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# Monolithic micro-immobilized-enzyme reactor with human recombinant acetylcholinesterase for on-line inhibition studies

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**Abstract**

The development and characterization of a human recombinant acetylcholinesterase (hrAChE) micro-immobilized-enzyme reactor (IMER), prepared by using an in situ immobilization procedure is reported. hrAChE was covalently immobilized on an ethylenediamine (EDA) monolithic convective interaction media (CIM) disk (12 mm × 3 mm i.d.), previously derivatized with glutaraldehyde. The optimal conditions for the immobilization were: 12 μg of enzyme dissolved in 800 μl of phosphate buffer (50 mM, pH 6.0). The mixture was gently agitated overnight at 4 °C. The resulting Schiff bases were reduced by cyanoborohydride and the remaining aldehydic groups were condensed with monoethanolamine. Under these conditions, 0.22 U of hrAChE were immobilized with retention of 3.0% of the initial enzymatic activity. The activity of the immobilized hrAChE was stable for over 60 days. The activity and kinetic parameters of the hrAChE micro-IMER were investigated by inserting the micro-IMER in a HPLC system and it was demonstrated that the enzyme retained its activity. The micro-IMER was characterized in terms of units of immobilized enzyme and best conditions for immobilization yield. IMERs were compared for their relative enzyme stability, immobilized units, yield and aspecific matrix interactions. The effect of AChE inhibitors was evaluated by the simultaneous injection of each inhibitor with the substrate. The relative $IC_{50}$ values were found in agreement with those derived by the conventional kinetic spectrophotometric method. In comparison with previously developed AChE-based IMERs, AChE monolithic micro-IMER showed advantages in terms of reduction of analysis time (2 min), lower aspecific matrix interactions and lower backpressure. Included in a HPLC system, it can be used for the rapid screening of new compounds' 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 analyzed in continuous.


*Keywords:* Immobilized enzymes; Monolithic disks; Acetylcholinesterase; Enzymes; Enzyme inhibitors

## 1. Introduction

Immobilization procedures have been found appropriate to covalently bind enzymes to modified silica matrices, with retention of enzymatic activity [1–7]. The inclusion of immobilized enzymes on a solid support in a chromatographic column has allowed preserving the enzymatic activity from inactivating processes, widely increasing the enzyme stability, with advantages in term of accuracy and reproducibility [8–15]. Considering the high cost and difficulty in over-expression, isolation and purification of recombinant enzymes, this analytical technique represents an extremely useful approach to preserve the activity of the small amount of enzyme available, to perform kinetic studies and to rapidly screen for potential drugs candidates. For example, the immobilization of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on a silica-based liquid chromatographic stationary phase increased the enzyme stability from hours to months [6].

Monoliths are considered a novel generation of stationary phases whose special feature is the fast separation and enzymatic conversion due to lack of diffusion resistance during mass transfer [16]. The large pores of monolithic materials allow high-speed analysis and low back pressure. Preliminary experiments carried out with enzymes immobilized onto compact porous supports have shown that such enzyme reactors have much higher rates of conversion than the reactors in which enzymes have been immobilized onto bulk porous supports [17].

In particular, monolithic disks based on a new polymeric macroporous material and available under the trademark convective interaction media (CIM) were chosen because

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promising for analytical application due to reduced time analysis and high enzyme efficiency [18]. CIM disks are characterized by small dimensions (12 mm in diameter and 3 mm in thickness) and consist of a monolithic stationary phase placed in a dedicated plastic housing [19]. This material has recently been studied as chromatographic support for immobilization of enzymes and ligands such as glucose oxidase [20–22].

In particular, an ethylenediamine (EDA) CIM disk is an amine monolithic activated support obtained by reacting the native epoxy groups with a convenient ethylendiamine spacer. Originally developed as weak ion exchange column, EDA monolithic disk can be used for bioconversion by coupling proteins, peptides or other ligands through crosslinking reaction with a suitable bifunctional reagent, i.e. glutaric dialdehyde. This matrix was selected for the immobilization of human recombinant acetylcholinesterase (hrAChE). Human acetylcholinesterase represents a widely studied target enzyme for Alzheimer's disease and is still object of research for the development of new drugs as enzyme inhibitors. In fact, the only four drugs approved for the clinical treatment of Alzheimer's disease (tacrine, rivastigmine, donepezil and galantamine) are acetylcholinesterase inhibitors, which act by maintaining high levels of acetylcholine at the muscarinic and nicotinic receptors in the central nervous system. In the present study, hrAChE was covalently immobilized on EDA CIM® disk to obtain a micro-immobilized-enzyme reactor (IMER with micrograms of immobilized enzyme) by a previous derivatization with glutaraldehyde. The hrAChE-micro-IMER was placed in a liquid chromatographic system and on-line chromatographic studies were performed. Results demonstrate hrAChE was immobilized with retention of enzymatic activity and increased stability. This new hrAChE-micro-IMER presented advantages over the previously performed immobilization of human erythrocytes AChE [7] in terms of very short analysis time (2 min versus 20 min), absence of backpressure, lower aspecific matrix interactions and immediate recovery of enzyme activity.

