The effect of time on the formation of fluorescence was determined by treatment of 20 mcg. of estradiol benzoate with 10 ml. of 88% sulfuric acid. The fluorescence was stabilized at room temperature after 1 hr. and was found to be practically stable for an additional hour.

To determine the precision of the procedures, analyses were carried out with standard steroid vegetable oil solutions prepared in this laboratory and containing exactly known quantities of steroids in approximately the same concentrations present in commercial preparations.

It is evident from the data in Table I that good reproducibility with a satisfactory standard deviation is obtained. The determination of steroids in commercial dosage forms showed that the results obtained are in good agreement with the labeled amount of the respective steroids. Results of these determinations are given in Table II.

REFERENCES

(1) Bićan-Fišter, T., *J. Chromatog.*, **22**, 465(1966).
(2) Umberger, E. J., *Anal. Chem.*, **27**, 768(1955).
(3) Szalikowsky, C. R., O'Brien, M. G., and Mader, W. J., *ibid.*, **27**, 944(1955).

Keyphrases

Steroid mixtures—analysis
Progesterone and estradiol benzoate in oil
Progesterone, testosterone propionate, and estradiol benzoate in oil
Testosterone butyrate and estradiol benzoate aqueous suspension
Progesterone and estradiol benzoate aqueous suspension
TLC separation
Fluorometric analysis
UV analysis

Miotic Activity Produced by Inhibitors of Acetylcholinesterase

By A. Z. BOOTH, J. P. LONG, and K. R. LONG

Various biological activities of bis-phenacyl derivatives were investigated. The picoline derivatives demonstrated potent activity as inhibitors of human red blood cell cholinesterase. The compounds were effective miotic agents in mice and rabbits. Marked differences in toxicity of the positional isomers were noted. The 3-picoline derivative is a very toxic agent in mice, however it is equal to 2-picoline in activity as an inhibitor of acetylcholinesterase. Since the 2-picoline derivative is much less toxic, some mechanism other than inhibition of acetylcholinesterase must be involved to explain the high toxicity of the 3-picoline derivative.

SEVERAL SERIES of bis-quaternary compounds have been prepared that demonstrate potent inhibition of acetylcholinesterase. In general these compounds are much more selective for acetylcholinesterase than they are for pseudo cholinesterase (1). Since the innervation of the circular muscles of the iris is cholinergic and thus the degree of muscle tone is indirectly controlled by functional acetylcholinesterase, the biological activity was evaluated for the potent inhibitors of acetylcholinesterase described in this report. Quaternary compounds that are inhibitors of cholinesterase (*e.g.*, neostigmine) have not been particularly effective as miotic agents, probably because of difficulty of penetration across the cornea. In this series of agents this difficulty apparently was overcome by obtaining compounds that are more active biologically than neostigmine and incorporating into the structure components that would be expected to increase the lipid solubility.

METHODS

The ability to inhibit human red blood cell cholinesterase was measured using a Radiometer titrator type TTTlc titrograph type SBR 2C with Alga syringe buret. The reagents used were as follows: NaCl, 21.6%; NaCl, 22.5%; NaCl, 0.9%; acetylcholine iodide, 0.250 *M*; acetyl-β-methylcholine bromide, 0.5 *M*; Potassium hydrogen phthalate, 0.7351 Gm./200 ml. (2.0 ml. = 36 μmoles NaOH); 0.1 *N* NaOH (carbonate free). Two milliliters of potassium hydrogen phthalate is added to 10.0-ml. distilled water and this solution is titrated under N_2 with 0.1 *N* NaOH. From this titration curve the ordinate of the titrigraph chart is calibrated directly in μmoles NaOH used, which is equivalent to μmoles of liberated acid (cholinesterase activity). The abscissa of the chart gives time in minutes. Values are read as μmoles/ml./min. Red cells are prepared by centrifuging the blood sample at a fixed RFC and then washing twice with normal saline. The washed cells are suspended in an equal volume of normal saline (*i.e.*, 50% solution). To 22.0 ml. of distilled water is added 1.0-ml. whole blood, washed red cells, or plasma. The mixture is allowed to stand for 10 min. at 37–38° to allow for hemolysis and temperature equilibration. A 1.0-ml. amount of 21.6% NaCl is added and the mixture is again allowed to stand for a few minutes so that the temperature will equilibrate. The reaction mixture is then placed on the titrator and N_2 is blown over the top. After adjusting to pH 7.4 (preliminary titration), 1.0 ml. of acetylcholine iodide (0.250 *M*) is added and the reaction rate followed for about 8 min. If the true cholinesterase only is to be measured, 1.0 ml. of acetyl-β-methylcholine (0.5 *M*) is added in place of the acetylcholine iodide. Some samples were found to have too much

