Identification of Urinary Catechol and Methylated Catechol Metabolites of Phenytoin in Humans, Monkeys, and Dogs by GLC and GLC–Mass Spectrometry

K. K. MIDHA *x, K. W. HINDMARSH ‡, I. J. McGILVERAY *, and J. K. COOPER *

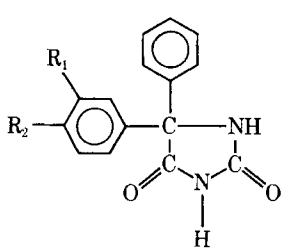
Abstract □ A catechol metabolite, 5-(3,4-dihydroxyphenyl)-5-phenylhydantoin, and a methylated catechol metabolite, 5-(3-methoxy-4-hydroxyphenyl)-5-phenylhydantoin, were identified as urinary metabolites in humans, monkeys, and dogs following the administration of phenytoin. These metabolites were separated from each other and from other known metabolites of phenytoin as *n*-butyl derivatives by GLC and positively identified by combined GLC–mass spectrometry.

Keyphrases □ Phenytoin metabolites—GLC and GLC–mass spectrometric analyses and identification, human, monkey, and dog urine □ Catechol and methylcatechol metabolites—of phenytoin, GLC and GLC–mass spectrometric analyses and identification, human, monkey, and dog urine □ GLC and GLC–mass spectrometry—analyses and identification of phenytoin metabolites in human, monkey, and dog urine □ Anticonvulsants—phenytoin, GLC and GLC–mass spectrometric analyses and identification of catechol and methylcatechol metabolites in human, monkey, and dog urine

Phenytoin (I) is a commonly prescribed anticonvulsant (1). The metabolism of I was reviewed recently (2–4). The presence of a catechol metabolite, 5-(3,4-dihydroxyphenyl)-5-phenylhydantoin (II), in rat urine was suggested (5) and later confirmed (6). This metabolite and the methylated catechol 5-(3-methoxy-4-hydroxyphenyl)-5-phenylhydantoin (III) (7) also were identified in the bile from isolated perfused rat liver (8).

Borga *et al.* (9) reported the identification of II in the urine of both rats and humans. However, an on-column methylation technique was used and, under these conditions, II and III would be converted to the same tetramethyl derivative 1,3-dimethyl-5-(3,4-dimethoxyphenyl)-5-phenylhydantoin. Therefore, the structure of the metabolite is tentative and may be II as suggested, III, or a mixture of both.

This report describes the positive identification of these two metabolites (II and III) in the urine of humans, monkeys, and dogs. These metabolites were separated from each other and from other known metabolites of I as their respective *n*-butyl derivatives by GLC and identified by combined GLC–mass spectrometry.



I: $R_1 = R_2 = H$
II: $R_1 = R_2 = OH$
III: $R_1 = OCH_3$, $R_2 = OH$
IV: $R_1 = H$, $R_2 = OH$
V: $R_1 = OH$, $R_2 = H$

EXPERIMENTAL

Ether[1] and methylene chloride[2] were distilled in glass prior to use. The hydrolytic enzyme used was β-glucuronidase[3]. Methanolic trimethylanilinium hydroxide (0.4 *M*) was synthesized according to the method of Barret (10). Tetraethylammonium hydroxide (0.62 *M*) and tetra-*n*-butylammonium hydroxide (0.4 *M*) were synthesized from their respective substituted ammonium iodides and silver oxide following the procedure described by Barret (10).

A gas chromatograph[4] equipped with a metal sleeve injection port and flame-ionization detector was employed. The column was coiled glass tubing[5], 1.8 m × 2.0 mm i.d., packed with 5% methyl phenyl silicone containing 75% phenyl silicone[6] coated on acid-washed, dimethyldichlorosilane-treated, 100–120-mesh, high-performance flux-calcined diatomite support[7]. The column was conditioned at 290° for 18 hr with a low nitrogen flow.

The operating temperatures were: injection port, 350°; column, 260° (for methyl and ethyl derivatives) and 280° (for butyl derivatives); and detector, 310°. The flow rate of the carrier gas (nitrogen) was 65 ml/min. Hydrogen and compressed air flow rates were adjusted to give maximum response.

GLC–Mass Spectrometry—GLC–mass spectrometry was carried out on a gas chromatograph[8] attached through a jet separator interface to a single-focusing mass spectrometer[9]. The ionization potential was 70 ev. The GLC column and conditions were the same as for direct GLC.

Human Studies—A 300-mg dose of phenytoin (3 × 100-mg capsules[10]) was administered orally to a healthy 80-kg male volunteer. All urine was collected for the first 72 hr and either used immediately or stored at −15° for later analysis. Urine samples were also collected from a 60-kg patient receiving 300 mg of phenytoin/day (3 × 100-mg capsules[10]).

Monkey Studies—A 125-mg dose of phenytoin suspension[11] (5 ml) was administered orally (30 mg/kg) to two healthy male Rhesus monkeys (*Macaca mulatta*), 4.74 and 3.76 kg, by stomach intubation. The animals were placed in metabolism cages, and total urine was collected for the 0–24- and 24–48-hr periods. The urine was stored at −15° prior to extraction.

Dog Studies—Phenytoin sodium was administered orally in a gelatin capsule to each of two healthy male Labrador dogs, 37.3 and 27.3 kg, at 20 mg/kg. The dogs were placed in metabolism cages, and urine collections were taken as described for the monkey studies.

Extraction of Urine—Aliquots of accumulated urine from humans, monkeys, or dogs (300 ml), after adjustment to pH 5.0 with 0.2 *M* sodium acetate–acetic acid buffer, were hydrolyzed at 37° for 20 hr following the addition of 1 ml of β-glucuronidase. The hydrolyzed urine samples were then extracted on a urine extraction apparatus[12] with 500 ml of methylene chloride–ether (11:14) for 24 hr. Extracts were concentrated under vacuum on a rotary evaporator[13] to approximately 20 ml using a 40° water bath temperature.

