

Measured Mass	Relative Intensity	Calculated Mass	Composition
103.0417	4.50	103.0422	C_7H_5N
104.0500	19.70	104.0500	C_7H_6N
105.0338	1.65	105.0340	C_7H_5O
146.0591	100.00	—	Background
147.0668	89.95	147.0684	C_9H_9NO
165.0770	14.75	165.0704	$C_{13}H_9$
180.0786	39.17	180.0813	$C_{13}H_{10}N$
208.0722	6.04	208.0762	$C_{14}H_{10}NO$
209.0784	6.00	—	$C_{14}H_{11}NO$
266.0674	31.00	266.0653	$C_{16}H_{14}N_2O_2$ (M^+)

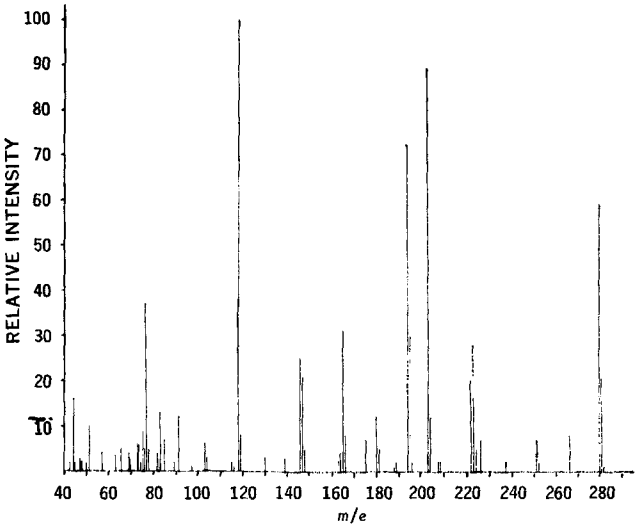


Figure 4—*Mass spectrum of dimethyldiphenylhydantoin.*

with the monomethyl derivative predominant. The use of sodium hydroxide also produced two products as indicated by the presence of two peaks in the chromatogram obtained on this mixture. The first product was predominant and had a shorter retention time (peak A, Fig. 1) relative to the second product (peak B). This second product was identical to that obtained with lower concentrations of potassium carbonate; both were the monomethyl derivative of diphenylhydantoin.

The product that corresponded to peak A (Fig. 1) was unstable if kept in organic solvents, such as chloroform, and decomposed to the methyl derivative corresponding to peak B. This was indicated by a change in peak intensity of Components A and B when the chromatograms of the same solution were obtained on separate days. This may indicate that Component A undergoes decomposition in solution to Component B. For this reason, methylation of diphenylhydantoin in the presence of potassium carbonate for routine GLC purposes is preferred over sodium hydroxide. However, for the determination of diphenylhydantoin metabolites, mainly the *p*-hydroxy derivative, sodium hydroxide is preferred over potassium carbonate for methylation. This is due to the presence of the phenolic hydroxyl group which was also methylated with dimethyl sulfate in the presence of sodium hydroxide but not potassium carbonate.

Mass Spectra of Methyl Derivatives—Samples of the mixture of methylated diphenylhydantoin were studied by the combination of GLC–mass spectroscopy. The two derivatives were separated by GLC, using OV-17 as the stationary phase, and were identified by mass spectroscopy.

The molecular ion (M^+) corresponding to peak A (Fig. 1) was at *m/e* 280 (60%). This molecular ion corresponds to the formula

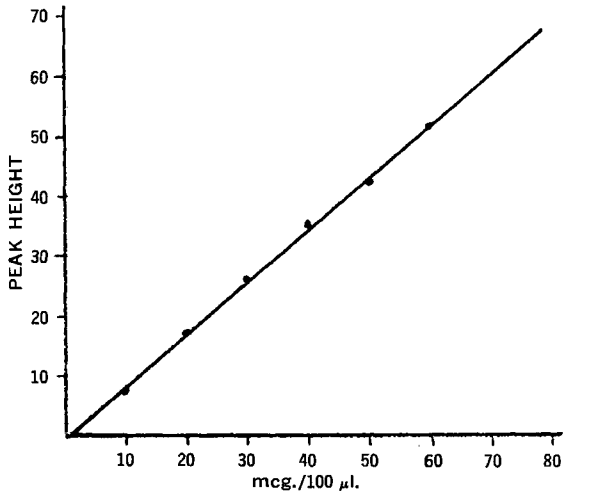


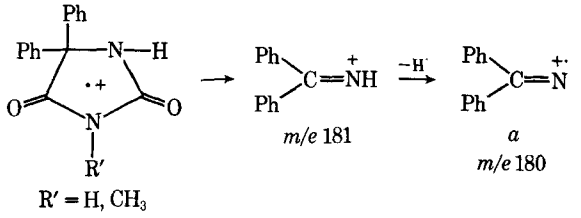
Figure 5—*Relationship between amount of monomethyldiphenylhydantoin injected and peak height. Five microliters injected in each case.*

