It is not considered likely that a set of process variables exists such that $R_i(t)$ will precisely equal $\bar{R}(t)$ for $i = 1, 2, \ldots, p$. However, one set will minimize the sum of the deviations for all dosage forms. One approach to determining an approximation to this set of process variables is to find the minimum value of Eqs. A15 and A16.

This alternative approach is seen to obviate open loop operation runs which would otherwise be required to be performed with $\bar{R}(t)$. It should be noted that with the alternative performance criterion for optimization, as expressed by Eqs. A15 and A16, the influence of the *in vivo* bioavailability data for each dosage form is implicit in $R_i(t)$ by virtue of its use in providing a reference for the closed operation of the apparatus. In any event, this alternative procedure is an indirect approach to the problem of minimizing the deviation of *in vitro* data from *in vivo* data. It may best be considered as a method for more rapidly attaining a set of process variables in the neighborhood of the optimum set. The fidelity of an *in vitro* test may best be gauged from a statistical comparison between drug release results obtained with the apparatus operated with optimal $R(t)$ and fixed process variables with *in vivo* bioavailability data using dosage forms previously unstudied *in vitro* and not included in the optimization procedure.

REFERENCES

(1) V. F. Smolen, B. D. Turrie, and W. A. Weigand, *J. Pharm. Sci.*, **61**, 1941(1972).

(2) V. F. Smolen, P. B. Kuehn, and E. J. Williams, *Drug Dev. Commun.*, **1**, 143(1975).

(3) *Ibid.*, **1**, 231(1975).

(4) V. F. Smolen, *J. Pharm. Sci.*, **60**, 878(1971).

(5) J. G. Wagner, "Biopharmaceutics and Relevant Pharmacokinetics," Drug Intelligence Publications, Hamilton, Ill., 1971, pp. 64–147.

(6) "Current Concepts in the Pharmaceutical Sciences: Biopharmaceutics," J. Swarbrick, Ed., Lea & Febiger, Philadelphia, Pa., 1970, pp. 265–294.

(7) W. A. Cressman, C. A. Janicki, P. C. Johnson, J. T. Dolusio, and G. A. Braun, *J. Pharm. Sci.*, **58**, 1516(1969).

(8) G. Levy, *ibid.*, **52**, 1039(1963).

(9) G. Levy and L. E. Hollister, *ibid.*, **53**, 1446(1964).

(10) H. Weintraub and M. Gibaldi, *ibid.*, **58**, 1368(1969).

(11) *Ibid.*, **59**, 1792(1970).

(12) V. F. Smolen, *Hosp. Pharm.*, **4**, 14(1969).

(13) J. E. Tingstad and S. Riegelman, *J. Pharm. Sci.*, **59**, 692(1970).

(14) F. Langenbucher, *ibid.*, **58**, 1265(1969).

(15) G. H. Elliot, *Pharm. J.*, **131**, 514(1933).

(16) E. Nelson, *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 297(1958).

(17) W. Brownell, S. Riegelman, and W. J. Mader, "Committee Report on Drug Dissolution Methodology," APhA Academy of Pharmaceutical Sciences, Pharmaceutical Analysis Control Section, 1968.

(18) A. C. Shah and J. F. Ochs, *J. Pharm. Sci.*, **63**, 110(1974).

(19) "Sartorius Membrane Filter: Absorption and Solubility Simulation," Tech. Bull. SM 2300, Beckman Instruments, Inc., Anaheim, Calif.

(20) G. Levy and E. J. Mroczak, *J. Pharm. Sci.*, **57**, 235(1968).

(21) T. Khalil and A. N. Martin, *ibid.*, **56**, 1225(1967).

(22) V. F. Smolen and D. E. Hagman, *J. Colloid Interface Sci.*, **42**, 70(1973).

(23) G. N. Ling, *Int. J. Neurosci.*, **1**, 129(1970).

(24) M. Gibaldi and S. Feldman, *J. Pharm. Sci.*, **56**, 1238(1967).

(25) P. Finholt and S. Solvang, *ibid.*, **57**, 1322(1968).

(26) C. R. Hicks, "Fundamental Concepts in the Design of Experiments," Holt, Reinhart, and Winston, New York, N.Y., 1964, pp. 224–245.

(27) V. F. Smolen and W. A. Weigand, *J. Pharmacokinet. Biopharm.*, 1, 329(1973).

(28) V. F. Smolen, E. J. Williams, and P. B. Kuehn, *Can. J. Pharm. Sci.*, **10**, 95(1975).

