Overall, the structural requirements for the production of fluorescence from pseudomorphine-like congeners include the following structural features: an unsubstituted C-3 phenolic group, a furan oxygen bridge, and lack of a C-6 carbonyl group. Thus, the observation by Weinstein *et al.* (8) that a C-6 hydroxyl group is necessary for formation of a fluorescent derivative of XVII under these conditions appears to be incomplete.

Certain structural changes modified λ_{em}, intensity, and the Stokes' shift constant (Table III). Substitution of a larger group for the methyl group at the *N*-substituent position generally produced a shift to longer λ_{em} with a concurrent increase in the Stokes' shift constant. The effect of this change on intensity, however, was varied and could increase or decrease. Saturation of the 7,8-double bond caused a sizable shift to shorter λ_{em}, as well as a decrease in intensity and the Stokes' shift constant.

Changes in the carbinol configuration at C-6 from the α to the β orientation consistently decreased λ_{em}. This change also produced a decrease in intensity and a large decrease in the Stokes' shift constant. Inspection of Dreiding molecular models indicates that the alcoholic group in the β orientation is *anti* to the biphenyl ring system and, thus, would exhibit an electron-withdrawing effect, which would result in a shift to shorter wavelengths and higher energy. However, substitution of an alcoholic group at C-14 for hydrogen caused little change in the fluorescence pattern, probably due to its isolation from the biphenyl system.

Substitution of bulkier groups at R_C in the oripavine series of compounds, XXVII–XXX, caused a decrease in λ_{em} as well as large decreases in intensity.

General trends for effects of substituent changes on the fluorescent properties of the series of morphine agonists and antagonists tested are shown in Table IV. The most pronounced effects resulted from (*a*) structural changes at C-3, where fluorescence was abolished by prevention of dimerization; (*b*) loss of the furan oxygen; and (*c*) structural changes in ring C, where fluorescence could be quenched by the presence of a carbonyl group at C-6. Diminution of intensity was produced by structural changes at C-6, C-7, and C-8.

The structural effects on λ_{em} were so pronounced by epimerization at C-6 that it could provide a means of distinguishing isomers in newly synthesized compounds and metabolites of this series with unknown configurations. Furthermore, the methods used for measurement of fluorescence are readily adaptable for quantitative measurements of drug in biological samples. Thus, a general understanding of the requirements for fluorescence of morphine analogs with the ferricyanide reagent may assist in the development of sensitive and specific assays for these compounds.

REFERENCES

(1) H. J. Kupferberg, A. Burkhalter, and E. L. Way, *J. Chromatogr.*, **16**, 558 (1964).

(2) K. W. Bentley and S. F. Dyke, *J. Chem. Soc.*, **1959**, 2574.

(3) H. J. Kupferberg, A. Burkhalter, and E. L. Way, *J. Pharmacol. Exp. Ther.*, **145**, 247 (1964).

(4) A. E. Takemorei, *Biochem. Pharmacol.*, **17**, 1627 (1968).

(5) S. J. Mulé and P. L. Hushin, *Anal. Chem.*, **43**, 708 (1971).

(6) I. Jane and J. F. Taylor, *J. Chromatogr.*, **109**, 37 (1975).

(7) E. J. Cone, *Tetrahedron Lett.*, **1973**, 2607.

(8) S. H. Weinstein, M. Pfeffer, J. M. Schor, L. Indindoli, and M. Mintz, *J. Pharm. Sci.*, **60**, 1567 (1971).

(9) S. Udenfriend, "Fluorescence Assay in Biology and Medicine," vol. II, Academic, New York, N.Y., 1969, p. 7.

(10) H. G. Leeman, K. Stich, and M. Thomas, in "Progress in Drug Research," vol. 6, E. Jucker, Ed., Birkhauser Verlag, Basel, Switzerland, 1963, pp. 160–185.

(11) E. P. Crowell and C. J. Varsel, *Anal. Chem.*, **35**, 189 (1963).

ACKNOWLEDGMENTS

The authors are grateful to Ms. L. J. M. Sturdivant for aid in preparation of this manuscript.

High-Performance Liquid Chromatographic Determination of Pilocarpine in Aqueous Humor: Derivatization by Quaternization of Methylimidazole Tertiary Amine Group

A. K. MITRA, C. L. BAUSTIAN, and T. J. MIKKELSON ˣ

Received July 23, 1979, from the *Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS 66044.* Accepted for publication October 1, 1979.

Abstract □ A derivatization procedure and a high-performance liquid chromatographic (HPLC) method of analysis for pilocarpine are described. The method is based on the quaternization of the 3′-tertiary amino group of the methylimidazole ring of pilocarpine with *p*-nitrobenzyl bromide. The HPLC system employs an RP-ODS column with a methanol–water mobile phase containing octanesulfonate as an ion-pairing agent. The sensitivity of the method permits the analysis of pilocarpine in biological fluids such as aqueous humor. The method is selective for pilocarpine in the presence of isopilocarpine. Its applicability to the analysis of aromatic heterocyclic and alkyl tertiary amines is demonstrated.

