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Determination of inhibitory potency of argatroban toward thrombin by electrophoretically mediated microanalysis

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ABSTRACT

Developing an EMMA method for enzymatic assay remains a challenge, particularly using UV detection. Indeed, it is necessary to optimize the separation conditions while allowing the enzymatic reaction to occur within the capillary respecting kinetic constraints and achieving enough sensitivity. In this work, such EMMA methodology was set up to evaluate the inhibitory potency of drugs toward thrombin, a serine protease implicated in the coagulation cascade. To achieve our goal, the separation buffer, the injection sequence, the internal standard and the chromogenic substrate were investigated. The newly developed system was then assessed determining the kinetic K_m constant for the selected substrate and compared with the results obtained with a continuous spectrophotometer cell assay. Secondly, the K_i inhibitory constant of the thrombin inhibitor argatroban was determined and found in agreement with the published value.

1. Introduction

Proteases are enzymes that selectively catalyze the hydrolysis of peptides and proteins. They are classified into six distinct classes according to the residue (an amino acid or a metal) implicated in the nucleophilic attack on the carbonyl atom of the peptide bond [1]. Among these classes, serine proteases are attractive targets for the design of inhibitors since they are involved in wide variety of pathological states including inflammation, allergic response and thrombosis [2,3]. Thrombotic diseases, in which a deregulated hemostatic activity occurs, remain a major cause of morbidity and mortality in the industrialized world. One of the strategies to address these diseases implies the treatment by anticoagulants targeting the coagulation cascade. This cascade comprises a series of serine proteases that ultimately leads to the conversion of fibrinogen to fibrin [2,4]. As the terminal enzyme of this cascade, thrombin (THR) plays a pivotal role in this process. Besides the cleavage of fibrinogen, THR amplifies its own generation by feedback activation of factors V, VIII, and IX and stimulates platelet activation. In contrast, when bound to thrombomodulin, it causes a feedback inhibition of the coagulation cascade by activation of protein C. This, in turn, inactivates factors Va and VIIIa by proteolytic degradation. Because of its importance in thrombosis, THR has been recognized early as a major target for the development of direct inhibitors and several are already approved for clinical use [5]. Indeed, the majority of anticoagulant drugs currently in clinical development specifically target THR or factor Xa, another serine protease of the coagulation cascade.

In the early stage of anticoagulant drug development, the activity of the candidates is usually tested on isolated enzymes. These in vitro bioassays are essential for the medicinal chemist to guide new chemical synthesis through the analysis of structure–activity relationships. Generally, they consist in measuring the activity of the enzyme with or without the inhibitors. Typically, with serine proteases, a fluorogenic or a chromogenic substrate is incubated with the enzymes. After incubation, the enzymatic reaction is stopped and the release of the chromophore or the fluorophore is measured spectrophotometrically. The main advantages of such assays are their ability to be transferred in High Throughput Screening (HTS) format using multiwell plate technologies. Whereas HTS allows the testing of thousands of compounds per day (10,000–100,000), it suffers from several drawbacks such as false negatives and false positives. As a result, HTS campaigns can be dominated by hits that ultimately turn out

Abbreviations: BGE, background electrolyte; CE, capillary electrophoresis; EMMA, electrophoretically mediated microanalysis; EOF, electro-osmotic flow; IS, internal standard; MEKC, micellar electrokinetic chromatography; NF, nitrofurantoin; SDS, sodium dodecyl sulfate; pNA, para-nitroaniline; THR, thrombin

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to be irrelevant [6,7]. Several mechanisms related to the tested molecules contribute to these artefacts including spectroscopic artefacts, absorption, and aggregation phenomena. Thus all assays need counter-screens and selectivity screens to enable the identification of quality hits [6].

Capillary electrophoresis (CE) has become an attractive analytical technique to study enzymatic reactions principally due to its high peak efficiency, low reagent consumption, integration of various detection modes (UV, fluorescence, mass spectrometry, etc.) and automation [8]. Two categories of assays can generally be distinguished: pre-capillary (offline) and in-capillary (online) assays [9]. In the former ones, the enzymatic reaction is performed outside the capillary and the reaction mixture is then analyzed in CE for the determination of the substrate and/or reaction products. On the contrary, CE in-capillary assays are developed in order to integrate the enzymatic reaction and the separation of the reactants and products in a single place, namely the capillary. Enzyme and substrate can be mixed into the CE capillary by diffusion or by electrophoresis, which gives rise to different modes for in-capillary homogeneous assays: zonal electrophoretically mediated microanalysis (EMMA) and plug–plug EMMA, at-inlet longitudinal and transverse diffusion assay. The loading and the mixing of the enzyme and the substrate during EMMA is usually performed using two main modes [10]. In the continuous mode, the capillary is entirely filled with either enzyme or substrate followed by the introduction of the other reactant as a plug or in continuous flow. In plug–plug EMMA (short contact mode), consecutive plugs of the reactants are injected into the CE capillary. In a very recent review, X. Hai et al. summarized recent developments and applications of EMMA in enzymatic reactions [11].