(29) V. F. Smolen, R. G. Barile, and T. G. Theophanous, *J. Pharm. Sci.*, **61**, 467(1972).

(30) V. F. Smolen and R. D. Schoenwald, *ibid.*, **60**, 96(1971).

(31) J. G. Wagner and E. Nelson, *ibid.*, **53**, 1392(1964).

(32) J. C. K. Loo and S. Riegelman, *ibid.*, **57**, 918(1968).

(33) W. J. Westlake, in "Current Concepts in the Pharmaceutical Sciences: Dosage Form Design and Bioavailability," J. Swarbrick, Ed., Lea & Febiger, Philadelphia, Pa., 1973, pp. 149–179.

ACKNOWLEDGMENTS AND ADDRESSES

Received September 25, 1972, from the *Interdisciplinary Drug Engineering and Assessment Laboratory, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907*

Accepted for publication February 12, 1976.

* Department of Chemical Engineering.

ˣ To whom inquiries should be directed (at the Department of Industrial and Physical Pharmacy).

Subnanogram Assay for Pilocarpine in Biological Fluids

W. F. BAYNE ˣ, L.-C. CHU, and F. T. TAO

Abstract □ A method for the determination of pilocarpine was developed in which the imidazole ring of pilocarpine was acylated with heptafluorobutyric anhydride, using triethylamine as a catalyst. After cleanup, the pilocarpine derivative was analyzed using GLC with electron-capture detection. The limit of sensitivity was 25–50 pg of pilocarpine, which had been subjected to the derivatization and cleanup procedures. The method was specific for pilocarpine, with the isopilocarpine derivative eluting prior to the pilocarpine derivative.

Keyphrases □ Pilocarpine—GLC analysis, biological fluids □ GLC—analysis, pilocarpine, biological fluids □ Ophthalmic cholinergic agents—pilocarpine, GLC analysis in biological fluids

Pilocarpine is used extensively in clinical ophthamology as a topical agent for lowering intraocular pressure of patients suffering from open- and closed-angle primary glaucoma. Much interest and analytical development (1) have recently been focused on this drug because of its incorporation into a new dosage form called an ocular therapeutic system (2, 3). This system is placed beneath the eyelid and provides continuous delivery of pilocarpine at a controlled low rate into the tear film, as opposed to intermittent pulses of drug into the tear film by conventional eyedrops.

Colorimetric methods based on the oxidation of the tertiary amino group or on opening the lactone ring with hydroxylamine have been reported (4). Pilocarpine has

successfully been chromatographed by GLC using several stationary liquid phases (5–7); one article (7) reported the separation of pilocarpine and isopilocarpine. This laboratory observed significant tailing and loss of resolution when pilocarpine and isopilocarpine were chromatographed and a tendency for pilocarpine to epimerize thermally to isopilocarpine, even with rigid control of the injector and column temperatures.

Repta and Higuchi (8) developed an analytical method for pilocarpine based on the catalytic action of its imidazole ring on the hydrolysis of 2,4-dinitrophenyl acetate. The product of hydrolysis, the 2,4-dinitrophenoxide ion, was determined spectrophotometrically. They suggested that the assay was capable of measuring 100 ng of pilocarpine with suitable modifications. Since the ability to measure absolute quantities of pilocarpine as low as 0.05 ng was desired, development of such an assay was undertaken with the intent to measure pilocarpine in biological fluids.

EXPERIMENTAL

Reagents—Methylene chloride, hexane, benzene, and ethyl acetate[1], used in the derivatization and cleanup procedures, were highly purified, commercially available solvents. Methylene chloride was distilled from phosphorus pentoxide prior to use; the other solvents were used as received. All other chemicals were reagent grade unless otherwise specified. Triethylamine[2] (I) and heptafluorobutyric anhydride[2] (II) were used for the acylation of pilocarpine.

Pilocarpine base, when used as the standard, was prepared by extracting pilocarpine with methylene chloride from an aqueous solution of pilocarpine nitrate adjusted to pH 9, followed by evaporation of the organic solvent, which had been dried with sodium sulfate. This pilocarpine base contained less than 1% isopilocarpine as determined using a liquid chromatographic procedure (1). A methanol[1] solution of the extracted pilocarpine was diluted down to a working standard and was stable at 0° for at least 1 month.

Borate buffer was made from 0.0625 *M* sodium tetraborate and 0.25 *M* H_3BO_3 adjusted to pH 8.5. Similarly, carbonate buffer was made from 1 *M* $KHCO_3$ adjusted to pH 9.25 with fresh concentrated potassium hydroxide. Hydrochloric acid, 0.5 *N*, was prepared by diluting the concentrated acid.