The immobilized enzyme can be placed in an on-line system for the rapid screening of compounds for inhibitory activity.

## 2. Materials and methods

### 2.1. Materials

EDA CIM Disks (12 mm × 3 mm i.d.) were purchased from BIA Separations (Ljubljana, Slovenia). (*S*)-Acetylthiocholine iodide, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB; Ellman's reagent), glutaraldehyde 70% aqueous solution, propidium iodide and hrAChE (EC 3.1.1.7) lyophilized powder were purchased from Sigma (Milan, Italy). Tacrine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride), edrophonium chloride and monoethanolamine were obtained from Aldrich Italia (Milan, Italy). Donepezil was a kind gift from Pfizer. Ambenonium chloride pentahydrate was purchased from Research Biochemicals International (Natick, MA, USA). Potassium chlorate and sodium cyanoborohydride were obtained from Fluka (Milan, Italy) and magnesium sulfate from Merck (Darmstadt, Germany). HPLC-grade methanol (Romil, UK) was used to prepare the inhibitors solutions. Purified water from a TKA ROS 300 system was used to prepare buffers and standard solutions. To prepare the buffer solutions, potassium dihydrogenphosphate, dipotassium hydrogenphosphate trihydrate of analysis quality and tris(hydroxymethyl)aminomethane (Carlo Erba, Milan, Italy) were used.

The buffer solutions were filtered through a 0.45 μm membrane filter and degased 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 BIP-I HPLC pump equipped with a Rheodyne Model 7125 injector with a 10 μl sample loop. The eluents 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 450 and 480 nm.

The chromatographic analyses on hrAChE-CIM disk were performed at 25 °C.

### 2.3. hrAChE immobilization

The EDA CIM Disk was inserted into the HPLC system and conditioned for 20 min with a mobile phase consisting of phosphate buffer (20 mM, pH 7.0) at 0.5 ml/min. Then, the CIM disk was removed and was placed in a glass beaker for derivatization. It was covered with 10 ml of a 10% glutaraldehyde solution in phosphate buffer (50 mM, pH 6.0) and kept under stirring for 6 h, in the dark. The reacted matrix was then washed with phosphate buffer (50 mM, pH 6.0). An aliquot of 7.0 μl of hrAChE solution in phosphate buffer (0.269 M, pH 8.0) (1.74 U/μl) was diluted to 800 μl with phosphate buffer (50 mM, pH 6.0), was added to the matrix and left to react overnight. After immobilization, the enzyme solution was analysed with the Ellman's assay in order to determine the unreacted enzyme units.

The Schiff bases were reduced by stirring the derivatized hrAChE-CIM disk in 10 ml of 0.1 M cyanoborohydride solution in phosphate buffer (50 mM, pH 6.0) for 2 h at 25 °C.

The matrix was then washed with phosphate buffer (50 mM, pH 6.0) and stirred for 3 h with 0.2 M monoethanolamine solution in phosphate buffer (50 mM, pH 6.0) at room temperature.

The EDA CIM disk was then washed, inserted in the appropriate holder, connected to the HPLC system and condi-

tioned with a mobile phase consisting of phosphate buffer (100 mM, pH 7.4) for 1 h at a flow rate of 0.8 ml/min and the enzymatic activity was determined as reported in the next paragraph.

The hrAChE-micro-IMER obtained was stored at 4 °C in phosphate buffer (100 mM, pH 7.4) containing 0.1% sodium azide.

### 2.4. Determination of immobilized hrAChE activity

The activity of hrAChE was determined by measuring the formation of the yellow anion (YA) obtained from the reaction between Ellman's reagent and the enzymatically obtained thiocholine from the substrate acetylthiocholine (ACth) [23]. One unit of enzymatic activity is defined as the amount of enzyme catalysing the hydrolysis of one μmol of ACth/min at pH 8.0 and 37 °C, i.e. the μmol formation of the stechiometrically correspondent YA.

The hrAChE-CIM disk was conditioned with 0.1 M phosphate buffer solution pH 7.4 and then with a mobile phase consisting of Tris–HCl buffer (0.1 M, pH 8.0) containing Ellman's reagent ($1.26 \times 10^{-4}$ M), $MgSO_4$ (10 mM), $KClO_3$ (100 mM) (buffer A). Aliquots of 10 μl acetylthiocholine aqueous solution at increasing concentration (range comprised between 3.1 and 250 mM), were injected in the HPLC system, at a flow rate of 1.0 ml/min and UV detection at 450 nm. Thiocholine, as the product of enzymatic reaction, reacted with Ellman's reagent in the mobile phase by forming a mixed disulfide and a yellow anion [23], which is stechiometrically related to the amount of the substrate hydrolyzed (Fig. 2).