The aim of this study was to develop an EMMA method for evaluating the inhibitory potency of potential inhibitors of thrombin using classical CE-UV detection. This methodology will use a chromogenic substrate in which the product, *para*-nitroaniline (pNA) is widely applied as a reporter for monitoring the activity of different proteases. Thus, the main advantages of our system would be the possibility to extend its applicability to a number of enzyme assays. However, the main drawbacks come from the relatively poor CE-UV sensitivity for pNA and the fact that this molecule remains uncharged making the development of the assay particularly challenging.

To achieve our goal, the optimal experimental conditions leading to the separation of each component of the reaction were first determined. After that, the enzymatic reaction was tested, first in offline assays and then in in-line assays using the EMMA principle. This second step consisted in the optimization of the enzymatic reaction within the capillary in terms of sensitivity and analysis time. Finally, the kinetic values (K_m, K_i) obtained by EMMA were compared to the ones obtained by standard spectrophotometric method.

2. Materials and methods

2.1. Chemicals and materials

Human thrombin stabilized (THR) was purchased from Roche (Mannheim, Germany), S-2238, S-2366 from Chromogenix (Milano, Italy) and T-3068, para-nitroaniline (pNA) and nitrofurantoin (NF) from Sigma-Aldrich (St. Louis, USA). Argatroban was obtained from Sequoia Research Products Limited (Pangbourne, United Kingdom) (cf. Fig. 1).

All solutions were filtered through a cellulose based membrane (0.20 μm) with Chromafil® syringe filters from Macherey-Nagel (Düren, Germany). Trishydroxymethylaminomethane hydrochloride (Tris–HCl), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium chloride (NaCl), polyethylenglycol (PEG), triethanolamine, sodium dodecyl sulfate (SDS), para-nitrophenol (pNP) and dimethylsulfoxyde (DMSO) were obtained from Acros Organics (NJ, USA). Ultra-pure water was supplied by a Milli-Q equipment (Millipore, Bedford, MA, USA).

