Rapid Method for Evaluation of Cholinesterase Inhibitors

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The fluorescence intensity of reversible inhibitor ethidium bromide fluorophore complex with equine blood butyryl cholinesterase decreases in the presence of inhibitor (tacrine) not fluorescing in the visible spectrum. An express method for tacrine evaluation is developed.

Key Words: *tacrine; butyryl cholinesterase; fluorescence*

Highly sensitive and highly selective biochemical method for evaluating activities of acetylcholinesterase or butyryl cholinesterase (BCE) [3,4] is widely used in the development of drugs for the treatment of Alzheimer's disease [4] and for evaluating their efficiency.

Ellman's colorimetric method is widely used in foreign countries [3]. It is based on evaluation of the rate of enzymatic hydrolysis of acetylcholine substrate with and without analyzed sample using Ellman's reagent as the indicator of 5,5'-dithiobis-(2-nitrobenzoic) acid thiol group, which, reacting with thiocholine, forms a colored anion RS^-. The main drawback of Ellman's method is easy oxidation of colored anion (at 412 nm) forming as a result of thiocholine reaction with Ellman's reagent.

The effects of BCE on the fluorescence (FL) of reversible fluorophore inhibitors was described [1,2]. We used these data for the development of a rapid method for evaluation of BCE inhibitors.

Indeed, the addition of nonfluorescent inhibitor I_{nfl}, characterized by higher affinity for the enzyme (or its presence in a significantly higher concentration) shifts the balance and leads to the release of the fluorophore inhibitor in accordance with the following system:

$$\begin{array}{c} E+I_{fl}\leftrightarrow EI_{fl} \\ \updownarrow\ I_{nfl} \\ EI_{nfl}\leftrightarrow E'+P_i, \end{array}$$

where E is cholinesterase, I_{fl} reversible fluorophore inhibitor, EI_{fl} is BCE complex with reversible fluorophore inhibitor, I_{nfl} nonfluorescent inhibitor, EI_{nfl} is BCE complex with nonfluorescent inhibitor, E' phosphorylated BCE, and P_i is the product released after BCE phosphorylation.

Addition of a nonfluorescent irreversible inhibitor (I_{irr}), *e.g.* organophosphorus insecticide, leads to a further shift of the balance towards the formation of phosphorylated BCE. FL in this system will also change more intensely.

The requirements to the fluorophore inhibitor were as follows: it was to be a strong cholinesterase inhibitor, with high quantum yield, well soluble in water media at pH 7.4-8.6 (as BCE exhibits maximum activity in this pH interval), be characterized by high photo- and thermostability and high Stokes' shift value (for reducing the interfering effect of scattered excitating light). One more important requirement: the fluorophore inhibitor was to possess an FL band in the visible spectrum, maximally distant from the BCE fluorescence band.

Ethidium bromide (EB), a BCE reversible inhibitor, meets these requirements: it has an FL band at 610 nm and forms a stable complex with BCE (the dissociation constant of the complex K_i=52 μM [5]).

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Tacrine, a potent reversible inhibitor, was selected as an inhibitor not fluorescent in the visible spectrum (K_i=0.17 μM); it is widely used for the treatment of Alzheimer's disease [1,5].

We studied the effects of nonfluorescent inhibitor (as exemplified by tacrine) on the fluorescence of EB—BCE complex and, based on the findings, developed a rapid method for evaluation of these inhibitors.

MATERIALS AND METHODS

Equine blood BCE (E.C. 3.1.1.8, 1 Unit enzyme activity corresponded to enzymatic hydrolysis of 1 μmol butyrylcholine/min at pH 8.0 and 37°C); EB (3.8-diamino-5-ethyl-6-phenyl-phenantridinium bromide) and 9-amino-1.2.3.4-tetrahydroacridine hydrochloride (tacrine; all reagents were from Sigma) were used.

The fluorescence intensity was recorded at λ=610 nm on a Hitachi spectrofluorometer (excitation at λ=470 nm).

BCE solution in phosphate buffer (pH 8.0; 10 U/ml), EB solution (10 μM), and tacrine solution in ethanol (100 μM) were used.