In order to account for μmol ACth hydrolyzed, the corresponding eluates for each substrate injection were collected in 5 ml volumetric flasks during 5 min of chromatographic elution. The absorbance at 412 nm of relative eluates were acquired by spectrophotometric analysis, using the mobile phase as blank. By dividing the absorbances for the contact time (0.34 min), the catalysis rates ($\Delta A$/min) were derived. By plotting the catalysis rates versus the injected substrate concentrations, a Michaelis–Menten plot was obtained and $K_m$ and $V_{max}$ derived. As already reported [7], from the $V_{max}$ value the immobilized active units were determined by applying the following equation:

$$U\ (\mu\text{mol/min}) = \frac{(\Delta A/\text{min})_{max}}{\varepsilon} \times 10^6 V_{collected}$$

A correlation between absorbance values and relative chromatographic peak areas was obtained (Fig. 5c)

### 2.5. Optimization of the chromatographic conditions

#### 2.5.1. Mobile phase composition

Two types of mobile phase buffers at increasing concentrations were tested: phosphate buffer and Tris–HCl buffer (10–100 mM). Potassium chlorate (0–100 mM) as a selective anion exchanger competitor for the protonated amine groups on the matrix, magnesium sulfate (0–20 mM) as enzyme activator and Ellman's reagent concentration ($0.079 \times 10^{-4}$ M − $2.52 \times 10^{-4}$ M) were evaluated. The peak symmetry and peak area obtained by the injection of a fixed saturating ACth concentration (150 mM) were determined for each buffer type and additive concentration, by using a flow rate of 1 ml/min and UV detection at 450 nm or 480 nm depending on the rate of the enzymatic reaction.

#### 2.5.2. Mobile phase pH

The enzyme column was equilibrated for 30 min with 0.1 M Tris–HCl containing 0.1 M $KClO_3$, 10 mM $MgSO_4$, $1.26 \times 10^{-4}$ M Ellman's reagent in a pH range comprised between 5.0 and 8.0.

A fixed acetylthiocholine concentration (150 mM) was injected in triplicate onto the HPLC with a flow rate of 1.0 ml/min with UV detection at 450 nm. The product peak area was integrated and plotted against the pH value of the mobile phase.

#### 2.5.3. Flow rate

A fixed acetylthiocholine concentration (250 mM) was injected in triplicate onto the HPLC with a flow rate range comprised between 0.2 and 1.4 ml/min with UV detection at 450 nm. The product peak area was integrated and plotted against the used flow rate.

### 2.6. Determination of inhibitory potency ($IC_{50}$)

Stock solutions of the test compounds (1–10 mM) were prepared in water or methanol. The assay solutions were prepared by diluting the stock solutions in water together with the substrate acetylthiocholine at a fixed concentration (150 mM). In particular five different concentrations of each compound were mixed together with the substrate in order to obtain inhibition of acetylcolinesterase activity comprised between 20 and 80%.

Aliquots of 10 μl of a solution containing 150 mM acetylthiocholine were injected into the chromatograph in triplicate and the areas of peaks eluting at $t_R = 0.67$ min were determined ($A_o$).

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 ($A_i$).

The peak areas were compared with those obtained in absence of inhibitor and % inhibition was calculated. The percent inhibition of the enzyme activity due to the presence of increasing test compound concentration was calculated by the following expression: $100-(A_i/A_o \times 100)$ where $A_i$ is the peak area calculated in the presence of inhibitor and $A_o$ 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.

### 2.7. Immobilized hrAChE stability

hrAChE-CIM disk stability was determined by using buffer A as mobile phase and injecting every day 10 μl of ACth saturating aqueous solution (150 mM) under optimized flow and detection conditions (1 ml/min and 450 nm).

## 3. Results and discussion

### 3.1. hrAChE immobilization

In a previous paper [7] we reported the immobilization of AChE from human erythrocytes on a chromatographic column (50 mm × 4.6 mm i.d.) containing epoxy silica. The yield of immobilization and the stability of the AChE–IMER were considered satisfactory, but some problems arose. The length of the IMER and the too large amount of enzyme covalently bound to the chromatographic support resulted in catalysis product long elution times, higher concentration of saturating substrate and consequently of inhibitors and some inhibitors aspecific matrix absorption with delayed enzyme activity recovery. In order to avoid these complications, we decided to reduce the dimension of the solid support for immobilization, hence the amount of immobilized enzyme, by selecting a monolithic matrix disk (12 mm × 3 mm i.d.) [18], specific in terms of very low backpressure and better accessibility of substrate to the AChE active site. For these reasons we choose the EDA CIM disk, an amine monolithic activated support obtained by reacting the native epoxy groups with a convenient ethylendiamine spacer.

By following the immobilization protocol suggested by the manufacturing company, we optimized the coupling of amine groups of the EDA disk with increased glutaraldehyde concentrations (from 1 to 10%). The scheme of the hrAChE immobilization on EDA CIM disk is reported in Fig. 1. These conditions were found suitable to condense a larger amount of amine groups with the aldehydic groups of glutaraldehyde, minimising the aspecific cationic interaction between the anionic product of enzymatic reaction with the residual protonated monolithic amine functions, once the hrAChE-micro-IMER was inserted in the chromatographic system. After the matrix activation, hrAChE was linked to the free aldehydic glutaraldehyde moiety by the reaction of the lysine primary amine functions. A subsequent reduction of the resulting double bonds was performed with sodium cyanoborohydride. Finally, the unreacted aldehydic groups were capped with monoethanolamine.