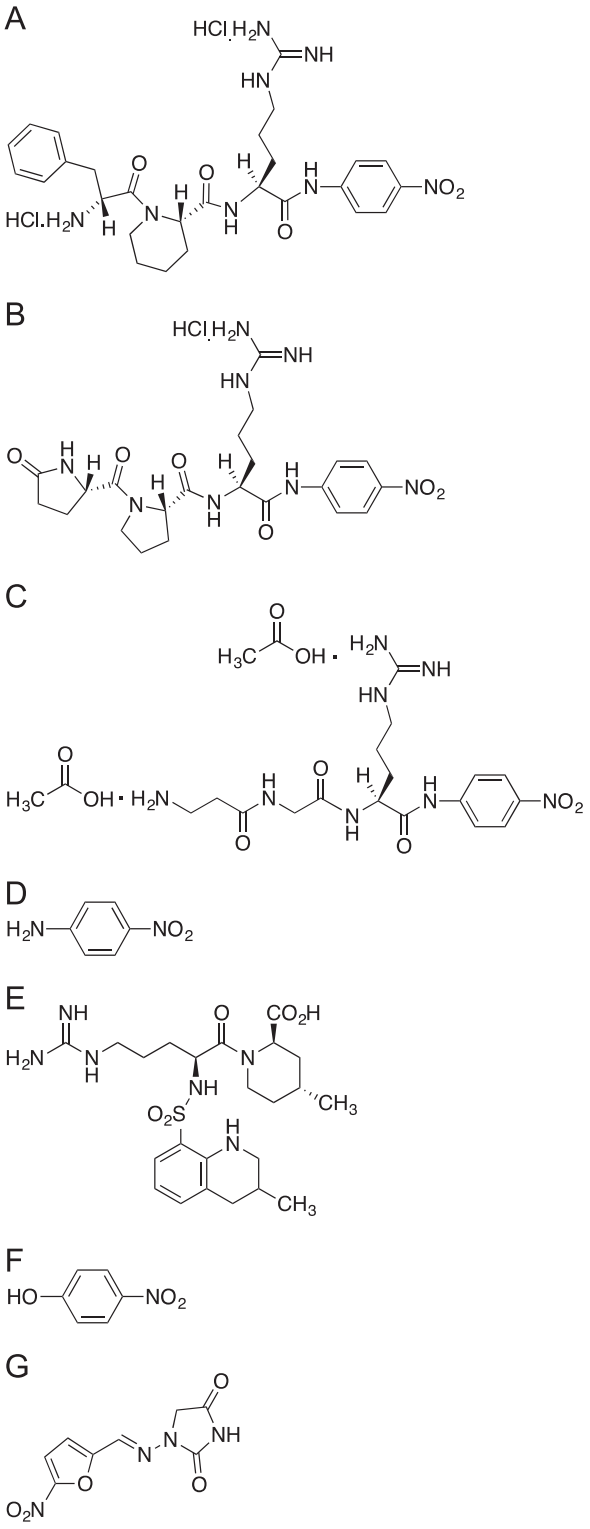

Fig. 1. Chemical structures of the tested substrates S-2238 (A), S-2366 (B) and T-3068 (C), the reaction product para-nitroaniline (D), the enzyme inhibitor argatroban (E), the tested internal standards: para-nitrophenol (F) and nitrofurantoine (G).

2.2. Instrumentation

All the experiments were carried out on a HP[3D]CE system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, an on-column DAD and a temperature control system (15–60 °C ± 0.1 °C). Chemstation (Hewlett-Packard, Palo Alto, CA, USA) was used for instrument control, data acquisition and data analysis. Fused silica capillaries were provided by ThermoSeparation Products (San Jose, CA, USA).

The spectrophotometer cell assays were run in semi-micro optical glass precision cells with a light path of 10 mm (Hellma) using a Lambda 20 bio-spectrophotometer equipped with a thermostated cell-holder (Perkin-Elmer).

2.3. Preparation of buffer and stock solutions

The buffer was made up of 0.01 M Tris–HCl, 0.01 M HEPES, 0.1 M NaCl, 0.1% PEG 6000 and adjusted to pH 7.5 with triethanolamine (kinetic buffer). For the separation, a surfactant, namely SDS, was added to the kinetic buffer at a concentration of 15 mM (background electrolyte, BGE).

A stock solution of thrombin (THR) was prepared in the kinetic buffer at a concentration of 5 μM. This stock solution was stored at −20 °C in aliquot of 20 μl in low adhesion Eppendorf. The working solution of thrombin was extemporaneously prepared by dilution with the kinetic buffer.

Stock solutions of S-2238, S-2366 and T-3068 were also prepared in the kinetic buffer at a concentration of 5 mM. A stock solution of para-nitroaniline (pNA) was prepared in a water/methanol (90/10; v/v) mixture at a concentration of 5 mM. Nitrofurantoine (NF) stock solution at 0.5 mM was prepared in a DMSO/kinetic buffer (10/90; v/v) mixture. Finally, a stock solution of argatroban at a concentration of 10 mM was prepared in a mixture of DMSO and kinetic buffer (30:70; v/v).

All these solutions were stored in the fridge protected from light and diluted with the kinetic buffer to reach the appropriate final concentrations.

2.4. Electrophoretic conditions

Electrophoretic separations were carried out on uncoated fused silica capillaries having 50 μm i.d. and 48.5 cm total length (8.5 cm effective length). The capillary was thermostated at 25 °C and the applied voltage used during the run was −17 kV (cf. Fig. 2).

At the beginning of each working day, the capillary was washed with 1 M NaOH, water and BGE for 10 min each. Before each injection, the capillary was successively washed with water for 1 min, 2 M NaOH for 3 min, water for 1 min and was then equilibrated with the BGE for 5 min. Capillary wash cycles were performed at a pressure of approximately 1 bar.

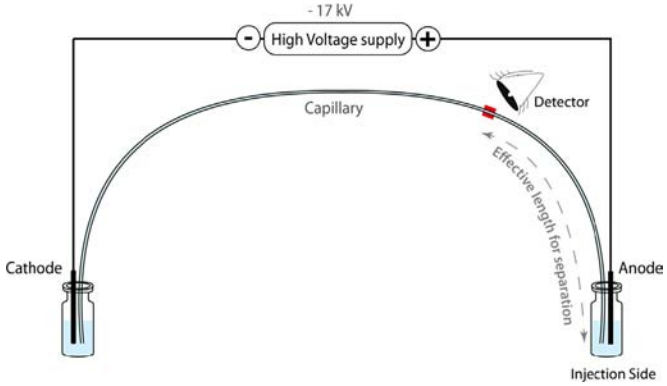

Fig. 2. Schematic representation of the capillary electrophoresis system used in this study.

For the short-end injection, the injection was made at the outlet vial by negative pressure (−50 mbar). The polarity was reversed relative to the traditional analysis so that the EOF migrates from the outlet vial toward the detector.