Keyphrases □ Pilocarpine—high-performance liquid chromatographic determination in aqueous humor, quaternization of the methylimidazole tertiary amine group □ High-performance liquid chromatography—determination of pilocarpine in aqueous humor, quaternization of the methylimidazole tertiary amine group □ Ocular agents—pilocarpine, high-performance liquid chromatographic determination in aqueous humor □ Tertiary amines—derivatization, quaternization, high-performance liquid chromatographic determination

Pilocarpine (I), a parasympathomimetic amine, is used widely as a topical ocular agent for the reduction of the elevated intraocular pressure associated with glaucoma. The UV absorption characteristics of pilocarpine are such that its direct spectrophotometric analysis is an alternative only when high sensitivity is not a requirement.

BACKGROUND

Various analytical methods are available for the determination of pilocarpine, but they are limited relative to sensitivity, specificity, selectivity, and/or reliability. The official colorimetric method (1) utilizes hydroxylamine to cleave the lactone ring. However, the official method is neither selective nor specific.

A kinetic method of analysis was reported (2). In the method, which is based on the imidazole- or substituted imidazole-catalyzed hydrolysis of 2,4-dinitrophenyl acetate, the extent of catalysis is dependent on the pilocarpine concentration. Microgram quantities of pilocarpine are measurable.

0022-3549/ 80/ 0300-0257$01.00/ 0

© 1980, American Pharmaceutical Association

GLC methods for pilocarpine have been reported. The most recent utilizes the heptafluorobutyric anhydride acylation of the methylimidazole ring of pilocarpine (3). The method is selective and sensitive and is readily capable of measuring pilocarpine at levels found in biological fluids such as aqueous humor. However, it requires extensive sample handling and workup.

High-performance liquid chromotographic (HPLC) methods for pilocarpine (4) and other imidazole bases (5) have been reported. These methods are based on the direct spectrophotometric measurement of pilocarpine (4) and other imidazole bases (5) at 217 and 208 nm, respectively. Although the approach of direct UV detection of pilocarpine (4) is selective and reliable and is suitable for the analysis of levels contained in pilocarpine dosage forms, it lacks adequate sensitivity for pilocarpine at levels found in aqueous humor following topical administration.

This report describes a derivatization and HPLC procedure for the analysis of pilocarpine in aqueous humor. The method is based on the quaternization of the 3′-tertiary amino group of the methylimidazole ring of pilocarpine with the chromophore *p*-nitrobenzyl bromide. The utilization of a reversed-phase chromatographic system with an ion-pairing agent is described. The method is reliable, because little sample handling is required; the method is selective since pilocarpine and isopilocarpine are separated and determined; and the method is sensitive in that it is applicable to the determination of pilocarpine levels encountered experimentally in aqueous humor. The applicability of the quaternization procedure to the analysis of other tertiary amines, such as aliphatic and aromatic heterocyclic amines, is also described.

EXPERIMENTAL

Materials—Pilocarpine nitrate[1] and isopilocarpine nitrate[2] were used as received. *p*-Nitrobenzyl bromide[2] was recrystallized from water–acetonitrile. The ion-pairing agent, 1-octanesulfonic acid sodium salt[3], was used as received, as was acetonitrile[4]. Methylene chloride[5] (certified ACS grade) was distilled from phosphorus pentoxide prior to use. HPLC grade methanol[5] also was used. All other reagents and chemicals used were analytical reagent grade or high purity.

Equipment—The HPLC system was comprised of a solvent delivery system[6], a septumless injector[7], a UV absorbance detector[8], and a 10-mv strip-chart recorder[9]. Absorbance was measured at 254 nm. Sensitivity settings to 0.005 aufs were used. The column was a 30-cm RP-ODS column with an inside diameter of 3.9 mm and a 10-μm (spherical) particle size[10].

NMR spectra of pilocarpine and the derivative were obtained on a spectrometer[11] in the pulse mode. Spectra were obtained using solutions in deuterium oxide and acetone-d_6, with tetramethylsilane as the internal reference at concentrations of 10–20%.

Chromatographic Conditions—All chromatography was conducted at ambient temperature. The mobile phase consisted of an 80% (v/v) methanol in water mixture containing 1×10^{-3} *M* sodium octanesulfonate. The mobile phase was filtered and degassed prior to use. The flow rate was 1.6 ml/min, which generated a column pressure of 1000–1500 psig.