Each concentrated extract was divided into two equal portions in polytef-lined screw-capped tubes (15 ml) and evaporated[14] to dryness

[1] Mallinckrodt Chemical Works Ltd., Montreal, Quebec, Canada.
[2] Caledon Laboratories Ltd., Georgetown, Ontario, Canada.
[3] No. G-0876, Sigma Chemical Co., St. Louis, Mo.
[4] Model 3920, Perkin-Elmer, Montreal, Quebec, Canada.
[5] Chromatographic Specialities, Brockville, Ontario, Canada.
[6] OV-25, Chromatographic Specialities, Brockville, Ontario, Canada.
[7] Chromosorb W, Chromatographic Specialities, Brockville, Ontario, Canada.
[8] Model 990, Perkin-Elmer, Montreal, Quebec, Canada.
[9] Model RMU-6L, Hitachi Perkin-Elmer.
[10] Dilantin Kapseals, 100 mg, Parke, Davis and Co., Montreal, Quebec, Canada.
[11] Dilantin-125 suspension, Parke, Davis and Co., Montreal, Quebec, Canada.
[12] Kontes Glass Co., Vineland, N.J.
[13] Buchler Instruments, Fort Lee, N.J.
[14] Thermolyne Dri-Bath, Fisher Scientific Co., Ottawa, Ontario, Canada.

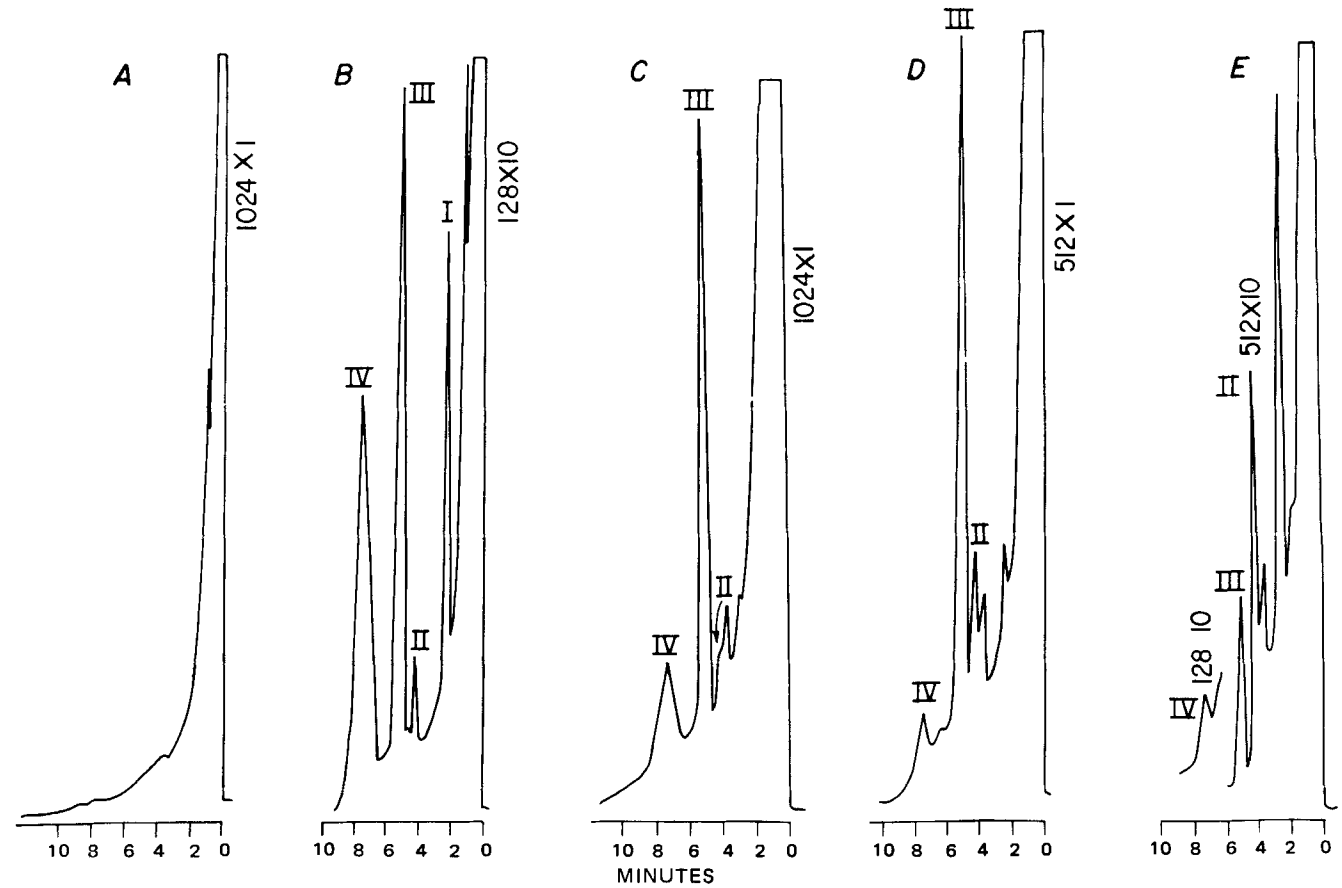


Figure 1—*Gas chromatograms of flash-heater methylated extracts of urine. Key: A, blank human urine; B, blank human urine spiked with I–V; C, urine from a patient receiving I; D, urine from a monkey dosed with I; E, urine from a dog dosed with I; peak I, methylated I; peak II, methylated V; and peak III, methylated IV.*

