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RESEARCH ARTICLES

Fluorescence Properties of Pseudomorphine and Congeners: Structure–Activity Relationships

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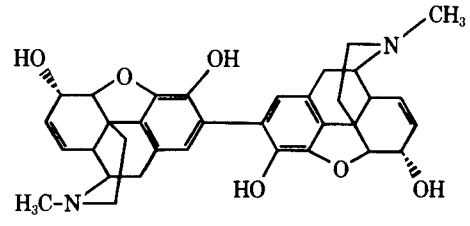
Abstract □ The fluorescence properties of morphine congeners oxidized with potassium ferro-ferricyanide are described. Fluorescence did not occur in congeners with the following structural features: an alkyl or acyl group at the 3-*O*-position, a C-6 carbonyl group, or absence of the furan oxygen. Masking the carbonyl group by ethylene ketal formation effectively restored fluorescence. Morphine congeners that fluoresced were most sensitive to structural changes at C-6, C-7, and C-8. Details of effects of structural changes on emission maxima, intensity, and Stokes' shift are reported.

Keyphrases □ Morphine congeners—ferro-ferricyanide oxidation, fluorescence characteristics □ Pseudomorphine—structural variations, fluorescence □ Fluorometry—structural effects, intensity, Stokes' shift, structural analysis □ Structure–activity relationships—pseudomorphine and congeners

Morphine (I, Table I) can be oxidized to the bimolecular base pseudomorphine (II) by reaction with potassium ferricyanide in aqueous solution (1). The structure of this derivative was shown to be 2,2′-bimorphine on the basis of spectral and chemical studies (2). Because of the extended conjugated system, the fluorescence properties of II are greatly enhanced over that of I.



II

Several fluorometric assays of I in biological material are based on the conversion of I to a pseudomorphine derivative (3–6). The occurrence of fluorescence from oxidized solutions containing morphine congeners also has been observed (1) and used for structural verification of metabolites of naltrexone (XVII) (7) and naloxone (XXII) (8). However, a detailed understanding of the structural requirements for fluorescence of pseudomorphine congeners is not available for prediction of fluorescence of new compounds, and there is some ambiguity in the literature concerning the requirements for fluorescence of morphine congeners (1, 8). Consequently, a study was made of the fluorescence properties of a series of morphine analogs to determine their basic structural requirements for fluorescence under oxidizing conditions. An explanation is also proposed for the unusual quenching properties of XVII and XXII.

EXPERIMENTAL

Apparatus—A spectrofluorometer[1] equipped with an *x–y* recorder[2] was used for determining excitation and emission spectra and for measuring fluorescent intensities. Spectra were not corrected for variations in lamp intensity or photomultiplier sensitivity but were calibrated with quinine sulfate at 350 and 450 nm before each measurement.

Chemicals[3]—All chemicals were reagent grade quality.

TLC—Glass fiber sheets[4] were developed with ethyl acetate–methanol–aqueous ammonia (70:10:5 v/v/v). The developing chamber was allowed to equilibrate 10 min prior to elution. After the sheets were developed and dried, they were inspected for fluorescence under 250- and 360-nm UV light. The plates were then sprayed with potassium iodoplatinate solution for visualization of morphine and pseudomorphine-like components.

Preparation of Standard Solutions—Quinine sulfate solutions (1 mg/liter) were prepared in 0.1 *N* H_2SO_4. Solutions of morphine (10 μg/ml) and congeners were prepared in distilled water. The oxidant solution was a mixture of potassium ferricyanide (28.85 mg) and potassium ferrocyanide (2.45 mg) in 50 ml of distilled water (1). The latter solution was prepared fresh daily, and an aliquot was diluted 10-fold before use.

Oxidation and Measurement of Fluorescence—Equal volumes of drug solution (5 ml, 10 μg/ml) and phosphate buffer (5 ml, 0.1 *M* K_2HPO_4, pH 8.5) were mixed and treated with the freshly prepared oxidant solution (0.5 ml). The mixture was allowed to remain at room temperature for 10 min. During the waiting period, the instrument was

[1] Model MK-1, Farrand Optical Co., New York, N.Y.
[2] Model F-100, Varian Associates, Palo Alto, Calif.
[3] Fisher Scientific Co., Fair Lawn, N.J.
[4] Gelman I.T.L.C., type S.G., Gelman Instrument Co., Ann Arbor, Mich.