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activity to measure even when 0.5 ml. of sample was used. In these instances 0.25 ml. of sample diluted with 0.25 ml. of normal saline was used. Inhibition studies were performed by allowing the enzyme reaction to proceed 3–5 min. to attain maximum activity and let the rate become linear. The inhibitors were added to 0.1-ml. amounts and the concentration in the reaction varied from 10^{-6} to 10^{-9} *M* depending upon the particular inhibitor employed.

The LD_{50} was calculated from data obtained with mice weighing 17–22 Gm. The compounds were administered intraperitoneally to three groups of 10 mice/group. Each compound was dissolved in distilled water and concentrations varied by 0.3 log intervals. The LD_{50}, 95% fiducial limits, and their relative activities were calculated by the methods of probits (2).

The miotic activities of the compounds were evaluated in mice and rabbits. The mice weighed 17–25 Gm. and 10 mice were used for each concentration of each drug. The compounds were prepared in distilled water and applied to the right cornea. The concentrations were varied by 0.48 log intervals. The solution was allowed to remain in contact with the cornea for 10 sec. and then the drug was washed off with distilled water (3). The amount of miotic activity was recorded at 10-min. intervals using a calibrated eyepiece and a dissecting scope (3). The relative activities of the compounds were calculated using a 2 × 2 parallel line assay (2).

Similar experiments were performed with Dutch rabbits weighing from 2–2.5 Kg. Seven rabbits were used for each dose of each compound. The pupil diameters were measured by a karatometer before and at 0.5-hr. intervals after the application of the compounds, which were dissolved in saline, with concentrations varied by 0.48 log intervals. The animals were observed for systemic effects such as diarrhea and salivation. The data were treated statistically as described above for mice.

A series of rabbits were anesthetized with sodium phenobarbital (200 mg./Kg.) administered intravenously and the influence of compounds *A* and *B* on pupil size and any alteration of arterial blood pressure responses to acetylcholine administered intravenously was evaluated.

RESULTS

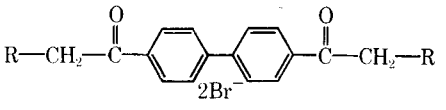
A summary of the structures and corresponding biological activity is shown in Table I.

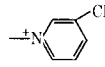
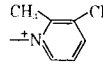
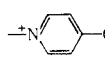
Toxicity—A wide variation in the LD_{50} following intraperitoneal administration was demonstrated by the compounds. One of the compounds, *B*, exhibited very high toxicity and it appeared to produce death by a mechanism other than inhibition of cholinesterase. The onset of observable symptoms and time for death with this compound in mice was slow, usually requiring 10 to 20 min. In contrast, the time for death for the remaining compounds was usually less than 8 min. The toxicity of the other compounds was less than that observed for neostigmine.

Inhibition of Acetylcholinesterase—The α- and β-picolines (compounds *A* and *B*) were approximately 1000 times more active than neostigmine bromide. Compound *C*, α,β-picoline, was somewhat less active. The γ-picoline derivative as well as the piperidine derivative (compounds *D* and *E*) were less active. Also the picoline compounds indicated a more rapid inhibition of the acetylcholinesterase than did neostigmine.

Miotic Activity—These quaternary compounds demonstrated miotic activity in both mice and rabbits. The duration of action was more than 2

TABLE I—BIOLOGICAL ACTIVITIES OF VARIOUS BIS-QUATERNARY INHIBITORS OF ACETYLCHOLINESTERASE



Compd.	R	LD_{50}[a] mg./Kg. (95% CI)	Acetylcholinesterase MI_{60}	Relative Miotic Activity (95% Fiducial Limits) Mice	Rabbits
Neostigmine	...	0.61 (0.46–0.80)	7.8×10^{-6}	...	...
A		6.7 (5.9–7.5)	8.2×10^{-9}	1[b]	1[c]
B		0.067 (0.047–0.101)	8.2×10^{-9}	0.65 (0.35–0.95)	0.55 (0.30–0.92)
C		0.88 (0.78–1.11)	3.0×10^{-8}	0.96 (0.76–1.22)	1.22 (0.87–1.75)
D		8.2 (6.6–12.4)	1.5×10^{-7}	0.11 (0.07–0.45)	0.43 (0.24–0.70)
E		8.4 (7.1–10.5)	4.7×10^{-6}	0.04 (0.03–0.06)	0.15 (0.08–0.36)