2.5. Enzymatic assays

2.5.1. Spectrophotometric assays for K_m determination

For these assays, stock solutions of S-2366 (2.5 and 5 mM) and thrombin (0.1 μM) were prepared in the kinetic buffer. The final substrate dilution in the same buffer was directly done in the cell just before the start of the enzymatic reaction by addition of 20 μl of 0.1 μM thrombin (final volume 500 μl). The OD increase was then continuously measured at 405 nm at 25 °C during 1 min in the spectrophotometer. The velocity was calculated from 0.1 to 0.25 min using Kinlab software v1.1 (Perkin-Elmer). In this experiment, each assay was done in triplicate and the final concentrations for thrombin and substrate were [thrombin]=4 nM and [S-2366]=1000, 500, 400, 200, 100, 50 or 25 μM.

2.5.2. Offline CE assay with S-2238 substrate

The offline assay was performed as follows: 20 μl of 0.1 μM thrombin was mixed with 140 μl of kinetic buffer. 40 μl of 5 mM S-2238 was then added and incubated for 5 min. After that, the mix was injected into the CE at the outlet vial (−50 mbar, 3 s) and the separation was performed by applying a voltage of −17 kV.

2.5.3. Electrophoretically mediated microanalysis (EMMA)

The injection sequence was the following: kinetic buffer (−50 mbar, 6 s), 0.01 μM THR (−50 mbar, 3 s), S-2238, S-2366 or T-3068 with the IS (−50 mbar, 3 s), kinetic buffer (−50 mbar, 6 s), BGE (−50 mbar, 2 s). One second injection at 50 mbar corresponds to 1.8 nl injected volume. After each injection, the outlet end of the capillary was dipped into kinetic buffer to avoid cross contamination. Then a voltage switch sequence was applied to favor compounds mixture: −0.5/+0.5/−0.5/+0.5 kV, each for 7 s. An incubation time was then started (1-5 min). After this period, a voltage of −17 kV was applied for analytes separation and the detection was performed at 200 nm and 367 nm.

For the in-line assays, a mix of 10 μl NF (0.5 mM), 10 μl S-2238, S-2366 or T-3068 (5 mM), and 30 μl kinetic buffer was prepared in a propylene vial. For the in-line assays in the presence of the inhibitor, 10 μl of argatroban replaced 10 μl kinetic buffer. In another propylene vial, 25 μl THR (0.02 μM) and 25 μl kinetic buffer were mixed together.

In the final conditions, concentrations were: 100 μM IS, 1 mM substrate, 0.01 μM THR.

2.6. Enzymatic kinetic

2.6.1. Determination of the K_m kinetic constant for substrate S-2366 with thrombin

Enzyme reaction incubations were carried out with S-2366 concentrations ranging from 0.05 to 1 mM at constant 0.01 μM enzyme concentration and 2 min incubation time. The nonlinear curve fitting program PRISM® 5.04 (GraphPad, San Diego, CA, USA) was used to determine K_m and V_{max} of the substrate hydrolysis according to Eq. (1):

$$v_i = \frac{V_{max}[S]}{K_M + [S]} \quad (1)$$

where v_i is the initial velocity, K_m is the Michaelis–Menten constant, V_{max} is the maximum reaction velocity and [S] is the substrate concentration.

2.6.2. Inhibition study of thrombin by argatroban: determination of IC50 and Ki by EMMA

The determination of the IC50 of argatroban on thrombin with the EMMA methodology was done by measuring the initial velocity for a range of inhibitor concentrations (5 nM to 2 mM) in the presence of 1 mM S-2366. Percent inhibition at each inhibitor concentration was calculated by dividing the initial velocity with inhibitor by the one without. IC50 values were calculated from a sigmoidal dose response nonlinear regression equation using PRISM® 5.04 (GraphPad Software). IC50, the concentration of inhibitor that causes 50 per cent inhibition of the enzymatic reaction, is then converted to the K_i inhibition constant using the Cheng-Prusoff equation Eq. (2):

$$K_i = \frac{IC_{50}}{1 + ([S]/K_m)} \quad (2)$$

where K_m is the Michaelis-Menten constant and [S] is the substrate concentration.

2.6.3. Determination of the IC50 and K_i values using the spectrophotometric assay

For these assays, stock solutions of S-2366 (2.5 mM) and thrombin (0.1 μM) were prepared in the kinetic buffer. 20 μL of argatroban in water (or water alone in the control), 40 μL of S-2366 (2.5 mM) and 420 μL of THR kinetic buffer were mixed. 20 μL of human thrombin (0.1 μM) were then added to the mixture and the OD increase at 405 nm was continuously measured at 25 °C during 1 min in the spectrophotometer. The velocity was calculated from 0.1 to 0.25 min using Kinlab software v1.1 (Perkin-Elmer). In this experiment, each assay was done in duplicate and the final concentrations for thrombin, substrate and argatroban were [thrombin]=4 nM, [S-2366]=200 μM and [argatroban]= 5000, 1000, 500, 100, 50, 25, 10, 5, 1, 0.5 or 0.1 nM. Percent inhibition at each concentration was calculated from the OD at 405 nm from the experimental and control samples. IC50 values were calculated from a sigmoidal dose response nonlinear regression equation using PRISM® 5.0 (GraphPad Software).

