GLC Determination of
Acetohexamide and Hydroxyhexamide in Biological Fluids

J. W. KLEBER [x], J. A. GALLOWAY, and B. E. RODDA

Abstract □ A sensitive and specific GLC assay was developed for ace-
tohexamide and hydroxyhexamide, its major metabolite, in plasma and
urine. The assay uses tolbutamide as a mass internal standard. Com-
pounds are extracted from acidified plasma or urine with toluene, con-
verted to methylated derivatives with dimethyl sulfate, and measured
by GLC using a flame-ionization detector. With GLC–mass spectrometry,
the compounds measured are the N-methylsulfonamides resulting from
GLC pyrolysis. Plasma and urine data are presented from a bioavail-
ability study to demonstrate the utility of this method.

Keyphrases □ Acetohexamide—GLC analysis, human plasma and urine
□ Hydroxyhexamide—GLC analysis, human plasma and urine □
GLC—analyses, acetohexamide and hydroxyhexamide, human plasma
and urine □ Antidiabetic agents—acetohexamide, GLC analysis, human
plasma and urine

Earlier methods for the determination of plasma con-
centrations of acetohexamide utilized isotope dilution
analysis (1–3), the Spingler (4) method, or the Toolan–
Wagner (5) procedure. None of these methods is suffi-
ciently sensitive or suitable for the assay of the multiple
samples needed in a bioavailability study, nor do they
permit the assay of acetohexamide and hydroxyhexamide
in urine.

The earliest GLC method for the determination of hy-
poglycemic sulfonylureas (6) involved conversion of tol-
butamide and chlorpropamide to their N-methylated
derivatives by treatment with dimethyl sulfate. Difficulty
in consistently reproducing this work was reported (7, 8),
and similar difficulties were experienced in this laboratory.
Prescott and Redman (9) later described a method for the
GLC estimation of tolbutamide and chlorpropamide,
which served as the basis for the present determination of
acetohexamide and hydroxyhexamide in plasma and
urine.
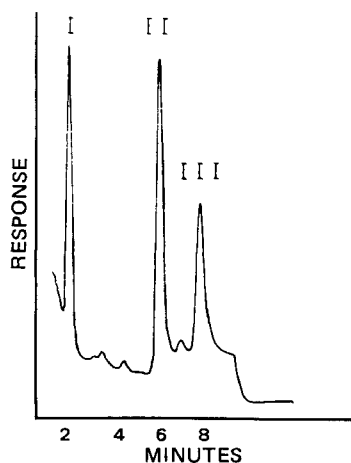
Figure 1—*Gas–liquid chromatograph of extract of plasma spiked with
10 µg of tolbutamide (I)/ml and 20 µg of acetohexamide (II) and hy-
droxyhexamide (III)/ml.*
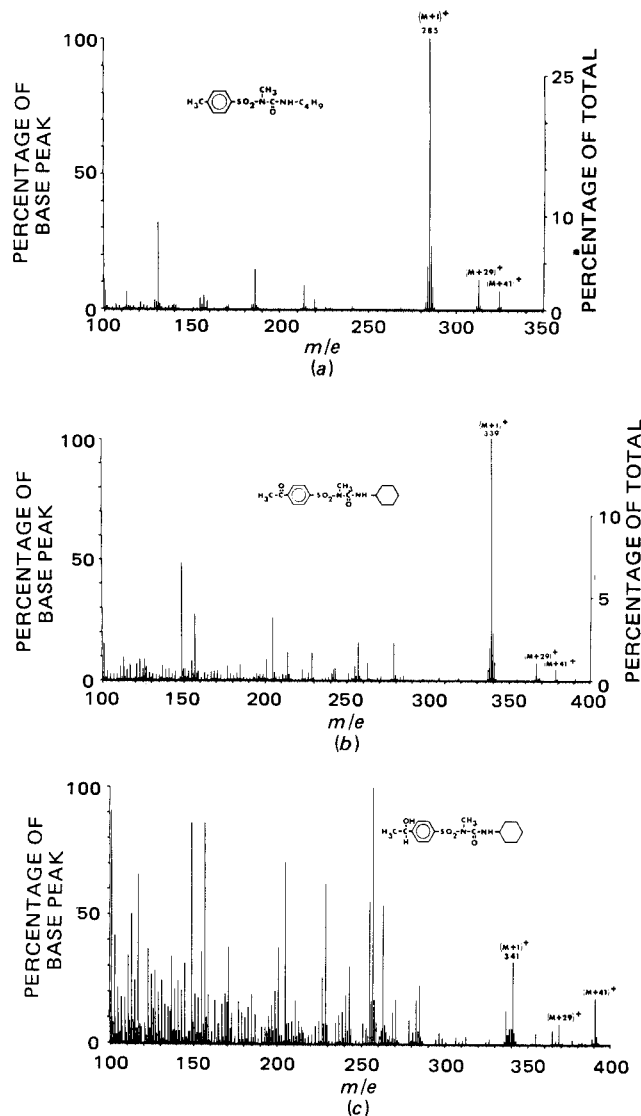
Figure 2—*Chemical ionization mass spectra of* N-*methyl derivatives
of tolbutamide* (a), *acetohexamide* (b), *and hydroxyhexamide* (c).

