Journal of Pharmaceutical and Biomedical Analysis 111 (2015) 64–70

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

A fast capillary electrophoresis method to assess the binding affinity of recombinant antithrombin toward heparin directly from cell culture supernatants

Anne-Lise Marie[a,b], Nguyet Thuy Tran[a,b], Elsa P. Bianchini[c], François Saller[c], Stéphane Pautus[c], Toufik Abache[d], Jean-Luc Plantier[d], Rémi Urbain[d], Delphine Borgel[c], Myriam Taverna[a,b,*]

[a] Université Paris Sud, Faculté de Pharmacie, 92290 Châtenay-Malabry, France
[b] CNRS UMR 8612, Institut Galien Paris Sud, 92290 Châtenay-Malabry, France
[c] Université Paris Sud, Laboratoire d'Hématologie, EA 4531, 92290 Châtenay-Malabry, France
[d] LFB, 91940 Les Ulis, France

ARTICLE INFO

Article history:
Received 14 October 2014
Received in revised form 13 February 2015
Accepted 20 February 2015
Available online 19 March 2015

Keywords:
Affinity capillary electrophoresis
Recombinant antithrombin
Heparin
Binding constant
Fluorescence spectroscopy

ABSTRACT

With the aim to determine the binding affinity of a new generation of recombinant antithrombin (AT) toward heparin, we developed a dynamic equilibrium-affinity capillary electrophoresis (DE-ACE) method. This method allows the determination of an AT-heparin binding constant (K_d) directly from the cell culture supernatant used to produce the AT variants. Eight measurements per AT variant are sufficient to determine an accurate K_d (uncertainty ≤22%, regression coefficient ≥0.97), which is not significantly different from the value obtained from a higher number of measurements. Due to the relatively short time required to determine the K_d of one AT variant (2 h), this method has the potential for being a low throughput screening method. The method was validated by analyzing five AT variants, whose K_d have been reported in the literature using fluorescence spectroscopy. Finally, the method was applied to estimate the K_d of one new AT variant and one AT conformer, a latent form, that exhibits a significant loss of affinity.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Antithrombin (AT) is a human plasma glycoprotein which belongs to the serine protease inhibitor (serpin) family. AT is the major physiological inhibitor of coagulation proteases, primarily thrombin and factor Xa. Efficient regulation of clotting proteases by antithrombin requires the polysaccharide cofactor heparin, which acts to increase the moderate rates of inhibition of AT by several thousand-fold [1–5]. Besides its anti-coagulant effect, AT also possesses anti-inflammatory properties, this anti-inflammatory effect being observed when a high concentration of AT is present in the blood. In order to produce AT variants with an improved therapeutic potential, a French pharmaceutical company currently produces a new generation of recombinant AT by directed mutagenesis. Owing to the great number of the produced AT variants (between 200 and 300), it has been necessary to develop a screening method to assess the affinity of these newly produced variants toward heparin. The analytical method had to be fast, to allow an accurate determination of the binding constant, and, above all, to afford the analysis of the variants directly from the cell culture supernatant used to produce them.

Currently, very few analytical methods are capable of measuring the binding constant between AT and heparin. Fluorescence spectroscopy has been widely used in the past for this purpose [6–11], since it is highly specific of AT-heparin interaction, and offers the advantage to be realized in free solution. However, this method requires considerable amounts (100 μg) of sample and the analysis of non purified antithrombin is not possible. Isothermal

Abbreviations: ACE, affinity capillary electrophoresis; AT, antithrombin; BGE, background electrolyte; CE, capillary electrophoresis; DE-ACE, dynamic equilibrium-ACE; EOF, electroosmotic flow; FA, frontal analysis; HEK, human embryonic kidney; K, binding constant; K_d, dissociation constant; μ_{ep}, electrophoretic mobility; NECEEM, non-equilibrium capillary electrophoresis of equilibrium mixtures; PAGE, polyacrylamide gel electrophoresis; PVA, polyvinyl alcohol; RSD, relative standard deviation; Serpin, serine protease inhibitor; SPR, surface plasmon resonance.

* Corresponding author at: Université Paris Sud, Faculté de Pharmacie, 92290 Châtenay-Malabry, France. Tel.: +33 1 46 83 54 62.
E-mail address: myriam.taverna@u-psud.fr (M. Taverna).

http://dx.doi.org/10.1016/j.jpba.2015.02.042
0731-7085/© 2015 Elsevier B.V. All rights reserved.

titration calorimetry (ITC) is an alternative method to fluorescence spectroscopy [12–14], but is less specific of AT-heparin interaction. Surface plasmon resonance (SPR) has also been used to study the interaction between AT and heparin [15–17]. Using this technique, the binding kinetics can be followed in real time (determination of k_{on} and k_{off}), with a low consumption of material. However, SPR requires the immobilization of heparin on a sensor chip surface, which hampers the conformational flexibility of the polysaccharide necessary to the formation of a stable AT-heparin complex. Affinity chromatography is also a good approach to study AT-heparin interaction [18–20], but, up to now, has not been exploited to determine binding constants between these two partners.