Table III—High-Resolution Mass Measurement of 1,3-Dimethyldiphenylhydantoin

Measured Mass	Relative Intensity	Calculated Mass	Composition
103.0418	3.90	103.0422	C_7H_5N
104.0512	3.20	104.0500	C_7H_6N
105.0365	0.50	105.0340	C_7H_5O
118.0660	100.00	118.0657	C_8H_8N
165.0684	31.15	165.0704	$C_{13}H_9$
180.0834	12.05	180.0813	$C_{13}H_{10}N$
194.0940	72.94	194.0970	$C_{14}H_{12}N$
209.0777	1.68	—	$C_{14}H_{11}NO$
223.1006	27.58	223.0997	$C_{15}H_{13}NO$
280.1180	60.00	280.1212	$C_{17}H_{16}N_2O_2$ (M^+)

$C_{17}H_{16}N_2O_2$, which indicates the addition of two methyl groups to diphenylhydantoin. The molecular ion coresponding to peak B (Fig. 1) was at *m/e* 266 (27%) which corresponds to the formula $C_{16}H_{14}N_2O_2$. This formula indicates the addition of one methyl group to diphenylhydantoin.

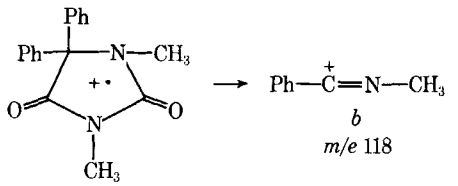
The mass spectra of diphenylhydantoin (Fig. 2) and of the monomethyl derivative (Fig. 3) show a very intense peak at *m/e* 180 ($C_{13}H_{10}N$), but it is very weak in the mass spectrum of the dimethyl derivative (Fig. 4). This peak may correspond to ion *a*. The formation of ion *a* upon electron impact of diphenylhydantoin monomethyl derivative may indicate that the methyl group may be attached to N-3 of the hydantoin ring (Scheme I) and that N-1 has no



Scheme I

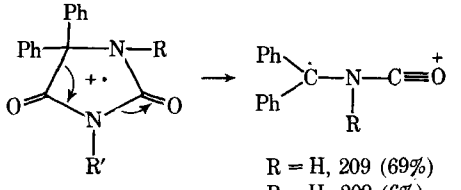
substituent as is the case with diphenylhydantoin itself. Similar results were reported by Grimmer *et al.* (10).

The presence of a very intense peak at *m/e* 118, C_8H_8N in the mass spectrum of the dimethyl derivative (Fig. 4), probably corresponds to ion *b* (Scheme II). Therefore, the additional methyl group



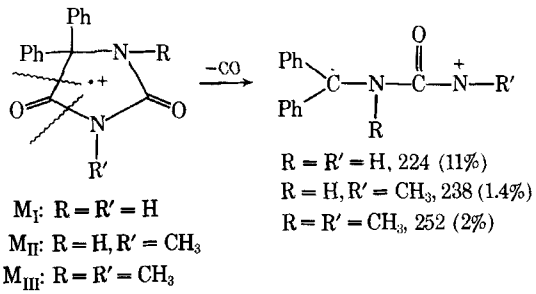
Scheme II

present in the compound corresponding to peak A is undoubtedly located on the nitrogen adjacent to the carbon carrying the two phenyl groups. Thus, it appears that the methyl groups were introduced at the nitrogen atoms of the hydantoin ring to form 3-methyl- and 1,3-dimethyldiphenylhydantoin. These two compounds have some common fragmentation patterns upon electron impact. Both compounds undergo loss of the C_2H_3NO fragment to give peaks at *m/e* 209 and 223, respectively. The most probable structure that corresponds to these ions is shown in Scheme III.



Scheme III

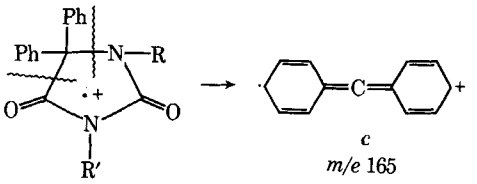
Both derivatives exhibit M—CO and M—HCO similar to that of diphenylhydantoin. Since there are two carbonyl groups in the hydantoin ring, either one could be lost upon electron bombardment. Thus, more than one possible structure could be drawn for such ions. However, loss of the carbonyl group at position 4 would give rise to a radical at C-5, which is highly stabilized by the two phenyl rings at that position. Thus, the possible structures of M—CO of the three compounds is shown in Scheme IV.