EXPERIMENTAL

Reagents—Acetohexamide, hydroxyhexamide, and tolbutamide were
synthesized[1]; tolbutamide was used as the mass internal standard for
GLC analysis. All solvents were analytical reagent quality except hexane[2],
which was distilled in glass. Commercially available GLC packing ma-
terial, 0.5% PEG 20M on Gas Chrom Q[3] (80–100 mesh), and the meth-
ylating reagent dimethyl sulfate[4] were used.

Apparatus—The basic instrument was a gas–liquid chromatograph[5]
with dual-column capability, equipped with a flame-ionization detector
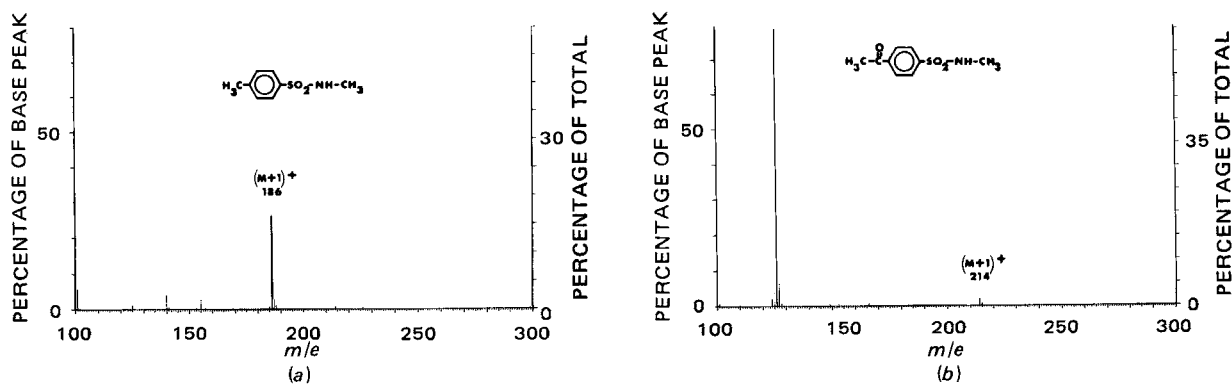
Figure 3—*Chemical ionization mass spectra of* N-*methyltoluenesulfonamide* (a) *and* N-*methyl-*p-*acetylbenzenesulfonamide* (b) *obtained after GLC.*

and 1-mv recorder[6]. Disposable 15-ml centrifuge tubes[7] were used for the extraction procedure; previously siliconized, tapered 15-ml glass centrifuge tubes[8] were used for the methylation step and hexane evaporation[9].

Chromatographic Conditions—The gas chromatograph was operated in the dual-column mode. The two glass columns, 0.61 m × 3 mm i.d., were operated initially at 190° with a programmed 5°/min increase to 240° during analyses. The recorder was operated at a chart speed of 0.63 cm/min, the detector temperature was 260°, and the oxygen and hydrogen flow rates were 90 ml/min. Helium carrier gas (flow rate of 90 ml/min) was filtered through a molecular sieve preconditioned overnight at 240° with dry nitrogen.

Under these conditions, the following retention times were observed: *N*-methyl-*p*-toluenesulfonamide, 90 sec; *N*-methyl-*p*-acetylbenzenesulfonamide, 300 sec; and *p*-(1-hydroxyethyl)-*N*-methylbenzenesulfonamide, 480 sec. When using the instrument in the dual-column mode, a satisfactory baseline was obtained as the oven was heated.