In contrast, capillary electrophoresis (CE) has proved to be very well suited to study protein–ligand binding interactions. In the past two decades, several modes with different operational protocols have been developed to study molecular interactions [21–23]. When binding kinetics are fast the ligand is rather introduced in the running buffer and the target injected. For slower kinetics the target and its ligand are preincubated before analysis. Frontal analysis (FA) based methods involve a large plug of sample or continuous sample introduction, and the measurement of a plateau height. FA implies therefore high consumption of sample. Alternatively, zonal elution methods, among which affinity capillary electrophoresis (ACE), can be used. ACE, which relies on the electrophoretic mobility measurement of either the complex or the target alone, is the most simple and frequently used mode. In ACE, the interactions can take place either at the surface of a capillary previously coated with the ligand or, more frequently, when both the target and the ligand are free in solution. Besides these modes, "non-equilibrium CE of equilibrium mixtures" (NECEEM) was developed by Krylov's group for intermediate equilibrium kinetics [24,25]. Although one single NECEEM electropherogram provides sufficient data for determining both K_d and k_{off}, this mode is much more complicated and, up to now, only a few publications have exploited it.

For the purpose of this study, we chose the ACE mode in dynamic equilibrium (DE-ACE) based on mobility shift measurements since it is relatively simple to implement, and does not require a high quantity of sample. Furthermore, in this mode, the binding assay is realized in free solution, with the preservation of the flexibility of the interacting molecules. DE-ACE has been exploited several times for the assessment of AT-heparin binding. Gunnarsson et al. were the first to report the binding study of AT to low-affinity heparins by DE-ACE. The analysis was performed at physiological pH, and used a polyacrylamide coated capillary to minimize the adsorption of AT to the capillary inner wall [26]. In 2006, Le Saux et al. described a DE-ACE method that allowed to assess the binding of AT toward fractionated heparins. This method, which used a non-coated capillary and a phosphate buffer, was suitable to determine weak to medium interactions between AT and short oligomeric heparin fragments [27]. Recently, Gotti et al. reported a DE-ACE method allowing a medium/highly throughput screening of the affinity of heparin batches from different sources toward AT [28]. The method used a polyvinyl alcohol (PVA) coated capillary, and a phosphate buffer as the separation medium. Even more recently, Dinges et al. reported the use of DE-ACE for the determination of the binding affinities between AT and low molecular weight heparins [29]. The analytical conditions were very similar to those employed by Le Saux et al. However, in all these works, the analyzed antithrombins were purified AT, obtained from human plasma. In order to analyze recombinant antithrombins, present in a complex matrix containing many other compounds (salts, amino acids, proteins, etc.), it was necessary to adapt these previously reported works.

The choice of a coated capillary, to minimize the adsorption of recombinant AT and also the cell culture components onto the capillary inner wall, was not retained since this kind of coating highly increases the analysis time due to the very low EOF. We therefore preferred using a non-coated capillary and optimizing the analytical conditions so as to minimize, as much as possible, the adsorption phenomena. For this purpose, capillary preconditioning, background electrolyte ionic strength and inter-run rinses were carefully studied. The development of a fast method, compatible with a screening, also required to adapt other parameters, like heparin concentrations. The accuracy of the method was proved by analyzing AT variants, whose binding constants have already been reported in the literature. The suitability of the method to detect a loss of affinity was also demonstrated. Finally, the developed DE-ACE method was applied to the analysis of one AT variant, whose K_d has not been reported yet, and results were compared with those obtained by fluorescence spectroscopy.

2. Materials and methods

2.1. Chemicals and sample preparations

Sodium hydroxide was purchased from VWR (Fontenay-sous-Bois, France). Ammonium hydroxide and thiourea were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). Sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$) was from Carlo Erba Reagents (Cornaredo, Italy).

Unfractionated Choay® heparin (5000 I.U./mL) was used for all the studies. The human antithrombin Aclotine® and the AT variants (ATWT, ATN135Q, ATN155Q, ATN96Q, ATN192Q) were provided by LFB Biotechnologies (Les Ulis, France). These variants were produced by using the FreeStyle™ 293 Expression System (Invitrogen), a suspension cell culture system generating large amounts of recombinant protein after a transient transfection in a protein-free cell culture medium. The variant ATN135Q-Pro394 was provided by the laboratory of hematology EA4531 (Châtenay-Malabry, France). This variant was produced in adherent HEK cells after stable transfection in DMEM-F12 (Invitrogen) supplemented with insulin, transferrin and selenium (Invitrogen), as previously described [30].

Latent and heterodimeric forms of AT were prepared as previously described by Karlsson et al. [31]. Briefly, Aclotine® was dialyzed against 5 mM HEPES, 0.9 M ammonium sulfate, pH 7.4 using 30000 MWCO Vivaspin 15R (Sartorius Stedim Biotech, Germany), and diluted to a concentration of 2.5 mg/mL in this buffer, followed by heating at 60 °C during 9 h and 72 h to prepare heterodimeric and latent forms, respectively. The reaction mixtures were then concentrated and dialyzed against serum-free DMEM buffer (Gibco) using 30000 MWCO Vivaspin 15R.

Human and recombinant ATs, and preparations enriched in latent and heterodimeric forms were all stored at −20 °C before use.

All buffers and samples used for CE analyses were prepared with Milli-Q water using a Direct-Q 3 UV purification system (Millipore, Bedford, MA, USA). Before use, all buffers were filtered through a 0.2 μm Millex membrane (Millipore).

2.2. PAGE analyses of latent antithrombin

The preparation of latent antithrombin was analyzed by polyacrylamide gel electrophoresis (PAGE) using a 10% polyacrylamide gel containing either 6 M urea (urea PAGE) or no urea (native PAGE), as previously described [32,33]. Urea PAGE analysis showed a clear separation of latent and native AT based on their own electrophoretic mobility, the latent conformer having a higher electrophoretic mobility under these experimental conditions. Native PAGE was used to evaluate the amount of heterodimeric forms in the preparation of latent AT. These heterodimeric forms exhibit a

Download English Version:

https://daneshyari.com/en/article/1220744

Download Persian Version:

https://daneshyari.com/article/1220744

Daneshyari.com