Glassware—The centrifuge tubes and ampuls used in the derivatization procedure were silanized by soaking for several hours in a solution of 5% dimethyldichlorosilane[1] in toluene and rinsing with toluene, methanol, and methylene chloride (3).

Derivatization—Volumes of 100 μl of either standard aqueous solutions of pilocarpine nitrate or aqueous humor samples were added to 500 μl of 0.3 *M* potassium bicarbonate solutions (pH 8.4) in silanized centrifuge tubes. The resulting solutions were each immediately extracted (vortexed for 1 min and centrifuged at 2000 rpm for 5 min at ambient temperature) with two 1.0-ml volumes of methylene chloride. Aliquots of the two extracts from each sample were combined in silanized glass ampuls and evaporated to dryness under nitrogen at 40°. To the dried samples, a 200-μl volume of a solution of *p*-nitrobenzyl bromide in acetonitrile (0.25 mg/ml) was added.

[1] Sigma Chemical Co.
[2] Aldrich Chemical Co.
[3] Eastman Kodak Co.
[4] Baker Analyzed HPLC reagent, J. T. Baker Chemical Co.
[5] Fisher Scientific Co.
[6] Model 6000A, Waters Associates.
[7] Model U6K, Waters Associates.
[8] Model 440, Waters Associates.
[9] Omniscribe model B-5117-1, Houston Instruments.
[10] μBondapak C_{18}, Waters Associates.
[11] Model R-24B, Hitachi/Perkin-Elmer Corp.

The ampuls were sealed at ambient pressure under a nitrogen atmosphere, using an oxygen torch flame, and reacted for 24 hr at 40°. Then they were cooled to ambient temperature and opened. The chromatography was conducted by direct injection of an aliquot of the reaction mixture or an internal standard solution was added, the mixture was vortexed, and an aliquot was injected. The internal standard solution was a 10-μl volume of phenylephrine hydrochloride in methanol at 2.0 mg/ml.

Milligram Quantities of Derivative—Pilocarpine nitrate, 50 mg, was dissolved in 25 ml of 0.3 *M* aqueous potassium bicarbonate and extracted with 25 ml of methylene chloride. The organic layer was separated, and the methylene chloride was removed under vacuum. The product obtained was a hydrated pilocarpine base as a clear or very slightly yellow, highly viscous fluid. The pilocarpine base, 50 mg, was combined with 500 mg of *p*-nitrobenzyl bromide in 10 ml of acetonitrile. The resulting solution was reacted at 40° for 24 hr, the reaction mixture was placed in a glass mortar, and the quaternary ammonium reaction product was precipitated by ether addition.

The precipitate was repeatedly washed and purified by trituration in the presence of ether. Then it was dried and stored under vacuum. After thorough drying, the solid was used for UV, mass spectral, and NMR analyses. In addition, the product was used to confirm the chromatographic peak used for quantitation of pilocarpine in the analytical procedure.

Standard Curves—Volumes of 100 μl of aqueous solutions of pilocarpine nitrate ranging in concentration from 0.3 to 10.0 μg/ml were used to develop standard curves. The previously described procedure for derivatization was followed. Standard curves were constructed on a basis of the measured peak heights of the analyte (II), which were compared to both an internal and an external standard. The internal standard, phenylephrine hydrochloride, was used in construction of the standard curve, which was applied to the analysis of samples obtained in *in vitro* transport studies across isolated, mounted corneal membranes.

In vivo pilocarpine absorption studies, where the test solutions were aqueous humor samples obtained as a function of time following topical dosing, and studies using spiked aqueous humor samples resulted in a contaminant that interfered with the internal standard. Therefore, all aqueous humor samples were measured against an external standard of II, which was pure analyte obtained in the milligram quantity preparation procedure described previously. When used, the internal standard was added to the reaction mixture after the derivatization reaction was completed and just prior to injection. This approach was suitable in view of the extraction efficiencies obtained.

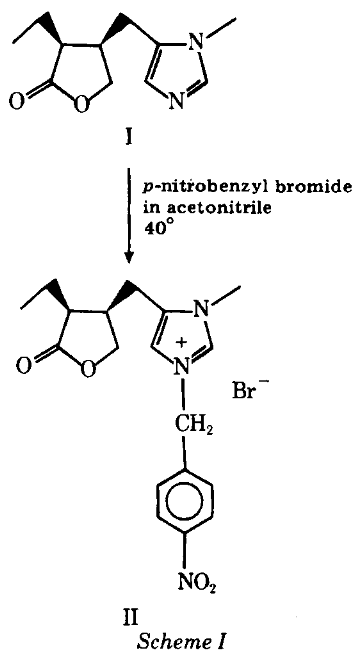
Aqueous Humor Samples—New Zealand albino rabbits were sacrificed by marginal ear vein administration of pentobarbital sodium solution. The corneal surface was rinsed with normal saline solution and patted dry with tissue paper. Aqueous humor samples were obtained from the anterior chamber by paracentesis by entering at the limbus with a 1-ml tuberculin syringe fitted with a 27-gauge needle.