Procedure—The 15-ml tapered centrifuge tubes were washed with chromic acid cleaning solution, rinsed with water, dried, and siliconized with a 1% solution of dimethyldichlorosilane in toluene[10]. Standard solutions of acetohexamide and hydroxyhexamide were added to 1 ml of blank plasma or urine to give calibration concentrations of 0, 5, 10, 20, and 40 µg of each drug/ml. To each tube were added 20 µl (10 µg) of the mass internal standard solution and 1 ml of 1.0 *N* HCl. The mixture was vortexed to ensure uniform distribution, and 5 ml of toluene was then added[11] to each tube.
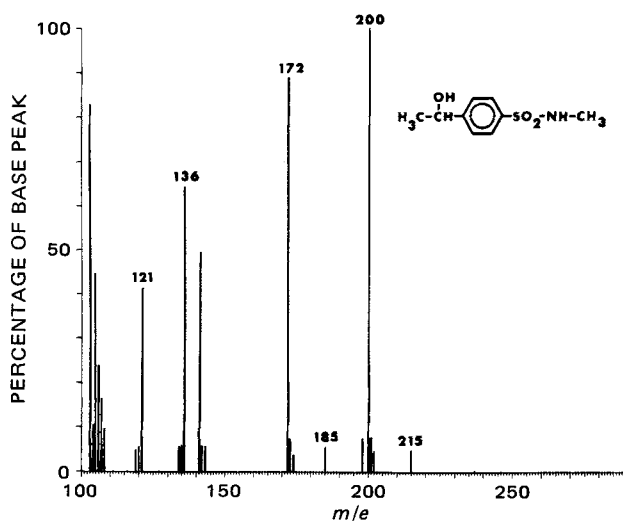
Figure 4—*Electron-impact mass spectrum of* p-*(1-hydroxyethyl)-*N-*methylbenzenesulfonamide obtained after GLC.*

The samples were shaken for 2 min and then centrifuged at 2000 rpm for 4 min. The upper organic phase was transferred to a conical screw-capped centrifuge tube containing 1 ml of 7.5% aqueous potassium carbonate solution, and the samples were again shaken and centrifuged. The toluene was aspirated and discarded. To the aqueous phase were added 1 ml of methanol and 0.1 ml of dimethyl sulfate. Then the tubes were loosely capped and placed in a water bath at 60° for 10 min.

After cooling, 1 ml of 1 *M* acetate buffer (pH 5.2) was added. The methylated sulfonylureas were extracted with 5 ml of *n*-hexane, which was transferred to a 15-ml siliconized, tapered centrifuge tube. The hexane was evaporated to dryness at 50° under a gentle stream of dry nitrogen. Dissolution of the residue in 25 µl of carbon disulfide–chloroform (1:1) was aided by vortex mixing, and 2 µl of each sample solution was then injected onto the GLC column (Fig. 1).

Following chromatography of the calibration standards, peak height ratios[12] of the *N*-methylsulfonamides to the *N*-methyl-*p*-toluenesulfonamide mass internal standard were calculated. These ratios, plotted against concentrations of acetohexamide and hydroxyhexamide in the 5.0–40.0-µg/ml range, gave straight lines passing through the origin (*r* = 1.0). Mean slope values of 0.080 ± 0.005 and 0.043 ± 0.004 were obtained for acetohexamide and hydroxyhexamide, respectively.

Chemical ionization mass spectra[13] of the *N*-methylated derivatives of tolbutamide, acetohexamide, and hydroxyhexamide were obtained before GLC; those of the respective *N*-methylated sulfonamides were determined after chromatography.

A bioavailability study was conducted to compare acetohexamide tablets[14] and to demonstrate the utility of this method. Tablets from three different lots[15], plus a placebo tablet to estimate basal response, were administered to eight subjects in a four-way crossover study. Each subject fasted for 8 hr before receiving a therapeutic dose of 1.5 tablets, 750 mg, of acetohexamide. Blood samples were drawn at −1, 0, 1, 2, 3, 4, 6, and 8 hr after administration, and urine was collected for the 0–8-hr period. In addition to the determination of acetohexamide and hydrox-
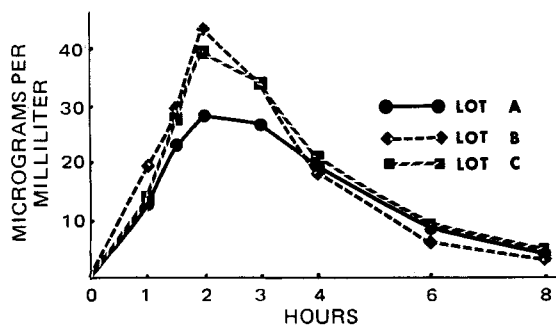
Figure 5—*Mean plasma concentrations of acetohexamide after administration of 750 mg of acetohexamide to eight subjects.*

