A SIMPLE METHOD OF ASSESSING COMPETITIVE INTERACTION OF REVERSIBLE INHIBITORS WITH CHOLINESTERASES

V. D. Tonkopii, V. B. Prozorovskii, and M. G. Konstorum

UDC 577.152.311.042.2

A method of determining the degree of competitiveness of interaction of reversible inhibitors with cholinesterases has been developed. The method is based on comparing I_{50} values determined by the usual method and on the basis of protecting the enzyme against inhibition by armin.* Characteristics of oxazyl,† neostigmine, tacrin, mytolon,‡ and galanthamine hydrobromide and iodomethylate obtained by the suggested method are given.

KEY WORDS: *cholinesterase; acetylcholine; cholinesterase inhibitors.*

To characterize interaction between reversible inhibitors and cholinesterases (CE) kinetic methods of investigation are used [5], by means of which not only is it possible to assess the degree of competition of the inhibitor with the substrate during their interaction with the enzyme, but also to determine the dissociation constant. However, these methods require special apparatus and they are quite laborious.

This paper describes a suggested method of assessing the degree of competition between reversible inhibitors by determining I_{50} values (the molar concentration of inhibitor at which CE activity is reduced by 50%). The value of I_{50} is determined by means of the formula suggested previously for calculating true inhibition of CE by reversible inhibitors independently of addition of the substrate [2].

The source of the enzyme in these experiments was rat brain homogenate. Physiological saline was added to a weighed sample of brain in the ratio of 1:19 and homogenized in a glass homogenizer. Residual CE activity was determined by Hestrin's method [4].

In the control tests 1 ml homogenate, 1 ml distilled water, 1.5 ml phosphate buffer, pH 7.5, and 1 ml acetylcholine (AC) in a final concentration of 4×10^3 M were taken as the sample. Anticholinesterase activity of the reversible inhibitors was assessed by comparing the results of two series of experiments. In series I 1 ml of a solution of corresponding concentrations of the preparations was added to 1 ml homogenate and the mixture was incubated at room temperature for 10 min, after which 1.5 ml of buffer solution and 1 ml AC were added. Incubation was carried out at 37°C for 30 min. The value of I_{50} in the presence of AC (I_{50} with AC) was calculated from the results of these experiments. In the experiments of series II the value of I_{50} described conventionally as I_{50} without AC was determined. The CE activity (in %) in

*Ethyl-p-nitrophenyl ester of ethylphosphinic acid.
†Ambenonium chloride.
‡Benzoquinonium chloride.

S. M. Kirov Military Medical Academy, Leningrad. (Presented by Academician of the Academy of Medical Sciences of the USSR S. V. Anichkov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 80, No. 8, pp. 120-122, August, 1975. Original article submitted October 22, 1974.

TABLE 1. Values of I_{50} (in M) for Reversible Cholinesterase Inhibitors

Inhibitor	Characteristics of anticholinesterase activity			
	I_{50} with AC	I_{50} without AC	I_{50} + AC/I_{50}	Type of inhibition
Galanthamine hydrobromide	$6{,}2 \cdot 10^{-6}$	$1{,}7 \cdot 10^{-7}$	36,4	Mainly competitive
Galanthamine iodomethylate	$4{,}9 \cdot 10^{-6}$	$1{,}2 \cdot 10^{-7}$	40,0	Ditto
Tacrin	$2{,}3 \cdot 10^{-7}$	$1{,}8 \cdot 10^{-8}$	12,7	Mixed
Neostigmine	$2{,}7 \cdot 10^{-8}$	$9{,}5 \cdot 10^{-9}$	2,8	Mixed with predominance of noncompetitive
Mytolon	$2{,}0 \cdot 10^{-8}$	$4{,}4 \cdot 10^{-9}$	4,5	Ditto
Oxazyl	$8{,}0 \cdot 10^{-10}$	$8{,}0 \cdot 10^{-10}$	1	Noncompetitive

