ELSEVIER

Journal of Chromatography B, 753 (2001) 375–383

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of inhibitors' potency (IC_{50}) by a direct high-performance liquid chromatographic method on an immobilised acetylcholinesterase column

V. Andrisano[a,*], M. Bartolini[a], R. Gotti[a], V. Cavrini[a], G. Felix[b]

[a] *Dipartimento di Scienze Farmaceutiche, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy*

[b] *E.N.S.C.P.B. University of Bordeaux, Talence, France*

Received 26 June 2000; received in revised form 18 October 2000; accepted 2 November 2000

Abstract

An immobilised acetylcholinesterase (AChE) stationary phase was prepared by using an in situ AChE immobilisation procedure. A stainless steel column packed with epoxide silica was connected to the HPLC system and the enzyme solution at pH 5.8 was recycled through the column at a flow-rate of 0.5 ml/min for 24 h. The activity of the immobilised AChE was determined by injecting the substrate acetylthiocholine, using as mobile phase 0.1 *M* phosphate buffer (pH 7.4) containing Ellman's reagent [5,5′-dithio-bis(2-nitrobenzoic acid)] and measuring the area of the obtained peak with UV detection at 412 nm. The effect of AChE inhibitors tacrine, edrophonium and donepezil were evaluated by the simultaneous injection of each inhibitor with the substrate. The resulting decrease in the AChE activity, as expressed by the decrease of the peak area detected at 412 nm, was related to the concentration and potency of the solutes. The obtained IC_{50} values were compared with those derived by the conventional spectrophotometric method. This immobilized enzyme reactor, included in a chromatographic system, can be used for the rapid screening for new inhibitors allowing for the on-line determination of a compound's inhibitory potency. The advantages over the conventional methods are the increased enzyme stability and system automation which allows a large number of compounds to be analysed continuously. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Column liquid chromatography; Inhibitors' potency; Acetylcholinesterase immobilisation; Activity evaluation

1. Introduction

Human acetylcholinesterase (AChE) is currently indicated as a target enzyme for drugs to be used in the treatment of Alzheimer's disease (AD). Presently, in the frame of the so-called ''cholinergic hypothesis'', a great deal of research is being devoted to the discovery of potent and selective AChE inhibitors able to maintain high levels of acetylcholine at the muscarinic and nicotinic receptors in the central nervous system [1–6]. The pharmaceutical interest in developing AChE inhibitors resides also in the fact that one of the most relevant neuropathological characteristics of AD brain is the senile plaques that are formed by a core of amyloid-β-peptide (Aβ) fibrils. AChE was found to accelerate the assembly of Aβ into Alzheimer fibrils and to be blocked in this induced action by peripheral anionic binding site inhibitors [7].

*Corresponding author.

E-mail address: analit@alma.unibo.it (V. Andrisano).

0378-4347/01/$ – see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(00)00571-5

In the case of AChE, as well as in the case of other enzymes of pathological and therapeutic relevance, it takes a great amount of effort to develop efficient inhibitors. These efforts must include preliminary studies aimed at accumulating the necessary knowledge about the potency and the mechanism of action of inhibitors.

One of the most widely used method to measure the activity of AChE and its decrease in the presence of inhibitors is the UV–Vis spectrophotometric method based on the rate of colour production monitored at 412 nm due to the formation of a yellow anion (5-thio-2-nitro-benzoic acid) derived from the dithiobisnitrobenzoate (Ellman's reagent) when it reacts with thiocholine (product of enzymatic hydrolysis of the substrate acetylthiocholine) [8].

One approach to the rapid screening for enzyme inhibitors is the immobilisation of the target enzyme and its inclusion in a chromatographic system. This allows for the on-line determination of a compound inhibitory potency as well as the mechanism of the observed inhibition. The utility of this approach was previously demonstrated using an immobilised α-chymotrypsin stationary phase [9].