Under the described conditions, the immobilization yield was found to be 3.0%, by determining the activity of the enzyme solution before and after immobilization. However if we consider that overnight the enzyme activity decreased by 50%, the immobilization yield can be considered between 3 and 6%. The optimized immobilization procedure was repeated twice.

The amount of hrAChE active immobilized units were assessed by inserting the resulting IMER into a HPLC system.

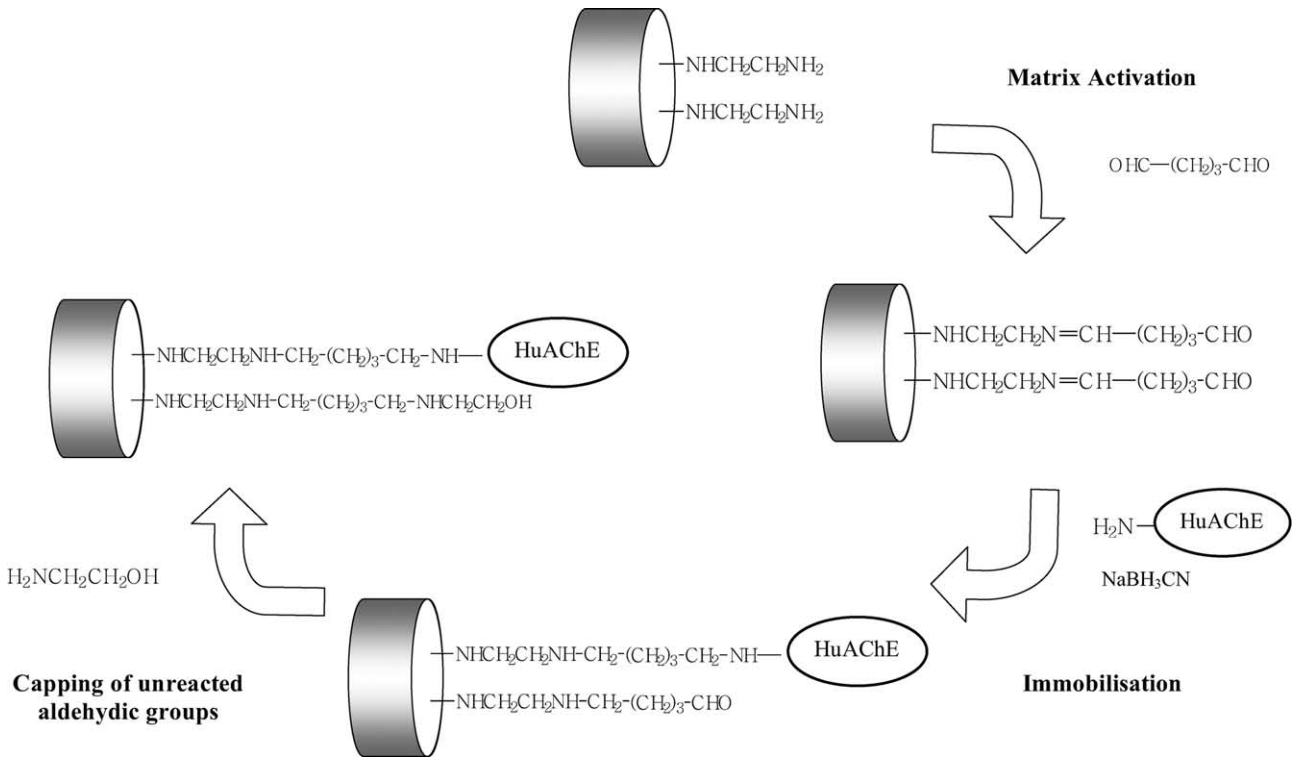


Fig. 1. Scheme for the hrAChE (HuAChE) immobilization procedure on an EDA CIM® disk.

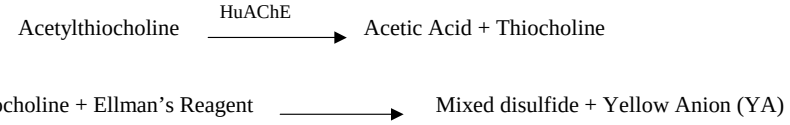


Fig. 2. hrAChE catalysis of acetylthiocholine and Ellman's reaction.

### 3.2. The on-line hrAChE CIM disk chromatographic system

As the product of the enzymatic hydrolysis, thiocholine does not present a significant chromophore for UV detection, the evaluation of enzyme activity was performed by injecting acetylthiocholine with the Ellman's reagent dissolved in the mobile phase and monitoring at 450 nm the amount of 5-thio-2-nitro-benzoic acid (yellow anion) (which is formed by the reaction of thiocholine and Ellman's reagent) (Fig. 2).

