Two New Methods Monitoring Kinetics of Hydrolysis of Acetylcholine and Acetylthiocholine

Šárka Štěpánková[a], Martina Vránová[a], Pavla Zdražilová[a], Karel Komers[a,*], Alena Komersová[a], and Alexander Čegan[b]

[a] Faculty of Chemical Technology, University of Pardubice, Department of Physical Chemistry, nám. Čs. legií 565, 53210 Pardubice, Czech Republic. E-mail: karel.komers@upce.cz

[b] Department of Biological and Biochemical Sciences, University of Pardubice, Štrossova 239, 53002 Pardubice, Czech Republic

* Author for correspondence and reprint requests

Z. Naturforsch. **60c**, 943–946 (2005); received May 6/June 8, 2005

Hydroxylamine and HPLC methods, measuring *in vitro* kinetics of enzymatic hydrolysis of acetylcholine or acetylthiocholine by cholinesterases, are described. The hydroxylamine method determines the dependence of substrate concentration vs. time, the HPLC method is able to measure simultaneously the time dependences of substrate and both primary products, choline or thiocholine, and acetic acid. Practical determinations are shown, comparison with known (above all Ellman's and pH-stat) methods, advantages and disadvantages are discussed.

Key words: Acetylcholine, Hydrolysis, Kinetics

Introduction

Acetylcholine (ACH) hydrolysis by acetylcholinesterase (ACHE) or butyrylcholinesterase (BCHE) plays an important role in impulse transmission at cholinergic synapses (Taylor, 1990). In the *in vitro* studies of these uninhibited and inhibited hydrolyses acetylthiocholine (ATCH) is often used instead of ACH because of its similar qualitative kinetic behaviour and the possibility of relative simple monitoring of the reaction course by on-line measurement of the time dependence of the actual concentration of the first product of ATCH hydrolysis, thiocholine (TCH), by the well known Ellman's spectral method (Ellman *et. al.*, 1961). Hanin and Dudas (2000) describe many methods measuring the cholinesterase activity. All of them could be principally used for monitoring of ACH and/or ATCH hydrolysis. The most common of them (usable principally for ACH as well as ATCH) is the pH-stat method, which determines on-line the time dependence of the second product of both hydrolyses, acetic acid (HA), by its continuous neutralisation with the analytic solution of strong alkali (*e.g.* KOH or NaOH). Unfortunately, in the case of ATCH hydrolysis, in the aqueous milieu the first product TCH behaves also as a weak acid and consumes the next, badly definable amount of the hydroxide solution at all reaction times. By Brestkin *et al.* (1974) a formula for correction of the titrant volume was given. We have used it in our attempts, but without great success. Our research group is now dealing with the study of *in vitro* kinetics and mechanism of hydrolysis of ACH and ATCH by ACHE and BCHE inhibited by new types of inhibitors with the goal to find a new, more effective and less toxic drug against Alzheimer disease. The course of the mentioned reactions was measured by the Ellman's and the pH-stat method, but we also looked for some methods determining not only the actual concentration of products [*i.e.* choline (CH) or TCH or HA)], but also of the reactant (*i.e.* ACH or ATCH), optimal substrate and product(s) in one measurement. Below we describe two methods having these properties, the hydroxylamine and HPLC method.

Materials and Methods

Hydroxylamine (HXA) method

The principle of this method was described by Hestrin (1949) for equilibrium (not reacting) mixtures: Hydroxylamine reacts in strongly alkaline aqueous medium with the substrate (ACH or ATCH) forming acethydroxamic acid and methanol. Acidification of this mixture with HCl and addition of Fe^{3+} ions result in a red-brown complex, ACH- or ATCH-acethydroxamic product, determined by colorimetry. We adapted this process for non-equilibrium mixures including time depending (actual) concentrations of ACH or ATCH.

Chemicals

Acetylcholine chloride (min. 99%) and acetylthiocholine iodide (p.a.), both from Sigma (Prague, CZ), were kept at 0 °C. Hydroxylamine hydrochloride (p.a.), potassium hydroxide (p.a.), hydrochloride acid conc., and ferric nitrate $Fe(NO_3)_3 \cdot 9H_2O$ (p.a.) were all from Lachema (Brno, CZ).