In this study, an immobilised AChE column was prepared and a direct high-performance liquid chromatographic (HPLC) procedure by using an in situ AChE derivatised column connected to a photodiode array detection (DAD) system has been developed. The final aim of this study is to perform on-line chromatographic screening for enzymatic inhibitors for the discovery and characterisation of new therapeutic agents. The screening technology involves the synthesis of an immobilized enzyme reactor in which the target enzyme has been immobilized with retention of its enzymatic activity. A known substrate of the enzyme is injected through the enzyme reactor and the resulting product directly determined. Known or suspected enzyme inhibitors are then added to the system and their effect on the product formation determined. The advantages over the conventional methods are the increased enzyme stability and the system automation which allows a large number of compounds to be analysed continuously.

Several LC methods have been published on the determination of choline and acetylcholine [10], α naphthyl acetate [11] using packed bed reactors. However, some of these methods required the immobilisation of the enzyme choline oxidase together with acetylcholinesterase, and an electrochemical detector was required to detect the released hydrogen peroxide [12].

The present approach is based on the use of the reactive substrate acetylthiocholine and involves a conventional UV detection system to detect thiocholine through its derivatisation with the Ellman reagent. Immobilised enzyme columns were prepared and the immobilised enzyme proved to remain active. This column was first characterised in order to assess the amount of active immobilised enzyme and the conditions for best performance in terms of activity, reproducibility and stability. Then the chromatographic system was used to determine the inhibition potency (IC_{50}) of known AChE inhibitors, whose activity was previously determined with the same enzyme in solution using the conventional Ellman spectrophotometric method [8].

2. Experimental

2.1. Materials

(*S*)-Acetylthiocholine iodide, 5,5′-dithio-bis(2-nitrobenzoic acid) DTNB (Ellman's reagent), acetylcholinesterase (0.5 U/mg) derived from human erythrocytes were purchased from Sigma (Milan, Italy). Tacrine (9-amino-1,2,3,4-tetrahydro acridine hydrochloride), edrophonium and donepezil were obtained from Aldrich Italia.

Buffer components and other chemicals were of the highest purity available commercially and supplied by Carlo Erba (Milan, Italy).

HPLC-grade methanol and ethanol (Promochem, Germany) were used to prepare the inhibitor solutions. Purified water from a TKA ROS 300 system was used to prepare buffers and standard solutions. To prepare the [0.1 *M*, pH 7.4] phosphate buffer solution, potassium dihydrogenphosphate and dipotassium hydrogenphosphate trihydrate of analysis quality (Carlo Erba) were used.

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

2.2. Apparatus

Spectrophotometric determinations 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.

The solvent delivery system was a Jasco PU-980 Intelligent HPLC pump equipped with a Reodyne Model 7125 injector with a 20-μl sample loop. The eluents were monitored by a Jasco MD 910 multiwavelength detection (DAD) system connected to a computer station. For routine analyses the detector wavelengths were set at 412 nm and 450 nm.

The HPLC stationary phase consisted of immobilised AChE column (50×4.6 mm I.D.). The chromatographic analyses on the AChE column were performed at 37°C and the temperature was maintained using a column heater/chiller (Model 7955; Jones Chromatography, UK)

2.3. AChE immobilisation

The enzyme was immobilised on a silica support by using the in situ derivatisation technique [13–15]. At room temperature, a stainless steel column packed with 0.7 g epoxide silica gel (Kromasil 200A-5 μm) was connected to the HPLC system and the enzyme solution [250 U dissolved in 80 ml of an aqueous solution containing 0.05 *M* KH_2PO_4 and 1.25 *M* $(NH_4)_2SO_4$ (pH 5.8)] was recycled through the column at a flow-rate of 0.5 ml/min for 24 h, flushing and back flushing until all the solution was used up. The amino groups of lysine residues of the enzyme reacted with the epoxide groups to give a covalent bond carbon–nitrogen and a vicinal hydroxy residue.

The amount of immobilised cholinesterase was determined by the UV absorbance decrease of the enzyme solution before and after the immobilisation procedure.