The dimethylated derivative of methazolamide, verified by mass spectrometry, was synthesized by an extractive alkylation technique using iodomethane in methylene chloride and tetrahexylammonium hydrogen sulfate[3] and used as an internal standard. This technique has been used to form a peralkylated derivative of chlorthalidone, which also contains a primary sulfonamide group (9). The suggestion (10) that silver sulfate be used to remove any tetrahexylammonium iodide formed during the alkylation from the organic phase was also followed.

Glassware—All glassware was silanized by soaking for several hours in 5% dimethyldichlorosilane in toluene and rinsing with toluene, methanol, and methylene chloride.

Apparatus—A gas chromatograph equipped with a scandium tritide detector[4] was used. A 2-mm i.d., 1.8-m (6-ft) silanized glass column was packed with 3% OV-17[5] on Chromosorb W[5] (100–120 mesh). This support was inactivated and coated with the liquid phase according to a published procedure (11). Nitrogen was used as the carrier gas.

Retention times were 5 min for dimethylmethazolamide (III) and 6 min for the pilocarpine derivative (IV) at a column temperature of 190° and a gas flow of 25 ml/min. The detector temperature was 250°. To prevent on-column formation of the isopilocarpine derivative, the injector temperature was kept below 210°.

Mass spectra of the pilocarpine derivative and the internal standard were obtained with a quadrupole mass spectrometer[6] interfaced with a gas chromatograph. The electron energy was 70 ev, and the emission current was 1 mamp. The column conditions and flow rate were identical with those employed in the GLC analysis, using either a flame-ionization detector or an electron-capture detector.

The PMR spectra were obtained on a spectrometer[7] in the pulse mode. The solutions analyzed were 20% (w/v), using chloroform-d_1 as a solvent with tetramethylsilane as an internal reference.

Derivatization and Purification for Electron-Capture Detection—An aliquot of a dilution of a pilocarpine nitrate aqueous solution (1 μg/ml) was added with a microsyringe to a stoppered[8] 15-ml centrifuge tube. Then 0.5 ml of borate buffer was added to the aliquot, and the aqueous solution was extracted twice with 1 ml of methylene chloride. The combined organic extract was evaporated at 40° under a stream of nitrogen. Then 0.5 ml of benzene was added to the residue, and the organic solvent was evaporated at 40° with nitrogen.

When an aliquot of pilocarpine base in methanol was used, the methanol was evaporated before the addition and evaporation of benzene since any residual methanol will consume II. In either case, 200 μl of methylene chloride, 2 μl of I, and 4 μl of II were added to the residue with careful mixing. After 15 min at 50°, 1 ml of borate buffer was added, and the solution was vortexed for 1 min and centrifuged. Excess II was thus removed from the organic phase by hydrolysis to the acid, with the pH chosen to minimize the breakdown of IV.

After the addition of 0.5 ml of 0.5 *N* HCl and vigorous vortexing, first nine volumes and then 2.5 volumes of hexane were used to wash the derivative to eliminate any organic soluble impurities. Complete removal of hexane was followed by the addition of 0.5 ml of benzene and approximately 0.8 ng of III. (Observation in this laboratory indicated that III had an appropriate retention time under similar chromatographic conditions for use as an internal standard.)

Adjusting the pH of the solution to 9 by addition of 1 ml of carbonate buffer permitted the partitioning of IV into the benzene layer. This benzene was transferred to a clean vessel, evaporated to dryness under a nitrogen flow, and then reconstituted in 50 μl of 20% ethyl acetate in hexane. Aliquots of this solution were injected into the gas chromatograph.

Preparation of Microgram Quantities of Derivative—Microgram samples were prepared in the same manner outlined for the submicrogram procedure, except that 4 μl of I and 8 μl of II in 200 μl of methylene chloride were used to derivatize pilocarpine. A few microliters of the final ethyl acetate–hexane solution was either injected onto a gas chromatograph equipped with a flame-ionization detector and chromatographed or onto a GLC–mass spectrometry system, which was employed to obtain the mass spectrum of IV.

Preparation of Milligram Quantities of Derivative—Milligram quantities of IV were prepared by reacting 100 mg of pilocarpine base with 2 ml of I and 4 ml of II in 90 ml of methylene chloride overnight at 40°. The solution was then transferred to a 250-ml separator and washed with 100 ml of carbonate buffer. After removal of the aqueous phase and addition of 90 ml of hexane to the methylene chloride, the organic layer was extracted three times with 50 ml of 1 *N* HCl.