3. Results and discussion

3.1. Development of the assay

In this study we intend to use CE as a microreactor to measure the activity of a thrombin inhibitor, namely argatroban, and to determine kinetic reaction parameters in a fully automatized approach. To achieve these goals, an in-capillary screening method (EMMA) was developed using short-end injection (effective capillary length: 8.5 cm). The CE method was optimized regarding selectivity, sensitivity, repeatability and speed of analysis. Three substrates for the enzymatic reaction were investigated, namely S-2238, S-2366 and T-3068. These substrates are known to be cleaved by thrombin (THR), releasing para-nitroaniline (pNA) as reaction product.

3.1.1. CE separation of the constituents

Even if in this particular case, the reaction product, namely pNA, has a relatively characteristic λ_{max} (367 nm), it is always interesting to use a separative technique to discriminate the different constituents of the reaction (enzyme, substrate, products, internal standard and inhibitor) and thus ensure a more selective response than the standard spectrophotometric method. As the enzymatic reaction has to occur in physiological conditions, we first used the classical reaction buffer made up of Tris–HCl, HEPES, NaCl and PEG (pH 7.5) [12,13] as CE background electrolyte (BGE). In this condition, the electroosmotic flow (EOF) is rather high and both pNA (pKa ≈ 1) and thrombin (pI ≈ 7.4), which are mainly uncharged, migrate with the EOF. On the contrary, all substrates are positively charged and migrate before the EOF. To separate co-migrating components, negatively charged cyclodextrins (CDs) (carboxymethyl-β-CD, sulfobutyl-β-CD and octakis(6-*O*-sulfo)-γ-CD) were tested but no interaction of pNA with these CD derivatives could be observed. The addition of SDS to the BGE was then investigated in order to bring selectivity. Using micellar electrokinetic chromatographic (MEKC) system, the electrophoretic behavior of the three substrates was found to be different in the presence of SDS even if all of them are positively charged at pH 7.5. Indeed, in the short-end injection mode, S-2366 migrates towards the detector in the negative polarity mode while S-2238 and T-3068, which have highly negative effective mobilities, are detected in the positive polarity mode (cf. Fig. 2). Therefore, it can be assumed that S-2238 and T-3068 have a very high affinity for the micelles, through hydrophobic bonds as well as ionic interactions [14]. 15 mM SDS was found to be the best compromise in terms of selectivity and analysis time. Indeed, in these conditions, pNA (MT: 1.7 min) is well-separated from the EOF (1.2 min), argatroban (MT: 3.9 min), S-2366 (MT: 4.6 min) and from thrombin slightly absorbing at 200 nm (MT: 2.5 min) while S-2238 and T-3068 migrate in the reversed-polarity mode.

For quantification purpose, para-nitrophenol (pNP) and nitrofurantoin (NF) were tested as internal standard because they absorb at 367 nm and are well-separated from the EOF, pNA and S-2366. NF (MT=2.2 min) was finally chosen because it provides good repeatability contrary to pNP in our final MEKC conditions. To estimate the sensitivity and the response function, normalized peak area ratio (pNA corrected-peak area (CA)/NF CA ratio) versus pNA concentration (from 5 to 1500 μM at seven concentration levels) was plotted. The regression line was found to fit the data optimally (r^2=0.996). The lowest pNA concentration studied (5 μM pNA) was estimated to be the LOQ (10 × S/N) in our experimental conditions.

3.1.2. EMMA with S-2238 substrate

Offline reaction was first tested in the kinetic buffer but also in the optimized BGE (kinetic buffer with SDS). After mixing the different reagents and incubating for 5 min, the solutions were analyzed in CE. On the contrary to the reaction performed in the kinetic buffer, pNA was not observed after incubation in the BGE, probably due to the denaturation of thrombin in the presence of SDS. This means that to allow the reaction to occur within the capillary, enzymatic reaction zone has to be isolated from the BGE. Van Schepdael et al. already developed the “partial-filling mode” in EMMA in which the BGE and the reactant plugs are separated by a plug of incubation buffer [15,16]. In this study we used two kinetic buffer plugs injected before and after the enzyme and substrate plugs to prevent any contact of the latter with the SDS-containing BGE. In these conditions pNA could be detected applying the EMMA method.

