Journal of Pharmaceutical and Biomedical Analysis 52 (2010) 355–361

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Development of a liquid chromatographic system with fluorescent detection for β-secretase immobilized enzyme reactor on-line enzymatic studies

Francesca Mancini, Vincenza Andrisano*

Department of Pharmaceutical Sciences, Via Belmeloro 6, University of Bologna, 40126 Bologna, Italy

ARTICLE INFO

Article history:
Received 15 June 2009
Received in revised form 4 July 2009
Accepted 9 July 2009
Available online 22 July 2009

Keywords:
hrBACE1–IMER
Fluorescence detection
Methoxycoumaryl peptide substrate
Inhibitors screening

ABSTRACT

A novel liquid chromatographic method has been developed for use in throughput screening of new inhibitors of human recombinant β-amyloid precursor protein cleaving enzyme (hrBACE1). The approach is based on the use of an immobilized enzyme reactor (IMER) containing the target enzyme (hrBACE1–IMER) and uses fluorescence detection. The bioreactor was prepared by immobilizing hrBACE1 on an ethylendiamine (EDA) monolithic disk (CIM) and a fluorogenic peptide (M-2420) containing the β-secretase site of the Swedish mutation of amyloid precursor protein (APP) was used as substrate. After injection into the hrBACE1–IMER system, M-2420 was enzymatically cleaved, giving rise to a fluorescent methoxycoumaryl-fragment (Rt = 1.6 min), which was separated from the substrate and selectively detected at λ_{exc} = 320 and λ_{em} = 420 nm. Product and substrate were characterized by using a post monolithic C18 stationary phase coupled to an ion trap mass analyser. A calibration curve was constructed to determine the immobilized hrBACE1–IMER rate of catalysis and kinetic constants. Specificity of the enzymatic cleavage was confirmed by injecting the substrate on a blank CIM-EDA.

The proposed method was validated by the determination of the inhibitory potency of five reference compounds with activities ranked over four order of magnitude (four peptidic inhibitors and a green tea polyphenol, (−)gallocatechin gallate). The obtained results were found in agreement with the data reported in literature, confirming the validity and the applicability of the hrBACE1–IMER as a tool for the fast screening of unknown inhibitors (more than 6 compounds per hour). Moreover, the hrBACE1–IMER showed high stability during the analysis, permitting its use for more than three months without affecting enzyme activity.

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1. Introduction

The aspartic protease β-secretase (human recombinant β-amyloid precursor protein cleaving enzyme, hrBACE1) is the rate-limiting enzyme for the generation of the amyloid beta protein (Aβ), a main and core component of Alzheimer's disease (AD) senile plaques [1]. Since the hydrolysis of the amyloid precursor protein (APP) is one of the first steps in the neurodegenerative cascade, hrBACE1 inhibition represents an alternative and potentially curative therapy for AD. In the last decade, hrBACE1 role in AD pathogenesis has been established [2,3] and the search for new inhibitors has become an intriguing challenge [4,5].

The most common methods used for medium- or high-throughput screening of hrBACE1 inhibitors involve fluorometric assays in multi-well plate format based on the fluorescence resonance energy transfer (FRET) principles [6–8]. The FRET substrate consists of a fluorescence donor linked by a peptide chain to a quenching acceptor. Upon hrBACE1 cleavage, the quantum yield of the donor is restored and enzyme activity is linearly related to the increase in fluorescence.

FRET assays are homogenous and sensitive but have inherent drawbacks such as long time analysis (usually more than 1 h), high enzyme consumption, variability due to different lots and source of enzyme, not re-usability of the enzyme together with the requirement of complex robotic liquid handling. Moreover, many synthetic and natural ligands show solubility limitations under the FRET assay conditions thus generating false positives or false negatives in the high or medium throughput screening [9]. In fact, non-specific effects of some class of compounds tested as inhibitors are due to their capacity to form aggregates able to incorporate or absorb enzymes within them. Thus, "promiscuous" inhibitors bind the target enzyme in a non-specific manner without an individual interaction [10,11]. Some of them can also carry in their structure fluorescent aromatic rings and/or eterocycles, thus interfering with the product fluorescence.

Immobilization of enzymes can be a valid solution to overcome these problems. In fact, the use of a chromatographic system in principle may allow the separation of the product of the enzymatic

* Corresponding author. Tel.: +39 051 2099742; fax: +39 051 2099734.
E-mail address: vincenza.andrisano@unibo.it (V. Andrisano).

