

ORIGINAL ARTICLE

SELECTIVE MONITORING OF ENZYMATIC ACTIVITY OF ACETYLCHOLINESTERASE BY FLOW INJECTION ANALYSIS WITH MASS SPECTROMETRIC DETECTION

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Summary

Flow injection analysis (FIA) is an analytical method where the reaction mixture is injected into flow of liquid. The reaction product is monitored by a suitable detector such as ultraviolet/visible (UV/VIS) spectrophotometric or electrochemical detector. Mass spectrometric detectors (MS) are coming to be a standard equipment of analytical laboratories in the present time. This work is focused on application of FIA-MS instrumentation for monitoring of Ellman's reaction where both reactants (acetylthiocholine and 5,5'-dithiobis-2-nitrobenzoic acid, DTNB) and the reaction product (5-mercapto-2-nitrobenzoic acid) are monitored. This reaction is usually used for monitoring of acetylcholinesterase and butyrylcholinesterase. Due to its simplicity, the developed method is generally applicable for monitoring of enzymatic reactions of cholinesterases. The main advantage of this method is high selectivity and reduction of influence of compounds, which are reacting with DTNB, resulting in a color product of Ellman's reaction.

Key words: flow injection analysis; mass spectrometry; acetylcholinesterase

INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7) is an enzyme which plays a crucial role in a cholinergic neural

system. It is localized in neuronal synapses and neuromuscular junctions. AChE hydrolyses neurotransmitter acetylcholine (ACh) producing choline and acetate, which leads to termination of synaptic transmission. The production of acetylcholine decreases with ageing due to the loss of basal cholinergic cells, which leads to the necessity of increasing of synaptic ACh for example by treatment with AChE inhibitors. On the other hand, the high level of inhibition of AChE may lead toward excess of ACh

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and thus permanent stimulation of receptors resulting in miosis, bronchospasm, muscular paralysis etc. Therefore, AChE is an important target of drugs used for treatment of neurodegenerative diseases such as Alzheimer disease [1], myasthenia gravis [2] or Parkinson disease [3]. Also, AChE is inhibited by chemical warfare nerve agents (sarin, soman, tabun, VX) [4] or organophosphorus pesticides (chlorpyrifos, parathion) [5].

Ellman's method [6] is the most often used tool for determination of AChE activity. The method is based on the hydrolysis of acetylthiocholine, an alternative substrate to ACh, to thiocholine followed by the reaction of the product with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) resulting in a yellow colored product of 5-mercapto-2-nitrobenzoic acid.

The chemical principle of Ellman's method is presented in Figure 1. The concentration of the final product is typically monitored spectrophotometrically after an appropriate time of reaction. There were various modifications of Ellman's method setup published including the flow injection analysis [7]. The method is relatively simple and cheap, however, it exerts a relatively low selectivity. Under the experimental conditions, the reagent DTNB can undergo reactions with sulphuryl group of various thiol compounds such as cysteine containing proteins. This non-selectivity may lead to false results especially when biological samples are analyzed. For this reason, the excess of acetylthiocholine and longer measurement period is preferred, because the influence of interfering compounds is lowered.