EMMA reaction was initiated by the electric field applied after injection of the enzyme and its substrate in separated plugs (short contact mode). To trigger the enzymatic reaction, the electric field intensity and its application time have to be optimized for the plugs interpenetration due to differences in the enzyme and substrate velocities. The procedure proposed by Sanders et al. in which successive mixing of plugs is carried out by rapid polarity switching was applied [17]. In this approach, the mixing quality of the injected plugs was found to be improved due to the application of a series of positive and negative voltages. After optimization, the injection sequence in EMMA was as followed: kinetic buffer (−50 mbar, 6 s), THR (−50 mbar, 3s), S-2238 with IS (−50 mbar, 3 s), kinetic buffer (−50 mbar, 6 s), BGE (−50 mbar, 2 s).

Then, a voltage switch sequence was applied to allow compounds mixture: −0.5/+0.5/−0.5/+0.5 kV, each for 7 s (cf. Figs. 3A–C).

After the optimization of EMMA conditions, we evaluated our methodology using S-2238 as chromogenic substrate. The substrate and enzyme concentration as well as the incubation time have to be selected so that the velocity of the reaction is in the linear domain.

The first experiments consisted in measuring the product pNA generated in the assay (incubation time 5 min) as a function of substrate concentration (0.25–4 mM). Whereas substrate concentration dependent effect with a saturation behavior was observed when plotting the reaction rate (amount of product by unit of time) vs. substrate concentration, it was not expected that this experiment would allow the determination of the Michaelis–Menten constant. Indeed, the Michaelis–Menten relationship implied the analysis of initial velocity vs. substrate concentration. It is commonly assumed that a good estimation of this initial rate could only be obtained if substrate consumption is less than 10–20% [18]. In our conditions, more than 50% of the substrate was found to be consumed when substrate concentration is lower than 2 mM.

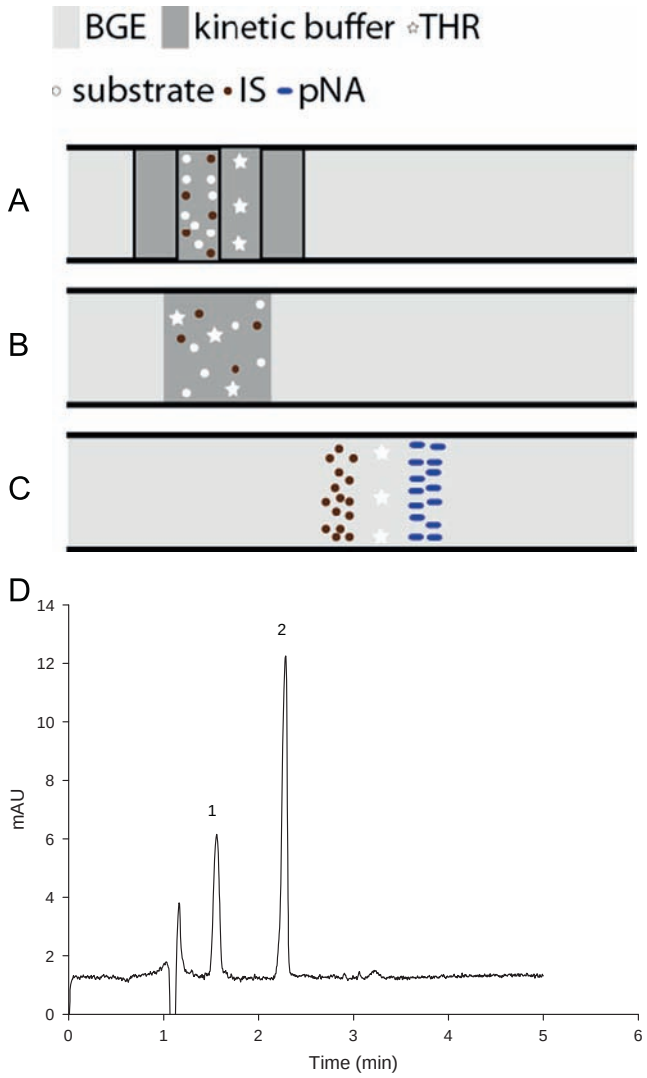

Fig. 3. Principle of the developed EMMA method. (A) Sequential injection of the kinetic buffer, THR, substrate with IS, kinetic buffer and the BGE. (B) Compounds mixture by applying a voltage switch sequence. (C) Mixture separation and detection of the product and IS. (D) Typical electropherogram at the optimal conditions: injection sequence: kinetic buffer (−50 mbar, 6 s), 0.01 μM THR (−50 mbar, 3 s), 1 mM S-2366 with the IS (−50 mbar, 3 s), kinetic buffer (−50 mbar, 6 s), BGE (−50 mbar, 2 s); switch of voltage after injection: −0.5/+0.5/−0.5/+0.5 kV, each for 7 s; incubation time: 2 min; separation voltage: −17 kV; effective length: 8.5 cm, λ: 367 nm; and peak identification: 1=para-nitroaniline, 2=IS.

