Fluorescent Method for Evaluation of Cholinesterase Inhibitors

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The sensitivity of detection of anticholinesterase compounds (*e.g.* tacrine) by the biochemical method significantly increased when fluorogenic compound N-(4-(7-diethylamino-4-methylcoumarine-3-yl)phenyl)maleimide was used instead of Ellman's reagent. A kinetic fluorescent method for evaluating cholinesterase inhibitors is proposed.

Key Words: *butyrylcholinesterase; fluorescence; tacrine*

Highly sensitive methods for detecting substances with anticholinesterase activity are needed for providing the safety of the staff at plants for extermination of chemical weapons. Organophosphorus pesticides possessing anticholinesterase activity in water and foodstuffs should also be controlled. In addition, cholinesterase activity measurements are widely used in the development and trials of new drugs intended for the treatment of Alzheimer's disease [6].

Ellman's colorimetric method [5] is most widely used all over the world; however, like other known modifications of the biochemical method for evaluation of cholinesterase inhibitors [1,3,4] it does not meet modern requirements to the sensitivity and rapidity of detection of highly toxic compounds with anticholinesterase effects.

We evaluated the sensitivity of detecting anticholinesterase compounds by Ellman's method, in which the colorimetric indicator of the thiol group was replaced with a fluorescent indicator. Good prospects of this approach are explained by high sensitivity of the fluorescent method detecting even individual molecules of a fluorescent compound [2].

MATERIALS AND METHODS

Equine serum butyrylcholinesterase (BCE, EC 3.1.1.8), butyrylthiocholine iodide (BTCI), 5',5'-dithio-bis(2-nitrobenzoic) acid (Ellman's reagent), and 9-amino-1,2,3,4-tetrahydroacridine hydrochloride (tacrine) were used in the study (all reagents were from Sigma). Tacrine was used as a strong reversible inhibitor, nonfluorescing in the visible spectrum (K_i=0.17 μM), and widely used for the treatment of Alzheimer's disease [6].

N-(4-(7-diethylamino-4-methylcoumarine-3-yl)phenyl)maleimide (CPM) [7] served as a fluorogenic indicator of the thiol group.

The product of CPM reaction with the compound containing the thiol group has a fluorescence maximum at λ=473 nm at excitation wavelength λ=390 nm (Fig. 1) [7].

BCE solution in phosphate buffer (0.37 U/ml, pH 7.5), 100 μM tacrine solution, 20 μM CPM solution, and 0.02 M BTCI solution were used.

Tacrine solution (2.5 ml; the same volume of water in the control), 0.5 ml aqueous solution of CPM, and 0.5 ml substrate were added to 0.5 ml enzyme solution in phosphate buffer; the mixture was poured into a quartz cuvette, and the fluorescence spectrum was recorded at λ=473 nm (excitation wavelength λ=390 nm), or the kinetics of enzymatic reaction was recorded on a Hitachi spectrofluorometer. All measurements were carried out in 0.02 M phosphate buffer (pH 7.5) at 20°C.

The degree of cholinesterase inhibition was estimated from the following ratio:

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$$I=(1-\frac{tg\alpha'}{tg\alpha''})\times 100\%,$$

where I is the degree of enzyme inhibition (%), tgα' and tgα'' are the slopes of kinetic curves of the enzymatic reaction in the presence and absence of the inhibitor respectively.

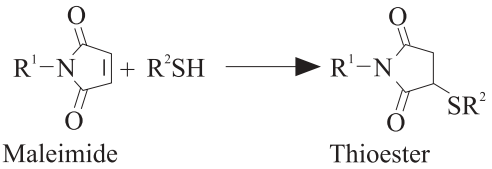

Fig. 1. Scheme of CPM reaction with the compound containing the thiol group.

RESULTS

Kinetic studies showed that the fluorescent product of thiocholine connection to CPM was stable for at least 10 h.

Optimization of the conditions of biochemical reaction showed that the substrate in a concentration above 0.01 M did not improve the analytical effect; neither did the addition of CPM in a concentration higher than 20 μM. CPM in a concentration of 20 μM was used in further studies.

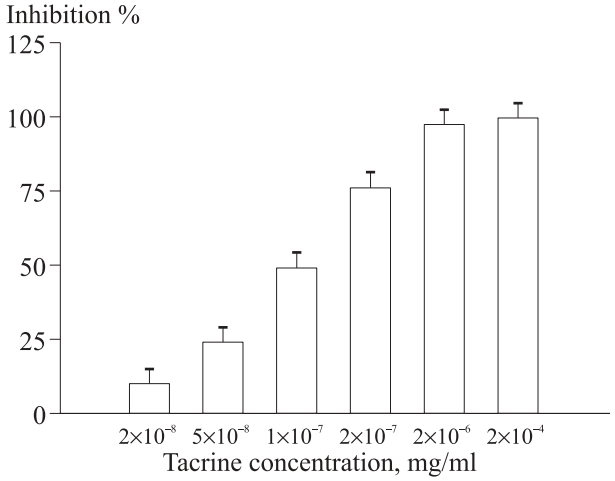

Fig. 2. Relationship between cholinesterase inhibition and tacrine concentration with CPM for the thiol group fluorogenic indicator and BTCI as the substrate.

TABLE 1. Tacrine Concentration (nM) Evaluated by Ellman's Method and Fluorescent Method (*M*±*m*)

Reference solution	Fluorescent method	Ellman's method
0.02	Not detected	0.020±0.002
1.00	Not detected	1.12±0.11
10.00	Not detected	9.10±1.01
100.00	95.00±10.23	101.00±9.05

The relationship between the degree of cholinesterase inhibition and tacrine concentration is shown in Fig. 2.

Comparison of Ellman's method and the proposed fluorescent method (Table 1) showed that the sensitivity of tacrine assay by the fluorescent method is 0.02 nM, which by more than 3 orders of magnitude surpassed the sensitivity of Ellman's method.

The sensitivity of biochemical analysis of organophosphorus compounds, irreversible inhibitors of cholinesterase, by two orders of magnitude surpasses the corresponding value for tacrine, and we therefore can expect that this method will evaluate organophosphorus toxins with a sensitivity of no less than 0.002 nM.

Hence, the proposed method has important advantages in comparison with widely used Ellman's method and its sensitivity meets the requirements to technologies for working zone air monitoring at plants for chemical weapons annihilation [5,6].

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