RESULTS AND DISCUSSION

Analyte Characteristics—The derivatizing reaction, which is the reaction of pilocarpine (I) with the chromophore *p*-nitrobenzyl bromide to form the quaternary ammonium analyte (II) *p*-nitrobenzyl pilocarpine bromide, is shown in Scheme I. The derivative II displayed a UV absorbance maximum at 263 nm with a molar absorptivity of 1.20×10^4. At 254 nm, this analyte displayed a molar absorptivity of 1.07×10^4.

Under the described chromatographic conditions, the capacity factor, k', for II was 4.87. In comparison, the capacity factor for derivatized isopilocarpine was 5.40. The resulting selectivity was evidenced by a separation factor, α, of 1.11; the degree of separation was evidenced by a resolution, R, of 0.80.

Kinetic experiments were conducted to optimize the reaction conditions in terms of the temperature required and the excess *p*-nitrobenzyl bromide required to achieve complete or near complete production of II in a reasonable time. Reactions forming quaternary salts from tertiary amines and alkyl halides, the Menschutkin reaction (6), are not fast (6–9). They are biomolecular and clearly second order (7). A significant sensitivity to the size and disposition of the nitrogen-substituted alkyl groups has been demonstrated; for example, quinuclidine, which has an exposed nitrogen, is 200-fold more reactive than triethylamine toward methyl chloride (7). Alkyl halide substitutions also significantly alter the quaternization rate. Approximately a 500-fold reduction in the conversion rate was detected through an alkyl series where the following reactivities were found: methyl > ethyl > higher normal alkyls > isopropyl (6, 8). The derivatizing agent, *p*-nitrobenzyl bromide, was chosen because of its UV



Scheme I

absorption characteristics. However, it was not expected to react rapidly with pilocarpine because of its molecular size, even though the 3′-tertiary nitrogen of the methylimidazole group of pilocarpine is an exposed nitrogen.

Preliminary kinetic studies were conducted at several temperatures, and 40° was chosen for the analytical derivatization. Molar ratios of *p*-nitrobenzyl bromide to I of 40, 125, 420, and 600 were studied. Pseudo-first-order kinetic behavior was observed in each instance, and a decreasing reaction time (10) was observed as a function of increasing molar ratios.

The conditions selected, as described in the procedure for derivatization, corresponded to a molar ratio of *p*-nitrobenzyl bromide to pilocarpine of approximately 600 for a pilocarpine sample solution containing 1.0 μg/ml. These particular conditions were selected because the reaction proceeded with a half-life of ~3 hr without any detectable side reactions. As a result, the reaction was essentially complete in the 24-hr reaction time. In addition, the selected level of *p*-nitrobenzyl bromide did not affect resolution of the analyte or the internal standard.

Comparisons of the NMR spectrum of pilocarpine and the analyte are presented in Table I (the proton numbering system is given in Structure III). The protons of pilocarpine were all accounted for in the derivative. The protons of the ethyl-substituted lactone ring and the methylene bridge were essentially unchanged. However, the protons of the methylimidazole ring demonstrated significant downfield shifts. The *N*-methyl protons, which appeared as a singlet, underwent a downfield shift in the analyte of 0.26 ppm due to the flow of electrons toward the quaternary nitrogen. The 2′- and 4′-protons underwent extensive deshielding. The 2′-proton was found downfield at 11.1 ppm in the analyte compared to 8.6 ppm in pilocarpine. The 4′-proton was at 7.3 ppm in pilocarpine and at 8.1 ppm in the analyte. However, the 4′-proton did not appear as a singlet in the analyte but overlapped as a multiplet with the aromatic protons.

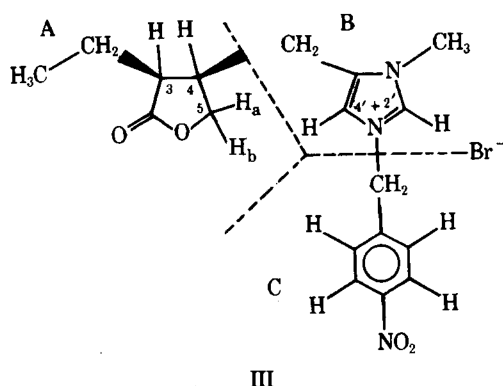


Table I—Chemical Shifts in Parts per Million Using Tetramethylsilane Standard for Pilocarpine in Deuterium Oxide and Analyte in Acetone-d_6 [a]