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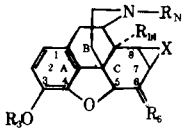
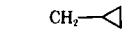
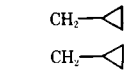
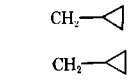
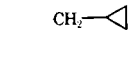
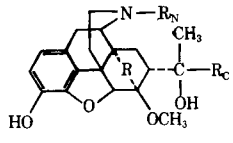
Table I—Structures and Fluorescence Properties of Morphine Congeners Oxidized with Potassium Ferro-ferricyanide



Compound	Structure					Fluorescence Characteristics[b]			
	R_3	R_6	R_{14}	R_N	X[a]	λ_{ex}	λ_{em}	I^{-1}	Stokes' Shift
I Morphine	H		H	CH_3	—	324	434	18.9	7823
III α-Isomorphine	H		H	CH_3	—	324	429	22.4	7554
IV Codeine	CH_3		H	CH_3	—	n.o.	n.o.		
V Normorphine	H		H	H	—	324	436	12.7	7928
VI Norcodeine	CH_3		H	H	—	n.o.	n.o.		
VII 6-Monoacetylmorphine	H		H	CH_3	—	323	433	22.0	7865
VIII Diacetylmorphine	CH_3CO		H	CH_3	—	n.o.[c]	n.o.[c]		
IX Nalorphine	H		H	$CH_2CH{=}CH_2$	—	323	437	127.1	8076
X Dihydromorphine	H		H	CH_3	H_2	321	427	22.5	7733
XI Dihydro-α-isomorphine	H		H	CH_3	H_2	322	422	28.6	7359
XII Hydromorphone	H	O	H	CH_3	H_2	n.o.	n.o.		
XIII Hydrocodone	CH_3	O	H	CH_3	H_2	n.o.	n.o.		
XIV Dihydrodesoxy-morphine-D	H		H	CH_3	H_2	322	422	17.0	7359
XV 6α-Naltrexol	H		OH		H_2	323	432	23.5	7812
XVI 6β-Naltrexol	H		OH		H_2	322	422	112.2	7359
XVII Naltrexone	H	O	OH		H_2	n.o.	n.o.		
XVIII *N*-Methylcyclo-propylnoroxymorphone cyclic ethylene ketal	H		OH		H_2	322	423	77.9	7415
XIX *N*-Methylcyclo-propylnoroxycodone	CH_3	O	OH		H_2	n.o.	n.o.		
XX 6α-Naloxol	H		OH	$CH_2CH{=}CH_2$	H_2	323	433	18.4	7865
XXI 6β-Naloxol	H		OH	$CH_2CH{=}CH_2$	H_2	322	423	30.3	7415
XXII Naloxone	H	O	OH	$CH_2CH{=}CH_2$	H_2	n.o.	n.o.		
XXIII 6α-Oxymorphol	H		OH	CH_3	H_2	323	431	38.9	7758
XXIV 6β-Oxymorphol	H		OH	CH_3	H_2	322	422	101.9	7359
XXV Oxymorphone	H	O	OH	CH_3	H_2	n.o.	n.o.		
XXVI Oxycodone	CH_3	O	OH	CH_3	H_2	n.o.	n.o.		



Compound	Structure			Fluorescence Characteristics[b]			
	R_N	R_C	R	λ_{ex}	λ_{em}	I^{-1}	Stokes' Shift
XXVII Etorphine	CH_3	$CH_2CH_2CH_3$	CH=CH	328	442	86.6	7863
XXVIII 6,14-*endo*-Etheno-7-(2-hydroxy-2-heptyl)-tetrahydrooripavine	CH_3	$(CH_2)_4CH_3$	CH=CH	327	441	500.1	7905
XXIX Diprenorphine		CH_3	CH_2CH_2	326	441	32.4	7999
XXX Buprenorphine		$C(CH_3)_3$	CH_2CH_2	326	439	298.3	7896

[a] The designations — and H_2 represent unsaturation and saturation of the 7,8-bond, respectively. [b] The constants λ_{ex} and λ_{em} are reported in nanometers, and Stokes' shift constant is reported in centimeters^{-1}. Intensity (I^{-1}) is equivalent to the concentration of the compound, in micrograms per milliliter, that will give the same fluorescence intensity as 1 μg/ml of quinine sulfate. The fluorescence of all compounds was measured at pH 8.5 at a concentration of 5 μg/ml, with the exception of XXVII–XXX which were measured at pH 7.0 due to insolubility at higher pH. n.o. indicates that fluorescence was not observed. [c] A small amount of fluorescence was observed due to contamination of VIII by hydrolysis to VII.