The effect of the pH and composition of mobile phase were studied. A mobile phase consisting of 0.1 M Tris–HCl pH 8.0 gave rise to YA symmetric peak, completely eluted in 2 min. With this type of monolithic support, pH 8.0 resulted compatible with the matrix composition improving enzymatic catalysis as already reported (Fig. 3) [23]. Moreover, the buffer concentration was not found to affect enzyme activity. The addition of potassium chlorate (0, 50, 100 mM) and magnesium sulfate (0, 10, 20 mM) were investigated. Potassium chlorate (100 mM) resulted to be a selective competitive anion for the cationic sites on the matrix and produced a displacement of the YA from the matrix with symmetric peaks and very short elution time. On the other hand, 10 mM magnesium sulfate slightly improved hrAChE catalytic activity (higher YA peak area). Finally, the Ellman's reagent concentration was increased in the mobile phase up to a stable YA peak area value.

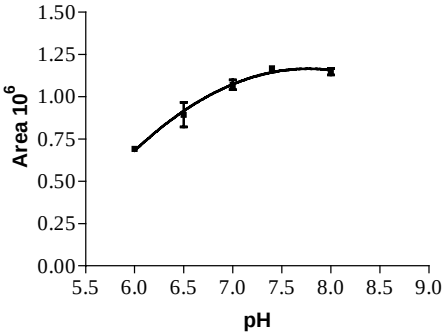


Fig. 3. Effect of mobile phase pH on YA peak area after 250 mM acetylthiocholine injection. Chromatographic conditions: hrAChE-micro-IMER; mobile phase consisting of 0.1 M Tris–HCl containing 100 mM $KClO_3$, 10 mM $MgSO_4$, $1.26 \times 10^{-4}$ M Ellman's reagent. UV detection at 480 nm; flow rate at 1 ml/min.

The optimized mobile phase consisted of 0.1 M Tris–HCl containing 100 mM $KClO_3$, 10 mM $MgSO_4$, $1.26 \times 10^{-4}$ M Ellman's reagent at pH 8.0.

By using the described mobile phase, the HuAChE-CIM disk required 5 min at 1 ml/min flow rate to be conditioned, i.e. same YA peak area for three subsequent injections of the same substrate concentration.

The effect of flow rate on the production of YA was determined using flow rates from 0.2 to 1.4 ml/min, reflecting

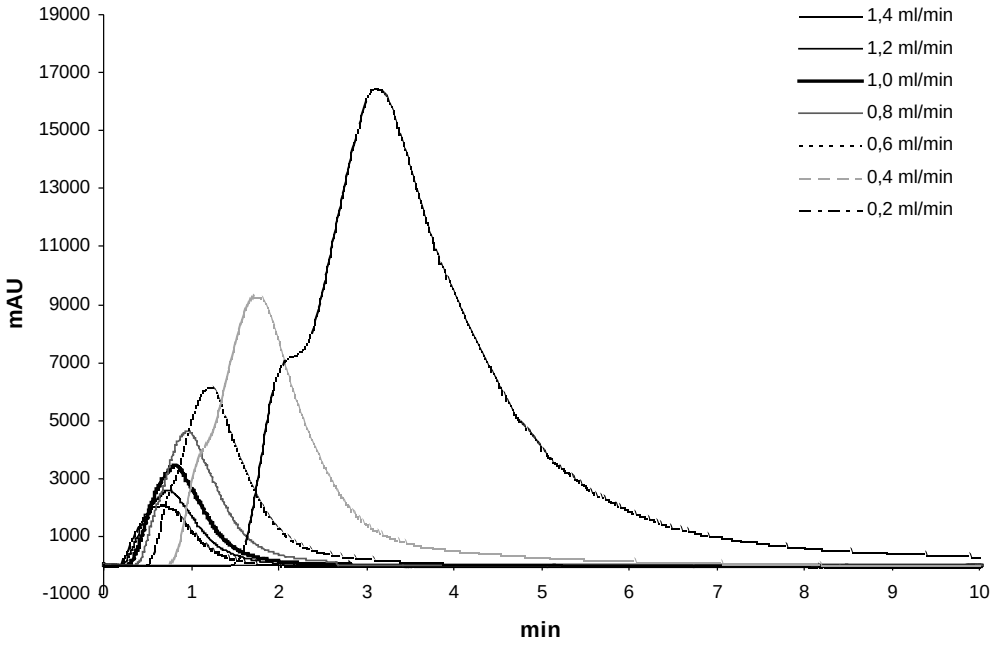


Fig. 4. Effect of mobile phase flow rate on YA peak area after 250 mM acetylthiocholine injection. Chromatographic conditions: hrAChE-micro-IMER; mobile phase consisting of 0.1 M Tris–HCl containing 100 mM $KClO_3$, 10 mM $MgSO_4$, $1.26 \times 10^{-4}$ M Ellman's reagent. UV detection at 480 nm.

substrate–enzyme contact times ranging from 1.7 to about 0.25 min, respectively. While the flow rates between 0.1 and 0.4 ml/min produced the greatest amount of YA (Fig. 4), they also produced the greatest variation in the areas of the YA peaks; for example, an increase in flow rate from 0.2 to 0.4 ml/min produced an over 200% decrease in the area of the YA peak. Thus, slight fluctuations in pump speed would produce significant variations and error in kinetic studies. The relationship between pump speed and the extent of YA production was not as great for flow rates ranging from 0.8 to 1.4 ml/min (Fig. 4), and these flow rates were easier for the pumping system to maintain. A flow rate of 1 ml/min was chosen because gave a high response in term of peak area (i.e. hydrolysis rate) and reasonable time for anion elution.

