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Cholinesterase assay by an efficient fixed time endpoint method

Mónica Benabent, Eugenio Vilanova, Miguel Ángel Sogorb, Jorge Estévez *

Instituto de Bioingeniería, Universidad Miguel Hernández de Elche (Alicante), Spain

A B S T R A C T

Many cholinesterase assays are performed to study the inhibition of cholinesterase (ChE) activity. Frequently a large number of samples are processed and Ellman's method [1] is the most commonly used [2,3]. Activity is estimated from the increment in absorbance between two reaction times when the reaction is not stopped. Bellino et al. [4] described a method based on Ellman's method whereby the reaction was stopped with SDS and then the absorbance was measured. In these methods, the chromogen reagent 5,5′-dithiobis nitrobenzoic acid (DTNB) is added with the substrate and colour is monitored. Some authors pointed that the chromogen can alter cholinesterase activity [5].

- A modification of Bellino's method is proposed for acetylcholine-hydrolyzing activity determinations that is based on stopping the reaction after a fixed substrate reaction time using a mixture of detergent SDS and DTNB.
- The method may be adapted to the user needs by modifying the enzyme concentration and applied for simultaneously testing many samples in parallel; i.e. for complex experiments of kinetics assays with organophosphate inhibitors in different tissues.
- The method allows to avoid undesired reactions with DTNB or TNB.

A R T I C L E I N F O

Method name: Cholinesterase assay by fixed time endpoint method

Keywords: Acetylcholinesterase, Assay, End-point, Fixed time, Sodium dodecyl sulphate

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Materials

Chemicals

Sodium dodecyl sulphate (SDS; purity 99%) was obtained from Panreac Química S.L.U. (Barcelona, Spain). Triton X-100 was obtained from Sigma–Aldrich Quimica SL (Madrid, Spain). Ellman's reagent, 5,5′-dithio-bis-2-nitrobenzoate (DTNB, purity 99%) was obtained from Sigma–Aldrich Quimica SL (Madrid, Spain). Acetylthiocholine iodide (purity ≥98) was obtained from Sigma–Aldridge Quimica SL (Madrid, Spain). Phenylmethylsulfonyl fluoride (PMSF) was purchased from Sigma (Madrid, Spain), diethyl *p*-nitrophenylphosphate (paraoxon, purity >99%) was acquired from Sigma (Madrid, Spain). Human butyrylcholinesterase (hButChE) was supplied by Palmer W. Taylor and Zoran Radić (Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, USA). Bovine serum albumin (BSA, purity 96%) was acquired from Sigma (Madrid, Spain). All the other reagents were obtained from Merck SL (Madrid, Spain) and were of analytical grade.

* Corresponding author. Tel.: +34 966658514.
E-mail address: jorge.estevez@umh.es (J. Estévez).

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Solutions

The "phosphate buffer" mentioned throughout the paper contained 0.1 M phosphate, pH 7.4, 1 mM EDTA.

The "phosphate buffer/6 mM DTNB" mentioned throughout the paper contained 0.1 M phosphate, pH 7.4, and 6 mM DTNB.

The "phosphate buffer/1% BSA" mentioned throughout the paper contained 0.1 M phosphate, pH 7.4, and 1% BSA.

The solution used to stop the AChE reaction contained 2% SDS and 6 mM DTNB, was prepared in the phosphate buffer, and is cited as "2% SDS/6 mM DTNB solution".

Acetylthiocholine iodide was dissolved in ultrapure water at the desired concentration.

Thiocholine was obtained from the chemical degradation of acetylthiocholine iodide. A solution of 15 mM acetylthiocholine, pH 10, was incubated at 37 °C for 5 h. The resulting thiocholine preparation was neutralised at pH 7.4 and was diluted with ultrapure water at the desired concentration before use.

Hen tissue preparation and subcellular fractioning

Hen tissues were obtained from a commercial slaughter house immediately after sacrifice. Brains were removed and stored in cold (0–5 °C). Tris buffer (50 mM Tris–HCl buffer at pH 8.0 containing 1 mM EDTA) until use (before 1 h). Brains were homogenised in a Polytron homogenizer (Kinematica GmbH, Germany) using a PTA 10S head at 70% power (3 × 3 s) in Tris buffer at a concentration of 200 mg fresh tissue/ml.

The homogenised tissue was centrifuged at 1000 × *g* for 10 min at 4 °C to yield a precipitate containing fibres and nuclei. The supernatant was then centrifuged at 100,000 × *g* for 60 min to precipitate mitochondrial and microsomal fractions. The pellet (containing fibres and nuclei) was resuspended with Tris–Triton buffer (50 mM Tris–HCl buffer at pH 8.0 containing 1 mM EDTA and 1% Triton X-100).

The supernatant (soluble fraction) and the resuspended pellet (membrane fraction) were kept in liquid nitrogen until use. Samples were thawed at room temperature before use. This concentrated enzyme preparation is cited through the paper as the "soluble enzyme preparation" or "membrane enzyme preparation" and was diluted with phosphate buffer at the desired concentration expressed as μl preparation/ml solution.

Method details

In the following described procedure, each step was performed in all the test tubes before starting the next step. In this way, a large number of samples and blanks were simultaneously tested in parallel.

A 20-μl volume containing phosphate buffer (for blanks), or another reagent, was added to 1 ml microtubes. This volume may contain inhibitors or other factors that need to be tested.

Then 200 μl of the diluted membrane or soluble enzyme preparation (phosphate buffer in blanks) were added.

The mixture was incubated at 37 °C for the desired (preincubation) time. This preincubation time can be shortened substantially if inhibitors or other factors are not tested.

After this time, 200 μl of substrate acetylthiocholine in water were added for a final concentration of between 1 and 14.3 mM in 420 μl of the reaction volume.

The mixture was incubated at 37 °C for 10 min to run the enzymatic reaction.

The reaction was stopped by adding 200 μl of 2% SDS/6 mM DTNB solution.

Then 200 μl of phosphate buffer (diluted enzyme preparation in blanks) were added. The final assay volume was 820 μl.

After mixing and waiting at least 5 min, a 300-μl volume from each microtube was transferred to a 96-well microplate, and absorbance was read at 410 nm.

An Automated Work Station (Beckman Biomek 2000) was employed, but the process can also be performed manually. By reducing all the volumes proportionally to 1/4, for a final volume of 205 μl, the full process can be performed directly in a thermostat 96-well microplate.

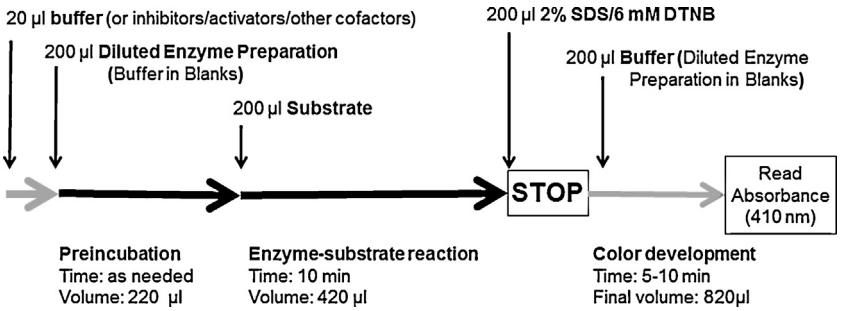

Fig. 1. Method scheme. The whole procedure was performed at 37 °C.