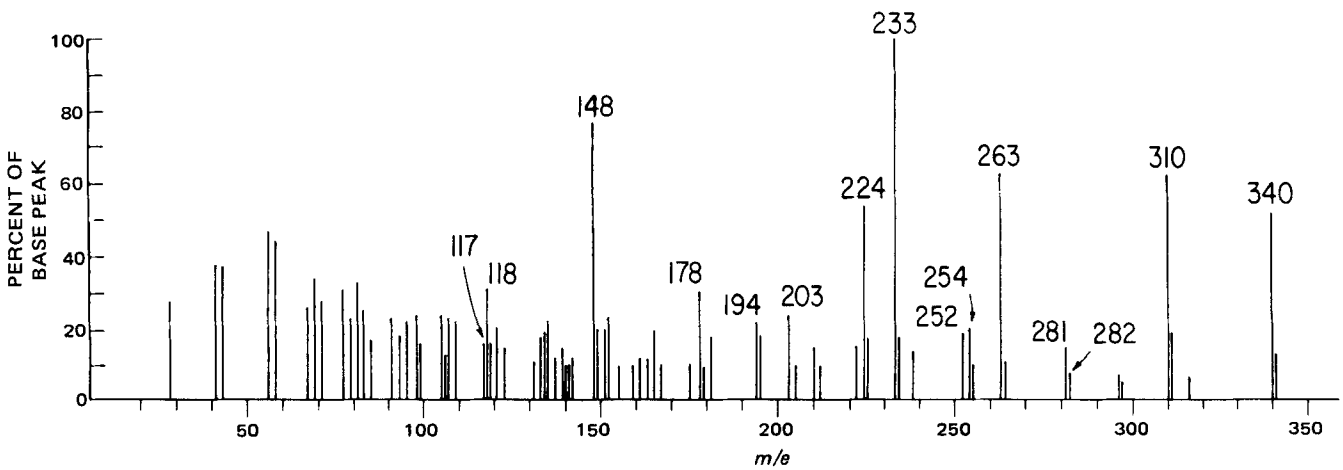


Figure 2—*Normalized electron-impact mass spectrum of the compound giving rise to peak IV (Fig. 1).*

under dry filtered nitrogen. Trimethylanilinium hydroxide (1 ml) was added to the first tube, and tetra-*n*-butylammonium hydroxide (1 ml) was added to the second tube. The contents were dissolved by swirling on a mixer[15] before injecting 1–2 μl into the gas chromatograph.

Aliquots of urine (300 ml) were also hydrolyzed with concentrated hydrochloric acid (300 ml) in a boiling water bath for 1 hr. The acid-hydrolyzed urine was adjusted to pH 5 with 6 *N* NaOH and then treated in a similar manner to that described for enzyme-hydrolyzed urine. Aliquots of urine (100 ml) were extracted similarly at pH 5.0 without prior enzymatic or acidic hydrolysis.

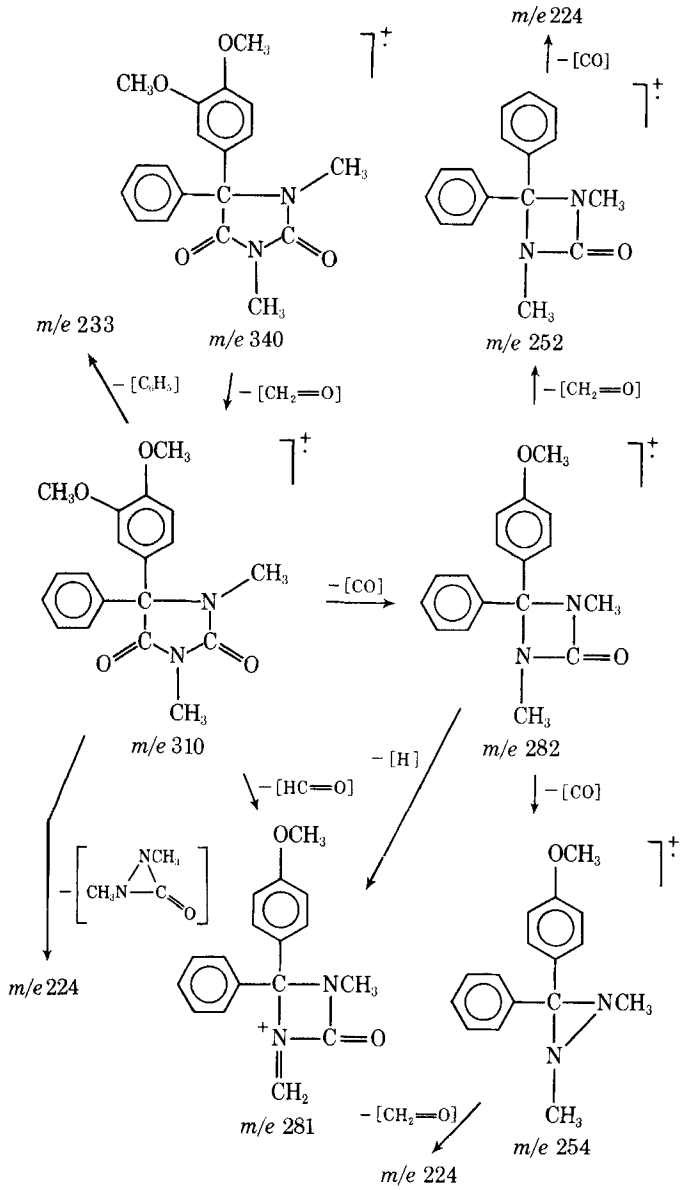
Control urine specimens (200 ml) collected prior to phenytoin administration were hydrolyzed with acid and β-glucuronidase and treated in parallel to the conditions described.

[15] Vortex Genie, Fisher Scientific Co., Montreal, Quebec, Canada.

