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Evaluation of flow injection analysis for determination of cholinesterase activities in biological material

Jiri Cabal*, Jiri Bajgar, Jiri Kassa

Department of Toxicology, Faculty of Military Health Sciences, University of Defense, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic

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ABSTRACT

The method for automatic continual monitoring of acetylcholinesterase (AChE) activity in biological material is described. It is based on flexible system of plastic pipes mixing samples of biological material with reagents for enzyme determination; reaction product penetrates through the semipermeable membrane and it is spectrophotometrically determined (Ellman's method). It consists of sampling (either in vitro or in vivo), adding the substrate and flowing to dialyzer; reaction product (thiocholine) is dialyzed and mixed with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) transported to flow spectrophotometer. Flowing of all materials is realised using peristaltic pump. The method was validated: time for optimal hydratation of the cellophane membrane; type of the membrane; type of dialyzer; conditions for optimal permeation of reaction components; optimization of substrate and DTNB concentrations (linear dependence); efficacy of peristaltic pump; calibration of analytes after permeation through the membrane; excluding of the blood permeation through the membrane. Some examples of the evaluation of the effects of AChE inhibitors are described. It was demonstrated very good uniformity of peaks representing the enzyme activity (good reproducibility); time dependence of AChE inhibition caused by VX in vitro in the rat blood allowing to determine the half life of inhibition and thus, bimolecular rate constants of inhibition; reactivation of inhibited AChE by some reactivators, and continual monitoring of the activity in the whole blood in vivo in intact and VX-intoxicated rats. The method is simple and not expensive, allowing automatic determination of AChE activity in discrete or continual samples in vitro or in vivo. It will be evaluated for further research of cholinesterase inhibitors.

1. Introduction

Inhibition of acetylcholinesterase (AChE, EC 3.1.1.7) is considered to be trigger mechanism of nerve agents action [1,2]. Thus, determination of AChE (and other cholinesterases) is a basic method for evaluation of toxic action of these agents and, simultaneously, it can be used for the research dealing with inhibition, reactivation or other influencing of these enzymes in biological material [3–5]. AChE determination in biological samples is an usual approach. It presupposes the preparation of the samples either in vitro or in vivo, e.g. monitoring of nerve agent concentration in the inhalation chamber and discontinual determination of cholinesterase activity in samples [3]—time and material consuming procedures. The choice of methods determining AChE activity is very broad and it was described many times, e.g. [4,5]. It appears from these studies that Ellman method [6] is simple and accurate.

Monitoring of intoxication by the evaluation of continual cholinesterase inhibition may bring new information that cannot be provided by analysis of biological material post-mortem. The technology providing such monitoring is sequential injection analysis. The topic of this study is to set up an apparatus and to optimize its process conditions for above-mentioned purpose.

2. Material and methods

2.1. Principle

The determination of cholinesterase activity was carried out by a common spectrophotometric method according to Ellman [6]. The substrate acetylthiocholine iodide is hydrolyzed by the enzyme to acetic acid and thiocholine; the free SH-group of thioocholine reacts with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) and the product formed (5-mercapto-2-nitrobenzoic acid) is determined photometrically.

2.2. Components

Experiment was carried out on apparatus that involves following components:

* Corresponding author. Tel.: +420 973 251 506; fax: +420 495 518 094.
E-mail address: cabal@pmfhk.cz (J. Cabal).

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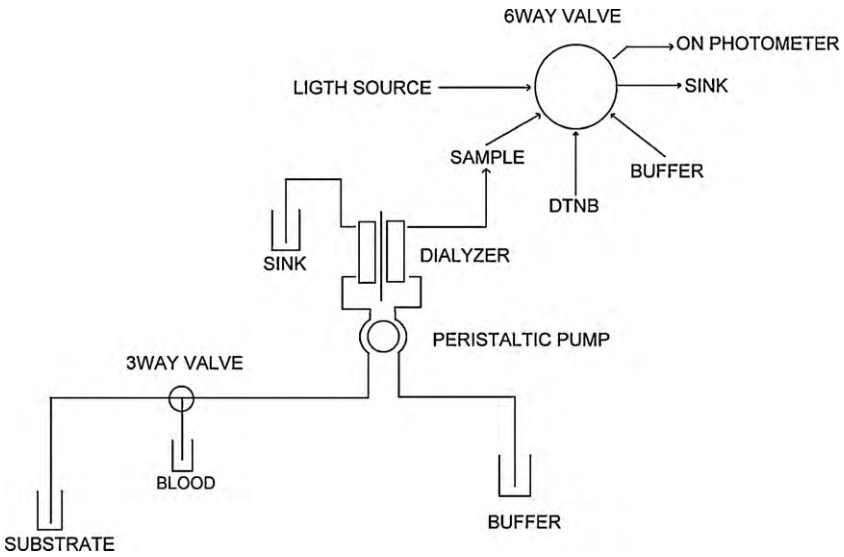