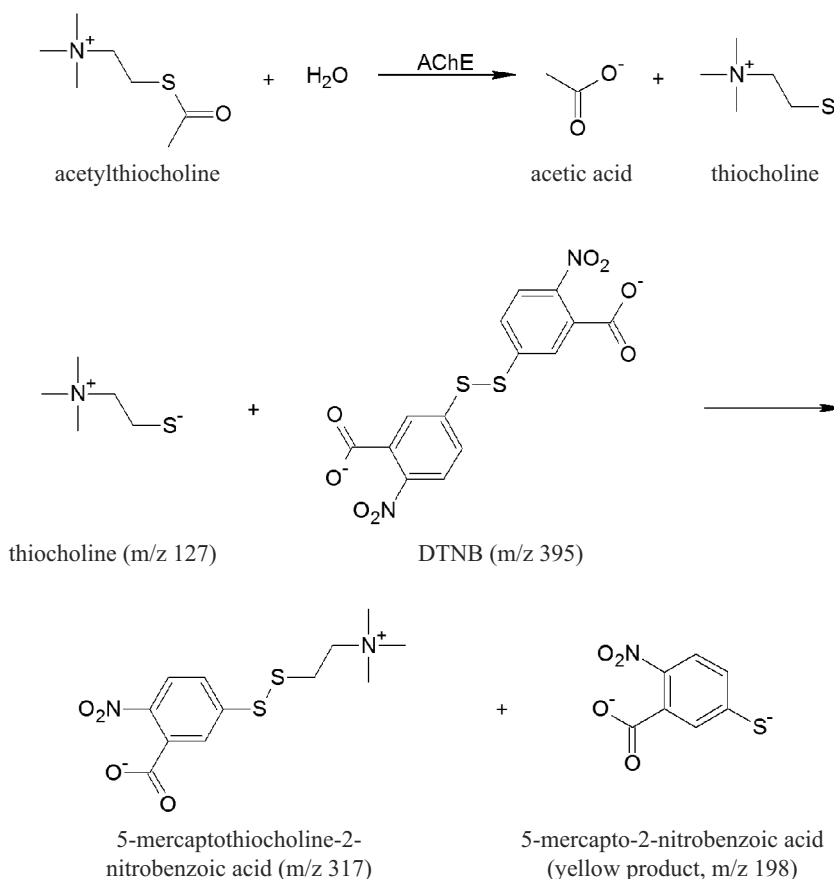


Figure 1. Scheme of Ellman's method

In this work, we took advantage of mass spectrometric selectivity in the detection of the reaction product of DTNB and thiocholine. The proposed method overcomes the disadvantages of the

standard setup of Ellman's reaction with spectrophotometric UV/VIS detection. In our method, the product of reaction between DTNB and exact analyte is quantified directly. It is in contrast

with UV/VIS detection, where the reaction is monitored through quantification of 5-mercapto-2-nitrobenzoic acid without any knowledge of reactant nature. This selectivity and sensitivity in the determination of the reaction product is a big advantage and allows determination of very low concentrations of the product. In addition, small changes in AChE activity can be monitored.

EXPERIMENTAL

Chemicals

Tris(hydroxymethyl)aminomethane hydrochloride (TRIS), hydrochloric acid, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCh) and acetylcholinesterase (AChE) were supplied by Sigma-Aldrich (Prague, Czech Republic).

Solutions

Enzymatic reaction was performed in 0.2M-TRIS-HCl buffer solution adjusted to pH 7.6 (24.2 g of TRIS was dissolved in 1 000 ml of redistilled water, 50 ml of this solution was mixed with 38.4 ml

of 0.2 M HCl and finally pH was adjusted to 7.6 and filled up to 200 ml by redistilled water). The reagent solution of DTNB was prepared by dissolution of 0.1 g DTNB in 50 ml 0.2 M TRIS-HCl buffer. Finally, the substrate ATCh solution was prepared in concentration 10 mM of redistilled water.

Instrumentation

The acetylthiocholine hydrolysis was monitored by HPLC system HP1100 (Agilent Technologies, Palo Alto, USA) which consisted of vacuum degasser unit (model G1322A), quaternary pump (G1311A), autosampler unit (G1313A), variable wavelength UV/VIS detector (G1314A), fluorescence detector (G1321A) and quadrupole mass detector (G1946VL) equipped with electrospray ion source.

The mass spectrometric detector parameters were optimized as follows: flow rate of drying gas 12 l/min, pressure of drying gas 55 psig, temperature of electrospray 350 °C, capillary voltage 3 000 V and fragmentor voltage 100 V. Mass spectrometer operated in both negative and positive ion scanning and in a single-ion monitoring mode, which allows higher sensitivity and lower detection limits in comparison with a scanning mode.

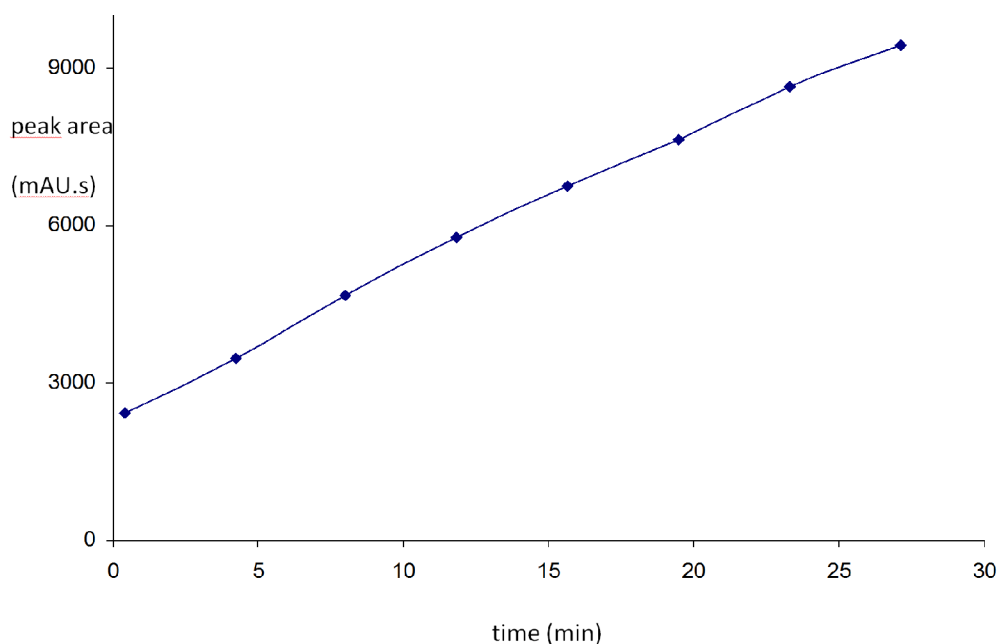


Figure 2. Kinetics of Ellman's reaction measured by UV/VIS detector at wavelength 412 nm.