[6] Model 7128A, Hewlett-Packard, Avondale, Pa.
[7] Corning Glass Co., Corning, N.Y.
[8] Matheson Scientific Co., Chicago. Ill.
[9] Model 106 N-Evap, Organomation Associates, Shrewsbury, Mass.
[10] This treatment appears to prevent adsorption of the *N*-methylsulfonylureas to the glassware.
[11] Repipet, Labindustries, Berkeley, Calif.

[12] Results were virtually unaffected when relative peak areas were used instead of relative peak heights.
[13] Using a Finnigan model 3100 Quadrupole M/S. Column was 45 cm long, contained 3% Dexsil, and was operated at 210°.
[14] Dymelor, Lilly, tablets 1843.
[15] Lot A was formulated with starch hydrolyzed powder and was 30 months old; Lots B and C were formulated with starch gelatinized powder and were 2 months old.

Table I—Precision and Accuracy in Measurement of Acetohexamide and Hydroxyhexamide Added to Plasma

Amount Added, μg/ml	Acetohexamide Measured[a]			Hydroxyhexamide Measured[a]		
	μg/ml	RSD, %	RE, %	μg/ml	RSD, %	RE, %
10.0	9.9 (8.1–11.5)	11.5	−1.0	14.1 (12.5–15.5)	7.2	41
20.0	19.8 (18.9–20.7)	3.2	−1.0	18.2 (14.9–21.6)	15.9	9
40.0	39.4 (37.7–41.5)	3.0	−1.5	40.0 (36.8–44.0)	7.4	1

[a] Mean of eight replicate samples (range).

Table II—Dissolution Studies[a] of Acetohexamide Tablets at 1 hr

Formulation	Mean, %	Range, %	RSD, %
Lot A	59.5	16.6	10.8
	58.5	17.7	11.3
Lot B	67.7	11.0	6.2
	72.7	25.5	12.4
	68.1	8.7	4.4
Lot C	51.2	—	11.5
	58.4	11.5	10.2
	68.0	14.6	7.7

[a] Using pH 7.6, 100 rpm, and 900 ml (USP XIX, 1975, p. 15).

Table III—Recovery of Acetohexamide and Hydroxyhexamide in 8-hr Urines

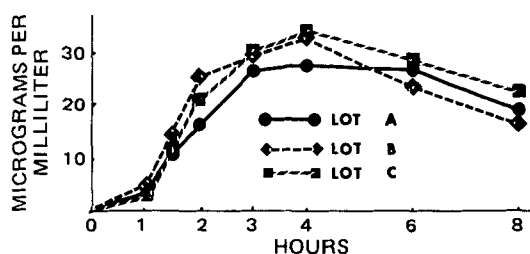| Lot	Acetohexamide, mg Mean	SD	Hydroxyhexamide, mg Mean	SD	Total Mean	Recovery, % Mean	SD
A	31	16	189	93	220	29.2	13.7
B	30	12	223	28	253	33.7	4.1
C	35	8	247	67	282	37.5	9.7

yhexamide concentrations in blood and urine, blood glucose (10) and serum insulin (11) concentrations were measured.

Dissolution of the acetohexamide tablets was determined by the method in USP XIX.

RESULTS AND DISCUSSION

To estimate the precision and accuracy of the procedure, plasma samples with known amounts of acetohexamide and hydroxyhexamide were assayed; the results are presented in Table I.