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doi:10.1016/j.jpba.2009.07.012

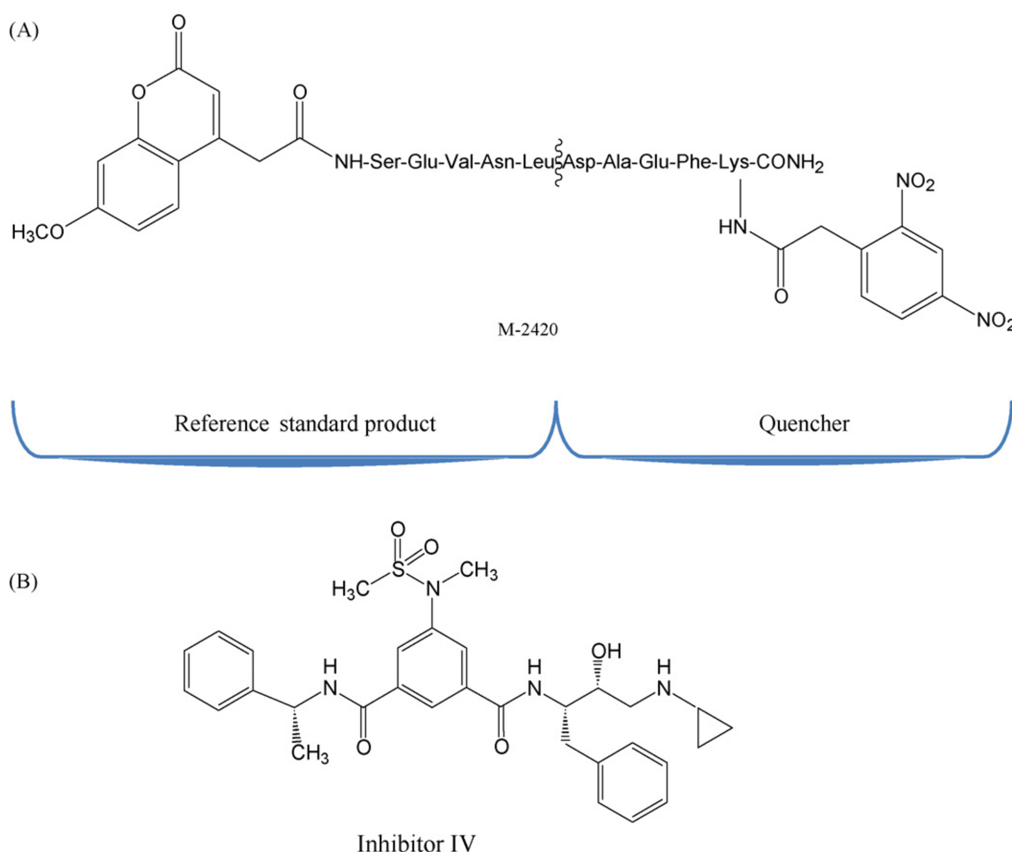

Fig. 1. Structures of substrate M-2420 (A) and inhibitor IV (B).

reaction and of the tested compounds from the substrate, thereby eliminating interferences. Moreover, in a flow through system, there is a lower risk of substrate-tested compound co-precipitation and/or aggregates formation.

Enzymes packed into chromatographic column (immobilized enzyme reactors, IMERs) have been widely used in flow through systems with a variety of protein and applied for inhibition studies [12–20]. IMERs prepared using monolithic supports, when inserted in LC systems, are characterized by lack of diffusion resistance during mass transfer. Thereby IMERs allow fast conversion of substrates and low backpressure. Thus, the specificity and sensitivity of an enzymatic reaction can be combined with rapidity and reproducibility of the chromatographic system [13,20,21].

In a previous paper, an hrBACE1–IMER was prepared with an *in situ* immobilization procedure on a CIM-EDA disk and characterized in terms of enzyme active units [21], by using a bi-dimensional LC system. Here, a novel fast and automated chromatographic method with fluorescence detection was developed for hrBACE1 enzymatic studies by using as substrate a fluorogenic peptide containing the β-secretase site of the Swedish mutation of APP (Mca-(Asn^{670}, Leu^{671})-APP$_{770}$ (667-675)-Lys(Dnp), named M-2420) (Fig. 1) [22].

The proposed method was validated by measuring hrBACE1–IMER inhibition from known compounds.

2. Experimental

2.1. Materials and methods

HrBACE1–IMER was prepared and characterized as previously reported [21]. Sodium acetate, dimethylsulfoxide (DMSO), sodium phosphate, CHAPS, acetic acid, sodium azide, (−)gallocatechin gallate, Pepstatin A, Casein-FITC and black microwell Corning plates were obtained from Sigma–Aldrich (Milan, Italy).

The peptidic substrate M-2420 (structure in Fig. 1A), the reference hydrolysis product (methoxycoumarin-Ser-Glu-Val-Asn-Leu-OH, MCA-fragment) and OM99-2 (H-Glu-Val-Asn-[(2R,4S,5S)-5-amino-4-hydroxy-2,7-dimethyl-octanoyl]-Ala-Glu-Phe-OH) were purchased from Bachem (Germany).

Statine-peptide derivative (H-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-[statine(3S,4S)]-Val-Ala-Glu-Phe-OH), inhibitor IV (structure in Fig. 1B) and Chromolith C18 Performance stationary phase (100 × 4.6 mm I.D.) were obtained from Merck (Darmstadt, Germany).

Panvera peptide was from Invitrogen (Milan, Italy).

All the other chemicals were of analytical reagent grade (Carlo Erba Reagenti and Sigma–Aldrich, Milan, Italy) and were used without further purification. Water used for the preparation of solutions and mobile phases was purified by a Milli-Rx apparatus (Millipore, Milford, MA, USA).