Then the column was rinsed with 100 ml of 0.05 *M* KH_2PO_4 (pH 6.0) solution. After that, the column was flushed with 50 ml of 1 *M* glycine in 0.05 *M* KH_2PO_4 (pH 7.0) solution and then rinsed with 20 ml of 0.05 *M* KH_2PO_4 (pH 7.0) solution. Before storage, 30 ml of the 0.05 *M* KH_2PO_4 (pH 7.0) solution containing 0.01% sodium azide was passed through the column.

2.4. AChE column activity determination

The enzyme column was connected between the HPLC pump and HPLC DAD system. It was equilibrated for 30 min with 0.1 *M* phosphate buffer, pH 7.4 and then conditioned with the same buffer containing $1.26 \cdot 10^{-4}$ *M* of Ellman reagent.

A 20-μl volume of aqueous solution of increasing acetylthiocholine concentration (range comprised between 5.8 and 900 m*M*) were injected onto the HPLC system with a flow-rate of 0.1 ml/min with UV detection at 412 nm. The amount of coloured species eluted from the AChE column was contained in the first 2.0-ml fraction of each eluate (20 min). Identification was confirmed by peak spectra acquisition by DAD. At the beginning of the study, each fraction was collected in a 10-ml volumetric flask (dilution factor, df=5) adjusting the volume with 0.1 *M* phosphate buffer (pH 7.4). The relative absorbance values were determined spectrophotometrically at 412 nm, by using as blank a solution of 0.1 *M* phosphate buffer (pH 7.4) containing $1.26 \cdot 10^{-4}$ *M* Ellman reagent. The rate of the enzymatic reaction (V) expressed as (ΔA/min) was calculated by:

$$V\ (\Delta A/\text{min}) = \frac{AU \times \text{df}}{\text{Time (min)}} = \frac{AU \times 5}{20}$$

To obtain estimates of the K_m and V_{max}, Lineweaver and Burk reciprocal plots of $1/V$ versus 1/[S] were constructed, were [S] is the concentration of injected acetylthiocholine solution (range from 5.8 to 900 m*M*).

To calculate the units (U) of immobilised enzyme the following equation was used:

$$\text{U}\ (\mu\text{mol/l/min}) = \frac{(\Delta A/\text{min})_{max}}{\epsilon} \cdot 10^6$$

One unit (U)=the amount of enzyme which hydrolyses 1 μmol of acetylthiocholine in 1 min.

In order to determine the enzyme product extinction coefficient for concentration assessment, increasing concentrations of acetylthiocholine (1.5–150 μ*M*) were hydrolysed by 0.035 U ml^{-1} of AChE in the presence of $1.26 \cdot 10^{-4}$ *M* Ellman reagent in solution and the increase of absorbance versus time spectrophotometrically recorded up to the maximum stable value. The extinction coefficient

of the coloured reaction product was the slope of the linear regression curve obtained by plotting the maximum absorbance values versus substrate concentrations.

To check the stability of enzyme activity, 20-μl volumes of aqueous solution of increasing acetylthiocholine concentration (range comprised between 5.8 m*M* and 432 m*M*) were injected onto the HPLC system (the AChE column connected to the DAD system) at a mobile phase flow-rate of 0.1 ml/min with UV detection at 450 nm. The area of the peak eluted at retention time (t_R) = 10 min was plotted against the corresponding substrate concentration.

2.5. Influence of pH on enzyme activity

The enzyme column was equilibrated for 30 min with 0.1 *M* phosphate buffer (pH 5.0–8.0) containing $1.26 \cdot 10^{-4}$ *M* of Ellman reagent (mobile phase).

A fixed acetylthiocholine concentration (5.21 m*M*) was injected in triplicate onto the HPLC system with a flow-rate of 0.1 ml/min with UV detection at 412 nm. The product peak area was integrated and plotted against the pH value of the mobile phase (Fig. 1).