The autosampler unit was thermostated to physiological temperature of 37 °C by thermostat KEVA (Pavel Krásenský, Brno, Czech Republic).

Sample preparation for analysis

A volume of 0.4 ml of DTNB solution was added to the vial with 0.2 ml of buffered solution of AChE (concentration 5 mg/mL) and 1.35 ml of TRIS buffer solution. The content of the vial was vortexed. The reaction was started by adding 0.2 ml of substrate (ATCh solution). A control sample containing redistilled water instead of substrate was analysed to inspect the purity of used chemicals. Reaction solution was vortexed and then tempered to 37 °C. After 3 min a 3 µl of aliquot of reaction mixture was automatically sampled to the flow of TRIS-HCl buffer (0.2 ml/min) each 4 min.

RESULTS AND DISCUSSION

All results were obtained under the conditions described in the Experimental section. The kinetics of ATCh hydrolysis was monitored both

spectrophotometrically at 412 nm (absorption maximum of the yellow product) and mass spectrometrically. The kinetic data obtained using the conventional spectrophotometric detector are presented in Figure 2. Increasing time of incubation with substrate linearly increased the signal (peak) area.

Figure 3 represents the same experimental conditions and the ATCh hydrolysis was monitored by mass spectrometric detection. Negatively charged ions of DTNB (m/z 395), yellow product of DTNB and thiocholine reaction (5-mercapto-2-nitrobenzoic acid, m/z 198), ATCh (m/z 127) and positively charged product with m/z 317 (5-mercaptothiocholine-2-nitrobenzoic acid, not included in Figure 3) were detected. It is obvious that the concentration of substrate ATCh was almost constant during the whole measurement. That is one of the requests for the appropriate measuring of enzymes kinetic profiles. Furthermore, the decreasing of DTNB concentration respectively increasing of the reaction product concentration is presented as well. The production of the second product (m/z 317) of the reaction of thiocholine with DTNB strongly corresponds with the first one (m/z 198).

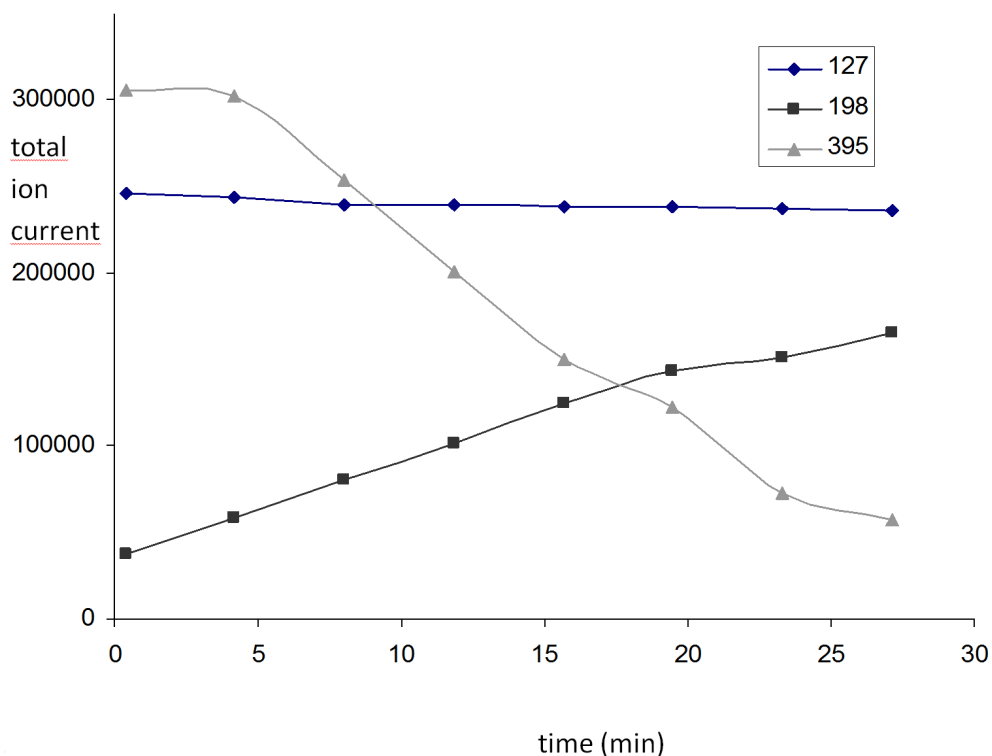


Figure 3. Kinetics of Ellman's reaction measured by mass spectrometric detection in the range of negative ions. Acetylthiocholine (m/z = 127), 5-mercapto-2-nitrobenzoic acid (m/z = 198) a DTNB (m/z = 395).