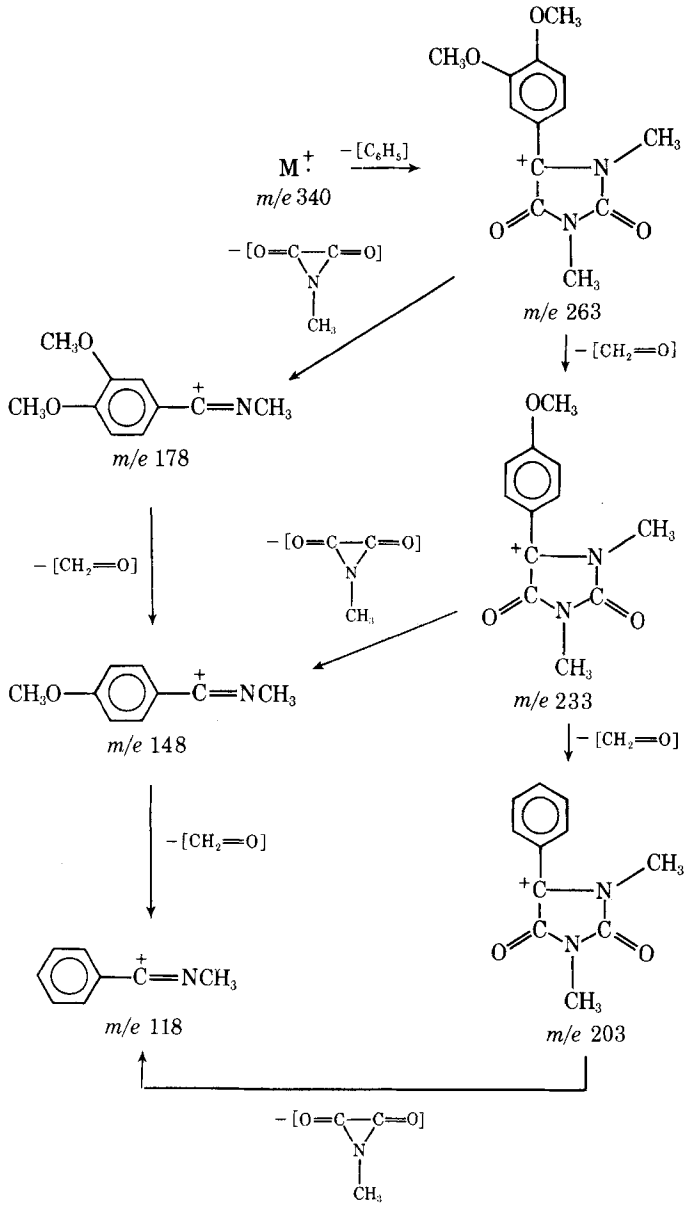
RESULTS AND DISCUSSION

Flash-heater methylation, with trimethylanilinium hydroxide, of enzymatically hydrolyzed human urine, obtained following phenytoin administration, showed three peaks on GLC (Fig. 1C) that were not present in the control urine (Fig. 1A). Previously (11, 12), phenytoin (I) and its metabolite 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (IV) were separated from each other using this methylation technique.

Peak II (Fig. 1C) had the same GLC retention time (4 min) as the methylated synthetic sample of 5-(*m*-hydroxyphenyl)-5-phenylhydantoin (V, Fig. 1B, peak II). Peak III (Fig. 1C) had the same retention time (4.8 min) as the methylated product of synthetic IV (Fig. 1B, peak III). GLC–mass spectrometric analysis of these two peaks indicated that they were the trimethylated derivatives with the structures 1,3-dimethyl-5-(*m*-methoxyphenyl)-5-phenylhydantoin and 1,3-dimethyl-5-(*p*-



Scheme I



Scheme II

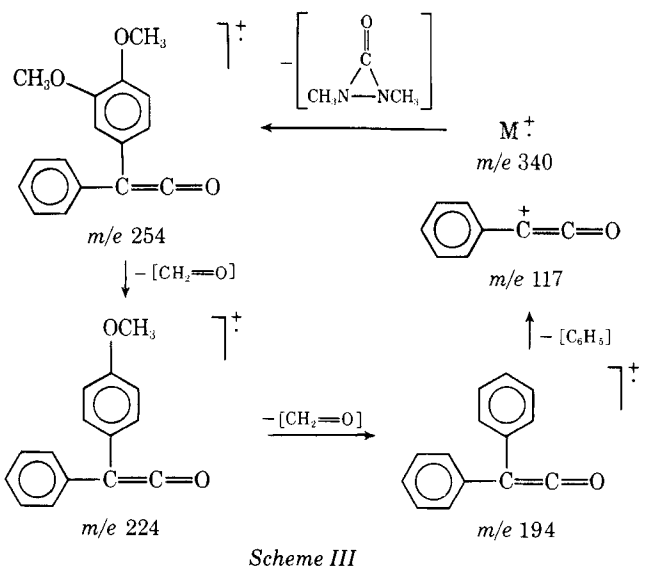
methoxyphenyl)-5-phenylhydantoin. A molecular ion at *m/e* 310 and characteristic ions at *m/e* 280, 233, 224, 148, and 118 are in agreement with two trimethylated structures reported earlier (11–13).

Peak IV (Fig. 1C, R_t 7.2 min) had the same retention time as the flash-heater methylated product of synthetic II and III (Fig. 1B, peak IV). GLC–mass spectrometric analysis of this peak indicated that it was a tetramethylated derivative, 1,3-dimethyl-5-(3,4-dimethoxyphenyl)-5-phenylhydantoin. The normalized spectrum (Fig. 2) reveals the presence of a molecular ion at *m/e* 340 and other characteristic ions at *m/e* 310, 282, 281, 263, 254, 252, 233, 224, 203, 194, 178, 148, 118, and 117. Formation of these ions is postulated in Schemes I–III.

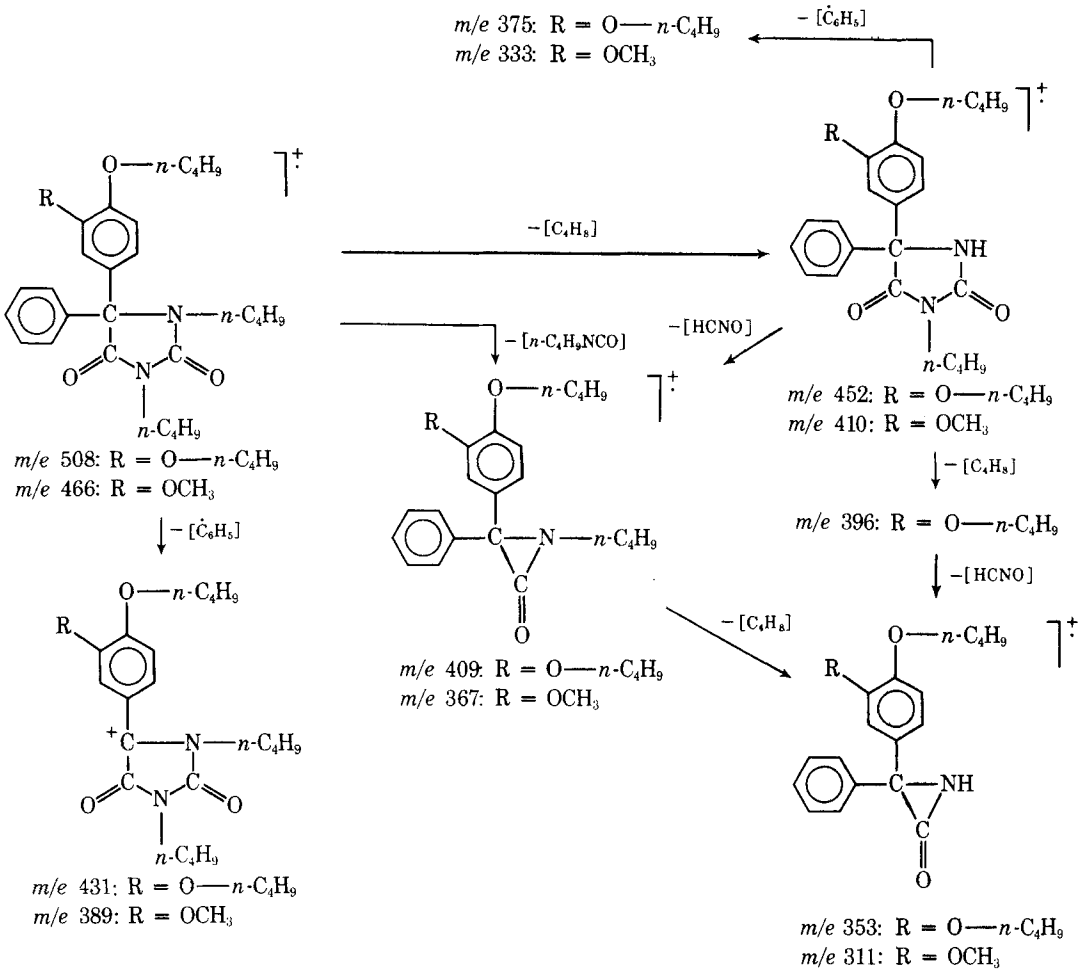
Scheme I depicts the formation of the diagnostic ions *m/e* 310, 282, 281, 254, 252, 233, and 224. Scheme II indicates that the ion at *m/e* 263 is formed by the loss of a phenyl radical from the molecular ion. The formation of this ion suggests that the two methoxy groups are on the same benzene ring. Formation of the ion at *m/e* 178 (Scheme II) appears to be the result of cleavage of the imidazolidine ring of the ion at *m/e* 263. Other characteristic ions at *m/e* 233, 203, 148, and 118 are rationalized in Scheme II.