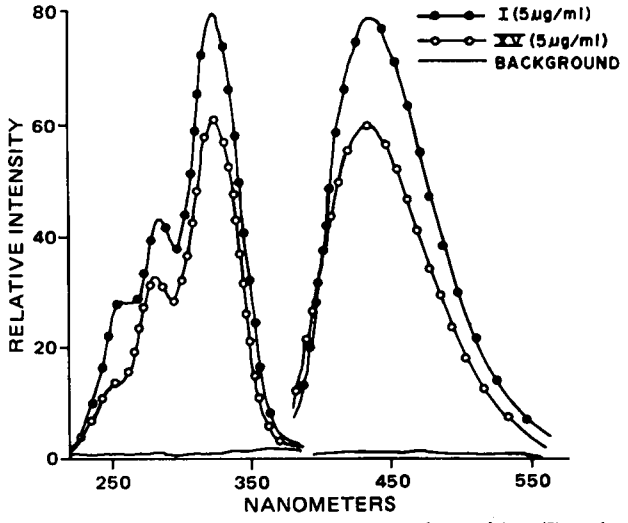


Figure 1—*Excitation and emission spectra of morphine (I) and 6α-naltrexol (XV) oxidized with potassium ferro-ferricyanide at pH 8.5. Both concentrations were 5 μg/ml; λ_{ex} = 324 nm and λ_{em} = 434 for I, and λ_{ex} = 323 nm and λ_{em} = 432 for XV.*

calibrated with quinine sulfate solution for wavelength and intensity corrections.

The excitation maximum, λ_{ex}, of the drug solution was located, and relative intensity was recorded. Background corrections were made using drug solutions treated similarly but without added oxidant. Contributions from background were generally <5%. All drugs were analyzed in triplicate, and the mean of the analyses is reported.

RESULTS AND DISCUSSION

Morphine congeners were oxidized with potassium ferro-ferricyanide solution to form pseudomorphine-like products similar to those formed from reaction of I. Fluorescence properties of these products were measured with a spectrofluorometer calibrated with quinine sulfate solution. The procedure used for the oxidation step was adapted from that of Kupferberg *et al.* (1) for the oxidation of I. Optimum conditions for production of fluorescence (pH and reaction time) were established prior to fluorescence measurement.

Typical excitation and emission spectra of the oxidation products of XV and I are shown in Fig. 1. The spectral similarities of XV to I are apparent. Three absorption bands were observed for all morphine congeners that exhibited fluorescence. Emission spectra characteristically showed a single maximum. The fluorescence properties of the oxidized morphine congeners are presented in Table I. The most intense absorption bands, λ_{ex} and λ_{em}, are reported. In addition, a measure of the reciprocal of the fluorescent intensity, I^{-1} (equivalent to the concentration of the compound in micrograms per milliliter that will give the same fluorescent intensity as 1 μg/ml of quinine sulfate), Stokes' shift constant (9, 10), and a structure are included. The Stokes' shift constant, a measure of the energy dissipated during the lifetime of the excited state before return to the ground state, also reflects energy differences in shifts of equal magnitude at different wavelengths.

Table II—Compounds that Did Not Fluoresce under Oxidizing Conditions and Associated Mechanisms Preventing Fluorescence

Compound	Apparent Structural Feature Involved in Prevention of Fluorescence
Codeine, α-isocodeine, norcodeine, 6α-hydrocodol, 6β-hydrocodol, 6α-oxycodol, 6β-oxycodol, hydrocodone[a], oxycodone[a], *N*-methylcyclopropylnoroxycodone[a], dextromethorphan[a], ethylmorphine, diacetylmorphine, 14-hydroxycodeine	Prevention of dimerization at C-2 by alkylation or acylation of the C-3 phenol group
Levallorphan, pentazocine, cyclazocine	Lack of a furan oxygen
Hydromorphone, naltrexone, naloxone, oxymorphone	Intramolecular quenching by C-6 keto group

[a] These compounds have additional structural features that would prevent fluorescence under these conditions.

