METHODS

Rapid Method for Measurement of Acetylcholinesterase Activity

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Fluorescence intensity of thioflavin T fluorogenic label increased significantly as a result of formation of enzyme-inhibitory complex with acetylcholinesterase of human blood erythrocytes. Thioflavin T is a reversible inhibitor, selectively reacting with acetylcholinesterase. Thioflavin T fluorescence intensity is proportional to acetylcholinesterase activity for the studied interval of enzyme activities. A rapid fluorescent method for measuring acetylcholinesterase activity is proposed.

Key Words: *acetylcholinesterase; fluorogenic label; fluorescence*

Measurements of human blood erythrocyte acetylcholinesterase (ACE) activity are widely used for the development of effective ACE reactivators for the treatment of victims of organophosphorus intoxications, for creation of drugs for the treatment of patients with Alzheimer's and Parkinson's disease, *etc.*

The structure of blood erythrocyte ACE is similar to that of synaptic ACE. Experiments demonstrated a correlation between blood erythrocyte ACE activity and neuromuscular transmission of the nerve pulse [5]. Clinically this relationship can be used for evaluating the severity of organophosphorus poisoning and efficiency of ACE reactivator oximes in the neuromuscular synaps, which is one of the most important problems of therapy in cases when atropine is ineffective [5]. Since blood erythrocyte ACE adequately reflects ACE activity in the synapse, it can be used for the diagnosis of Alzheimer's and Parkinson's diseases.

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Ellman's colorimetric method is most widely used for measurements of ACE activity all over the world [3]. However, like all the known modifications of the biochemical measurement of ACE activity [1, 2], this method does not meet modern requirements as regards the rate of analytical response.

We studied the reactions between human blood erythrocyte ACE and thioflavin T fluorogenic label (TF) and developed a method for ACE measurements based on fluorescent registration of the analytical effect, which is more rapid in comparison with the known methods for ACE measurements.

Good prospects of this approach are supported by published data on TF interactions with human recombinant ACE with the formation of intensely fluorescing complex with the dissociation constant of 1 μM within 1 second [4].

MATERIALS AND METHODS

Sigma reagents were used in the study: human blood erythrocyte ACE (EC 3.1.1.7) and TF.

The fluorescence spectra were recorded on a Hitachi spectrofluorometer. The TF fluorescence was

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measured at excitation wavelength λ=448 nm and emission wavelength λ=490 nm. All measurements were carried out in phosphate buffer (pH 7.0) at 20°C.

Human erythrocyte ACE (20 U/ml) in 0.08 M phosphate buffer (pH 7.0) and TF solution (20 μM) were used.

Solution of ACE (1 ml) in phosphate buffer of appropriate dilution was added to 3 ml of TF solution, the reaction mixture was put into a quartz cuvette after mixing, and the fluorescence intensity was recorded.

RESULTS

Thioflavin T under the selected reaction conditions (pH 7.0) fluoresced in the visible spectrum at λ_{max}= 490 nm at stimulation λ=448 nm. The TF fluorescence was modified significantly by ACE. Addition of ACE to TF solution led to an increase of the fluorescence intensity without shifting its maximum (λ=490 nm). The fluorescence intensity linearly increased with increasing ACE activity (from 0.5 to 4 U/ml enzyme activity; Fig. 1). We concluded from this that there was no concentration quenching within the chosen interval of concentrations of initial reagents.

The TF fluorescence intensity in the presence of ACE changed within 1 sec in our experiment. The fluorescence intensity which was attained by this moment remained stable for more than 15 min.

Selective effect of ACE on the TF fluorescence was confirmed by studies of its fluorescence spectra in the presence of human serum cholinesterase and BSA. Addition of human serum cholinesterase and BSA, which do not fluoresce at λ=490 nm, did not modify the TF fluorescence spectrum. Increase of TF fluorescence intensity at λ=490 nm in the presence of ACE can be explained by an increase of its quantum release as a result of reduced torsion relaxation during binding to the ACE peripheral site [4].

We compared the results of human blood erythrocyte ACE measurements by Ellman's method and our fluorescent method (Table 1). The activity of ACE in the reference solution was measured by potentiometric titration of acetic acid released as a result of enzymatic hydrolysis of acetylcholine.

The results indicate that the error in evaluation of human blood erythrocyte ACE by the proposed fluorescent method is no higher than the error of measurements by Ellman's method.

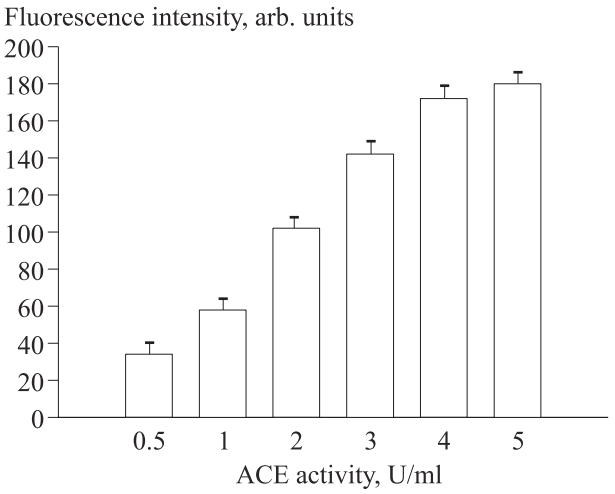

Fig. 1. Relationship between TF solution (15 μM) fluorescence intensity and human blood erythrocyte ACE (U/ml).

TABLE 1. Analysis of Activity of Human Blood Erythrocyte ACE in the Reference Solution by Ellman's Method [3] and Fluorescent Method (*M*±*m*)

ACE activity in reference solution, U/ml	ACE activity measured by Ellman's method, U/ml	ACE activity measured by fluorescent method, U/ml
0.50	0.49±0.05	0.51±0.02
1.00	0.90±0.10	1.05±0.02
2.00	1.85±0.20	1.90±0.10
5.00	4.80±0.50	4.95±0.10

Hence, the suggested method for measurement of ACE activity is more rapid, selective, and less difficult in comparison with the commonly used Ellman's method.

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