The chemical ionization mass spectra by which the structures of the N-methylated sulfonylureas were identified are shown in Fig. 2. When these compounds were chromatographed using the described conditions, they were quantitatively pyrolyzed to the respective N-methylsulfona-mides. The GLC–mass spectra for N-methyl-p-toluenesulfonamide and N-methyl-p-acetylbenzenesulfonamide are shown in Fig. 3. p-(1-Hy-droxyethyl)-N-methylbenzenesulfonamide did not elute from the column under the conditions used and was investigated by GLC–electron-impact mass spectrometry[16]. The fragmentation pattern shown in Fig. 4 is con-
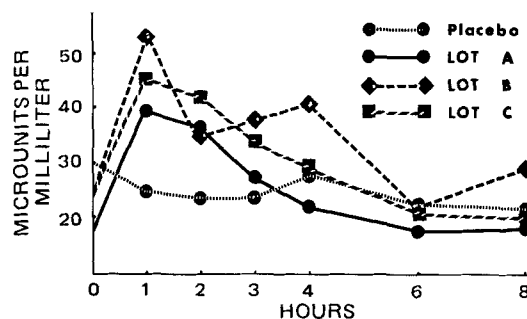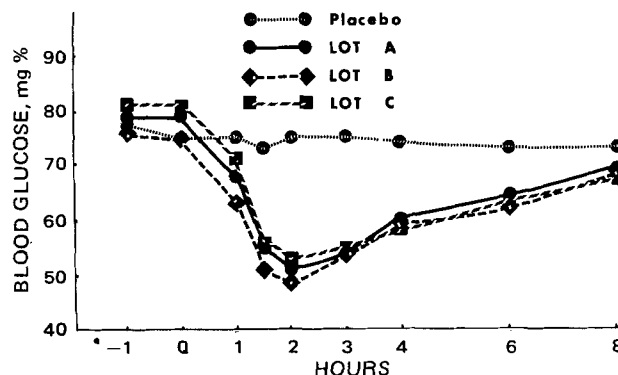
Figure 7—*Mean insulin levels in serum after administration of 750 mg of acetohexamide to eight subjects.*

sistent with that expected for the N-methyl-substituted pyrolysis product of N-methylhydroxyhexamide.

Dissolution profiles are shown in Table II; all lots met the USP XIX dissolution requirement. Although Lot B appeared to have more rapid dissolution, the variability was such that all lots were judged to have relatively similar dissolution patterns.

The average plasma concentration–time curves observed for aceto-hexamide and hydroxyhexamide are given in Figs. 5 and 6, respectively. Recovery of drug and metabolite in the 0–8-hr urines is shown in Table III, and mean serum insulin and blood glucose responses associated with these three tablet formulations are shown in Figs. 7 and 8. The three lots were compared with respect to the area under the plasma concentra-tion–time curves (AUC) and the peak plasma concentration responses for both acetohexamide and hydroxyhexamide. Analysis of variance suggested a lower bioavailability of acetohexamide for Lot A than for B or C. While it is difficult to be certain why Lot A appeared poorer than the other lots, the formulation used in preparing Lot A tablets was dif-ferent from that used for Lots B and C, and Lot A tablets were approxi-mately 30 months old at the time of this study while the other lots were only 2 months old.

The physiological responses (elevation of serum insulin and lowering of blood glucose) may more truly reflect the bioavailability of acetohex-amide from the three tablet formulations, because the metabolite, hy-droxyhexamide, is not only physiologically active but also has a more prolonged effect than the parent drug. Since no substantial differences were found in the plasma concentrations of hydroxyhexamide from these tablet lots, the physiological responses may more closely reflect the plasma metabolite concentrations or a combination of the plasma con-centrations of drug and metabolite.

Figure 6—*Mean plasma concentrations of hydroxyhexamide after administration of 750 mg of acetohexamide to eight subjects.*

[16] Using an LKB 9000S GC–mass spectrometer with column and conditions used in this procedure.

Figure 8—*Mean blood glucose responses after administration of 750 mg of acetohexamide to eight subjects.*

REFERENCES

(1) D. L. Smith, T. J. Vecchio, and A. A. Forist, *Metabolism*, **14**, 229 (1965).

(2) B. D. Cohen, J. A. Galloway, R. E. McMahon, H. W. Culp, M. A. Root, and K. J. Henriques, *Am. J. Med. Sci.*, **254**, 608 (1967).

(3) R. E. McMahon, F. J. Marshall, and H. W. Culp, *J. Pharmacol. Exp. Ther.*, **149**, 272 (1965).

(4) H. Spingler, *Klin. Wochenschr.*, **35**, 533 (1957).

(5) T. J. Toolan and R. L. Wagner, *Ann. N.Y. Acad. Sci.*, **74**, 449 (1959).

(6) K. Sabih and K. Sabih, *J. Pharm. Sci.*, **59**, 782 (1970).

(7) S. B. Matin and M. Rowland, *J. Pharm. Pharmacol.*, **25**, 186 (1973).

(8) D. L. Simmons, R. J. Ranz, and P. Picotte, *J. Chromatogr.*, **71**, 421 (1972).