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

2.2. Apparatus and chromatographic conditions

Chromatographic analyses of M-2420 digestion were performed on the hrBACE1–IMER inserted into the HPLC system consisting of a Jasco PU-1580 solvent delivery system connected to a Jasco auto sampler model AS-2055 and a Jasco FP-2020 detector system (Jasco, Cremella, Italy). Fluorescence detector was set at 320 nm and 420 nm for excitation and emission wavelengths, respectively. The mobile phase consisted of a mixture composed of 25 mM sodium phosphate buffer containing CHAPS (0.1%, w/v) at pH 5.5 and DMSO in the volumetric ratio 95/5 (v/v), the flow rate was set at

$1.0\,mL\,min^{-1}$ and the injection volume was 10 μL. Chromatographic analysis on the hrBACE1–IMER were performed at controlled temperature (37 ± 1.0 °C).

LC–MS analysis were performed with a Chromolith C18 column (100 × 4.6 mm I.D.) on a Jasco PU-1585 (Jasco, Cremella, Italy) equipped with a Reodyne Model 7125 injection valve (sample loop of 20 μL) connected to a LCQ DUO ion trap mass spectrometer (MS) with electrospray ionization (ESI) ion source, controlled by Xcalibur software 1.3 (Thermo Finnigan, San Jose, CA, USA). The mobile phase consisted of 10 mM ammonium acetate/acetic acid pH 4.5 and acidic methanol (35/65, v/v) with a flow rate of $0.4\,mL\,min^{-1}$. A postcolumn T-splitter (split ratio: 1/3) was used to direct a lower amount of the mobile phase into the mass spectrometer via the ESI interface.

Nitrogen was used as sheath gas and helium (0.1 Pa) served as damping and collision gas. Data acquisition and analysis were conducted using Xcalibur software (version 1.0 SR1, Thermoquest, U.S.A.). The ESI system employed a 4.5 kV spray voltage (positive polarity), a capillary temperature of 200 °C and a cone voltage of 14 V.

The detection was performed with an ion trap mass spectrometer in positive polarity (full scan 700–1600 m/z) and in single ion monitoring (SIM) mode on the generated cations at m/z = 1534 (M-2420), m/z = 777.3 (hydrolysis product).

When not in use, the hrBACE1–IMER was maintained at 4 °C in sodium acetate buffer (50 mM, pH 6.0) containing 0.1% (w/v) sodium azide as storage buffer.

In solution studies were carried out by using a Fluoroskan Ascent spectrofluorimeter (beam diameter: 3 mm) by using black microwells (96 wells) Corning plates.

Stock solutions of M-2420 and inhibitors were prepared in DMSO and the following dilutions obtained in DMSO.

2.3. In solution studies

2.3.1. Fluorogenic substrate selection

Hydrolysis of the fluorogenic substrates was monitored in a 200 μL reaction volume by measuring the fluorescence increase in black microwell plates. $\lambda_{exc}/\lambda_{em}$ pairs were set at 320/405 nm, 485/538 nm and 544/590 nm for substrates 1 (M-2420), 2 (Invitrogen peptide) and 3 (Casein-FITC), respectively (Table 1). Specificity constants (k_{cat}/K_m) were determined under pseudo-first-order conditions, via the 'progress curve method' [23] using a substrate concentration (0.04 μM or 0.05 μM) far below K_m, and a final enzyme concentration of 10 nM or 34 nM (E_0). The {time; fluorescence} data pairs were fitted to equation (1): ($F(t) = \Lambda F[1 - \exp(-k_{obs}t)] + F_{init}$), and the apparent first-order rate constant (k_{obs}) was calculated. The second-order rate constant, k_{cat}/K_m values were calculated according to the following equation (2): $k_{cat}/K_m = k_{obs}/[E_0]$. The program used to analyse these data was GraphPad Prism 4.0.

Quenching efficiency was determined according to the equation (3): q.e.(%) = $(1 \times F_0/F_1) \times 100$, were $F_0 = F_{init} - F_{buffer}$ and $F_1 = F_{max} - F_{buffer}$.

Table 1
k_{cat}/K_m ($M^{-1}\,s^{-1}$) and quenching efficiency (%) values obtained in solution with the free enzyme.

Substrate	Fluorophore	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	Quenching efficiency (%)
M-2420 (1)	Methoxycoumarin	6221.61	91.38
Invitrogen peptide (2)	Rhodamine	1286.13	98.51
Casein-FITC (3)	Fluorescein	3298.23	83.55

Values are the mean of two independent measurements, each performed in duplicate (SEM: standard error of the mean).

2.3.2. Inhibition studies

Inhibition studies were performed using the following procedure: 5 μL of test compound (or DMSO) were pre-incubated with 175 μL of enzyme (20.7 nM, final concentration) in 20 mM sodium acetate pH.4.5 containing CHAPS (0.1% w/v) for 1 h at room temperature. M-2420 (3 μM, final concentration) was then added and left to react for 15 min. The fluorescence signal was read at λ_{em} = 405 nm (λ_{exc} = 320 nm). DMSO concentration in the final mixture was maintained below 5% (v/v) to guarantee no significant loss of enzyme activity.