		Pilocarpine[b]	Analyte[b]
Lactone ring with bridge	CH_3–CH_2 (CH3 italic)	1.02 (t)	1.03 (t)
	CH_3–CH_2 (CH2 italic)	1.71 (m)	1.64 (m)
	–CH_2-bridge plus 3H,4H	2.81 (m)	3.15 (m)
	5H_a	4.17 (dd)	4.31 (dd)
	5H_b	4.19 (dd)	4.32 (dd)
Methylimidazole group	N–CH_3	3.77 (s)	4.04 (s)
	2′H	8.60 (s)	11.1 (s)
	4′H	7.30 (s)	8.1 (m)[c]
Chromophore	–CH_2–	—	5.96 (s)
	Aromatic	—	8.10 (m)[c]

[a] See Structure III for numbering. [b] s = singlet, dd = double doublet, t = triplet, and m = multiplet. [c] The 4′-proton in the analyte occurs as a multiplet with the aromatic protons.

The integration of the analyte spectrum clearly demonstrated the formation of a one-to-one reaction product. The multiplet at 8.1 ppm was indicative of five protons, the four aromatic protons of the chromophore plus the 4′-proton of the imidazole ring. The singlet at 5.96 ppm, which is the methylene group of the *p*-nitrobenzyl moiety, was indicative of two protons. In comparison, the *N*-methyl group, the lactone–imidazole methylene bridge, the methylene group of the lactone ethyl group at the 3-position, and the methyl group of the lactone ethyl group at the 3-position clearly were indicative of three, two, two, and three protons, respectively.

The mass spectrum of the analyte III did not display a molecular ion peak. The lability of the bond between the quaternary nitrogen of the methylimidazole ring and the methylene of the *p*-nitrobenzyl group was demonstrated by the abundance of *m/e* 208 and 136 fragments, corresponding to a fragment of A + B (pilocarpine), and a fragment of C in III. Further fragmentation was apparent as a function of the abundance of *m/e* 113 (A in III), 84 (A − –C_2H_5), 95 (B in III), and 80 (B − –CH_3) fragments.

The product sample used to obtain NMR and mass spectral data was isolated and purified from a reaction run at 40° for 24 hr, which indicated that the reaction conditions used to generate the analyte did not result in unexpected side reactions. *p*-Nitrobenzyl bromide and pilocarpine are stable under the conditions of the reaction in that all protons are accounted for and other structural integrity is observed. The reaction follows the expected bimolecular behavior, resulting in the quaternary derivative.

Standard Curve and Detection Limits—Following the procedure for derivatization, plots of calculated peak height ratios, where the analyzed solutions were sample aqueous solutions of pilocarpine nitrate in the concentration range of 0.3–10.0 μg/ml and the internal standard was phenylephrine hydrochloride, were constructed. These plots were linear and reproducible with typical results of $n = 10$, slope = 0.144, intercept = 0.018, and correlation coefficient = 0.995.

Figure 1*a* is the chromatographic result of the derivatization of a standard sample of an aqueous solution containing 6.0 μg/ml of pilocarpine nitrate, approximately equivalent to an injection of 100 ng of pilocarpine. The excess *p*-nitrobenzyl bromide did not interfere with the internal standard or the analyte peaks. Figure 1*b* is the chromatographic result obtained from spiked aqueous humor containing 1.8 μg/ml of pilocarpine nitrate. The internal standard was not added to aqueous humor or spiked aqueous humor samples because of overlapping and interfering substances. For aqueous humor samples, an external standard was employed. Standard curves of peak height plotted against concentration, which were constructed from standard pilocarpine nitrate solutions, from spiked aqueous humor samples, and directly from solutions of known concentrations of analyte, were linear.

The initial step in the derivatization procedure is the double extraction with methylene chloride of the aqueous solutions or samples to which bicarbonate has been added. The efficiency of this extraction was evaluated in two ways. First, several standard pilocarpine solutions were carried through the derivatizing procedure, including the extraction step. The chromatographic results or peak heights obtained were compared to the peak heights obtained from solutions prepared directly with known concentrations of analyte. The chromatographic results were within ±2%, which confirms the extraction efficiency.