Fig. 1. Flow diagram for the determination of AChE activity.

Analyser Microsia, FIAlab Instruments Inc.
Peristaltic pump Watson–Marlow 403U
Flow-Thru Dialyzers 150 and 300 μl Harvard Apparatus or Sandwich Membrane Flowcell, FIAlab Instruments Inc.

2.3. Flow diagram

Flow diagram for the determination of AChE activity is shown in Fig. 1. Before sampling, intact blood or blood mixed with VX was given into the system. Then it was mixed with flow of substrate and transported to the dialyser. Buffered solution of DTNB is sucked to the secondary dialyser site. Then other chemicals (substrate) are passed through the dialyser and reaction product (thiocholine) is reacting with DTNB and formed yellow product is photometrically detected.

3. Results and discussion

3.1. Arrangement

The whole arrangement was set up to fulfill following functions:

- piston pump distributes solutions of agents;
- six way valve mixes agents and samples;
- fibre optics photometer determines Ellman indicator;
- dialyser separates macromolecules from analytes;
- peristaltic pump distributes solutions in the dialyser;
- software for control of apparatus.

3.2. Optimalization

Optimized parameters of device setup will be as follows:

- concentration of DTNB and substrate;
- influence of membrane thickness on peak height;
- influence of direction and velocity of flow by dialyser on peak height;
- influence of dialyser construction on peak height;
- influence of velocity of flow by dialyser on peak uniformity;
- debugging of software for apparatus operations.

3.3. Final conditions

After optimization, following parameters were used for operations:

- wavelength 400 nm;
- flow velocity in dialyser 0.44 ml/min;
- flow velocity of blood sample 0.014 ml/min;
- substrate concentration 0.001 M;
- salinity on primary side of dialyser 0.1 M phosphate buffer, pH 7.5;
- salinity on secondary side of dialyser 0.025 M phosphate buffer, pH 7.5;
- concentration of DTNB 0.0001 M;
- dose of blood for one analysis - 37 μl;
- duration of one cycle of measurement: 4–5 min.

Concentrations of analytes passed through the membrane show that the heights of peaks are linear (Fig. 2). For calibration, hydrolysate of acetylthiocholine was used, in concentrations as it

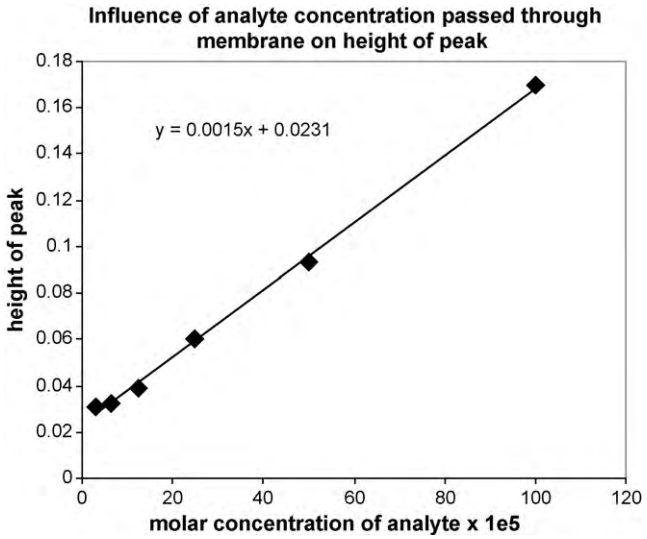

Fig. 2. Influence of analyte concentration passed through membrane on height of peak.