### 3.3. Enzymatic activity of the hrAChE-CIM disk

The enzymatic activity of the hrAChE-micro-IMER was determined by following the production of YA. In order to accurately quantify the amount of YA produced by the on-line system, a calibration curve was obtained by injecting standard solutions of ACth onto the chromatographic system. The areas of the YA peaks were correlated to the concentrations (Fig. 5c). To this purpose, the spectrophotometric absorbance values at 412 nm of the collected eluates from the hrAChE-CIM disk were divided by the YA extinction coefficient [7]. The concentration of the injected acetylthiocholine solutions ranged from 3.1 to 250 mM and the eluting YA concentrations (following 10 μl injections) ranged from 5.18 to 15.28 μM (from 25 to 75 nmol). The correlation between YA peak areas and absorbances was linear ($y = 1.630 \times 10^{-7}x + 0.02479$; $r^2 = 0.9709$). By this correlation the YA concentration was then directly extrapolated from the obtained chromatographic peak area (Fig. 5c). The kinetic parameters of the hrAChE-micro-IMER were determined by varying the substrate concentrations. YA production was correlated to the concentration of substrate to obtain Michaelis–Menten plot, Fig. 5a and b, respectively. Saturation in the Michaelis–Menten plot of μmol YA produced was reached at 150 mM acetylthiocholine concentration. This concentration was used in the following activity assays and kinetic studies. Lineweaver–Burk reciprocal plots of 1/activity and 1/[substrate] allowed to estimate the value of $K_m$ and $V_{max}$ for ACth ($y = 0.9911x + 10.42$; $r^2 = 0.9854$). The apparent affinities ($K_m$) of ACth for hrAChE as free enzyme and in the IMER format, respectively, were

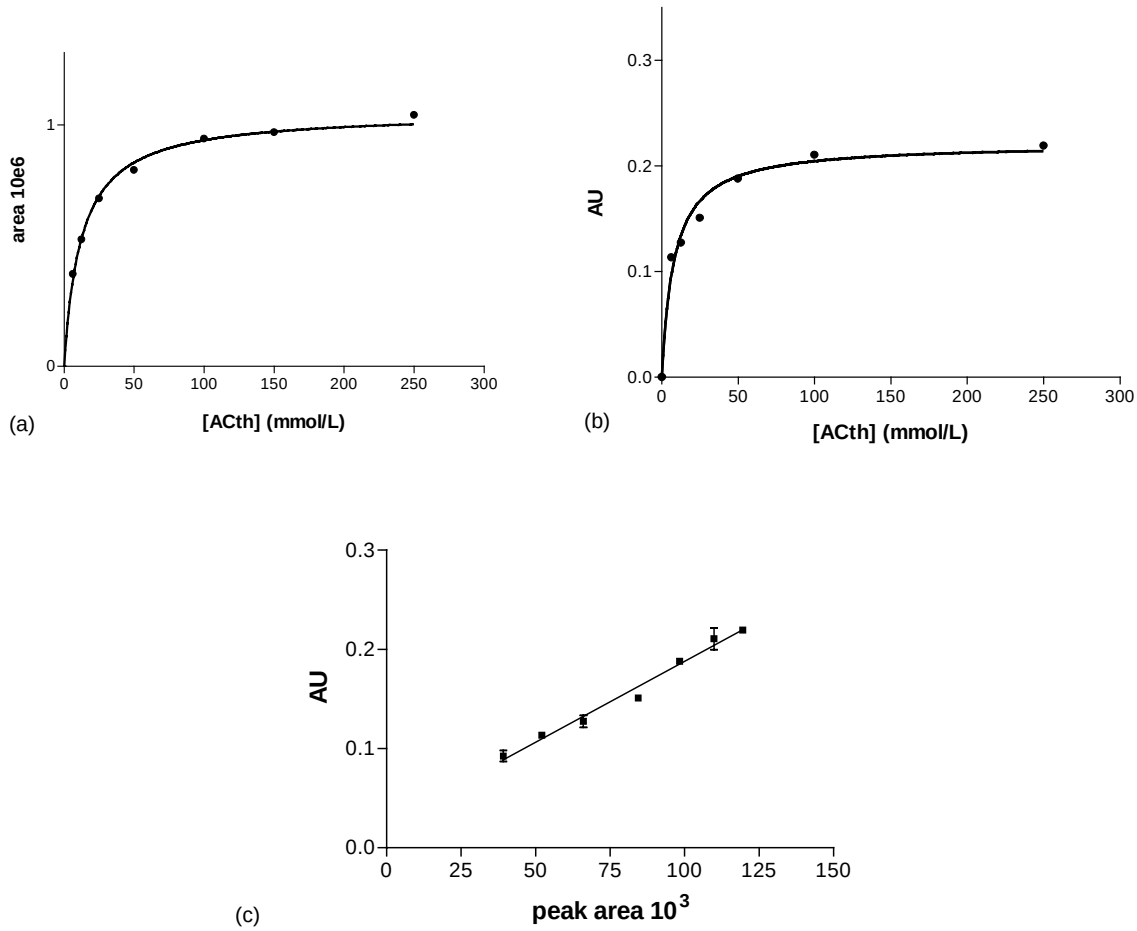


Fig. 5. Michaelis–Menten plots by HPLC analysis with hrAChE-micro-IMER: (a) YA peak areas vs. acetylthiocholine injected concentrations, (b) absorbance at 412 nm of collected YA eluates vs. acetylthiocholine injected concentrations, (c) correlation between YA peak areas and absorbance values.