The pooled acid extract was adjusted to pH 9 with sodium carbonate followed by two extractions of IV with 75 ml of carbon tetrachloride. The organic solvent was then removed with a rotary evaporator. The resulting 93 mg of yellow, oily residue was reconstituted with chloroform-d_1 to make a 20% (w/v) solution for PMR analysis.

Derivatization of Base-Catalyzed, Hydrolyzed Pilocarpine—A methanol solution (1 μg/μl) of a pilocarpine sample that had been subjected to base-catalyzed hydrolysis in butanol was prepared. Aliquots of this working solution, representing approximately 100 μg of hydrolyzed pilocarpine, were either derivatized directly after removal of methanol or extracted twice with 1 ml of methylene chloride from 0.5 ml of borate buffer after removal of the methanol. The conditions employed were those described in the microderivatization procedure.

Standard Curve and Calibration—The standard curve was constructed by transferring microliter quantities of pilocarpine nitrate aqueous solution, ranging from 0.05 to 10 ng of pilocarpine base, to the centrifuge tubes. The derivatization was then conducted as al-

[1] Nanograde solvents, Mallinckrodt, St. Louis, Mo.
[2] Sequanal grade, Pierce Chemical Co., Rockford, Ill.
[3] Synthesized by a method supplied by Labkemi AB, Sweden.
[4] Varian model 2100, Walnut Creek, Calif.
[5] Applied Science Labs, State College, Pa.
[6] Finnigan 1015 SL, Sunnyvale, Calif.
[7] Varian XL-100 FT, Palo Alto, Calif.
[8] Lined with Teflon (du Pont).

Table I—Proton Chemical Shifts (Parts per Million) Relative to Tetramethylsilane for Pilocarpine and Derivative in Chloroform-d_1 [a]

	CH_3CH_2—	CH_3CH_2—	—CH_2 (Bridge) 3H, 4H	$5H_a$	$5H_b$	Imidazole Protons 4'H	Imidazole Protons 2'H	N—CH_3
Pilocarpine	1.12 (t)	1.71 (m)	2.64 (m)	4.05 (dd)	4.19 (dd)	6.76 (s)	7.42 (s)	3.63 (s)
Derivative	1.14 (t)	1.72 (m)	2.70 (m)	4.04 (dd)	4.26 (dd)	7.22 (s)	(2'H or 4'H)	4.00 (s)

[a] s = singlet, t = triplet, m = multiplet, and dd = doublet of doublet.

ready described; a constant amount of III, approximately 0.8 ng, was added after the addition of benzene in the cleanup procedure. The standard curve was constructed by measuring the area, peak height times width at half-height, for IV and III. The ratio of the area of IV to III was then plotted against the amount of pilocarpine and yielded a linear plot (slope, 0.37; intercept, 0.008; and r^2, 0.997).

Sampling of Aqueous Humor in Rabbits—Samples of aqueous humor, 24 hr after instillation of 50 μl of a 2% pilocarpine nitrate[9] solution or insertion of a membrane-controlled delivery system[10], were obtained using a 1-ml syringe with a 27-gauge needle. The cornea of anesthetized rabbits was punctured near the limbus, and 50–100 μl of fluid was withdrawn. A few microliters of aqueous humor was added to 0.5 ml of borate buffer, and the assay was conducted as already described.

RESULTS

Samples containing subnanogram quantities of pilocarpine were derivatized and analyzed with good detection (Fig. 1). The minimum detectable quantity was between 25 and 50 pg/25 μl of 20% ethyl acetate–hexane when 9 μl of solution was injected. This limit is practical since 25–50 pg of pilocarpine can be derivatized and carried through the cleanup procedure.

The mass spectrum of IV (Fig. 2) exhibited a molecular ion peak, *m/e* 404, which was consistent with a monoacylated heptafluorobutyryl derivative of pilocarpine. The *m/e* 291 fragment resulted from the loss of the lactone ring from the molecular ion. Further cleavage with the loss of the heptafluorobutyryl group resulted in the *m/e* 94 fragment, which was the base peak. The *m/e* 235 fragment arose from the loss of the C_3F_7 radical from the molecular ion and underwent further loss of the lactone ring to give the *m/e* 122 fragment. Thus, the mass spectrum indicated the loss of one imidazole proton during the acylation.