[a] The compounds were administered intraperitoneally. [b] The minimal concentration used for assay in mice was 0.011%. [c] The minimal concentration used for assay in rabbits was 0.033%.

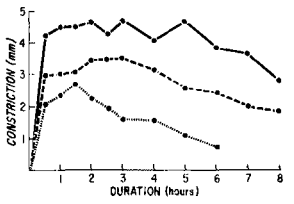


Fig. 1—Duration response curves in unanesthetized Dutch rabbits following topical application of compound A. Key: top, 0.44%; middle, 0.15%; bottom, 0.033%.

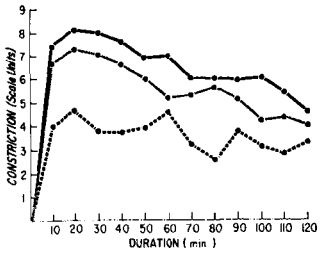


Fig. 2—Duration response curves for miosis in mice for compound A following topical application. Key: top, 0.1%; middle, 0.033%; bottom, 0.011%.

hr. in mice and 8 hr. in rabbits. The time to reach maximal inhibition in mice was 20 min. and approximately 0.5 hr. in rabbits. The dose-effect curves for compound *A* in rabbits and mice are shown in Figs. 1 and 2. Five rabbits were anesthetized and the right carotid artery was cannulated for blood pressure measurements. Control responses to acetylcholine, epinephrine, and norepinephrine were obtained. Compound *A* (0.6%) was applied to the cornea. During the following hour no alteration in blood pressure occurred and no potentiation or antagonism of the acetylcholine depressor response was observed. Also there was no alteration in the pressor responses to epinephrine or norepinephrine. With compound *B*, topical application of 0.5% solution to the anesthetized rabbits produced respiratory failure after a period of about 1 hr.

DISCUSSION

If heterocyclic substitution is placed on the bisphenacyl moiety, potent inhibitors of acetylcholinesterase are obtained. Compound *B* is of interest because of the high toxicity that apparently cannot be explained by its inhibition of cholinesterase. Since these are analogs of hemicholinium (4) it may be that this compound is exhibiting hemicholinium-type action.

Apparently these compounds cross the "corneal barrier" and reach acetylcholinesterase in concentrations sufficient to produce miosis of considerable duration. With the biphenyl nucleus as well as the substituted pyridine derivatives, the compounds may have components that contribute considerably to lipid solubility.

With the exception of compound *B* there was no evidence of systemic absorption of the compounds. Therefore it may be that the compounds can be localized effectively to the site of application, namely the cornea and anterior chamber of the eye.

SUMMARY

It has been demonstrated that a series of bis-quaternary phenacyl derivatives are potent inhibitors of acetylcholinesterase and are active miotic agents when applied topically to the cornea of both mice and rabbits. There is a wide variation in the LD_{50} of the compounds and the mechanism of toxicity is unknown for compound *B*. The high toxicity of this compound does not appear to be related to its ability to inhibit cholinesterase. The compounds, with the exception of compound *B*, do not appear to be absorbed systemically in quantities sufficient to potentiate the depressor response of acetylcholine.

REFERENCES

(1) Long, J. P., "Handbuch per Experimentellen Pharmakologie," Erganzungsiverk, Springer-Verlag, Berlin, Germany, 1963, p. 374.

(2) Finney, D. J., "Statistical Method in Biological Assay," Hafner Publishing Co., New York, N. Y., 1952.

(3) Armaly, M. F., and Long, J. P., *Arch. Intern. Pharmacodyn.*, **161**, 423(1966).

(4) Long, J. P., and Schueler, F. W., *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 79(1954).

Keyphrases

Acetylcholinesterase inhibitors
Bis-quaternary ammonium compounds
Miotic activity
LD_{50} values