The methodology is proposed not only for the measurement of cholinesterases reaction kinetics, but it can also be used for monitoring an inhibitory effect of various compounds.. In addition, it can find applicability in the activity of potential drugs measurement, which can be used in the treatment of poisoning by organophosphorus pesticides or

chemical warfare nerve agents. For this purpose, prior to the measurement, AChE is inhibited by pesticide or nerve agent and, as a result, the increase of activity of AChE reactivation is monitored. The reaction is started by adding substrate. The proposed time scheme is presented in Figure 4.

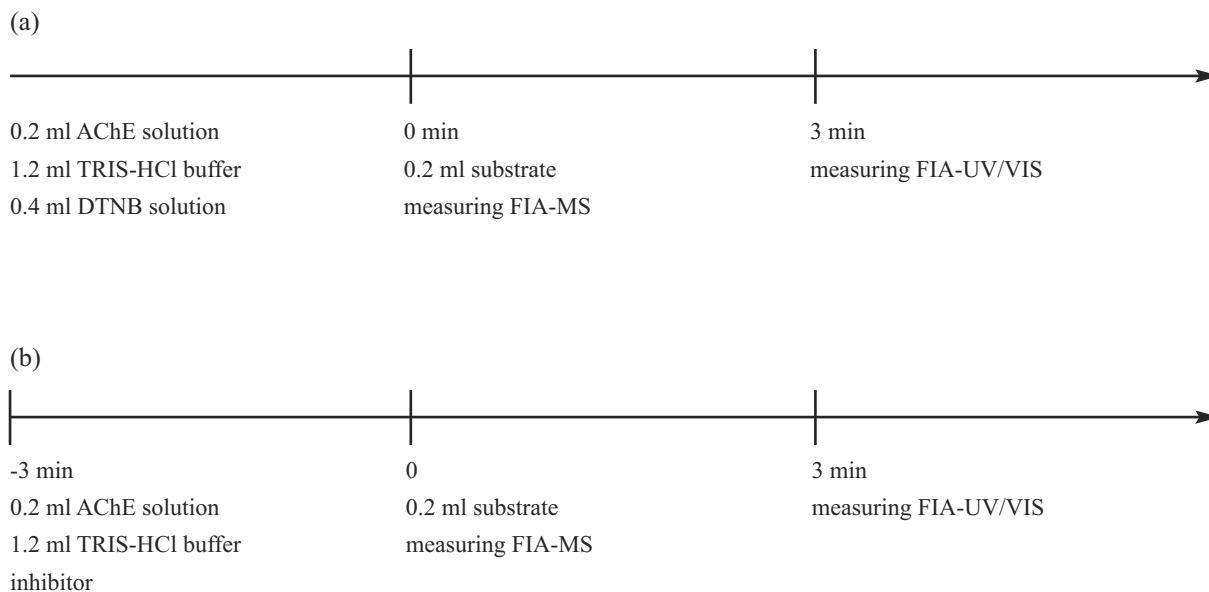


Figure 4. Comparison of time scheme of measurement (a) enzyme kinetics or (b) kinetics of acetylcholinesterase inhibition by FIA-UV/VIS and FIA-MS.

The selectivity in detection of specific product of thiocholine with DTNB reaction is a big advantage compared to commonly used spectrophotometric detection, such was performed e.g. by Cabal et al.[7]. Even, when fast processes are monitored in biological samples and only small amounts of thiocholine are produced, non-specificity of a UV/VIS detector can play an important role in the correctness of results and should not be applied. Biological samples may contain small concentrations of interfering compounds which react with DTNB under the formation of yellow 5-mercapto-2-nitrobenzoic acid. Shorter times of reaction increase the influence of the interfering compounds. In case of longer incubation, the interfering compounds completely react with DTNB and they form a relatively minor amount of the total 5-mercapto-2-nitrobenzoic acid. Thus,

the incubation times should be in order of minutes when UV/VIS detection is applied and this can be very limiting.

CONCLUSION

The monitoring of enzymatic or chemical reaction kinetics is of a high importance in the study of their mechanism or in their controlling. Flow injection analysis in combination with mass spectrometric detection is a very perspective tool for these purposes. In our work this methodology was applied for the monitoring of enzymatic hydrolysis of ATCh by AChE and compared with measurement using a standard spectrophotometric method. The advantages of mass spectrometric

detection are high selectivity and lower detection limits in comparison with standard UV/VIS detection. They allow a more accurate study when very short time of analysis is necessary in biological applications.

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