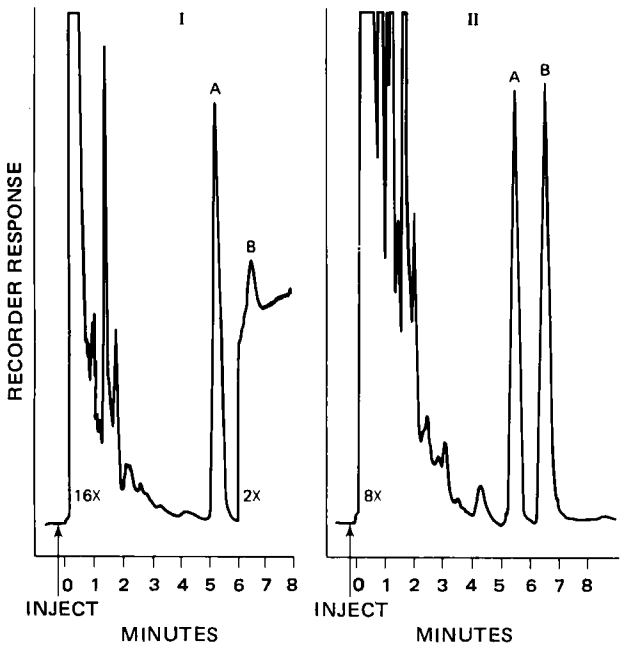


Figure 1—*Representative chromatograms of pilocarpine derivative prepared from aqueous pilocarpine nitrate solutions carried through extraction, derivatization, and cleanup steps. Key: A, internal standard, methazolamide derivative; B, pilocarpine derivative; I, pilocarpine base equivalent to 50 pg; and II, pilocarpine base equivalent to 1 ng.*

The PMR spectrum of the derivative contained only one imidazole proton, whereas pilocarpine had two distinct resonances for the two imidazole protons (Table I). The PMR spectrum also showed a large downfield shift of the *N*-methyl protons in the derivative relative to pilocarpine. This shift can readily be explained by the withdrawal of electron density from the nitrogen by the fluorinated acyl group. It has been reported that a direct correlation between the electron density on the nitrogen and the chemical shift of the attached methyl group exists for a large number of azoles (12). No coupling of the 4'-imidazole proton to the methylene bridge protons was observed in pilocarpine; such coupling has been shown to occur in some 1,5-disubstituted imidazoles (13). Therefore, it is not possible to assign unequivocally the acyl group to either the 2'- or 4'-position of the imidazole ring.

When 100 μg of pilocarpine, which had been subjected to base-catalyzed hydrolysis, was derivatized, only isopilocarpine was obtained. When an extraction from borate buffer (pH 8.5) was performed prior to derivatization, no derivative was obtained. When a sample of pilocarpine was either derivatized directly or extracted and derivatized, the same response was obtained. This response was the same as that obtained for the derivatized sample of pilocarpine subjected to hydrolysis. Thus, it may be concluded that:

1. The sample subjected to hydrolysis was isopilocarpic acid or isopilocarpate.
2. Isopilocarpic acid was not extracted with methylene chloride from a pH 8.5 aqueous solution.
3. Isopilocarpic acid underwent quantitative ring closure to isopilocarpine under the reaction conditions of the derivatization procedure.
4. Pilocarpine can be extracted quantitatively.

The derivatives of pilocarpine and isopilocarpine elicited the same responses from the flame-ionization detector used in this study. Therefore, an extraction from a borate buffer should be performed for biological samples to separate the acids from the lactones if pilocarpine and isopilocarpine are to be specifically determined.

When the described procedure was used to measure pilocarpine concentrations in the aqueous humor of rabbits 24 hr after administration of 50 μl of a 2% solution of pilocarpine nitrate, no pilocarpine was detected (<3 ng/g). However, 24 hr after insertion of the membrane-controlled delivery system, the average concentration in the aqueous humor was 520 ± 16 (*SE*) ng/g (n = 5). This value compares favorably with results obtained using N-$^{14}CH_3$-pilocarpine (14) incorporated into the membrane-controlled delivery system; after appropriate corrections for metabolism (15), an average concentration of 450 ± 16 (*SE*) ng/g (n = 5) was obtained during steady-state delivery.