Second, aqueous humor samples were spiked with several known concentrations of tritiated pilocarpine and split into two sets. One set

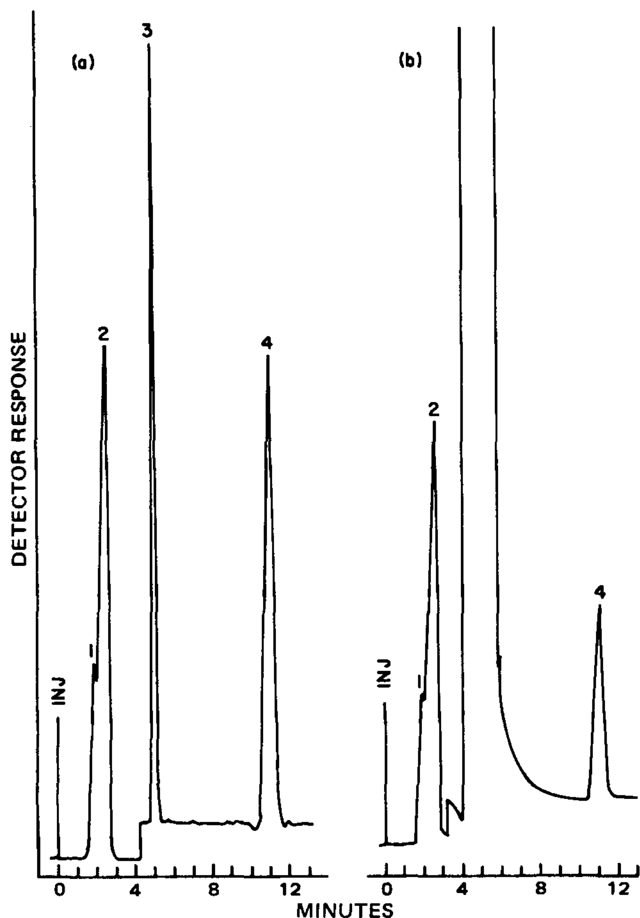


Figure 1—(a) *Chromatogram resulting from a standard pilocarpine nitrate solution. The peaks are: 1, solvent front; 2, excess* p*-nitrobenzyl bromide; 3, internal standard; and 4, analyte.* (b) *Chromatogram resulting from a spiked aqueous humor sample. No internal standard was added because of the broad overlapping contaminant peak.*

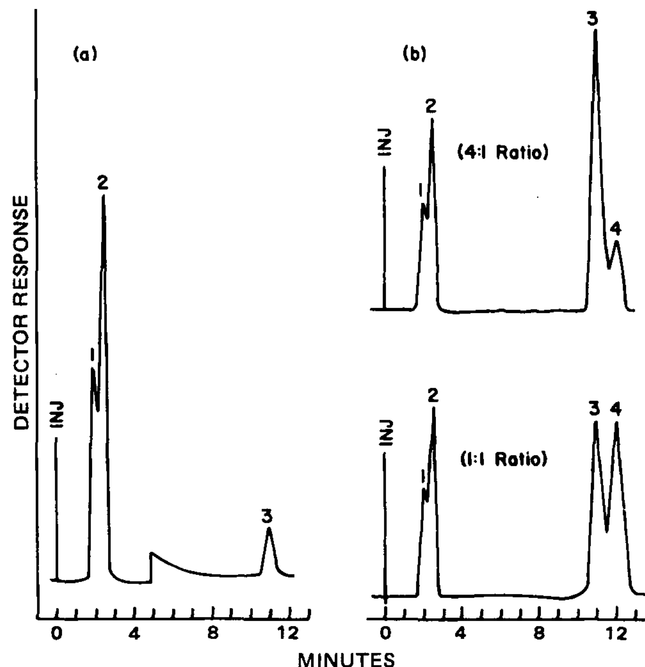


Figure 2—(a) *Chromatogram resulting from an initial solution of pilocarpine nitrate containing 0.05 μg/ml where 0.005 aufs was employed. This amount represents the equivalent of a 3.8-ng injection of pilocarpine. Peak height response linearity was observed at this level.* (b) *Chromatogram resulting from samples containing pilocarpine and isopilocarpine at ratios of 4:1 and 1:1. The peaks in both* a *and* b *are: 1, solvent front; 2, excess* p*-nitrobenzyl bromide; 3, analyte or derivatized pilocarpine (II); and 4, derivatized isopilocarpine.*

was assayed by the derivatization procedure, including the extraction step, and the other set was counted for radioactivity (11). The two sets of analytical results were within ±5%. In the procedure, the solutions containing pilocarpine to which a bicarbonate solution has been added are twice extracted with methylene chloride. The two extractions achieved a total transfer in excess of 98% of the pilocarpine, which is consistent with a previously reported extraction procedure for pilocarpine (2).

The results of the experiments conducted to determine the extraction efficiency support the use of the internal reference standard that is added just prior to injection and not added to the system prior to extraction. Also supported is the use of an external reference standard prepared directly from purified analyte rather than the use of standard solutions of pilocarpine nitrate which are carried through the complete derivatization procedure.

Of the pilocarpine nitrate solutions used to construct standard curves as outlined in the procedure for derivatization, the lowest concentration used was 0.3 μg/ml. To enhance sensitivity, experiments were conducted with 100-μl volumes of the *p*-nitrobenzyl bromide solutions in acetonitrile at 0.5 mg/ml (rather than 200 μl at 0.25 mg/ml). Decreasing the derivatizing reaction volume permitted the volume of the injected aliquot to approach the total volume of the reaction mixture.