Fluorescence intensities with and without inhibitor were compared and the percent inhibition due to the presence of test compounds was calculated. The background signal was measured in control wells containing all the reagents, except hrBACE1 and subtracted. The % inhibition due to the presence of increasing test compound concentration was calculated by the following expression: $100 - (IF_i/IF_0 \times 100)$, where IF_i and IF_0 are the fluorescence intensities obtained for hrBACE1 in the presence and in the absence of inhibitor, respectively. Inhibition curves were obtained for each compound by plotting the % inhibition versus the logarithm of inhibitor concentration in the assay sample. The linear regression parameters were determined and the IC_{50} extrapolated (GraphPad Prism 4.0, GraphPad Software Inc.).

2.4. Optimization of the chromatographic conditions

A fixed substrate concentration (M-2420, 2 μM, normalised concentration) was injected into the HPLC–hrBACE1–IMER system, by using sodium acetate and sodium phosphate at different pHs (ranging between pH 4.0 and pH 6.0) as mobile phases. The resulting chromatographic peak area was integrated, and the stability of the baseline was evaluated. The presence of an organic modifier (DMSO) at various percentage (1–10%) and of magnesium chloride, potassium chlorate and CHAPS in the mobile phase was also evaluated.

2.5. M-2420 Michaelis–Menten on hrBACE1–IMER

The activity of the hrBACE1–IMER was determined by employing M-2420 as substrate and evaluating the amount of the hydrolysis product at λ = 420 nm (excitation at 320 nm). M-2420 solutions, in the range 0.5–20 μM, were injected onto the hrBACE1–IMER under the optimized chromatographic conditions described in Section 2.2. Normalized substrate concentration was calculated by the following formula:

$$[\text{M-2420}]_{\text{normalized}} = \frac{C_{inj} \times V_{inj}}{BV}$$

where C_{inj} is the injected substrate concentration, V_{inj} is the injected volume and BV is the bed volume of the hrBACE1–IMER.

Peak product area was plotted against the correspondent substrate concentration. Each concentration was analysed in duplicate. K_m and v_{max} values were derived by using GraphPad Prism Software.

To evaluate the amount of hydrolysis product obtained from enzymatic catalysis, a calibration curve was built by injecting onto the hrBACE1–IMER standard solutions of MCA-fragment at increasing concentrations in the range: 0.15–3.0 nM (normalised concentration).

2.6. Characterization of substrate and product from hrBACE1–IMER hydrolysis

To characterize peaks obtained from on-line enzymatic digestion, multiple injections of M-2420 solution (2 μM, normalised concentration) were performed onto the hrBACE1–IMER under the following chromatographic conditions: 10 mM ammonium

acetate/acetic acid pH 4.5, flow rate 1 mL min^{-1}, detection at 320/420 nm ($\lambda_{exc}/\lambda_{em}$). Resulting peaks were separately collected by multiple injections, evaporated and the obtained concentrated solutions were injected onto the Chromolith C18 column and analysed with the LC–MS method described in Section 2.2.

2.7. Inhibition studies

To obtain estimates of the inhibitory potency, IC_{50} values were obtained by injecting in duplicate solutions containing a fixed M-2420 concentration (2 μM, normalised concentration), together with the tested inhibitor at increasing concentrations. In particular, five different concentrations of each compound were mixed together with the substrate to obtain inhibition of hrBACE1–IMER activity ranging between 20% and 80%. Chromatographic conditions were set as reported in Section 2.2. The % inhibition was calculated by evaluating product peak area with and without inhibitor. The following expression was used: $100 - (A_i/A_0 \times 100)$, where A_i is the peak area calculated in the presence of the tested inhibitor and A_0 is the peak product area obtained with the substrate solution only. The percent inhibition values were then plotted against normalized inhibitor concentration (considering the injected amount in 0.34 mL CIM disk void volume) ad the IC_{50} value was extrapolated.

3. Results and discussion

The aim of the present study was the development, application and validation of a rapid chromatographic method with fluorescence detection for the hrBACE1–IMER on-line enzymatic studies. HrBACE1 is a key rate-limiting enzyme that initiates the formation of β-amyloid peptide (Aβ), a main and core component of toxic plaques found in the brain of patients with AD. Therefore, this enzyme represents an attractive drug target for AD [24,25].

In a previous work, a bi-dimensional chromatographic method was developed to characterize an hrBACE1–IMER [21]. This method was useful for the determination of enzyme active units and advantageous to optimise the conditions for increasing enzyme stability and efficiency, but it showed some limitations for the application in an automated and fast screening process. In fact, the coupling of two chromatographic system with the mass analyser required multiple steps lengthening the overall analysis time.

On the basis of these premises, the main object of the present study was to decrease the time of analysis in order to perform on-line fast screening for hrBACE1 inhibitors. Thus, a novel LC method based on fluorescence detection was developed and validated. The substrate employed (M-2420, structure in Fig. 1A), contains methoxycoumaryl and dinitrophenyl moieties as the fluorescent and the quencher groups, respectively, linked by a peptide chain which contains the aminoacid sequence (Leu-Asp) of the Swedish mutated APP. The intrinsic fluorescence of uncleaved M-2420 is reduced because of intramolecular resonance energy transfer to the quenching group.