Solutions

The solutions were as follows: 2 M aqueous hydroxylamine hydrochloride (a), 3.5 M aqueous KOH (b), conc. HCl/H_2O 1:2 (c), 0.37 M Fe^{3+} (as ferric nitrate) in aqueous 0.1 M HCl (d). A standard aqueous solution of 4 mM ACH (ATCH) was kept at 0 °C.

Measuring procedure

From the actual reaction mixture (including substrate with initial concentration $[S]_0 = 4$ mM, enzyme preparation, products, phosphate buffer, pH 8, water and eventually inhibitor) samples of 1 ml volume were taken in the chosen time intervals. Every sample was immediately and vigorously mixed with 2 ml of the mixture of (a)/(b) 1:1 (volumetrically). The rapid change of pH stopped the hydrolysis. The resulting mixture was mixed 2 min to convert ACH (ATCH) totally to acethydroxamic acid. Then the pH value was changed again to ca 1.2 by adding of 1 ml of (c) and 1 ml of (d). The absorbance A(540 nm) of this final solution was measured in the glass cuvette with optical path of 2 cm. The comparative solution had the composition of the reaction mixture without substrate. A diode array spectrograph Hewlett-Packard 8453 with a PC Chemstation was used.

Calibration procedure

From the standard solution described above 6 solutions with the ACH (ATCH) concentrations ranging from 4–0.1 mM were prepared by dilution with water. From these mixtures samples were taken and executed as described above. From the calibration lines A(540 nm) – [ACH] ([ATCH]) the molar absorption coefficients of both complexes were calculated: ε(ACH, 540 nm, 25 °C) = 785 $(\text{M cm})^{-1}$, ε(ATCH, 540 nm, 25 °C) = 857 $(\text{M cm})^{-1}$ with correlation coefficients $R = 0.9982$ and 0.9981. Using the ε coefficients the original dependencies A(540 nm) *vs.* time (t) were converted to dependencies [ACH] or [ATCH] *vs.* t and used then for kinetic analysis of the given hydrolysis. Precision of the HXA method is ± 0.1 mM, the detection limit is 0.05 mM of the substrate.

HPLC method

The greatest advantage of this method is the possibility of simultaneous determination of ACH, CH and HA (or ATCH, TCH and HA) in one measurement. The already described HPLC methods (Kaneda *et al.*, 1986; Tirefors and Gillfsberg, 1987; Damsma and Flentge, 1988; Tsai, 2000; Guirierri and Palmisano, 2001; Bielavská and Kassa, 2003) deal only with the equilibrium mixtures, separate substrate and product in a special column with a special mobile phase (mostly by means of a special ion-pair reagent) and transform them then in the consecutive column by immobilized suitable enzymes biochemically to hydrogen peroxide, which is detected amperometrically.

We developed a more simple HPLC method, including only the separation of substrate and products and their refractometric detection. Unfortunately the separation of peaks of substrate and products was not good enough using the columns and mobile phases recommended by the papers mentioned above. We were successful with this original combination: column ZORBAX SB-Aq, 4.6 × 250 mm, 5 μm (Agilent Technologies Prague, part no. 880975–914), mobile phase distilled water acidified to pH 2.2 by HCl (p.a.), 5 μl sample loop, flow rate 0.5 ml/min, pressure ca 1.5 MPa, 25 °C. The isocratic HPLC apparatus consisted of the high pressure pump HPP 5001, differential refractometer RIDK-102 and integrator CI-105 (all Laboratory Apparatus Prague, CZ).

The measuring procedure was as follows: 5 ml sample from the actual reaction mixture (with the same initial composition described in the HXA method) were most quickly acidified with 0.2 ml of 1 M aqueous HCl. The pH change stops immediately the enzyme activity and therefore also the hydrolysis. The volumetric change of the sample was included in the calibration curve. This acidified sample was then injected to the column.