2.6. Influence of flow-rate on enzyme activity

A fixed acetylthiocholine concentration (5.21 m*M*) was injected in triplicate onto the HPLC system at different flow-rates (0.07–0.30 ml/min) of the mobile phase with UV detection at 412 nm.

2.7. Determination of inhibitory potency (IC_{50})

Stock solutions of the test compounds (1 m*M*) were prepared in ethanol. The assay solutions were prepared by diluting the stock solutions in water together with the substrate acetylthiocholine at a fixed concentration. In particular five different concentrations of each compound were mixed together with the substrate (261 m*M*) in order to obtain inhibition of acetylcolinesterase activity comprised between 20 and 80%.

Aliquots of 20 μl of a solution containing 261 m*M* acetylthiocholine were injected into the chromatograph in triplicate and the peaks eluting at $t_R = 10$ min determined.

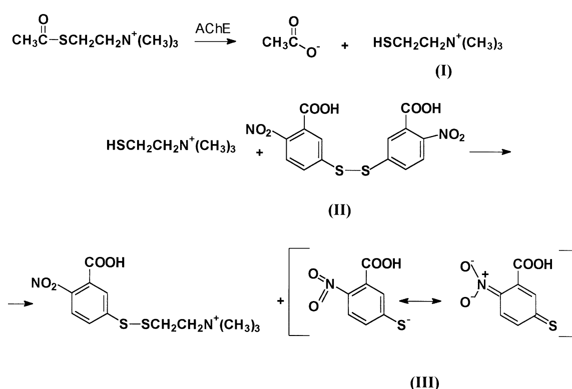


Fig. 1. AChE catalysed hydrolysis of acetylthiocholine and thiocholine (I) reaction with Ellman reagent (II) with yellow anion (III) production.

The assay solutions containing increasing inhibitor concentration and a fixed substrate concentration were then injected into the chromatographic system and the relative peak areas integrated.

The peak areas were compared with those obtained in absence and presence of inhibitor and % inhibition was calculated. Each concentration was analysed in triplicate. The percent inhibition of the enzyme activity due to the presence of increasing test compound concentration was calculated by the following expression: $100 - (A_i / A_0 \cdot 100)$ where A_i is the peak area calculated in the presence of inhibitor and A_0 is the peak area obtained with the substrate solution only. Inhibition curves were obtained for each compound by plotting the % inhibition versus the logarithm of inhibitor concentration in the assay solution. The linear regression parameters were determined for each curve and the IC_{50} extrapolated.

3. Results and discussion

3.1. AChE immobilisation and activity determination

Following the described immobilisation procedure, by the decrease of the UV absorbance due to enzyme

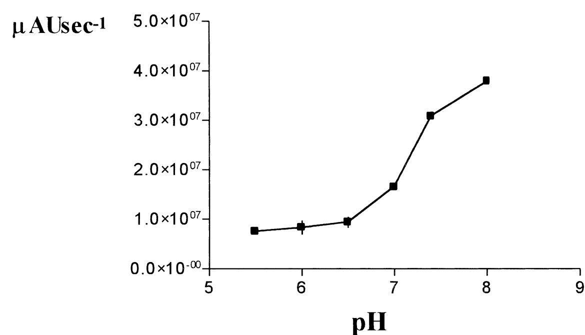


Fig. 2. Mobile phase pH dependence of AChE activity. The values are the mean of three independent measurements (SE±2.5%).

immobilisation, it was possible to calculate the amount of immobilised units which were found to be 63. The yield of immobilisation was 25%.

However, it was considered necessary to determine the amount of active immobilised enzyme and the saturating substrate concentration in order to perform inhibition experiments.

As the product of the enzymatic hydrolysis, thiocholine (I), does not present a significant chromophore for UV detection; the evaluation of enzyme activity was performed by injecting acetylthiocholine with the Ellman reagent (II) dissolved in the mobile phase. The enzymatic hydrolysis and the subsequent derivatisation reaction are shown in Fig. 2.