Tacrine (3 ml; 3 ml water in control) and 0.5 ml EB aqueous solution (0.6 μM) were added to 0.5 ml enzyme solution (0.72 U/ml) in phosphate buffer, the mixture was placed into quartz cuvette (10×10×40 mm), and the fluorescence spectrum was recorded. All measurements were carried out in 0.02 M phosphate buffer (pH 8.0 at 20°C except the experiment on evaluation of the impact of temperature for the analytical effect.

RESULTS

The intensity of EB fluorescence increases in the presence of BCE [2]. The relationship between EB fluorescence and activity of BCE in phosphate buffer is presented by a linear curve for a wide range of values [2].

Addition of tacrine to the EB—BCE complex led to reduction of the fluorescence intensity in proportion to tacrine concentration (Fig. 1). The threshold detectable tacrine concentration was 0.53 μM.

TABLE 1. Results of Tacrine Measurement by Ellman's Method and Fluorescent Method (*M*±*m*)

Tacrine concentration in reference solution, μM	Tacrine concentration, μM (Ellman's method)	Tacrine concentration, μM (fluorescent method)
0.53	0.50±0.05	0.51±0.03
5.00	5.10±0.06	4.90±0.05
10.00	9.90±0.05	9.85±0.04
15.00	14.80±0.04	15.10±0.03

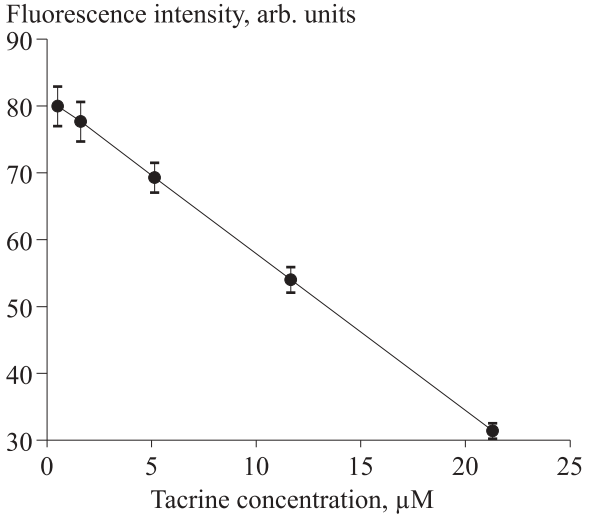

Fig. 1. Relationship between the fluorescence intensity of ethidium bromide complex with butyryl cholinesterase and tacrine concentration.

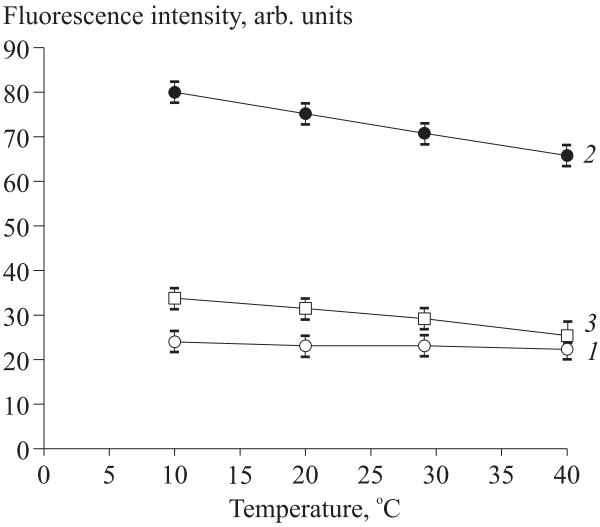

Fig. 2. Relationship between temperature and analytical effect in tacrine treatment of ethidium bromide (EB) complex with butyryl cholinesterase (BCE). *1*) EB; *2*) EB—BCE; *3*) tacrine+EB—BCE.

Selecting the optimal conditions for analysis, we studied the impact of temperature for the analytical effect.

The intensity of EB fluorescence virtually did not change at 10-40°C (Fig. 2, *1*), while the fluorescence of EB—BCE complex slightly monotonously increased (Fig. 2, *2*) with decreasing the temperature, which was due to the decrease in complex dissociation degree. The variations of the temperature within 10-40°C had also negligible effect on the response of EB—BCE complex to addition of tacrine (Fig. 2, *3*).

The error of tacrine evaluation by the fluorescent method was no higher than by its evaluation by Ellman's method (Table 1). In contrast to Ellman's method, in which analysis has to be performed at 38-40°C, our method can be used at 10-40°C (Fig. 2).

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