The chromatograms of the samples included the following order of peaks of the reaction components and their approximate retention times: ACH hydrolysis: buffer and HCl (5.4 min), CH (5.8 min), HA (7.9 min), ACH (8.5 min); ATCH hydrolysis: buffer and HCl (5.4 min), HI (6.3 min), TCH (7.0 min), HA (8.3 min), ATCH (ca. 14 min, broad peak). In the chromatogram of the ATCH hydrolysis sample the peaks of (HI + TCH), HA and ATCH were clearly separated and all could be directly integrated. From the common signal (HI + TCH) the constant area of HI must be substracted. In the chromatogram of the ACH hydrolysis sample the HA and ACH peaks generally interfered with high ACH levels present at the start of the reaction. To calculate [ACH] the area of HA had to be substracted (according to [CH] and [HA] calibration curves) from this complex peak. At smaller ACH concentrations the HA and ACH peaks were separated as described above. Precision of the described HPLC method is ± 0.1 mM, the detection limit is 0.05 mM of the substrate.

Results and Discussion

Both methods described above were used (together with Ellman's and pH-stat method) in the research of kinetics and mechanism of the *in vitro* enzymatic hydrolyses of ACH and ATCH by ACHE and BCHE uninhibited and inhibited with new types of inhibitors. Examples of the practical use of the described methods are given in Figs. 1 and 2. The actual molar concentrations of the determined reaction components are expressed by brackets [ ], *t* is the reaction time in seconds.

Fig. 1 describes the dependence [ACH] *vs.* *t* determined by the HXA method and dependences [ACH] and [CH] *vs.* *t* determined simultaneously by the HPLC method for the uninhibited total hydrolysis of ACH by ACHE (from electric eel, Sigma Prague, CZ). The reaction was realized in a glass batch reactor at following initial conditions: 25 °C, pH 8 (phosphate buffer), $[ACH]_0 = 4$ mM and ACHE catalytic activity 3.5 U in the reaction mixture.

Remark: The catalytic activity of 1 unit (U) has such amount of the given enzyme preparation which converts 1 μmol of the given substrate in 1 min at the given reaction conditions.

Fig. 2 shows the dependencies [ATCH], [TCH] and [HA] *vs.* *t* determined simultaneously for hydrolysis of ATCH by BCHE (from bovine erythrocytes, Sigma Prague, CZ) under identical conditions as described for Fig. 1. The assignment of single types of points to individual reaction components is given in both figures.

It can be seen that both curves [ACH] *vs.* *t* in Fig. 1 and dependencies [TCH] and [HA] *vs.* *t* in Fig. 2, both determined by independent methods, are well comparable. The practically constant values (≈ 4 mM) of the points representing the sum of actual [ACH] and [CH] (Fig. 1), [ATCH] and [TCH] or [ATCH] and [HA] (Fig. 2), indicate, that 1.) during the whole reaction time ACH (ATCH) is hydrolyzed only to CH (TCH) and HA; 2.) CH (TCH) and HA are formed in one step.

Figs. 1 and 2 demonstrate good applicability and comparability of the results obtained by the described new methods at the same reaction conditions. The application of these methods is simpler

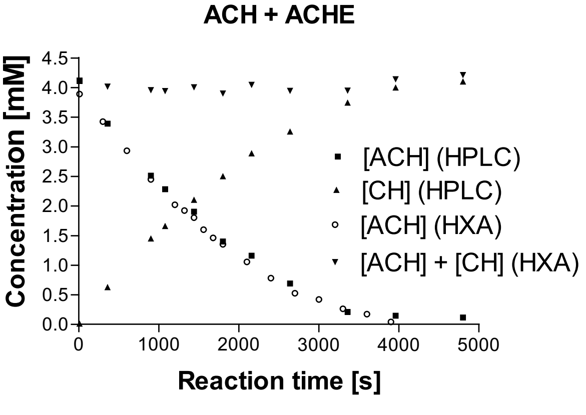

Fig. 1. Total uninhibited hydrolysis of ACH by ACHE (electric eel) monitored by the HXA and HPLC method. Detailed descriptions are in the figure and text.