Fig. 3. Michaelis–Menten plots of (A) immobilised AChE, (B) free AChE rate versus acetylthiocholine concentration. Lineweaver and Burk reciprocal plots for (C) immobilised AChE, (D) free AChE. The values are the mean of two independent measurements (SE±4%).

The active immobilised units were calculated by following the reported method, i.e., injecting the substrate onto the AChE column, collecting the product of the enzymatic hydrolysis, reading the absorbance of the product solution. By using the yellow anion calculated extinction coefficient the number of units were determined. The yellow anion extinction coefficient was determined by subjecting low concentration of substrate (1.5–150 μ*M*) to complete enzymatic hydrolysis in solution. A linear correlation ($r^2=0.9999$) was found between the anion absorbance (412 nm) and the concentration of acetylthiocholine, stoichiometrically equal to the concentration of yellow anion. The resulting slope provided the extinction coefficient value ($\epsilon=13\,900\pm17$ cm^{-1} M^{-1}) according to the literature [6].

The product of the acetylcholine enzymatic hydrolysis had to be collected because it is not commercially available, so it was the only way to determine its concentration and hence the K_m and V_{max} and from the V_{max} the active immobilised units. The amount of anion from AChE catalysed hydrolysis was found to be dependent from the concentration of the injected acetylthiocholine, following the Michaelis–Menten equation (Fig. 3). To determine the amount of active immobilised enzyme and the saturating substrate concentration, the rate of acetylthiocholine hydrolysis at increasing injected acetylthiocholine amounts was plotted against the corresponding injected substrate concentration. Lineweaver and Burk reciprocal plot of $1/V$ and $1/[S]$ [17] allowed one to estimate the value of K_m (247± 25 m*M*) and V_{max} (1.351±0.135 ΔA/min) (Fig. 3). The K_m and V_{max} found with 0.035 units/ml of free AChE in solution were, respectively, 0.170±0.005 m*M* and 0.130±0.010 ΔA/min (Fig. 3). The amount of immobilised enzyme is about 2000-times the amount used in solution, the immobilised enzyme K_m value was found comparably high.

The amount of active immobilised enzyme was found to be 96±9 units which is comparable to the value of the immobilised enzyme. So all the immobilised enzyme was found to be active after the immobilisation procedure. However, over time, the column was found to slowly loosing activity, down to 70% of the initial value, its saturating concentration and K_m decreasing.

In order to check the enzyme stability over time, aliquots of 20 μl of aqueous solution of increasing acetylthiocholine concentration (range comprised between 5.8 and 432 m*M*) were injected into the chromatograph at a mobile phase flow-rate of 0.1 ml/min with the AChE column connected to the DAD system. The area of the peak eluted at $t_R=10$ min was plotted against the corresponding substrate concentration.

The amount of the resulting coloured species (III) was selectively monitored fixing the detection wavelength at either 412 nm (λ_{max}) or higher wavelengths such as 450 nm in order to reduce interference from Ellman reagent excess or from the disulfide side-product and to keep in scale the absorbance value of the eluted anion. The retention time of the yellow coloured anion was 10 min and the time required for its complete elution was 20 min (equivalent to 2 ml of retention volume).

The Michaelis–Menten trend was found by plotting the peak area against substrate concentration, with UV detection fixed at 450 nm in order to keep the scale of absorbance below the value of 2, because at the beginning of the study, the amount of active immobilised enzyme was such that the saturating substrate concentration was out of scale in the HPLC detector. A progressive loss of enzyme activity was followed over time down to 70% of its initial value in 4 months. This was followed by a progressive reduction of peak area for injecting the same amount of saturating substrate concentration. The detection limit was found to be 0.02 m*M* at 412 nm.

The pH value of the mobile phase was found to influence the enzymatic acetylthiocholine hydrolysis. The dependence of peak area related to the sulfide anion on the mobile phase pH value is shown in Fig. 1. A pH 7.4 value was chosen, because as already reported [8] this pH value was used to study the human AChE at its physiological pH, to maintain the stability of the chromatographic support and in order to maximise enzyme activity. It is also suitable for the reaction with Ellman reagent.