In the optimized conditions of analysis, a significant amount of fluorescent product obtained from hrBACE1–IMER hydrolysis was detected, after injection and short contact time (CT) between hrBACE1–IMER and M–2420 (0.34 min at the flow rate of 1.0 mL min^{-1}). Kinetics constants were determined and the method validated by testing standard reference compounds.

3.1. Choice of the substrate: in solution studies

It is well known that hrBACE1 has a relatively loose substrate specificity [26]. In fact, a variety of peptide substrates are usually employed in hrBACE1 FRET assays; the most common of them are fluorogenic peptides mimicking the Swedish mutated sequence of APP, which is cleaved by hrBACE1 100 times faster than the wild type APP [27]. In FRET assay, the fluorophore group of the substrate emits only after cleavage of the peptide chain and the removal of the terminal quencher [28].

By following our previous studies [29], in the present work, a preliminary evaluation of substrates affinity for hrBACE1 was carried out with the free enzyme in solution. Three FRET substrates characterized by the presence of different fluorophores (methoxycoumaryl, rhodamine and fluorescein for substrates 1, 2 and 3, respectively, Table 1) were chosen for this study. k_{cat}/K_m values were determined as described in Section 2.3, by using low substrate concentrations [23]. The increase in fluorescence intensity was indicated by the ratio F_1/F_0 (quenching efficiency, QE) (where F_1 = fluorescence intensity after hydrolysis and F_0 = fluorescence of the intact substrate). k_{cat}/K_m values and QE values are reported in Table 1. It is possible to note that the highest k_{cat}/K_m value was found for substrate 1 (M-2420), thus indicating this substrate is processed by hrBACE1 in the most efficient way. QE values were found >90% for both substrates 1 and 2. On the basis of these results, M-2420 was chosen as a suitable fluorogenic substrate to be used in the on-line hrBACE1–IMER LC studies.

3.2. Optimization of the chromatographic conditions and evaluation of kinetic parameters

Chromatographic parameters were optimized in order to find the best conditions for determining enzyme activity, together with a good stability of the baseline and a complete elution of substrate, hydrolysis product and inhibitors. In particular, a critical choice was the pH of the mobile phase. HrBACE1 shows maximal activity at acidic pH (pH 4.5), being mainly present in cellular compartments such as Golgi [30], endosomes and the cell surface [31]. In the present work, the pH value of the mobile phase buffer was set slightly higher to overcome the baseline instability at lower pHs (data not shown). The data indicate that pH 5.5 represented the best compromise between enzyme activity and chromatographic stability. In addition, a mobile phase composed of DMSO and buffer (5:95) (v/v) was required to allow the complete elution of the enzymatic product without affecting enzyme activity and CHAPS, a tensioactive agent which is used in the FRET assay to increase tested compounds solubility, was also employed at a concentration of 0.1% (w/v).

Under the optimized conditions of analysis, a Michaelis–Menten plot was obtained by injecting increasing concentration of M-2420 onto the hrBACE1–IMER LC system.

In order to determine the amount of product (pmol min^{-1}) produced in the hrBACE1–IMER system, a calibration curve of commercial standard corresponding to the hydrolysed peptide, was constructed (range 0.15–3.06 nM, normalised concentration) by plotting peak area versus standard peptide concentration. The resulting curve was linear, $y = 340.3(\pm 18.7)x + 36{,}400$, $r^2 = 0.9910$. The reported statistical data represent the average correlation coefficient, slope, and intercept for calibration curves obtained in three different days. 95% confidence intervals of the y-intercepts were found to be −57,240 to 130,000. Peak product area obtained for increasing M-2420 concentration was converted in the correspondent peptide concentration and finally in the amount of product obtained per min (Fig. 2), used to determine v_{max}^{app} value (pmol min^{-1}) and the immobilized active units.

To compare data obtained with the hrBACE1–IMER with those from the FRET assay, a calibration curve (range: 26–173 nM) was also obtained in solution as described in Section 2.3. The obtained relationship was found $y = 0.054(\pm 0.0013)x + 0.913$, $r^2 = 0.9981$. Michaelis–Menten plot for M-2420 was built in the range: 0.15–20 μM and maximum fluorescence value was converted in amount of product obtained per min.

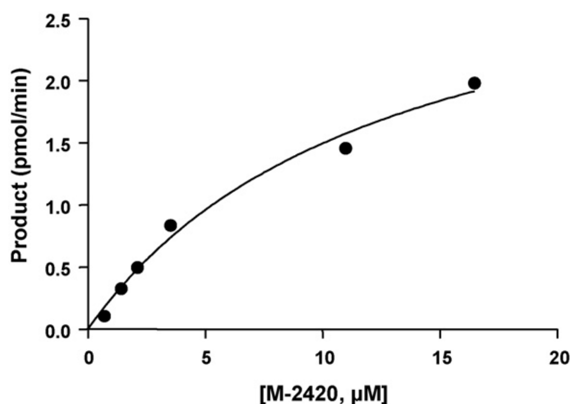

Fig. 2. Michaelis–Menten plot showing the rate (pmol min^{-1} of fluorescent product vs. injected M-2420 concentration) of hrBACE1–IMER catalysis. Reported values are the mean of two independent experiments in which each injection was performed in duplicate.