$0.159 \pm 0.002$ mM and $14.39 \pm 2.06$ mM. Considering the different amount of enzyme used in the two systems (around 20 times higher in the IMER), the immobilized enzyme $K_m$ value for the hrAChE-CIM disk was found comparably high. The amount of immobilized units was found to be $0.22 \pm 0.01$ U.

### 3.4. Stability of immobilized and free enzyme

The stability of hrAChE was also examined for the free enzyme and the hrAChE-micro-IMER. The activity was evaluated daily at saturating substrate concentration.

The studies with the free enzyme confirmed the rapid inactivation of hrAChE upon dilution in phosphate buffer. During the first 10 h, the solution lost 30% of its activity and more than 80% after 1 day (data not shown).

The optimal conditions of storage for the immobilized hrAChE were investigated and phosphate buffer (pH 7.4) containing 0.1% (w/v) sodium azide, resulted the most convenient. Buffers were freshly prepared every day. Under these storage conditions, over 80% of the initial activity was retained up to 2 months. After an initial lost of 15% of the enzymatic activity, hrAChE-CIM disk enzymatic activity remained almost unchanged for over 60 days (Fig. 6).

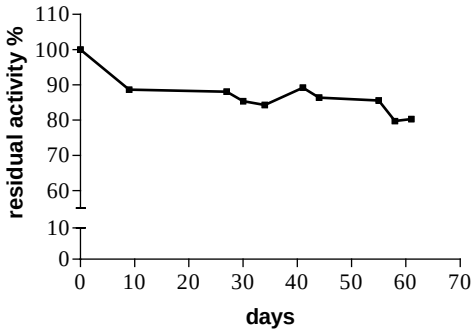


Fig. 6. Stability study on hrAChE-micro-IMER. Residual activity calculated as % initial activity (YA peak area) after 150 mM acetylthiocholine injection over time.

### 3.5. Determination of inhibitory potency ($IC_{50}$)

The sensitivity of the immobilized hrAChE in the hrAChE-micro-IMER format to hrAChE inhibitors was examined. Inhibitors whose potency is distributed over four orders of magnitude were analysed. Their $IC_{50}$ was first determined by the conventional spectrophotometric Ellman's method [24], using the same type of hrAChE which was used for immobilization.

Then the $IC_{50}$ of five known inhibitors (tacrine, edrophonium, ambenonium, donepezil and propidium) was assessed by using the immobilized enzyme column, by extrapolation from the inhibition curves (Fig. 7). The inhibition curves 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 YA peak area (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

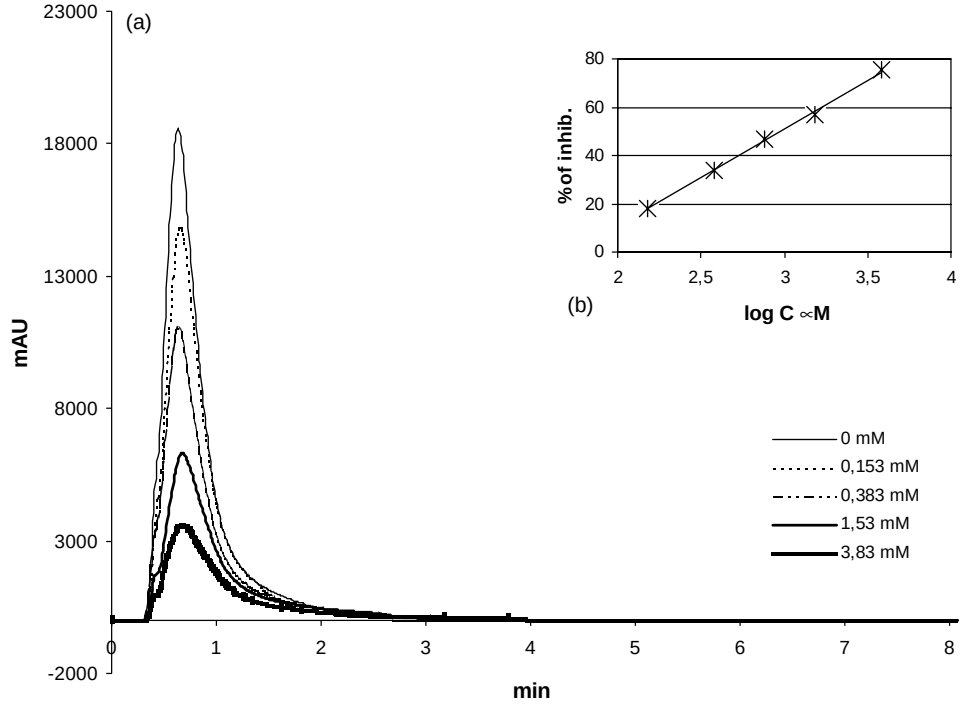


Fig. 7. (a) Overlaid chromatograms obtained after injection of substrate at saturating concentration (150 mM) and substrate plus increasing concentration of edrophonium, (b) edrophonium inhibition plot.

was plotted against the logarithm of inhibitor concentration to obtain the inhibition curves.