Scheme IV

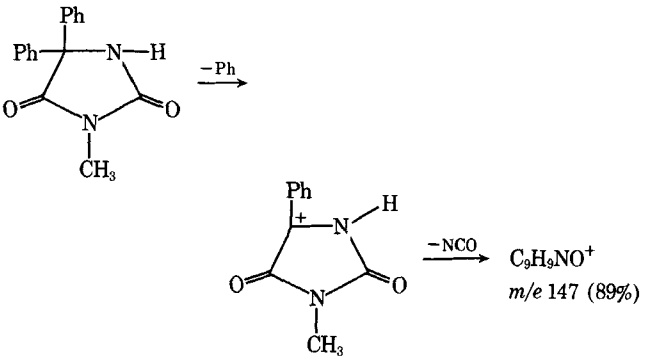
The mass spectra of diphenylhydantoin and its methylated derivatives show a peak at *m/e* 105, which corresponds to C_7H_5O, which may correspond to benzoyl ion (Ph—C≡O$^+$). This ion may originate only if one of the two phenyl groups migrates to the adjacent carbonyl group to produce a benzoyl urea derivative which may give rise to the benzoyl ion. However, the intensity of the peak at *m/e* 105 is relatively weak. Skeletal rearrangements of this type were reported for several other compounds (11–16).

The mass spectra of diphenylhydantoin and its methyl derivatives show a peak at *m/e* 165 ($C_{13}H_9$). This peak may correspond to ion *c*. The possible origin of this ion is shown in Scheme V.



Scheme V

The mass spectrum of 3-methyldiphenylhydantoin shows an intense peak at *m/e* 147 (89%). The possible origin of this peak is shown in Scheme VI.

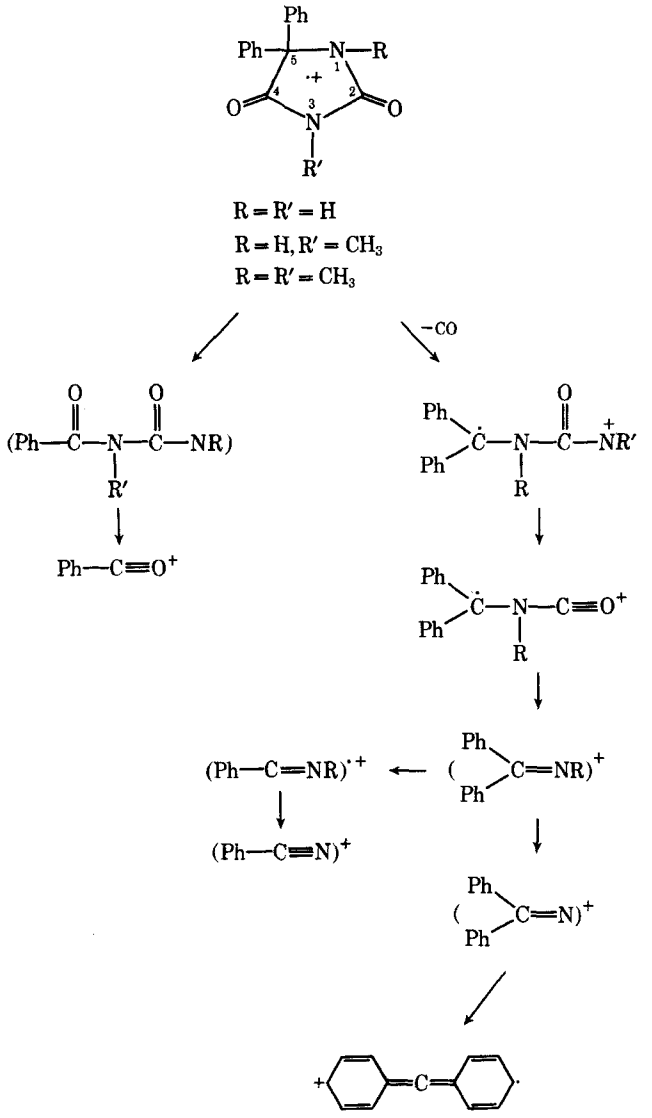


Scheme VI

The possible overall common fragmentation pattern of diphenylhydantoin and its methyl derivatives is shown in Scheme VII.