Table 2
K_m^{app} and v_{max}^{app} values obtained with the free enzyme and with hrBACE1–IMER. Values are the mean of two independent measurements, each performed in duplicate (SEM: standard error of the mean).

	K_m^{app}	v_{max}^{app}
HrBACE1 free	1.91 ± 0.42 μM	1.62 ± 0.15 pmol min^{-1}
HrBACE1–IMER	8.32 ± 0.49 μM	3.48 ± 0.09 pmol min^{-1}

Final results for in solution studies and on-line studies are shown in Table 2. Kinetic values obtained in solution were found to be in accordance with a previous characterization of this substrate [27]. It is of interest that a higher amount of product was obtained with the hrBACE1–IMER (2.15 times more than in solution), after a CT between immobilized enzyme and substrate of only 0.34 min, as compared to a CT of 15 min when the free enzyme is employed. Taken together, these data strengthen the advantages of this fast IMER. It was therefore demonstrated that in the immobilized format hrBACE1 efficiency is increased, which is consistent with previous reports [12–14,21]. In fact, the use of monolithic matrix enhances reaction rate due to the high local concentration of immobilized enzyme, together with a better accessibility of the active site for the substrate.

It should be noted that immobilized active units (3.48 pmol min^{-1}, Table 2) are 30 times lower than the value we found in our previous work (100 pmol min^{-1}) immediately after immobilization [21]. This is mainly due to a loss of enzyme activity after long time usage (more than one year use and 1000 injections). Anyway, this sensitive method allows to detect a revealable amount of product even when a low number of enzyme active units are retained on the monolithic disk.

3.3. Characterization of hrBACE1 hydrolysis product

A typical chromatogram of M-2420 enzymatic hydrolysis on hrBACE1-IMER system is reported in Fig. 3a. As shown, two peaks are present, with retention times of 0.52 min and 1.47 min, respectively. The second peak was found corresponding to the fluorescent methoxycoumaryl fragment obtained when M-2420 peptide bond between leucine and aspartate is catalytically cleaved by hrBACE1–IMER. This product showed the same retention time (Rt = 1.6 min) as the reference peptide (Fig. 3c). The first element of specificity of product formation was acquired by injecting the same substrate concentration both on the hrBACE1–IMER and on a blank CIM-EDA disk. As demonstrated in Fig. 3, the fluorescent peak eluting at Rt = 1.6 min was obtained from the catalysed hydrolysis