(9) L. F. Prescott and D. R. Redman, *J. Pharm. Pharmacol.*, **24**, 713 (1972).

(10) W. S. Hoffman, *J. Biol. Chem.*, **120**, 51 (1937).

(11) L. Heding, *Diabetologia*, **8**, 260 (1972).

Quantitative Analysis of Degradation Products in Pilocarpine Hydrochloride Ophthalmic Formulations

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Abstract □ The presence of isopilocarpine, an epimer of pilocarpine, and of pilocarpinic acid, a hydrolytic degradation product of pilocarpine, was established and all three substances were assayed in various commercial ophthalmic formulations of pilocarpine hydrochloride by ¹³C-Fourier transform spectroscopy. Assay was based upon integrated intensities of selected resonances from any formulation calibrated against the intensity of tetramethylammonium bromide, used as a common external reference. The normalized intensities were then related to those of a reference solution of pilocarpine hydrochloride, thereby eliminating any factor arising from variability of ¹³C-relaxation times. The ¹³C-resonance for the *N*-methyl group, being common to all products, provides a convenient basis for the assay of the total alkaloid content whereas the C-8 resonances are best suited for assaying residual pilocarpine and its degradation products. This procedure, estimated as accurate to ±5%, constitutes the first comprehensive analytical method to differentiate between pilocarpine and its degradation products.

Keyphrases □ Pilocarpine hydrochloride—and degradation products, ¹³C-NMR analysis, commercial ophthalmic formulations □ Isopilocarpine—¹³C-NMR analysis in commercial ophthalmic formulations of pilocarpine hydrochloride □ Pilocarpinic acid—¹³C-NMR analysis in commercial ophthalmic formulations of pilocarpine hydrochloride □ ¹³C-NMR spectroscopy—analysis, pilocarpine hydrochloride and degradation products, commercial ophthalmic formulations □ Ophthalmic formulations, commercial—¹³C-NMR analysis of pilocarpine hydrochloride and degradation products

No satisfactory methodology[1] is available for the analysis of the degradation products, isopilocarpine (II) and pilocarpinic acid[2] (III), in aqueous formulations of pilocarpine (I). The analysis of such impurities has received little attention because of: (*a*) their apparent relatively low concentrations in pilocarpine formulations, (*b*) the great difficulty in effecting any separation, and (*c*) the difficulty in realizing unequivocal characterization. However, numerous procedures for the determination of pilocarpine, including colorimetric, volumetric, polarimetric, and polarographic (3, 4), have been developed.

All commonly used procedures for the assay of pilocarpine have drawbacks (5). A common deficiency is the inability to distinguish between the parent alkaloid and its degradation products. For example, various colorimetric methods use the ferric hydroxamate reaction in aqueous (4, 6) or alcoholic (7) media, the sodium nitroprusside reaction (8), the hydrogen peroxide dichromate reaction (9), and the phosphomolybdic acid reaction (10). Although none of these colorimetric procedures excludes the contribution from degradation products from the total assay,

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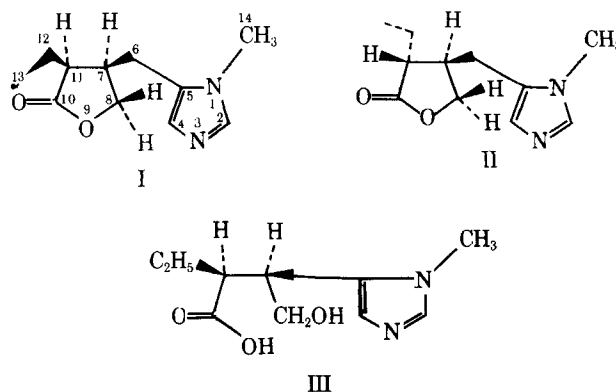
[1] The simultaneous determination of pilocarpine and isopilocarpine in pharmaceutical preparations (1) was published after this paper was submitted. The same method had been used to analyze isopilocarpine and pilocarpine in the synthesis of *d*-pilocarpine-*N*-¹⁴CH₃ (2).

[2] Pilocarpinic acid has also been called pilocarpic acid (T. A. Henry, in "The Plant Alkaloids," 4th ed., Blakiston, Philadelphia, Pa., 1949) and pilocarpoic acid (R. H. F. Manske, in "The Alkaloids," vols. 3 and 5, Academic, New York, N.Y., 1953 and 1955).