DISCUSSION

Grimison and Ridd (16) studied the kinetics and deuterium isotope effects of the reaction of imidazole with the electrophiles formed from iodine and iodide and diazotized sulfanilic acid. The 4- or 5-position was the initial site of attack by the iodonium electrophile, while the 2-position was attacked by the diazonium electrophile. The difference in site of attack was rationalized in terms of the position of the transition states along the reaction coordinate, attack by the diazonium species being governed by charge density of imidazole and attack by the iodonium species being controlled by the localization energy of

[9] P. V. Carpine Liquifilm, Allergan, Irvine, Calif.
[10] Ocusert P-20, Alza Corp., Palo Alto, CA 94304

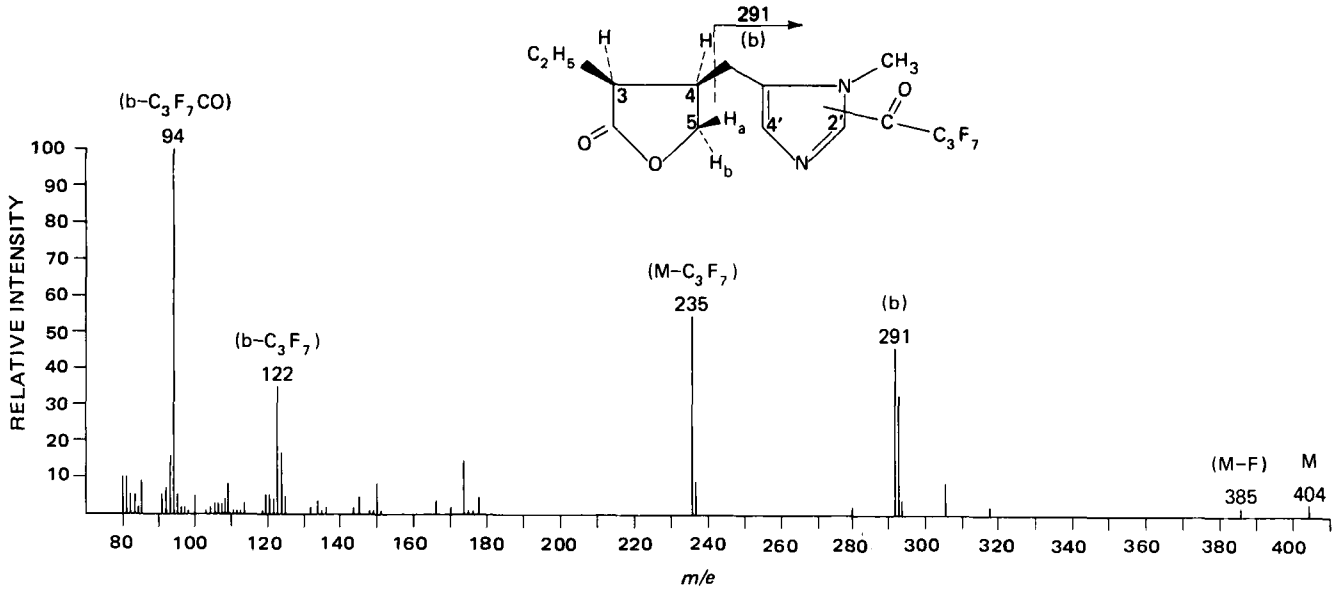


Figure 2—*Mass spectrum of pilocarpine derivative obtained with electron energy of 70 ev and emission current of 1 μamp.*

a high energy intermediate (17). The investigators postulated that the conjugate base of imidazole was the species that reacted with the electrophiles. No reaction occurred when 1-methylimidazole was used.

However, in this laboratory, the acylium electrophile generated from I and II acylated, in methylene chloride or benzene, the imidazole ring of pilocarpine, which has a tertiary amino group. Sulfonation and nitration of imidazole have been reported to occur in the 4- or 5-position, the conjugate base not existing in the acidic medium (18). Blake *et al.* (19) indicated that aromatic compounds can be acylated with fluorinated anhydrides in ethyl acetate, using aluminum chloride as a catalyst. This approach was not successful with pilocarpine in this laboratory.

The derivative (IV) could be made with no apparent epimerization of pilocarpine to isopilocarpine. When microgram samples of pilocarpine were derivatized and chromatographed, separation between the isopilocarpine and pilocarpine derivatives was achieved. The small peak proceeding IV in Fig. 3 had the same retention time as a derivatized sample of isopilocarpine. Figure 3 is a chromatogram of derivatized pilocarpine base, reported to contain less than 1% isopilocarpine. The GLC analysis indicated that the sample contained 0.65% isopilocarpine. Therefore, the derivatization can be conducted without significant epimerization to the more thermodynamically stable *trans*-configuration of the 3- and 4-protons of the lactone ring (isopilocarpine).