these experiments was determined as follows: 1) the initial enzyme activity, equal to 100% (V_0) was found; 2) the residual activity (V_t) was determined after incubation of the homogenate with a concentration of organophosphorus inhibitor (in this case armin) with which CE activity was reduced to 20-5%. For this purpose, 0.1 ml armin in the appropriate concentration was added to a mixture of 1 ml homogenate and 1 ml distilled water, the mixture was kept for 30 min at 37°C, after which buffer and substrate were added; 3) CE activity was determined by the usual method after treatment with reversible inhibitors (V_i); 4) activity of the enzyme was determined after the addition of 0.1 ml armin to the mixture (1 ml homogenate and 1 ml reversible inhibitor) in the same concentration as for incubation with the intact enzyme (V_{ti}). The values of the activities thus obtained were used to calculate the true inhibition of CE (j) by the equation suggested previously:

$$j = 100 - \frac{\lg (V_i/V_{ti}) \cdot 100}{\lg (V_0/V_t)}.$$

The value of I_{50} without AC was found graphically from the values of CE inhibition thus determined. This value is the concentration of the preparation at which CE activity is reduced by 50% in the case when no substrate is added to the incubation mixture.

The results of determination of I_{50} for several reversible inhibitors are given in Table 1. It will be noted that all the inhibitors differed in their corresponding values of the ratio I_{50} with AC/I_{50} without AC. For galanthamine hydrobromide and iodomethylate, these ratios were maximal, namely 36.4 and 40. This difference in the values of I_{50} is evidently connected with the ability of AC to displace galanthamine from the active centers of CE. It was in fact shown previously that galanthamine is an inhibitor with a mainly competitive type of action [1]. For oxazyl the values of I_{50} calculated by the two methods were identical. The noncompetitive mechanism of interaction between oxazyl and CE is confirmed by the results of kinetic investigations [6]. Mytolon, similar in structure to oxazyl, is a mixed inhibitor with a marked noncompetitive action, in agreement with other observations [8]. Neostigmine, judging from the values of its I_{50} ratios, is an inhibitor with a mixed type of action and a mainly noncompetitive mechanism of inhibition. Carbamates are in fact converted into predominantly noncompetitive inhibitors at the stage of carbamylation of the enzyme [7]. Tacrin is another inhibitor with a mixed mechanism of action. Its competitive part is more marked than that of mytolon and neostigmine. In the presence of high AC concentrations, this inhibitor has been shown to develop partly competitive relations with the substrate [3].

The suggested method can thus be used to assess the degree of competitiveness of interaction between reversible inhibitors and CE. The value of the ratio

I_{50} with AC/I_{50} without AC > 30 corresponds under these circumstances to a reversible inhibitor with a predominantly competitive type of action: a value of this ratio close to 1 is noncompetitive, and an intermediate value denotes a mixed reversible inhibitor. The method described cannot completely replace kinetic investigations, but it does enable the mechanism of interaction of reversible inhibitors with CE to be assessed in the presence of substrate.

LITERATURE CITED

1. É. T. Vasilenko and V. D. Tonkopii, "Characteristics of galanthamine as a reversible cholinesterase inhibitor," Biokhimiya, No. 4, 701 (1974).
2. V. D. Tonkopii, N. V. Savateev, A. P. Brestkin, et al., "Determination of cholinesterase activity in animal tissues after treatment with reversible inhibitors," Dokl. Akad. Nauk SSSR, 207, 736 (1972).
3. V. A. Yakovlev, The Kinetics of Enzymic Catalysis [in Russian], Moscow (1965), p. 248.
4. E. Heilbronn, "Inhibition of cholinesterase by tetrahydroaminoacrine," Acta Chem. Scand., 15, 1386 (1961).
5. J. Hestrin, "The reaction of acetylcholine and carboxylic acid derivatives with hydroxylamine, and its analytical application," J. Biol. Chem., 180, 249 (1949).
6. J. Jensen-Holm, H. H. Lausen, K. Milthers, et al., "Determination of the cholinesterase activity in blood and organs by automatic titration," Acta Pharmacol. (Copenhagen), 15, 384 (1959).
7. H. H. Stein and J. J. Lewis, "Noncompetitive inhibition of acetylcholinesterase by eserine, Biochem. Pharmacol., 18, 1679 (1969).
8. J. D. Webb, "Affinity of Benzoquinonium and ambenonium derivatives for the acetylcholine receptor, tested on the electroplax, and for acetylcholinesterase in solution," Biochim. Biophys. Acta, 102, 172 (1965).