Moreover, when designing enzymatic inhibition assay, it is essential to select balanced assay conditions to be able to identify lead compound [19]. Considering our limit of quantification (5 μM pNA) and the 10% consumption limit, the minimum substrate concentration that we might use for enzymatic assay would be 50 μM. Besides, if we want to be able to spot a 10-fold decrease in enzyme activity due to the presence of a reversible inhibitor (i.e. argatroban), the substrate concentration has to be set at 500 μM. In this condition and given the reported K_m value (7 μM) for S-2238, the ratio [S] on K_m would be around 70 and the bioassay would not be very sensitive [19]. Thus, to improve the ability of our assay to detect inhibitor with lower activity (K_i 100 μM), it is necessary to select a chromogenic substrate with a higher K_m.

3.1.3. EMMA with S-2366 and T-3068 substrates

With this aim in view, two substrates, i.e. S-2366 and T-3068, were found to show less affinity for thrombin with K_m of 150 μM and 1.95 mM respectively (data from the manufacturer). The enzymatic reaction with 1 mM substrate in the previously optimized EMMA conditions gave a very small quantity of pNA with T-3068. This low production of pNA could be explained by the kinetic properties of THR with T-3068. Indeed, the reported k_{cat}, which represents the number of substrate molecules turned over per enzyme molecule per second, is very low for this substrate (1.9 s^{-1}, manufacturer data). It could be assumed that a higher incubation time would be necessary to produce enough pNA. In this assay, increasing the incubation time is not an option due to the risk of plug diffusion during a prolonged incubation.

On the contrary, the reaction with S-2366 (k_{cat}=330 s^{-1}, manufacturer data) gave rise to appropriate pNA concentration and was thus selected for further kinetic studies. Fig. 3D shows a typical electropherogram under the optimized EMMA conditions using S-2366 as chromogenic substrate.

3.2. Kinetic studies

3.2.1. Initial velocity determination

As stated above, when designing enzymatic bioassays to evaluate the inhibitory potency of a compound, it is important to restrict the assay time to the initial velocity phase of the enzymatic reaction. Indeed, in this phase, the velocity is almost constant, and the time-course of product formation should be linear. Thus to optimize the incubation time in our assays, we measured the concentration of product vs. incubation time. As can be seen from Fig. 4A, the product formation in the presence of S-2366 is almost linear until 5 min whereas an additional measurement at 10 min showed a marked decrease in the velocity. From this experiment, the incubation time was set to 2 min since in this condition we are still in the initial velocity phase while maintaining enough generated product for the sensitivity of the bioassay.

3.2.2. K_m measurement for S-2366

To ensure the validity of our assay, the Michaelis constant K_m was determined by measuring the initial velocity vs. substrate concentration (0.05–1 mM; Fig. 4B). It is generally accepted that a good estimation of the K_m value could be obtained with substrate concentration ranging from 0.4K_m to 4K_m. In our experiments, the K_m value, determined by non-linear regression according to the Michaelis–Menten equation, was 308 ±25 μM for the S-2366. For comparison purpose, we determined this value in an off-line spectrophotometric assay. In this experiment, the enzymatic reaction is continuously monitored by measuring the absorbance at

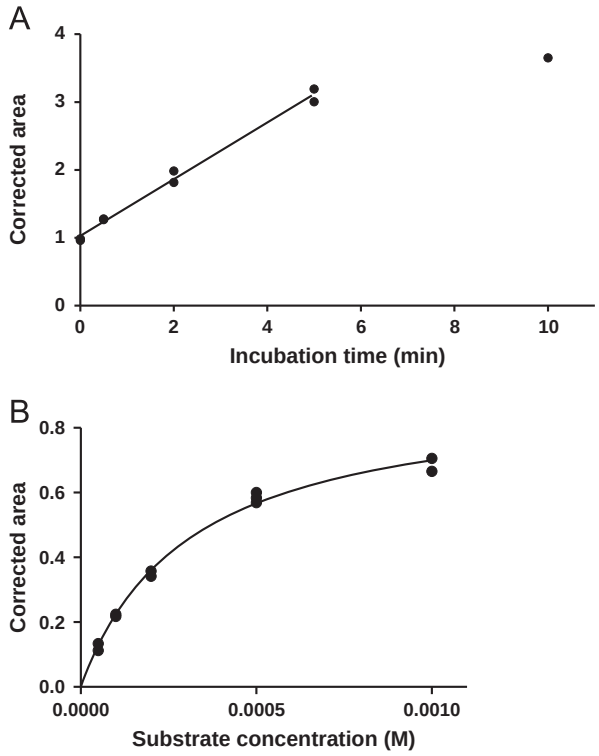

Fig. 4. Effect of the incubation time on the product formation (A). Experimental conditions as in Fig. 3D. Michaeli–Menten curve (B). S-2366 concentration was varied between 0.05 mM and 1 mM. Other conditions as in Fig. 3D.

