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Characterization of reversible and pseudo-irreversible acetylcholinesterase inhibitors by means of an immobilized enzyme reactor

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Abstract

The aim of the present study was the application of a human AChE-CIM-IMER (enzyme reactor containing acetylcholinesterase immobilized on a monolithic disk) for the rapid evaluation of the thermodynamic and kinetic constants, and the mechanism of action of new selected inhibitors. For this application, human recombinant AChE was covalently immobilized onto an ethylenediamine (EDA) monolithic Convective Interaction Media (CIM) disk and on-line studies were performed by inserting this IMER into a HPLC system. Short analysis time, absence of backpressure, low nonspecific matrix interactions and immediate recovery of enzyme activity were the best characteristics of this AChE-CIM-IMER. Mechanisms of action of selected reversible inhibitors (tacrine, donepezil, edrophonium, ambenonium) were evaluated by means of Lineweaver–Burk plot analysis. Analyses were performed on-line by injecting increasing concentrations of the tested inhibitor and substrate and by monitoring the product peak area. AChE-CIM-IMER kinetic parameters (K_m^{app} and v_{max}^{app}) were derived as well as inhibitory constants (K_i^{app}) of selected compounds. Moreover, noteworthy results were obtained in the application of the AChE-CIM-IMER to the characterization of the carbamoylation and decarbamoylation steps in pseudo-irreversible binding of carbamate derivatives (physostigmine and rivastigmine). AChE-CIM-IMER appeared to be a valid tool to determine simultaneously the kinetic constants in a reliable and fast mode. The obtained values were found in agreement with those obtained with the classical methods with the free enzyme. Furthermore, after inactivation by carbamates, activity could be fully recovered and the AChE-CIM-IMER could be reused for further studies. Results showed that the AChE-CIM-IMER is a valid tool not only for automated fast screening in the first phase of the drug discovery process but also for the finest characterization of the mode of action of new hit compounds with increased accuracy and reproducibility and with saving of time and materials.

Keywords: Acetylcholinesterase; Immobilization; CIM monolithic disk; Liquid chromatography; Mechanism of action; Reversible and pseudo-irreversible inhibitors; Kinetic constants

1. Introduction

Fast, accurate and reproducible analyses, alternative to the classic methods are required in new lead selection and drug discovery. Lately, immobilization of target proteins and their insertion in flow-through systems have been quite spread out, as ligand high-throughput screening methodologies [1–7]. Recent applications concern the frontal affinity chromatography (FAC) methodologies [8,9], continuous flow microfluidic systems [10], displacement [7] and zonal elution chromatography [11,12].

In this context, the preparation of an AChE-CIM-IMER (immobilized human recombinant acetylcholinesterase-based micro enzyme reactor) for inhibitor screening was previously optimized by us [5,6]. This bioreactor (3 mm × 12 mm I.D.) was characterized in terms of rate of immobilization, stability, conditioning time for HPLC analyses, optimum mobile phase and peaks shape as well as nonspecific interactions and costs. Covalent immobilization through Schiff bases linkage gave a stable reactor without any significant change in the enzyme behavior. The chosen Convective Interaction Media (CIM) monolithic matrix guaranteed very short conditioning time (5 min), suitable time of analysis (complete elution of the product in 2 min) and fast recovery of the enzymatic activity that represent very important features in high-throughput analysis. The usability of the AChE-CIM-IMER in inhibition studies was assessed by studying selected well-known AChE inhibitors. In particular, IC_{50} values were assessed by injecting increasing concentrations of the tested inhibitor and saturating concentration of substrate

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(conversion of substrate into product lower than 2%). The reduction of the product area was correlated to the inhibition of the enzymatic activity.

However, in a second stage following the screening step, selected hits need to be further characterized in terms of mechanism of action (reversible inhibition) and kinetic parameters (pseudo-irreversible inhibition).

The understanding of selected potent AChE inhibitors mechanism of action is a key information to rationally design new compounds, as candidates for the treatment of Alzheimer's disease. In particular, inhibition mode is of key importance in the case of reversible AChE inhibitors because of the potential role of the AChE peripheral binding site in inducing beta-amyloid aggregation and senile plaques formation [13–17]. To this purpose, we here report the application of the AChE-CIM-IMER to the determination of both the mechanism of action and inhibitory constants of selected AChE reversible in a highly reliable and automated mode.

On the other hand, inhibition by pseudo-irreversible inhibitors is time dependent, therefore, the determination of the kinetic parameters is of utmost importance to assess time of action [18–20]. Although this need, at our knowledge no specific online kinetic study on pseudo-irreversible inhibitors is present in literature, which might be related to the difficulties in regenerating inhibited enzyme.