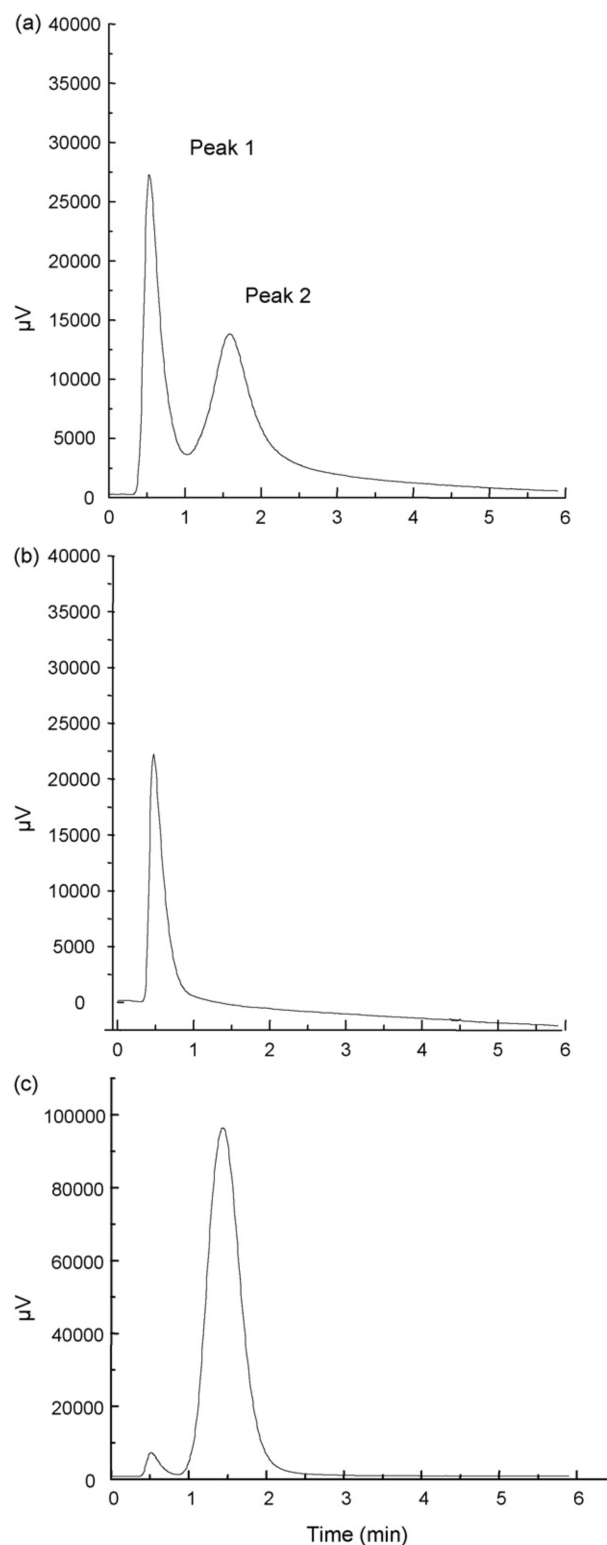

Fig. 3. (a) A typical chromatogram obtained by injecting M-2420 onto the hrBACE1–IMER. Chromatographic conditions: mobile phase composed of a mixture between 25 mM sodium phosphate buffer (pH 5.5) containing CHAPS 0.1% (w/v) and DMSO (95:5) (v:v). Flow rate was 1.0 mL min^{-1} and injection volume was 10 μL. (b) A typical chromatogram obtained by injecting M-2420 onto the blank CIM-EDA; chromatographic conditions as in (a). (c) A typical chromatogram obtained by injecting MCA-fragment onto the hrBACE1–IMER; chromatographic conditions as in (a).

of M-2420, which occurred only in the hrBACE1–IMER, and showed the same retention time as the commercial standard (Fig. 3c).

Peak 1 was assumed to be the uncleaved substrate. To confirm the identity of hrBACE1 substrate and product and to complete their characterization, a LC–MS analysis was carried out. Peaks 1 and 2 were separately collected, concentrated and injected on a monolithic C18 stationary phase under the optimized chromatographic conditions described in Section 2.2. The peak apex mass spectra were recorded within 700–1600 m/z full scan (positive polarity), providing the total ion current (TIC) chromatograms and the pseudomolecular mass of the analytes. As showed in the overlaid chromatograms in Fig. 4, peaks were eluted in this system with retention times of 4.3 (peak 2) and 5.8 min (peak 1), respectively. This elution was found consistent with their lipophilicity. Mass spectra of MCA-fragment (SIM mode at m/z = 777.3) and M-2420 (SIM mode at m/z = 1534) are reported in Fig. 4b and c, respectively.

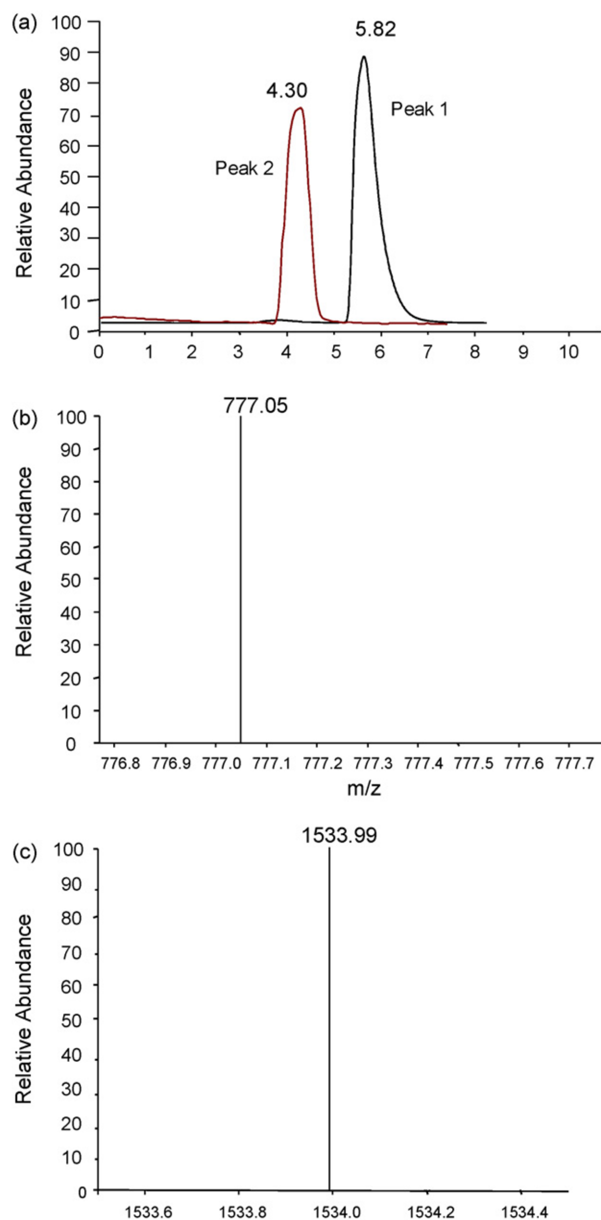

Fig. 4. LC-ESI–MS analysis of peaks 1 and 2. (a) Overlaid chromatograms of peaks 1 and 2. Analysis were carried out on a Chromolith C18 column (100 × 4.6 mm I.D.) under the following chromatographic conditions: mobile phase consisting in 10 mM ammonium acetate/acetic acid pH 4.5 and acidic methanol (35/65, v/v), flow rate 0.4 mL min^{-1}, injection volume 20 μL. (b) MS spectra of peak 2 (MCA-fragment). SIM mode at m/z = 777.3. (c) MS spectra of peak 1 (M-2420). SIM mode at m/z = 1534.