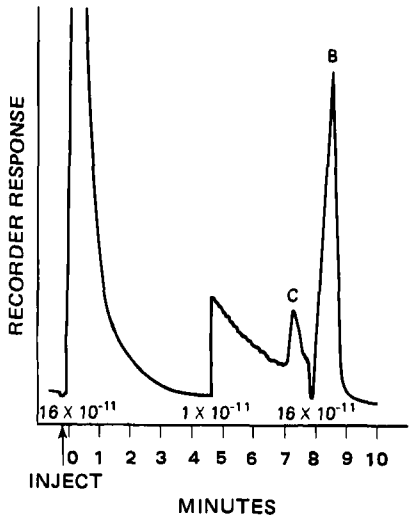


Figure 3—*Chromatogram of derivatized pilocarpine base, showing presence of isopilocarpine. Key: B, pilocarpine derivative; and C, isopilocarpine derivative. The column temperature was lowered to 170° to ensure complete resolution of the two peaks.*

The base-catalyzed epimerization of pilocarpine to isopilocarpine and the base-catalyzed ring opening to pilocarpate, reported previously (20), were minimized due to the high lipophilicity of IV. The derivative (IV) cannot be reextracted to any measurable extent from benzene or methylene chloride with pH 4.5 acetate buffer. Thus, when the benzene and internal standard are added to the aqueous acid and the aqueous phase is made alkaline, IV overwhelmingly favors the organic phase, minimizing residence time in the basic medium. Thus, the extraction procedure could be conducted with little or no epimerization. Furthermore, a linear standard curve resulted.

The cleanup procedure was checked by derivatizating microgram samples of pilocarpine, injecting a microliter of the solution, and comparing the response after the cleanup. The procedure was nearly 100% efficient.

The internal standard was a matter of convenience. Any compound that has favorable partitioning toward benzene relative to an aqueous basic phase, a favorable retention time relative to the pilocarpine derivative, good chromatographic properties in the subnanogram range, and reasonably good sensitivity toward electron-capture detection can be used.

REFERENCES

(1) T. Urbányi, A. Piedmont, E. Willis, and G. Manning, *J. Pharm. Sci.*, **65**, 257(1976).

(2) D. M. Worthen, T. J. Zimmerman, and C. A. Wind, *Invest. Ophthalmol.*, **13**, 296(1974).

(3) M. G. Holland, *Ann. Ophthalmol.*, **6**, 447(1974).

(4) M. S. Karawya and M. G. Ghourab, *J. Assoc. Offic. Anal. Chem.*, **55**, 1180(1972), and references cited therein.

(5) E. Brochmann-Hanssen and A. B. Svendson, *J. Pharm. Sci.*, **51**, 1095(1962).

(6) E. Brochmann-Hanssen and C. R. Fontan, *J. Chromatogr.*, **19**, 296(1965).

(7) J. L. Massingill, Jr., and J. E. Hodgkins, *Anal. Chem.*, **37**, 952(1965).

(8) A. Repta and T. Higuchi, *J. Pharm. Sci.*, **60**, 1468(1971).

(9) M. Ervik and K. Gustavii, *Anal. Chem.*, **46**, 39(1974).

(10) H. Ehrsson, *ibid.*, **46**, 922(1974).

(11) R. J. Leibrand and L. L. Dunham, *Res. Dev.*, **Sept. 1973**, 32.

(12) R. N. Butler, *Can. J. Chem.*, **51**, 2315(1973).

(13) H. R. Matthews nd H. Rapoport, *J. Am. Chem. Soc.*, **95**, 2297(1973).

(14) J. I. DeGraw, J. S. Ingstrom, and E. Willis, *J. Pharm. Sci.*, **64**, 1700(1975).
(15) L. Sendelbeck, D. Moore, and J. Urquhart, *Am. J. Ophthalmol.*, **80**, 274(1975).
(16) G. Grimison and J. H. Ridd, *J. Chem. Soc.*, **1959**, 3019.
(17) G. Grimison and J. H. Ridd, *Proc. Chem. Soc.*, **1958**, 256.
(18) R. J. Gillespie, A. Grimison, J. H. Ridd, and R. F. M. White, *J. Chem. Soc.*, **1958**, 3228.
(19) J. W. Blake, R. Huffman, J. Noonan, and R. Ray, *Am. Lab.*, **May 1973**, 63.
(20) M. A. Nunes and E. Brochmann-Hanssen, *J. Pharm. Sci.*, **63**, 716(1974).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 24, 1975, from *Alza Research, Palo Alto, CA 94304*

Accepted for publication February 2, 1976.