The best flow-rate to give a higher response in term of peak area (i.e., hydrolysis rate) and reasonable time for anion elution was found to be 0.1 ml/min.

The yield of the enzymatic hydrolysis under the described chromatographic conditions was also evaluated.

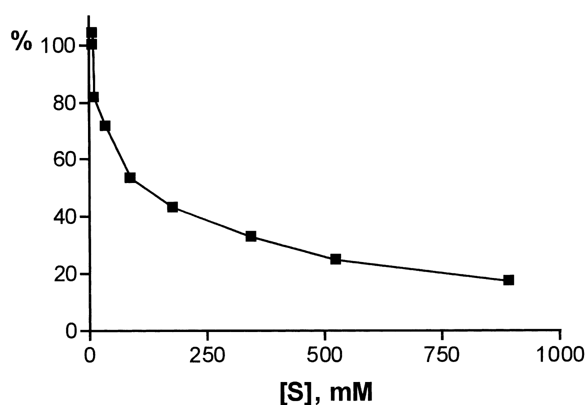


Fig. 4. Yield of AChE catalysed hydrolysis of acetylthiocholine [S] as a function of injected substrate concentration. The values are the mean of three independent measurements (SE±4%).

For each injected substrate concentration the yield was determined by collecting 10 ml of the eluate from the enzyme column, measuring the absorbance value at 412 nm and hence determining the related anion concentration by the value of the extinction coefficient ($\epsilon = 13\,900 \pm 17$ cm^{-1} M^{-1}). The resulting anion concentrations were then compared to the theoretical concentration from completely hydrolysed substrate and the obtained percent yield are reported in Fig. 4. As shown, the enzymatic substrate hydrolysis was found to be quantitative at low substrate concentration, while by increasing the

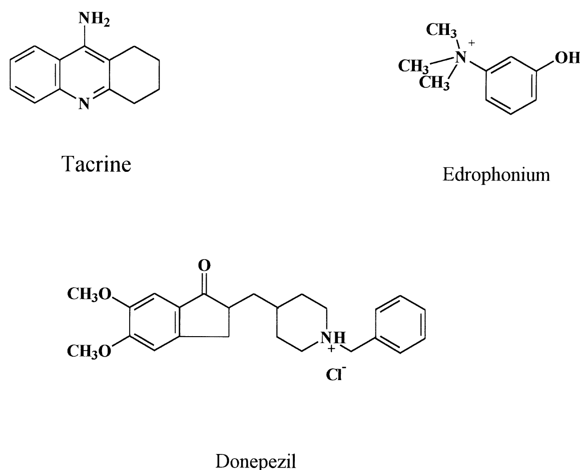
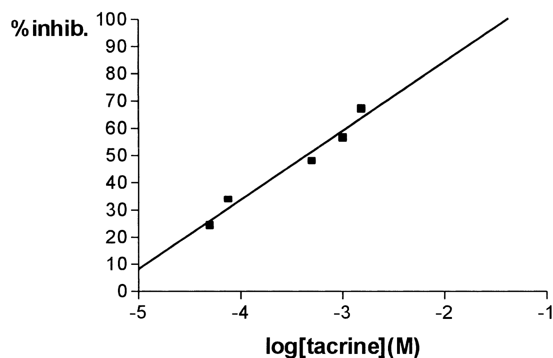
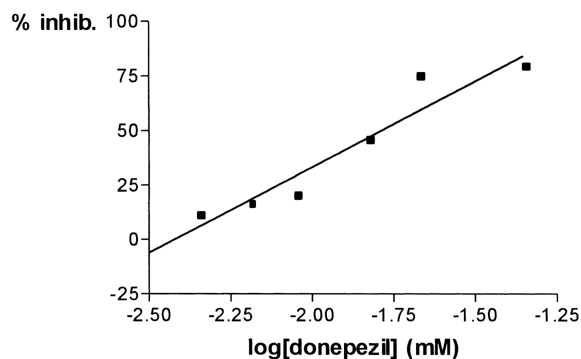


Fig. 5. Structures of the analysed inhibitors.