An alternative pathway for the formation of ions at *m/e* 254 and 224 is shown in Scheme III. The ion at *m/e* 224 in Scheme III further fragments, by the loss of formaldehyde, to give an ion at *m/e* 194 which, in turn, loses a phenyl radical to give the ion at *m/e* 117.

The GLC analysis of the enzyme-hydrolyzed extracts of monkey and dog urine (Figs. 1D and 1E) showed peaks for methylated V and IV and



Scheme III



Scheme IV

the catechol metabolites (Peak IV). In the dog, V was the major metabolite, in agreement with previously reported findings (12).

Similar peaks were obtained on GLC analysis of the acid-hydrolyzed urine of humans, monkeys, and dogs. However, the amounts of the methylated derivatives of V and IV had increased. This increase may be due to the decomposition of the 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin, as observed by Chang and Glazko (4).

To determine whether peak IV (Figs. 1C–1E) was due to II or III or a combination of both, synthetic samples of these metabolites were flash-heater butylated on GLC with tetrabutylammonium hydroxide.

Flash-heater butylation of synthetic II and III, under the experimental conditions described, gave two peaks on GLC in each case. Figure 3B shows the separation of these peaks. Peak V (R_t 7 min) was shown by GLC–mass spectrometric analysis to be the tributyl monomethylated derivative, 1,3-di-*n*-butyl-5-(3-methoxy-4-*n*-butoxyphenyl)-5-phenylhydantoin. Peak VI was due to a dibutyl monomethylated derivative.

It appeared from the mass spectrum that either the N-1 or N-3 position of the imidazolidine ring was not butylated. Peak VII was the tetrabutyl derivative, 1,3-di-*n*-butyl-5-(3,4-di-*n*-butoxyphenyl)-5-phenylhydantoin. Peak VIII was a tributylated derivative of II. Several different conditions, such as increasing the injection port temperature of the chromatograph and adding excess butylating agent, did not result in complete butylation.

Figure 3A shows the chromatogram of the flash-heater butylated urine extract obtained after enzymatic hydrolysis from a patient receiving I. Comparison of GLC retention times and GLC–mass spectra with those of the synthetic butylated metabolites (V, IV, II, and III) indicated that peak I (R_t 4.5 min) was 1,3-di-*n*-butyl-5-(3-*n*-butoxyphenyl)-5-phenylhydantoin. Peak II (R_t 4.9 min) was shown to be the incompletely butylated product of V. Peak III was identified as 1,3-di-*n*-butyl-5-(4-*n*-butoxyphenyl)-5-phenylhydantoin, and peak IV was due to the incomplete butylation of IV.

The shoulder indicated that peaks V and VI had the same retention times as the completely and incompletely butylated derivatives, respectively, of III (Fig. 3B). Peaks V and VI (Fig. 3A) were collected at their respective retention times on a methyl phenyl silicone column[6] from the detector end, after extinguishing the flame and rechromatographing on columns packed with phenyl methyldimethyl silicone[16] and cyanopropylmethyl phenyl methyl silicone[17], respectively. The retention times of the two peaks on these columns corresponded to those of synthetic III when flash-heater butylated. Similarly, the shoulder shown as peaks VII and VIII had retention times corresponding with those of the completely and incompletely butylated derivatives, respectively, of II.