The authors acknowledge N. Keller, L. Sendelbeck, and D. Moore of the Biological Sciences Department, Alza, for obtaining the biological samples.

Note added in proof: The authors of a recent Communication in *J. Pharm. Sci.* [**65**, 1262(1976)], accepted after this research article, reported the formation of a heptafluorobutyramide derivative of pilocarpine. The chemical structure of pilocarpine would seem to preclude formation of an amide. The evidence presented in this research article indicates that either the 2′- or 4′-position of the imidazole ring is acylated with heptafluorobutyric anhydride when the reaction is catalyzed with triethylamine. Since the authors of the Communication employed reaction conditions similar to those presented in this article, it is likely that they formed the same acylated derivative of pilocarpine.

[x] To whom inquiries should be directed.

Circular Dichroism Spectra of Tetracycline Complexes with Mg^{+2} and Ca^{+2}

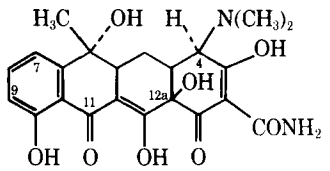
E. C. NEWMAN [*] and C. W. FRANK [x]

Abstract □ The study of Ca^{+2} and Mg^{+2} complexes of tetracycline in buffered solution was undertaken to determine their stoichiometry and the chelation sites. Circular dichroism was used to follow complex formation. Modified tetracyclines, in which potential complexation sites were blocked, were used to determine the participation of particular sites in complexation. Calcium formed a 2:1 metal-ion to ligand complex, while the magnesium complex formed at a 1:1 ratio. Formation of the calcium complex involved addition of one metal ion to the C-10, C-11 site with subsequent addition of a second metal ion at the C-12, C-1 site. The magnesium chelate occurred at the C-11, C-12 β-diketone site.

Keyphrases □ Tetracycline—complexes with Ca^{+2} and Mg^{+2}, circular dichroism spectral investigation of binding properties □ Metal-ion complexes—Ca^{+2} and Mg^{+2} with tetracycline, circular dichroism spectral investigation of binding properties □ Complexes, metal ion—Ca^{+2} and Mg^{+2} with tetracycline, circular dichroism spectral investigation of binding properties □ Spectrometry, circular dichroism—investigation of binding properties of tetracycline complexes with Ca^{+2} and Mg^{+2} □ Antibiotics—tetracycline, complexes with Ca^{+2} and Mg^{+2}, circular dichroism spectral investigation of binding properties

The mode of action of the tetracycline (I) antibiotics is dependent upon the presence of certain metal ions, and the metal-ion complexation of these drugs has been the subject of numerous investigations. The complex chemistry of the tetracyclines and the large number of potential binding sites have led to much confusion as to the location of these sites. A major contributing factor in this problem has been the failure of the commonly used spectroscopic techniques to pinpoint the complexation centers. Circular dichroism, however, seems to be capable of filling this void, as indicated by recent work (1–4).

From an antimicrobial standpoint, the tetracycline complexes with Mg^{+2} and Ca^{+2} are probably the most important, because the concentrations of these cations



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and the large formation constants of their complexes with tetracycline dictate that *in vivo* tetracycline would exist as one of these two complexes.

The tetracycline complexes of Mg^{+2} and Ca^{+2} were studied in pH 7.4 buffered 90% methanol, and differences found in the circular dichroism (as well as fluorescence and UV–visible) spectra were attributed to different conformations and thus different binding sites for the two complexes (1). A four-coordinate binding involving positions C-11, C-12, C-2, and C-3 was proposed for the Ca^{+2} complex; the Mg^{+2} complex was thought to be at a different, unspecified site.

Mitscher *et al.* (2) concluded that, in aqueous solutions of pH 7.4 or below, Ca^{+2} and Mg^{+2} both bind somewhere at the B, C, or D complexation site (C-10, C-11; C-11, C-12; or C-12, C-1) of tetracycline. Above pH 7.5, Ca^{+2} binds between the C-4 dimethylamine group and the C-12a hydroxyl group, but Mg^{+2} cannot.

EXPERIMENTAL

Circular Dichroism Spectra—All circular dichroism spectra were recorded on an optical rotatory dispersion–circular dichroism instrument[1]. Concentrations of tetracyclines were always 1×10^{-4} *M*.

[1] Cary 60.