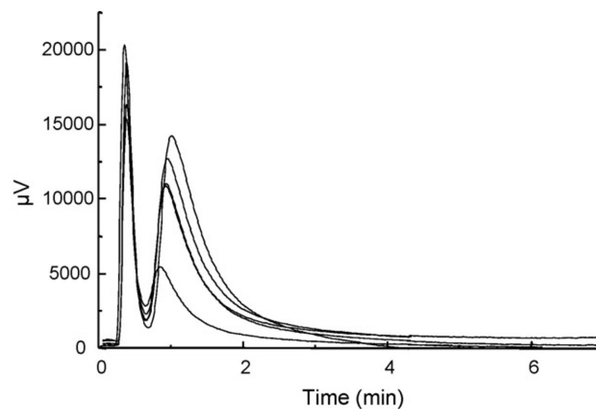

Fig. 5. Overlaid chromatograms obtained after injection of substrate (2 μM, normalised concentration) and substrate plus increasing concentrations of inhibitor IV.

Table 3
IC_{50} values for five standard inhibitors obtained with the FRET assay in solution and with the hrBACE1–IMER. Values are the mean of two independent measurements, each performed in duplicate (SEM: standard error of the mean).

	IC_{50} (μM), FRET assay	IC_{50} (μM), hrBACE1–IMER
Pepstatin A	21.19 ± 0.35	481.95 ± 10.70
(−)Gallocatechin gallate	3.11 ± 0.16	127.54 ± 15.34
Statine derivative	0.14 ± 0.05	11.38 ± 5.70
OM99-2	0.04 ± 0.00	0.70 ± 0.07
Inhibitor IV	0.02 ± 0.00	0.46 ± 0.06

3.4. Inhibition studies

In the process of drug discovery, the linear correlation between on- and off-line studies is a key requisite in order to compare data from on-line studies with data from literature [13,20]. For this reason, five compounds were tested for their ability to inhibit hrBACE1–IMER activity. Three of them (Inhibitor IV, OM99-2 and statine derivative) are well known potent inhibitors of hrBACE1 while (−)gallocatechin gallate and Pepstatin A are less active compounds [32,33].

Each inhibitor was also tested in solution with the free enzyme as reported in Section 2.3.2.

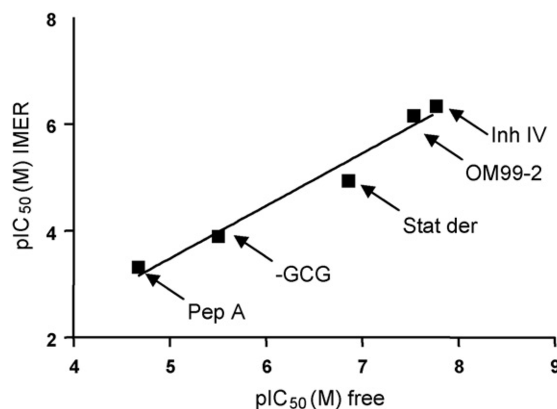

Fig. 6. Correlation plot for inhibitory potency (pIC_{50}) of five well known inhibitors obtained with hrBACE1–IMER and with the enzyme in solution. pIC_{50} values [$-\log IC_{50}(M)$] represent the concentration of inhibitor required to decrease enzyme activity by 50% and are the mean of two independent measurements. Inh IV: inhibitor IV; OM99-2; Stat der: statine derivative; (−)GCG: (−)gallocatechin gallate and Pep A: pepstatin A.

In the on-line studies, by comparing peak 2 area in the presence and in the absence of inhibitors, an increasing product area reduction was observed for increasing inhibitor concentrations. As an example, overlaid chromatograms are reported in Fig. 5 for the most active compound, inhibitor IV [34]. The % inhibition was plotted against the logarithm of injected normalized inhibition concentrations and the IC_{50} values were obtained by interpolation. IC_{50} values obtained with the free hrBACE1 and with the hrBACE1–IMER are reported in Table 3.

The pIC_{50} values obtained with the IMER were compared with those obtained with the free enzyme, the correlation graph is reported in Fig. 6. Correlation factor was found to be 0.9694 ($n = 2$). The satisfactory correlation value makes the hrBACE1–IMER a valid tool to assess the inhibitory potency of unknown inhibitors.

4. Conclusions

A fast, reproducible and feasible chromatographic method was developed and applied for the evaluation of hrBACE1–IMER activity and inhibition. This method was found to be very sensitive; in fact the product of *on-line* enzymatic reaction was selectively detected and inhibition studies were performed even with a low number of immobilized enzyme active units. The immobilization of hrBACE1 on monolithic disk allowed to increase enzyme stability and efficiency and to perform fast and reproducible analysis.

Fluorogenic substrate and product obtained from on-line enzymatic hydrolysis were characterized through a LC–MS analysis. Further inhibition studies were performed to confirm the applicability of this hrBACE1–IMER for the high-throughput screening of new inhibitors. Compared to standard off-line methods (requiring more than 1 h for each analysis), the method proposed allowed to get more reliable results. Moreover, one batch of enzyme was used continuously in the immobilized format for an extended period of time, with the final advantage in terms of enzyme stability, reproducibility, costs and time of analysis.

Acknowledgements

University of Bologna (RFO) and MIUR (PRIN 2007) are gratefully acknowledged for their financial support.

The authors thank Nicola Petragnani for his valuable and helpful technical assistance. We wish also to thank Prof. Irving Wainer for his suggestions regarding this manuscript.

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