405 nm immediately after mixing the enzyme and the chromogenic substrate in the spectrophotometric cell. The initial velocities obtained with substrate concentrations ranging from 0.025 to 1 mM were used to determine K_m according to the Michaelis–Menten equation. The calculated K_m (194 ± 17 μM) is closely related to the one reported by the manufacturer (150 μM). Beside, when compared with the value obtained by EMMA methodology (308 ± 25 μM), only a small difference is observed (K_m by EMMA 1.6 fold higher than by continuous spectrophotometric method). The difference could be explained by several reasons such as a dilution during plug mixing or a modification of the ionic strength in the plug.

3.2.3. *IC50 and K_i determination of argatroban*

The ability of the developed EMMA method to evaluate the inhibitory potency of thrombin inhibitor was investigated with argatroban, the first direct reversible thrombin inhibitor that entered into clinical practice. This compound was shown to be a competitive inhibitor with a K_i value of 19 nM [20]. IC50 value was first determined using the EMMA methodology by performing concentration-response plot (cf. Fig. 5) with various inhibitor concentrations (5 nM–2 mM). This IC50 value together with the previously calculated K_m is used in the Cheng–Prussof relation [21] in order to calculate the inhibition constant K_i which was found to be 22.7 ± 2.4 nM using the developed EMMA method. The IC50 value was also determined using the classical spectrophotometric assay (inhibitor concentrations from 0.1 nM to 5 μM) and the resulting K_i value was 10.8 ± 1.6 nM. Beside, in a previous study using S2238 as substrate, we estimated a K_i value for argatroban of 13 nM [22]. Thus, the K_i values obtained using both EMMA and spectrophotometric methodologies are in very good agreement with the data published by Kikumato, demonstrating the usefulness of our bioassays in the evaluation of inhibitory potency.

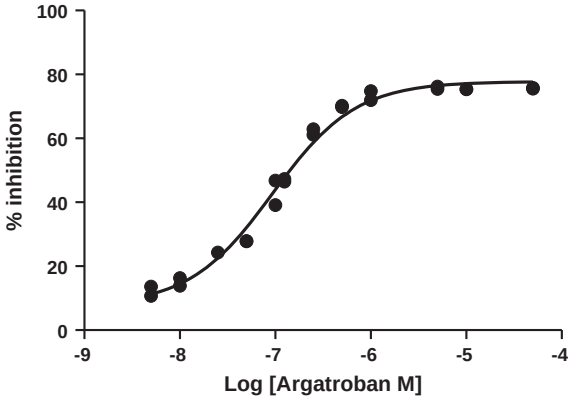

Fig. 5. Inhibition curve for argatroban inhibition of THR. Argatroban concentration was varied between 5 nM and 2 mM. Other conditions as in Fig. 3D.

4. Conclusion

As stated in the introduction, the difficulty of developing an EMMA assay for monitoring enzyme activity with a chromogenic substrate that liberates pNA comes from the uncharged nature and the relatively poor CE-UV sensitivity for pNA. The first hurdle was overcome by using the MEKC mode. SDS was used for this purpose. However, to prevent the denaturation of THR, it was necessary to separate its plug with the one containing SDS. Secondly, recent development on EMMA enzyme assay usually employs a fluorogenic substrate to get enough sensitivity. In our assay, we wanted to select UV detection mode, not only because the accessibility of appropriate substrates is higher but also because it is the most available detection system in CE. In this paper, we showed that it is possible to develop such assay by carefully selecting the substrate. Since it is usually necessary to set the substrate concentration around the K_m value to be able to detect even weak inhibitor, the key is to select a substrate with a higher K_m. The k_{cat} for the substrate is also important to take into consideration since a too slow enzymatic reaction could not be appropriate for in-capillary reaction assay. Indeed, in this case, the incubation time for the reaction should be importantly increased which is not recommended due to the risk of diffusion in the plugs before the separation steps.

In conclusion, the enzymatic activity of thrombin and its inhibition were measured successfully by an EMMA combined with short-end injection and partial-filling methodology. The use of pNA as the reporter molecule would make possible to adapt this assay to any assay using the same reporter. Finally, compared to the spectrophometric method, such method has the advantage to be fully automatized and to reduce the amounts of analytes (sample and enzyme) to few tens of nanoliters and to reduce the incubation time.

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