The $pIC_{50}$ values obtained on the hrAChE-micro-IMER were compared with the values obtained for the free enzyme and a valid correlation was obtained ($r^2 = 0.9900$). This will allow a direct comparison between on-line determined inhibition potencies and $pIC_{50}$ values determined with the classical spectrophotometric method. The results indicate that the hrAChE-micro-IMER could be used to on-line screen for new hrAChE inhibitors.

It is remarkable the time reduction for such a determination: a chromatographic run of 2 min gives a preliminary indication of the inhibitors potency. If we consider that the development of new inhibitors require a large number of compounds to be tested for the lead selection and optimization, provided that an autosampler is put on-line, hundreds of compounds can be processed in continuous. Due to these peculiarities, this method can be considered suitable for high throughput screening in drug discovery.

## 4. Conclusions

The proposed immobilization procedure was found appropriate to covalently bind hrAChE to a modified monolithic matrix (EDA CIM disk) maintaining enzyme activity. Moreover, the inclusion of hrAChE-micro-IMER in a chromatographic column allowed preserving the enzymatic activity from inactivating processes, widely increasing the stability of the enzyme with advantages in term of accuracy and reproducibility. The small amount of hrAChE immobilized on a reduced size monolithic CIM disk was found appropriate for a 10 times increase in the inhibitors' testing speed. The same immobilization procedure can be potentially applied to prepare IMERs containing enzymes from different sources. Considering the high cost and difficulty in over-expression, isolation and purification of enzyme from recombinant source, this analytical technique represents an extremely useful approach to preserve the activity of the small amount of enzyme available, to perform kinetic studies and for the high throughput screening of potential drugs candidates.

## References

[1] C. Bertucci, A. Petri, G. Felix, B. Perini, P. Salvadori, Tetrahedron: Asymm. 10 (1999) 4455.
[2] G. Felix, V. Descorps, Chromatographia 49 (1999) 606.
[3] G. Massolini, E. Calleri, A. Lavecchia, F. Loiodice, D. Lubda, C. Temporini, G. Fracchiolla, P. Tortorella, E. Novellino, G. Caccialanza, Anal. Chem. 75 (2003) 535.
[4] N. Markoglou, I.W. Wainer, J. Chromatogr. A 948 (2002) 249.
[5] N. Markoglou, I.W. Wainer, J. Chromatogr. B 766 (2002) 145.
[6] M. Bartolini, V. Andrisano, I.W. Wainer, J. Chromatogr. A 987 (2003) 331.
[7] V. Andrisano, M. Bartolini, R. Gotti, V. Cavrini, G. Felix, J. Chromatogr. B 753 (2001) 375.
[8] M.D. Greaney, D.L. Marshall, B.A. Bailey, I.N. Acworth, J. Chromatogr. 622 (1993) 125.
[9] P.C. Gunaratna, G.S. Wilson, Anal. Chem. 62 (1990) 402.
[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] J.-F. Cloix, I.W. Wainer, J. Chromatogr. A 913 (2001) 133.
[14] B. Zhang, M.M. Palcic, D.C. Schriemer, G. Alvarez-Manilla, M. Pierce, O. Hindsgaul, Anal. Biochem. 299 (2001) 173.
[15] D.C. Schriemer, D.R. Bundle, L. Li, O. Hindsgaul, Angew. Chem. Int. Ed. Engl. 37 (1998) 3383.
[16] D. Josic, A. Buchacher, A. Jungbauer, J. Chromatogr. B 752 (2001) 191.
[17] H. Abou-Rbyeh, F. Koerber, K. Schubert-Rehberg, J. Reusch, Dj. Josic, J. Chromatogr. 566 (1991) 341.
[18] A. Strancar, P. Koselj, H. Schwinn, D. Josic, Anal. Chem. 68 (1996) 3483.
[19] www.biaseparations.com.
[20] Dj. Josic, H. Schwinn, A. Štrancar, A. Podgornik, M. Barut, Y.P. Lim, M. Vodopivec, J. Chromatogr. A 803 (1998) 61.
[21] A. Podgornic, M. Vodopivec, H. Podgornik, M. Barut, A. Štrancar, Progress in Biotechnology, vol. 15, Elsevier, Amsterdam, 1998, p. 541.
[22] M. Vodopivec, M. Berovič, J. Jančar, J. Podgornic, A. Štrancar, Anal. Chim. Acta 407 (2000) 105.
[23] G.L. Ellman, K.D. Courtney, V. Andres Jr., M. Featherstone, Biochem. Pharmacol. 7 (1961) 88.
[24] M. Bartolini, C. Bertucci, V. Cavrini, V. Andrisano, Biochem. Pharmacol. 65 (2003) 407.