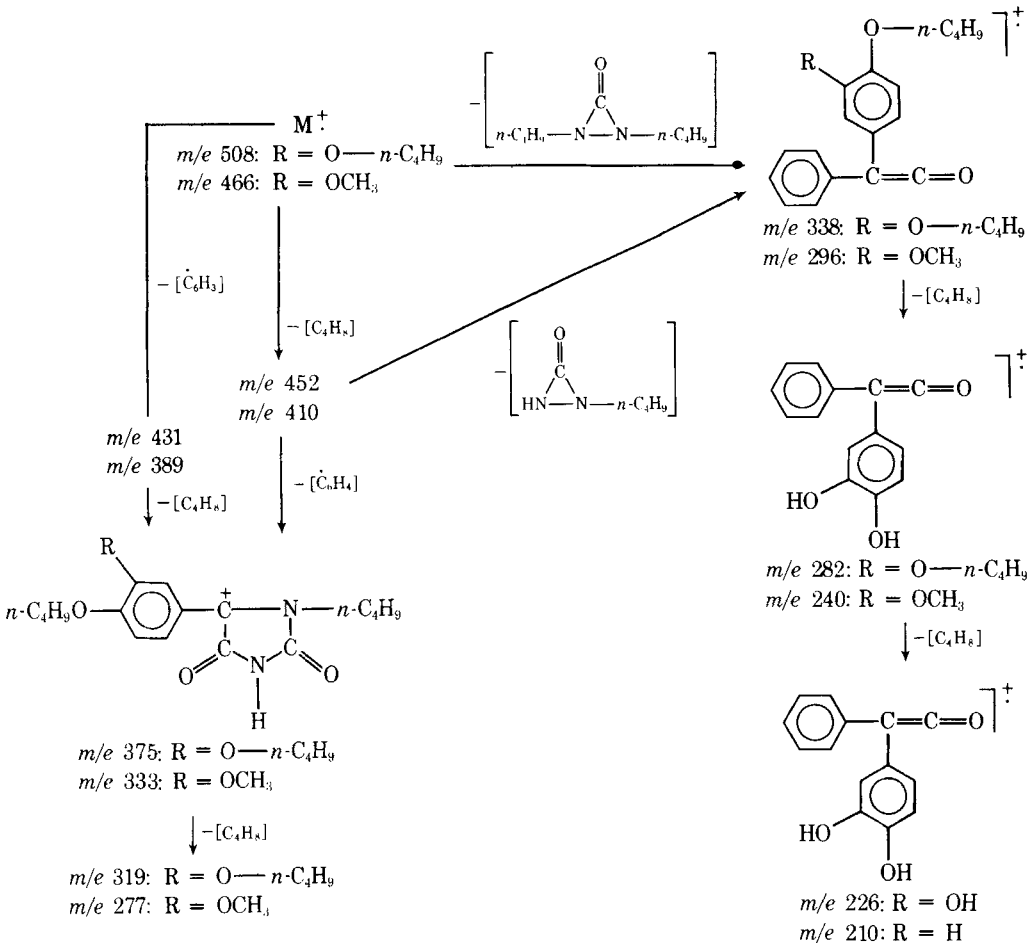
Figures 4 and 5 show the normalized mass spectra of peaks V and VII, respectively (Fig. 3A). The mass spectra of these peaks show molecular ions at *m/e* 466 and 508, respectively. Formation of most of the diagnostic ions for both derivatized metabolites is proposed in Schemes IV–VII.

These mass spectra (Figs. 4 and 5) were virtually identical to the spectra of completely butylated derivatives of synthetic III and II, respectively. The molecular ions for both compounds lose C_4H_8 to give ions at *m/e* 410 and 452. The loss of the C_4H_8 unit can be rationalized either by a McLafferty rearrangement or by a 1,4-shift, as shown for ions at *m/e* 235 and 193 in Scheme VII.

Ions at *m/e* 410 and 452 (Scheme IV) can lose HNCO, as a neutral molecule, to give ions at *m/e* 367 and 409, respectively. The ions at *m/e* 367 and 409 may also be formed from the molecular ions by the loss of C_4H_9NCO as a neutral molecule. The ions at *m/e* 311 and 353 (Scheme IV) are the result of the loss of C_4H_8 from ions at *m/e* 367 and 409, respectively. Alternatively, the ion at *m/e* 452 may lose C_4H_8 to give an ion at *m/e* 396 and then, by the loss of HNCO, may go to *m/e* 353.

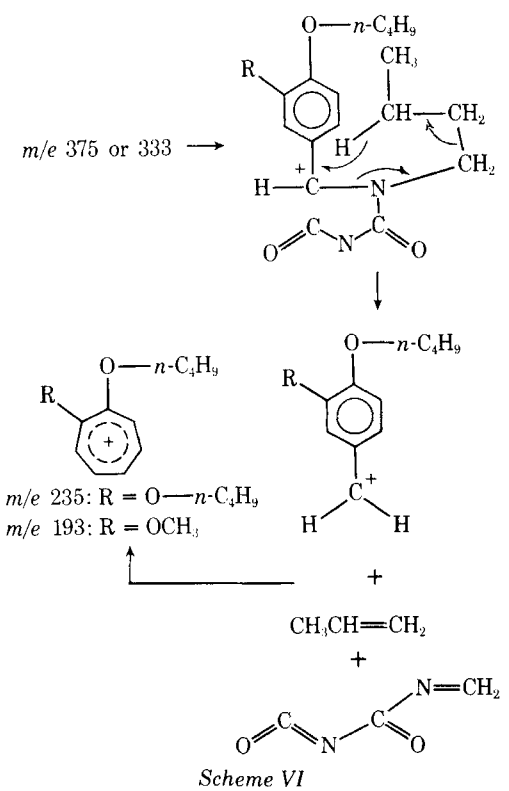
Ions at *m/e* 389 and 431 (Scheme V) can be formed from their respective molecular ions by the loss of a phenyl radical, (*cf.*, ion at *m/e* 263, Scheme II). The occurrence of these and subsequent ions at *m/e* 333, 375, 277, and 319 are indicative of the presence of both substituents on one

[16] OV-7, Chromatographic Specialities, Brockville, Ontario, Canada.
[17] OV-225, Chromatographic Specialities, Brockville, Ontario, Canada.



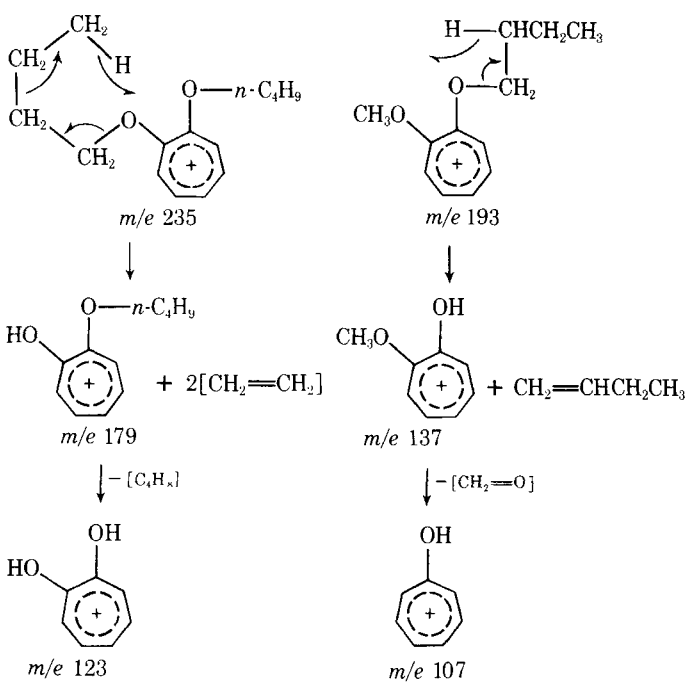
Scheme V

aromatic ring. Ions at *m/e* 333 and 375 seem to give rise to ions at *m/e* 193 and 235, respectively, by a rearrangement. A rationale for this rearrangement is suggested in Scheme VI.