The here presented automated, accurate, precise method, is the first reported HPLC-based method for the on-line kinetic determination of pseudo-irreversible inhibitors mode of action. The fact that the amount of enzyme is stable for months guaranteed the fast and reliable characterization of new inhibitors.

2. Materials and methods

2.1. Materials

EDA-CIM disks (3 mm × 12 mm I.D., 0.34 mL internal volume) were kindly donated by BIA Separations (Ljubljana, Slovenia). 5,5′-Dithio-bis(2-nitrobenzoic acid) (DTNB; Ellman's reagent), glutaraldehyde 70% aqueous solution, physostigmine and human recombinant acetylcholinesterase (AChE, EC 3.1.1.7) lyophilized powder and its substrate (S)-acetylthiocholine iodide (ATCh) were purchased from Sigma (Milan, Italy). Tacrine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride), edrophonium chloride and monoethanolamine were obtained from Aldrich (Milan, Italy). Donepezil was a kind gift from Pfizer. Ambenonium chloride pentahydrate was purchased from Tocris Cookson (UK). Potassium chlorate and sodium cyanoborohydride were obtained from Fluka (Milan, Italy) and magnesium sulfate from Merck (Darmstadt, Germany). Rivastigmine was extracted from Exelon 3 mg, tablets (Novartis, Basel, Switzerland), and its purity confirmed by HPLC (98%).

HPLC-grade methanol (Romil, UK) or bidistilled water was used to prepare inhibitors' solutions. Purified water from Milli-RX system (Millipore, Milford, MA, USA) was used to prepare buffers and standard solutions. To prepare buffer solutions potassium dihydrogenphosphate, dipotassium hydrogenphosphate trihydrate, Tris–HCl (Carlo Erba, Milan, Italy) of analysis quality were used.

The buffer solutions were filtered through a 0.45 μm membrane filter and degassed before their use for HPLC.

Stock solutions of reference inhibitors were prepared in water or methanol (1–10 mM) and further diluted in water.

2.2. Apparatus

Spectrophotometric determinations with AChE in solution were performed using a Jasco double beam V-530 UV–vis spectrophotometer, with a slit width of 2 nm and 0.5 s data pitch.

AChE-CIM-IMER was inserted in a HPLC system consisting of a Jasco BIP-I HPLC pump equipped with a Rheodyne Model 7125 injector with a 10 μL sample loop. The eluates were monitored by a Jasco 875-UV Intelligent UV–vis detector connected to a computer station (JCL 6000 program for chromatographic data acquisition). For routine analyses the detector wavelength was set at 412 or 450 nm in order to monitor the yellow product of the enzymatic reaction.

2.3. Chromatographic conditions

The chromatographic analyses on AChE-CIM-IMER were performed at 25 °C unless otherwise stated.

Optimal chromatographic conditions were obtained with a mobile phase consisting of 0.1 M Tris–HCl pH 8.0 containing 100 mM $KClO_3$ as selective competitive anion for the cationic sites on the matrix, 10 mM $MgSO_4$, 1.26×10^{-4} M Ellman's reagent (Buffer A) [5,6].

2.4. AChE immobilization

AChE-CIM-IMER was prepared by linking human recombinant AChE to a previously activated EDA-CIM disk [6]. In brief, the EDA-CIM disk was first activated by a 10% glutaraldehyde solution in phosphate buffer (50 mM, pH 6.0) (6 h, in the dark). The reacted matrix was then washed with phosphate buffer (50 mM, pH 6.0). 12 U of AChE in 800 μL of phosphate buffer (50 mM, pH 6.0) were added to the matrix and left to react overnight.

The Schiff bases were reduced by a 0.1 M cyanoborohydride solution in phosphate buffer (50 mM, pH 6.0) (2 h at room temperature). The matrix was then washed and unreacted aldehydic groups were quenched by a 0.2 M monoethanolamine solution in phosphate buffer (50 mM, pH 6.0) (3 h at room temperature) [5].

The AChE-CIM-IMER was then washed with phosphate buffer 0.1 M pH 8, inserted in the appropriate holder and connected to the HPLC system.

The amount of active units retained after immobilization resulted to be 0.22 ± 0.01.

2.5. Apparent kinetic constants variation on flow rate

Apparent K_m^{app} and v_{max}^{app} values for AChE-CIM-IMER were determined at increasing flow rates (0.2–1.2 mL/min) by