Table 1
Inhibition of AChE by known inhibitors

Compound	$IC_{50} \pm SE$ (μM) (reference method)	$IC_{50} \pm SE$ (mM) (AChE column)
Tacrine	0.25±0.01	0.45±0.03
Edrophonium	5.22±0.57	4.33±0.22
Donezepil	0.005±0.002	0.0092±0.0015





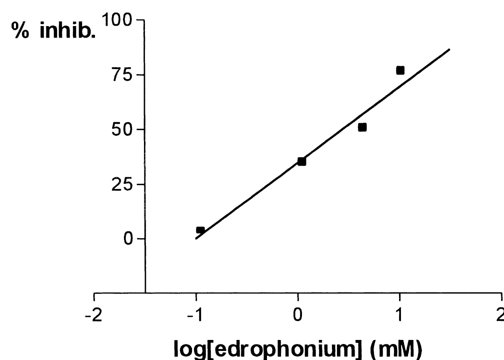


Fig. 6. Inhibition curves for tacrine, donepezil and edrophonium. The values are the mean of three independent measurements (SE±6%).

amount of injected substrate the product yield exponentially decreased to 20% for a 900 m*M* substrate concentration.

3.2. Determination of inhibitory potency (IC_{50})

Three reversible inhibitors of AChE were examined: tacrine, donepezil, edrophonium (Fig. 5) [1,3,16]. These inhibitors were chosen due to their difference in potency and mode of action (Table 1) as regards the AChE binding sites. Tacrine is a mixed mode competitive inhibitor, edrophonium is a pure competitive inhibitor. Tacrine is more active than edrophonium by one order of magnitude, while donezepil is two order of magnitude more potent than tacrine.

Their IC_{50} values were first determined by the conventional spectrophotometric Ellman method [6], using the same type of AChE (from human erythrocytes) which was immobilised on column.

Then the IC_{50} values of the three inhibitors were assessed by using the immobilised enzyme column, by extrapolation from the inhibition curves (Fig. 6). The inhibition curve were obtained by injecting simultaneously both the substrate at a fixed saturating concentration, as determined by the Michaelis–Menten plot and inhibitors at increasing concentration. Increasing reduction of the peak area of the coloured anion (i.e., inhibition of enzyme rate of hydrolysis), when compared to the area obtained by the sole substrate, was observed for increasing inhibitors concentration (Fig. 7). The percent inhibition was plotted against inhibitor concentration to obtain the inhibition curves (Fig. 6).

The IC_{50} values of the three inhibitors on the enzyme column are reported in Table 1. They correlate well with the reference method ($r^2=0.99$), their relative potency being respected.

4. Conclusion

The proposed immobilisation procedure was found appropriate to covalently bind AChE to silica matrix, maintaining enzyme activity. Therefore the AChE column was tested for determining the immobilised enzyme kinetic parameters and was used to assess the potency of inhibitors.

The proposed method was found appropriate for discriminating the potency of compounds whose activity varies among three-orders of magnitude.

Moreover the system has the potential of being automated, allowing a large numbers of compounds to be analysed continuously. By using an autosampler, potential inhibitors can be screened by injecting them together with the substrate at a saturating concentration and monitoring reduction of the peak area of the coloured anion (i.e., inhibition of enzyme rate of hydrolysis) when compared to the area obtained by the sole substrate injection. Another advantages in term of accuracy and precision is the fact that the enzyme does not constitute a variable.