Figure 2*a* gives the result of an experiment approximating the practical sensitivity limits of the procedure. The result was obtained from a pilocarpine nitrate solution at 0.05 μg/ml. The injection contained the equivalent of 3.8 ng of pilocarpine. Detection at this level was quantitative. The absolute limits of detection were not determined but are less than the 3.8 ng (or 0.05 μg/ml of pilocarpine nitrate) used previously.

Selectivity—To assess method selectivity, solutions were prepared that contained different ratios (4:1, 1:1, and 1:4) of pilocarine (I) to isopilocarpine (IV). These solutions were run through the described procedure and compared to samples of pure I and IV. A sample result is shown in Fig. 2*b*. The peak height ratios obtained (peak height of I to peak height of IV) were in excellent agreement with the concentration ratios of the initial solutions.

The results suggest that: (*a*) conversion of pilocarpine to isopilocarpine does not occur as a result of the procedure, (*b*) the method is quantitative for isopilocarpine and is applicable to the determination of the amount of isopilocarpine present in pilocarpine as a contaminant, and (*c*) the absorptivity of the pilocarpine and isopilocarpine derivatives is the same at 254 nm. In experiments conducted at the higher pilocarpine nitrate concentrations, *e.g.*, 10 μg/ml, the presence of contaminating isopilocarpine nitrate in the commercial pilocarpine nitrate was observed.

Other Nucleophiles—The primary intent of this study was to develop a method for the low level analysis of pilocarpine in aqueous humor. The method described is based on the reaction between the alkyl halide *p*-nitrobenzyl bromide and the 3′-tertiary amino group of the methylimidazole ring of pilocarpine, which results in the formation of the quaternary ammonium analyte II. Methods are readily available in the literature for the derivatization of primary and secondary amines for purposes of analysis (10, 12, 13), but this is not the case for tertiary amines.

The applicability of this derivatization procedure was investigated for other tertiary amines. Milligram samples of triethylamine, 4-methoxypyridine, and 1-methylimidazole were derivatized and chromatographed. The capacity factors, k', obtained for the resulting quaternary amine derivatives were 5.93, 6.47, and 6.73, respectively. The results indicate that the quaternization reaction using an alkyl halide, such as *p*-nitrobenzyl bromide, can be readily applied to the HPLC analysis of aromatic, heterocyclic, and alkyl tertiary amines in general. There is also flexibility relative to the choice of alkyl halide. An alternative for enhanced UV detection would be the selection of a chromophore with greater molar absorptivity. Another alternative would be the selection of an alkyl halide fluorophore, which would afford the advantage of greater sensitivity of fluorescence.

REFERENCES

(1) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975, pp. 385, 386.

(2) A. Repta and T. Higuchi, *J. Pharm. Sci.*, **60,** 1465 (1971).

(3) W. F. Bayne, L.-C. Chu, and F. T. Tau, *ibid.*, **65,** 1724 (1976).

(4) T. Urbanyi, A. Piedmont, E. Willis, and G. Manning, *ibid.*, **65,** 257 (1976).

(5) E. Gaetani and C. F. Laureri, *Farmaco, Ed. Prac.*, **33**, 26 (1978).
(6) N. Menschutkin, *Z. Phys. Chem.*, **5**, 589 (1890).
(7) C. G. Swain and N. D. Hershey, *J. Am. Chem. Soc.*, **94**, 1901 (1972).
(8) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," 2nd ed., Cornell University Press, New York, N.Y., 1969, p. 435.
(9) R. B. Wagner and H. D. Zook, "Synthetic Organic Chemistry," Wiley, New York, N.Y., 1953, p. 665.
(10) W. Morozowich and M. Cho, in "GLC and HPLC Determination of Therapeutic Agents, Part 1," K. Tsuji and W. Morozowich, Eds., Dekker, New York, N.Y., 1978, pp. 222–224.
(11) T. Patton, *J. Pharm. Sci.*, **66**, 1058 (1977).
(12) T. H. Jupille, *Am. Lab.*, **1976**, 85.
(13) J. F. Lawrence and R. W. Frei, "Chemical Derivatization in Liquid Chromatography," Elsevier, New York, N.Y., 1976.

ACKNOWLEDGMENTS

Supported in part by National Eye Institute Grant EY02328, by National Institute of General Medical Sciences Grant GM22357, and by Biomedical Research Support Grant SO7-RR-5606.