Compounds that did not fluoresce under the mild oxidizing conditions of the potassium ferro-ferricyanide solution are listed in Table II. It is apparent that at least three structural features are important in the prevention of fluorescence of morphine congeners under these conditions. The dimerization process can be blocked by introduction of an alkyl or acetyl group at the 3-*O*-position. TLC analyses of the oxidized products of I, X, XII, XIII, XV, XVII, and 6β-hydrocodol for polar, pseudomorphine-like adducts provided evidence for their occurrence for I, X, XII, XV, and XVII. Thus, it is likely that the C-2 position of these compounds is activated by the presence of the C-3 phenolic group through tautomerization to a quinone-like structure, which may then undergo a coupling reaction. Blockade of this process with an alkyl or acyl group would prevent dimerization.

A second structural feature important in the prevention of fluorescence involves quenching by a carbonyl group located at C-6. Both XII and XVII reacted under the standard oxidizing conditions to produce a more polar product, as evidenced by iodoplatinate-positive spots on TLC. Although dimerization apparently occurred for XII and XVII, neither compound fluoresced. In contrast, fluorescence was observed for the 6α- and 6β-carbinol homologs of XII and XVII and for the ethylene ketal XVIII. Protection of the carbonyl group as a ketal, thus providing a fluorescent derivative, is a similar effect to that observed by Crowell and Varsel (11), who reported that the fluorescence quenching effect of aromatic aldehydes could be overcome by acetal formation.

The quenching effects of the carbonyl group result from the nonbonded electrons undergoing $n \rightarrow \pi^*$ transitions at lower energy levels and with lower molar absorptivity than the $\pi \rightarrow \pi^*$ transitions. In addition, the greater degree of *s* character in the sp^2 hybridized nonbinding orbital of the carbonyl oxygen than in the π orbitals of the biphenyl system results in a higher degree of spin-orbit coupling. Intersystem crossing is increased, thereby decreasing fluorescence and increasing the possibility of phosphorescence at lower temperatures. The conversion of XVII to XVIII results in less *s* character for the carbon–oxygen bond at C-6 (sp^3 orbitals) and eliminates the π bond, thereby restoring fluorescence.

The possibility that fluorescence quenching by the carbonyl group was occurring *via* an intermolecular process rather than by intramolecular quenching was ruled out by mixing the fluorescent species and measuring the resultant fluorescence. Three solutions were prepared: A, XVII (20 μg/ml); B, XV (20 μg/ml); and C, XVII (10 μg/ml) and XV (10 μg/ml). The compounds were oxidized and an additional solution, D, was prepared from equal parts of the oxidized Solutions A and B. This procedure gave the same concentration of drug in Solutions C and D.

The fluorescence of each solution was measured (Fig. 2). The fluorescent intensity of Solution D was approximately one-half of that observed for Solution B, as would be expected if intermolecular quenching

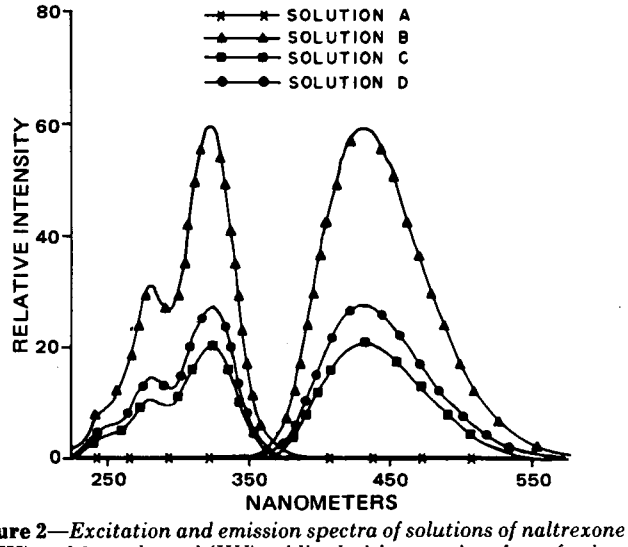
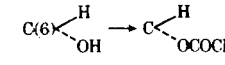
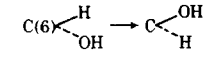
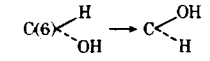
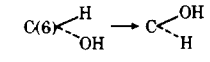
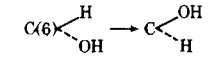
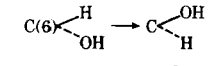
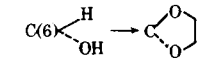
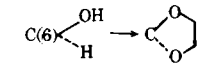
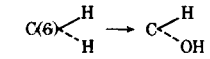
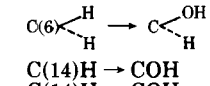