Scheme VI

Scheme VII presents a postulation of the formation of ions at *m/e* 179 and 123 observed in the spectrum of the tetrabutylated catechol metabolite (II). Ions at *m/e* 179 and 123 further demonstrate that the two hydroxyl groups are on the same ring. Similarly, the ion at *m/e* 137 observed in the spectrum of the tributylated derivative of the 3-*O*-methylcatechol metabolite (III) suggests that the methoxy and hydroxy sub-



Scheme VII

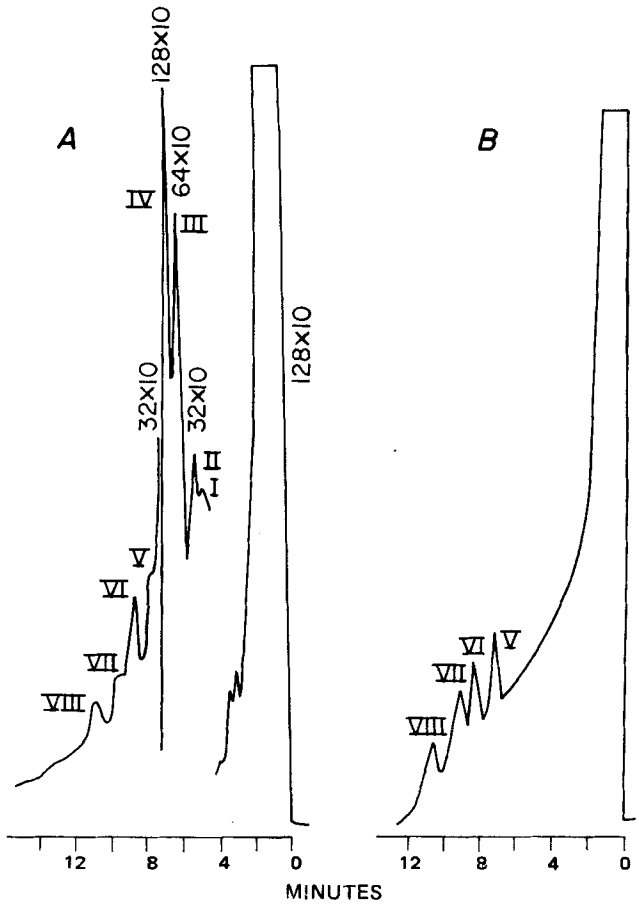
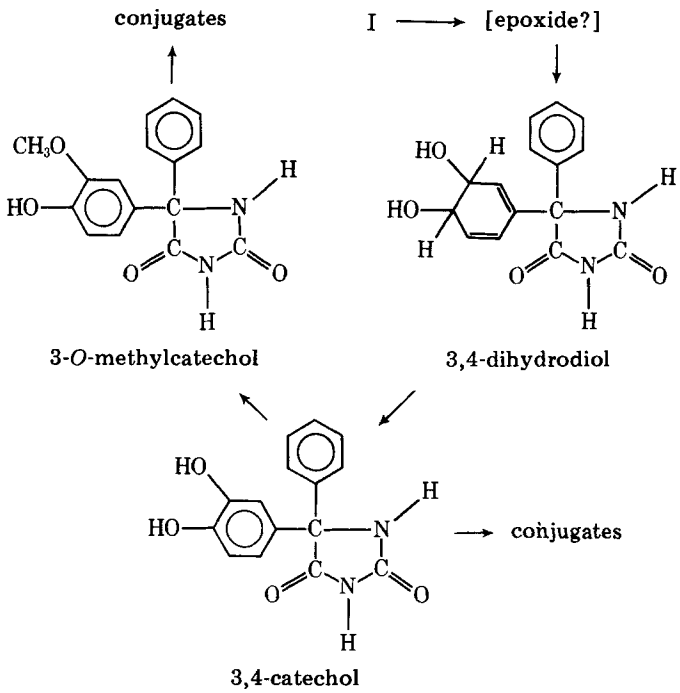


Figure 3—*Gas chromatograms of a flash-heater butylated extract of urine from a patient receiving I (A) and that of synthetic catechol II and methylated catechol III (B). Key: peak V, tributylated monomethylated III; peak VI, dibutylated monomethylated III; peak VII, tetrabutylated II; and peak VIII, tributylated II.*



Scheme VIII

stituents are attached to the same ring. Comparison of the retention times and mass spectra with those of the authentic butylated metabolites demonstrates that peak V was due to the butylated derivative of III and peak VII was due to the butylated derivative of II.

Minute amounts of both of these metabolites were observed in the unhydrolyzed urine from humans, monkeys, and dogs. However, the amount of each metabolite increased on hydrolysis. Quantitative determination of II and III is under investigation.

The possible biotransformation pathway for the formation of these metabolites (II and III) in humans, monkeys, and dogs is shown in Scheme VIII and is in agreement with that suggested by Glazko (2). The