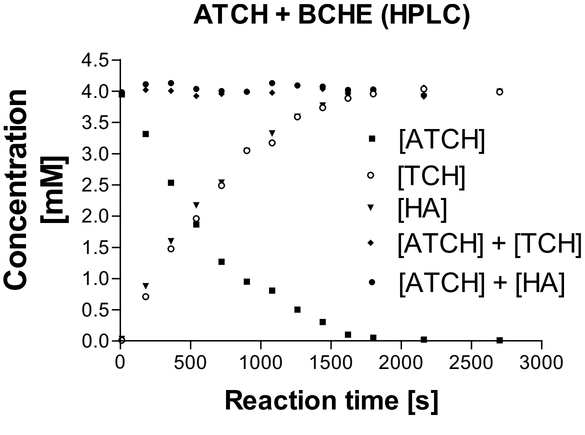

Fig. 2. Total uninhibited hydrolysis of ATCH by BCHE (bovine erythrocytes) determined by the HPLC method. Detailed descriptions are in the figure and text.

than that of the Ellman's or the pH-stat method, and requires only currently used laboratory equipment. The methods have two disadvantages: 1.) They cannot be realized on-line; the samples must be taken from the actual reaction mixture in suitable intervals. 2.) They are not as sensitive as the Ellman's method, working with the initial concentration of substrate in order of 10^{-5} M. In case of the HPLC method, the sensitivity could be probably increased using UV spectral detection. On the other hand, the composition of the starting reaction mixture (which can substantially affect the reaction mechanism) is fully identical in both methods (*e.g.* water, buffer, substrate, enzyme), while *e.g.* the Ellman's method requires still the surplus of Ellman's reagent and in the pH-stat method no buffer can be used.

Acknowledgement

This work was financially supported by Ministry of Education, Youth and Sport as a part of the research project MSMOO21627502.

Bielavská M. and Kassa J. (2003), Determination of the level of acetylthiocholine and choline in the rat hippocampus by liquid chromatography with electrochemical detection. Chem. Lett. **97**, 1190–1192 (in Czech).

Brestkin A. P., Žukovskij J. G., and Sipenkova T. M. (1974), Hydrolysis of cholin- and thiocholin esters by cholinesterases. Biochimia **39**, 13–18 (in Russian).

Damsma G. and Flentge F. (1988), Liquid chromatography with electrochemical detection for the determination of choline and acetylcholine in plasma and red blood cells. J. Chromatogr. **428**, 1–8.

Ellman G. L., Courtney K. D., Andres V. Jr., and Featherstone R. M. (1961), A new rapid colorimetric determination of acetylthiocholinesterase activity. Biochem. Pharmacol. **7**, 88–95.

Guirierri A. and Palmisano F. (2001), An acetylcholinesterase/choline oxidase based amperometric biosenzor as a liquid chromatography detector for acetylcholine and choline determination in brain tissue homogenates. Anal. Chem. **73**, 2875–2882.

Hanin I. and Dudas B. (2000), Measurement of cholinesterase activity. In: Cholinesterases and Cholinesterase Inhibitors (Giacobini E., ed.). Martin Dunitz, London, pp. 139–144.

Hestrin S. (1949), The reaction of acetylcholine and other carboxylic acid derivates with hydroxylamine, and its analytical application. J. Biol. Chem. **180**, 249–261.

Kaneda N., Asano M., and Nagatsu T. (1986), Simple method for the simultaneous determination of acetylcholine, choline, noradrenaline, dopamine and serotonin in brain tissue by high-performance liquid chromatography with electrochemical detection. J. Chromatogr. **360**, 211–219.

Taylor P. (1990), Acetylcholinesterase and butyrylcholinesterase. In: The Pharmacological Basis of Therapeutics, 5th. ed. (Gilman A. G., Nies A. S., Rall T. W., and Taylor P., eds.). MacMillan, New York, pp. 131–150.

Tirefors N. and Gillfsberg P. G. (1987), Determination of acetylcholine and choline in microdialysates from spinal cord of rat using liquid chromatography with electrochemical detection. J. Chromatogr. **423**, 85–91.

Tsai T. H. (2000), Separation methods used in the determination of choline and acetylcholine. J. Chromatogr. B **747**, 111–122.

Satz und Druck: AZ Druck und Datentechnik GmbH, Kempten