Figure 2—*Excitation and emission spectra of solutions of naltrexone (XVII) and 6α-naltrexol (XV) oxidized with potassium ferro-ferricyanide at pH 8.5. The contents of the solutions were: A, XVII (20 μg/ml); B, XV (20 μg/ml); and C, XVII (10 μg/ml) and XV (10 μg/ml). Solutions A–C were oxidized, and Solution D was prepared from equal parts of the oxidized Solutions A and B.*

Table III—Effects of Structural Changes on Fluorescence Characteristics

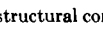
Comparison	Structural Change[a]	Effect[b]		
		$\Delta\lambda_{em}$, nm	Intensity Ratio	Δ Stokes' Shift, cm^{-1}
I → V	(N)CH_3 → H	+2	0.67	+105
I → IX	(N)CH_3 → $CH_2CH{=}CH_2$	+3	6.72	+253
XXIII → XX	(N)CH_3 → $CH_2CH{=}CH_2$	+2	0.47	+107
XXIV → XXI	(N)CH_3 → $CH_2CH{=}CH_2$	+1	0.30	+56
XXIII → XV	(N)CH_3 → 	+1	0.60	+54
XXIV → XVI	(N)CH_3 → 	0	1.10	0
V → IX	(N)H → $CH_2CH{=}CH_2$	+1	10.01	+148
XX → XV	(N)$CH_2CH{=}CH_2$ → 	−1	1.28	−53
XXI → XVI	(N)$CH_2CH{=}CH_2$ → 	−1	3.70	−56
I → X	HC(7)=C(8)H → CH_2CH_2	−7	1.19	−90
III → XI	HC(7)=C(8)H → CH_2CH_2	−7	1.28	−195
I → VII		−1	1.16	+42
I → III		−5	1.18	−269
X → XI		−5	1.27	−374
XV → XVI		−10	4.77	−453
XX → XXI		−10	1.65	−450
XXIII → XXIV		−9	2.62	−399
XV → XVIII		−9	3.31	−397
XVI → XVIII		+1	0.69	+56
XIV → X		+5	1.32	+374
XIV → XI		0	1.68	0
X → XXIII	C(14)H → COH	+4	1.73	+25
XI → XXIV	C(14)H → COH	0	3.56	0
XXIX → XXX	$CH_3(R_c)$ → $C(CH_3)_3$	−2	9.21	−103
XXVII → XXVIII	$CH_3CH_2CH_2(R_c)$ → $CH_3(CH_2)_4$	−1	5.77	+42

[a] The molecular site at which a structural comparison is made is indicated in parentheses. [b] $\Delta\lambda_{em}$ and ΔStokes' shift are expressed as differences in the fluorescence constants of the second compound minus those of the first compound. The intensity ratio is I^{-1} of the second compound divided by that of the first compound. When the intensity ratio is less than unity, the fluorescence intensity of the second compound is increased by the molecular change.

were not occurring. The fluorescence of Solution C was somewhat greater (75% of the intensity of Solution D) than would be accounted for by the dimers of XV formed in this mixture. This finding indicates that the mixed dimer forms (XV–XVII adduct) exhibit some fluorescence, although less intense than that of XV.

The third structural feature that was important in the production of fluorescence by pseudomorphine-like compounds was the furan oxygen bridge. Compounds lacking this group (*e.g.*, levallorphan, pentazocine, and cyclazocine) exhibited no fluorescence under oxidizing conditions. Delocalization of the nonbonding electrons of the furan oxygen into the biphenyl ring system, thereby extending the conjugated system, possibly accounts for the importance of this group.

Table IV—Effects of Substituents on the Fluorescence of Morphine Congeners Oxidized with Potassium Ferro-ferricyanide

Structural[a] Group	Substituent Change	Effect on λ_{em}	Effect on Intensity	Effect on Stokes' Shift
C(3)-OH	C—O-alkyl	Fluorescence not observed	—	—
	C=O	Fluorescence not observed	—	—
Furan oxygen bridge	Elimination	Fluorescence not observed	—	—
		Decrease	Decrease	Decrease
HC(7)=C(8)H	CH_2CH_2	Decrease	Decrease	Decrease
C(14)H	C—OH	None to small increase	Decrease	None to small increase
(N)H	NCH_3	Decrease	Decrease	Decrease
(N)CH_3	$CH_2CH{=}CH_2$ or 	None to small increase	Increase or decrease	None to large increase

[a] The molecular site at which a structural comparison is made is indicated in parenthesis.

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.

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

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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.

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