GLC—For routine work, methanolic potassium carbonate was utilized to methylate diphenylhydantoin. This procedure gave the monomethyl derivative in yields ranging from 90 to 100%. Analysis of the methyl derivative was carried out on the DC-200 column. Figure 1 shows a typical chromatogram of the monomethyl and dimethyl derivatives of diphenylhydantoin.

The methylation procedure rendered the GLC method more sensitive over the published method (7). Subtherapeutic levels were easily detected using the procedure mentioned here.



Scheme VII

The method is applicable to determine diphenylhydantoin in biological fluids, such as blood, and is also applicable to tissues such as liver.

The extraction procedure of diphenylhydantoin from plasma and tissues was adequate and gave maximum recovery, as reported earlier (7). A plot of peak height against the amount of the drug injected (0.1–1.0 mcg.) gave a straight line passing through the origin (Fig. 5), indicating that the method is suitable for quantitative analysis.

Other commonly used drugs, *e.g.*, barbiturates and glutethemide, showed no interfering peaks in the same region of diphenylhydantoin. These drugs can also be methylated and analyzed using the procedure described in this article.

CONCLUSIONS

Methylation of diphenylhydantoin with dimethyl sulfate in the presence of a base produces monomethyl and dimethyl derivatives of the drug, depending on the reaction conditions. The structure of the derivatives was identified by high-resolution mass spectrometry and was found to be *N*(3)-methyl- and *N,N'*-dimethyldiphenylhydantoin. Similar results were obtained when other methylating agents, such as diazomethane, were used (9).

These derivatives, together with diphenylhydantoin itself, undergo mainly ring cleavage upon electron impact, accompanied by loss of groups such as CO, HCO, RNCO, or phenyl groups. Skeletal rearrangements were also observed.

The GLC method described is very sensitive and can be used for clinical and toxicological studies of diphenylhydantoin.

REFERENCES

(1) O. Svensmark and P. Kristensen, *J. Lab. Clin. Med.*, **61,** 501(1963).

(2) J. Wallace, J. Biggs, and E. V. Dahl, *Anal. Chem.*, **37,** 410(1965).

(3) J. Huisman, *Clin. Chim. Acta*, **13,** 323(1966).

(4) W. A. Dill, A. Kazenko, L. M. Wolf, and A. J. Glazko, *J. Pharmacol. Exp. Ther.*, **118,** 270(1956).

(5) J. Wallace, *Anal. Chem.*, **40,** 978(1968).

(6) D. J. Sandberg, G. L. Resnick, and Z. Bacallo, *ibid.*, **40,** 736(1968).

(7) K. Sabih and K. Sabih, *ibid.*, **41,** 1452(1969).

(8) J. MacGee, *ibid.*, **42,** 421(1970).

(9) P. M. Krueger and J. A. McCloskey, *ibid.*, **41,** 1930(1969).

(10) V. G. Grimmer, J. Jacob, and H. Schafer, *Arzneim.-Forsch.*, **19,** 1287(1969).

(11) S. Meyerson, H. Drews, and K. Fields, *Anal. Chem.*, **36,** 1294(1964).

(12) J. B. Thomson, P. Brown, and C. Djerassi, *J. Amer. Chem. Soc.*, **88,** 4049(1966).

(13) E. Dynesen, S. O. Lawesson, G. Schroll, J. H. Bowie, and R. G. Cooks, *J. Chem. Soc. B*, **1968,** 15.

(14) M. F. Grostic, R. J. Wnuk, and F. A. MacKellar, *J. Amer. Chem. Soc.*, **88,** 4664(1966).

(15) E. K. Fields and S. Meyerson, *ibid.*, **88,** 2836(1966).

(16) J. H. Bowie, B. K. Simmons, and S. O. Lawesson, *Rev. Pure Appl. Chem.*, **19,** 61(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 25, 1970, from the *Department of Pharmacology, University of Kansas Medical Center, Kansas City, KS 66103*

Accepted for publication March 12, 1971.

The microanalysis was supplied by the Medicinal Chemistry Department, University of Kansas. This work was supported by funds from Public Health Service Grant GM15956 and the National Institutes of Health, Division of Research and Resources, Contract No. NIH-69-2226. The high-resolution mass spectra were furnished by Battelle Memorial Institute, Columbus, Ohio. The authors thank Dr. Rodger L. Foltz of the Battelle Memorial Institute for his valuable discussion and assistance in preparing the mass spectra.

* Present address: College of Pharmacy, University of Baghdad, Baghdad, Iraq.