Dosage Form Design for Improvement of Bioavailability of Levodopa II: Bioavailability of Marketed Levodopa Preparations in Dogs and Parkinsonian Patients

KUNIHIRO SASAHARA ˣ, TAKASHI NITANAI, TAEKO HABARA, TADASHI MORIOKA, and EIICHI NAKAJIMA

Received March 16, 1979, from the *Product Development Laboratories, Sankyo Company, Ltd., 1-2-58, Hiromachi, Shinagawa, Tokyo, Japan.* Accepted for publication September 14, 1979.

Abstract ☐ To estimate the absolute bioavailability of oral levodopa, plasma concentrations and urinary excretion of levodopa and its metabolites were determined in beagle dogs and in parkinsonian patients after intravenous and oral drug administration. The absolute bioavailability of orally administered levodopa was estimated to be about 35% in both dogs and patients; however, the total amount absorbed of intact drug and levodopa metabolites was estimated to be 80–90% of the administered dose. Due to the similarities of the pharmacokinetic characteristics of levodopa found in beagle dogs and in humans, beagle dogs can serve as a model to study bioavailability, absorption, and metabolic mechanisms.

Keyphrases ☐ Levodopa—bioavailability, dosage form design, dogs and humans ☐ Bioavailability—levodopa, dosage form design, dogs and humans ☐ Antiparkinsonian agents—levodopa, bioavailability, dosage form design, dogs and humans

Much higher oral doses than intravenous doses of levodopa are required to achieve a therapeutic effect in parkinsonian patients, and the resultant plasma levels after oral levodopa are markedly lower than those in patients receiving the drug intravenously.

Rivera-Calimlim *et al.* (1, 2) reported that when levodopa was administered orally to parkinsonian patients, a considerable portion apparently was metabolized *via* reactions in the stomach and intestines before it was absorbed. Other investigators (3–6) reported that 50–70% of the oral doses appeared to be metabolized in the intestinal mucosa of parkinsonian patients.

This paper reports part of a continuing study of levodopa to delineate mechanisms involved in the absorption profile in humans and to develop new dosage form designs to improve the bioavailability of levodopa. The absolute bioavailability of levodopa from marketed conventional preparations was determined by measuring the plasma levels and urinary excretion of levodopa and its metabolites after intravenous and oral administration of single doses to beagle dogs and to parkinsonian patients.

EXPERIMENTAL

Single Intravenous Doses in Dogs—After six healthy male beagle dogs, 10.3–12.5 kg, had been fasted for ~16 hr, levodopa[1] at a 50-mg dose was injected over 30 sec into the brachial vein. Blood samples were withdrawn with a heparinized syringe from the contralateral brachial vein. Samples were obtained 0, 2, 5, 15, and 45 min and 1, 1.5, 2, 3, 4, and 6 hr after dosing. Sodium metabisulfite solution (5% in a saline solution, 0.1 ml/5 ml of blood) and disodium ethylenediaminetetraacetate (2% in a saline solution, 0.1 ml/5 ml of blood) were added to the freshly drawn heparinized blood, and the plasma was separated immediately by centrifugation at 4°. All procedures were conducted in an ice bath.

Urine was collected before dosing and for 48 hr in bottles containing 5 ml of 6 *N* HCl and 3 ml of 0.2 *M* disodium ethylenediaminetetraacetate. The pH of the collected urine was adjusted to 2.0, and the urine was stored at −20° until assayed.

Single Oral Doses in Dogs—The same six beagle dogs used in the single intravenous dose studies were forcefully administered 250 mg of levodopa in capsule form[2] orally with 10 ml of warm water. The dogs' mouths were closed by hand to prevent emesis. Blood samples were withdrawn with a heparinized syringe 0, 0.5, 1, 2, 3, 4, and 6 hr after oral administration.

The blood samples were treated using the same procedures as in the intravenous study. The urine also was collected before dosing and for 48 hr after oral administration following the same procedure used for intravenous administration.

Single Intravenous Doses in Patients—Five parkinsonian patients (three men and two women) were studied. Their mean age was 63 years (range 51–71). Three days prior to the experiment, all levodopa preparations and other drugs that had been administered for the treatment of Parkinson's disease were withdrawn. Levodopa[1], 50 mg, diluted with 200 ml of physiological saline, was infused for 20 min at a constant rate into the brachial vein of these patients. Blood samples were withdrawn with a heparinized syringe at 0, 10, and 20 min during infusion and at 15 and 30 min and 1, 2, and 3 hr after infusion. The blood and urine samples were collected and processed in the same way as were those taken from the dogs.

Single Oral Doses in Patients—Six parkinsonian patients (three men and three women) were studied. Their mean age was 68 years (range 58–74). Three days before drug administration, all levodopa preparations and other drugs that had been administered for the treatment of Par-

[1] Dopaston Injection, Sankyo Co. Ltd., Tokyo, Japan.

0022-3549/ 80/ 0300-0261$01.00/ 0
© 1980, American Pharmaceutical Association