The stability of the immobilised enzyme was found to be maintained for more than 4 months with

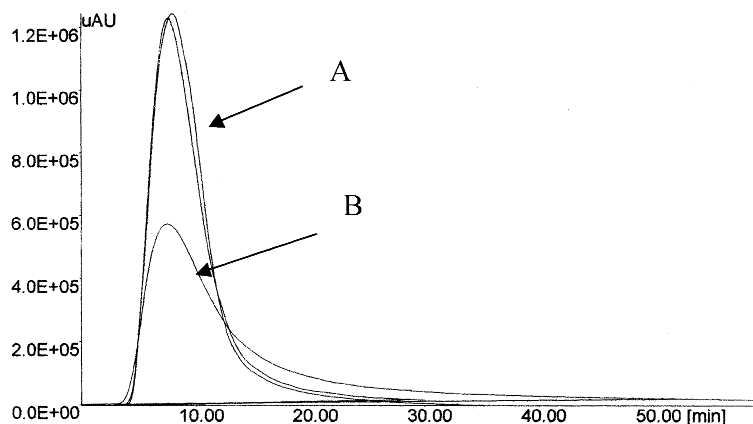


Fig. 7. Overlaid chromatograms of (A) substrate at saturating concentration, (B) substrate and 9 μ*M* donepezil.

daily use, even if during this length of time a progressive continuous loss of enzyme activity was observed down to 70% of the initial one. However the determination of inhibitory potency was still possible in term of sensitivity, accuracy and precision.

Acknowledgements

Investigation supported by University of Bologna (funds for selected rersearch topics) and by MURST (Cofin '98). We thank Mr. M. Baraldi for technical assistance.

References

[1] M. Brufani, L. Filocamo, S. Lappa, A. Maggi, Drugs Future 22 (1997) 397.
[2] D.R. Rosemberg, B.A. Wright, S. Gershon, Drugs Today 26 (1990) 44.
[3] M. Williams, R.E. Davis, Expert Opin. Invest. Drugs 6 (1997) 735.
[4] F. Gualtieri, S. Dei, D. Manetti, M.N. Romanelli, S. Scapecchi, E. Teodori, Farmaco 50 (1995) 489.
[5] C. Melchiorre, V. Andrisano, M.L. Bolognesi, R. Budriesi, A. Cavalli, V. Cavrini, M. Rosini, V. Tumiatti, M. Recanatini, J. Med. Chem. 41 (1998) 4186.
[6] A. Rampa, A. Bisi, F. Belluti, S. Gobbi, P. Valenti, V. Andrisano, V. Cavrini, A. Cavalli, M. Recanatini, Bioorg. Med. Chem. 8 (2000) 497.
[7] N.C. Inestrosa, A. Alvarez, C.A. Perez, R.D. Moreno, M. Vicente, C. Linker, O.I. Casanueva, C. Soto, J. Garrido, Neuron 16 (1996) 881.
[8] G.L. Ellman, K.D. Courtney, V. Andres Jr., M. Featherstone, Biochem. Pharmacol. 7 (1961) 88.
[9] I.W. Wainer, P. Jadaud, G.R. Schombaum, Chromatographia 25 (1988) 903.
[10] P.E. Potter, J.L. Meek, N.H. Neff, J. Neurochem. 41 (1983) 188.
[11] M.E. Leon-Gonzalez, A. Townshend, J. Chromatogr. 539 (1991) 47.
[12] J. Salamoun, J. Remien, J. Pharm. Biomed. Anal. 10 (1992) 931.
[13] G. Felix, V. Descorps, Chromatographia 49 (1999) 595.
[14] G. Felix, V. Descorps, Chromatographia 49 (1999) 606.
[15] C. Bertucci, A. Petri, G. Felix, B. Perini, P. Salvadori, Tetrahedron Asymmetry 10 (1999) 4455.
[16] Y. Kawakami, A. Inoue, T. Kawai, M. Wakita, H. Sugimoto, A.J. Hopfinger, Bioorg. Med. Chem. 4 (1996) 1429.
[17] M. Dixon, E.C. Webb, in: Enzymes, 2nd ed., Longmans, Green and Company, 1964, p. 54.