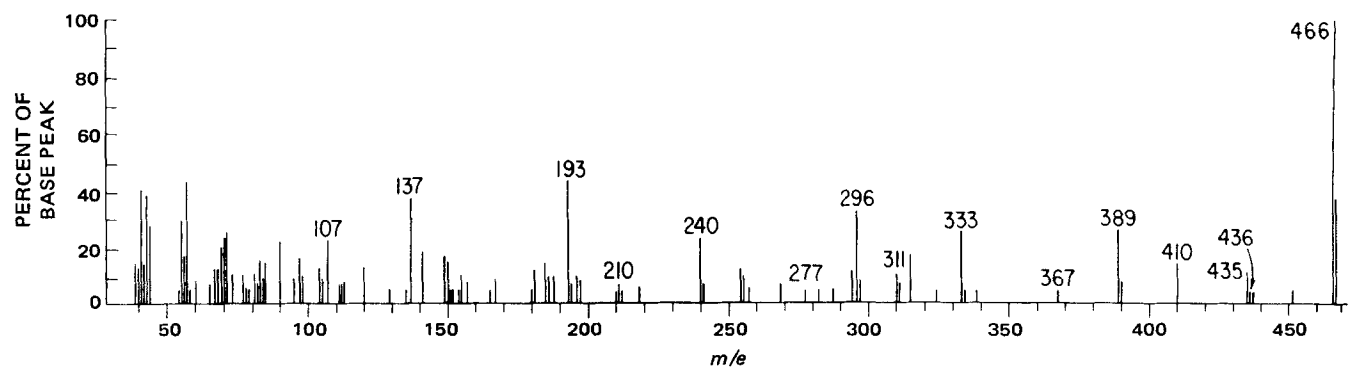


Figure 4—*Normalized electron-impact mass spectrum of the compound giving rise to peak V (Fig. 3).*

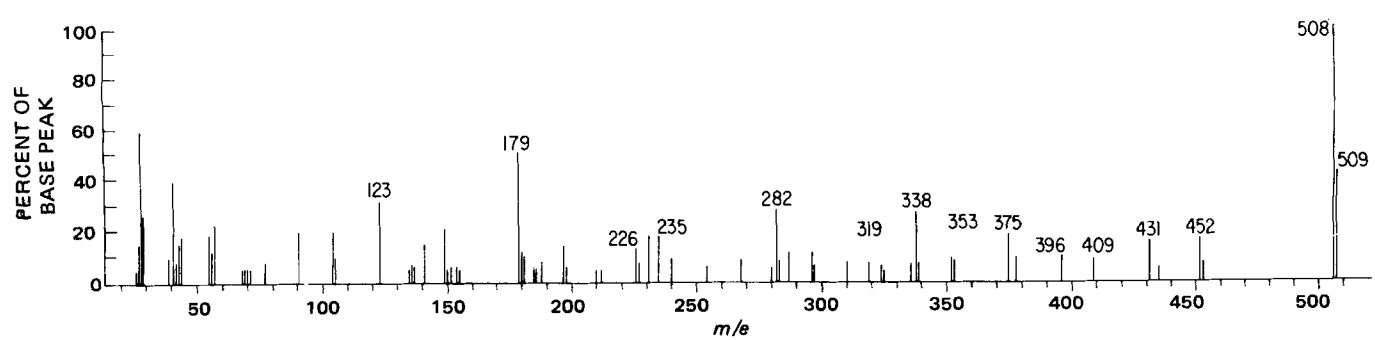


Figure 5—*Normalized electron-impact mass spectrum of the compound giving rise to peak VII (Fig. 3).*

dehydrogenation of dihydrodiols to catechols was observed previously (14–16). The formation of the methylated catechol from 3,4-catechol may be due to the enzyme, catechol O-methyltransferase, since other catechols are known to be methylated *via* this enzyme (8, 17, 18).

In conclusion, it was demonstrated that the catechol metabolite in humans, reported by Borga *et al.* (9), is a mixture of the catechol Metabolite II and the methylated catechol Metabolite III. These metabolites are also formed in minor amounts in other species such as the monkey and dog.

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ˣ To whom inquiries should be directed.

Synthesis and Structure–Activity Relationships of Selected Isomeric Oxime *O*-Ethers as Anticholinergic Agents

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Abstract □ A series of isomeric (*Z*)- and (*E*)-oxime *O*-β-dimethylaminoethyl ether methylhalide derivatives was synthesized, and their (*Z*)- and (*E*)-assignments were made on the basis of chemical and spectral data. The respective (*Z*)- and (*E*)-isomers were evaluated as anticholinergic agents on the rat ileum. The antimuscarinic potencies of the respective (*Z*)- and (*E*)-isomers were compared to determine the effect upon potency of this type of geometric isomerism. Three general structure–activity relationships are discernible among the synthesized compounds: (*a*) among oxime *O*-ethers derived from aromatic aldehydes, the higher potency consistently resides in the isomer where the aryl substituent is (*E*) to the ammonium ether substituent; (*b*) among oxime *O*-ethers derived from diaryl ketones, the (*Z*)- and (*E*)-isomers are approximately equipotent; and (*c*) oxime *O*-ethers derived from diaryl ketones are the most potent of the synthesized compounds.

Keyphrases □ Oxime *O*-ethers, various—isomers synthesized, anticholinergic activity of methylhalide salts evaluated on rat ileum □ *O*-Dimethylaminoethyl oxime ethers, various—isomers synthesized, anticholinergic activity of methylhalide salts evaluated on rat ileum □ Anticholinergic activity—evaluated on rat ileum for isomers of various oxime *O*-dimethylaminoethyl ethers □ Structure–activity relationships—isomers of various oxime *O*-dimethylaminoethyl ethers evaluated for anticholinergic activity on rat ileum

The parasympathetic postganglionic cholinergic receptor site is of considerable interest. In particular, among anticholinergics the muscarinic receptor exhibits stereospecificity toward optical isomers, and this stereospecificity contributes to a knowledge of the receptor (1). The relationship between anticholinergic potency and geometric isomerism also may be informative, but it has not been investigated extensively. Therefore, such an investigation was undertaken to determine whether or not the receptor was stereospecific toward geometric isomers and to yield further information about the receptor.

Oxime *O*-ethers were selected for study since they possess anticholinergic activity (2, 3) and the oxyimino moiety allows the preparation of geometric isomers.

DISCUSSION

The ketone oximes were prepared by the method of Lachman and Noller (4) (Table I). The method involves the generation of hydroxylamine from the hydrochloride salt with excess base in the presence of the ketone, with water as the solvent for low molecular weight ketones and with alcohol for higher molecular weight ketones. The isomer ratios produced are easily predicted qualitatively based on the general rule that the more nearly equal they are in bulk the more nearly equal will be the isomer ratio (5). Thus, the ratio is 89:11 (*E*)–(*Z*) for acetophenone oxime. As can be expected for the diaryl compounds, the isomer ratio is not great.

Separation by fractional crystallization was successful for only one of the 3,4-dimethylbenzophenone oxime isomers. Differences in chelating