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Short communication

# Affinity capillary electrophoresis in binding study of antithrombin to heparin from different sources

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ABSTRACT

Heparin, a highly sulfated polydispersed glycosaminoglycan (GAG), is the most widespread clinical anticoagulant; it binds antithrombin III (AT), a member of serine proteinases superfamily, accelerating its antagonist effect on blood coagulation. The binding interaction with AT is an important aspect in characterization of physicochemical properties of GAGs. With the aim at profiling several clinical and experimental heparin batches from different sources (porcine, bovine and ovine mucosa), a quantitative investigation of the binding heparin–AT, was undertaken by means of Affinity Capillary Electrophoresis (ACE). In dynamic-equilibrium ACE, the electrophoretic mobility of the receptor (AT), analyzed in a BGE containing the ligand (the considered GAG), is correlated to ligand concentration and binding constant. In particular, a 20 mM sodium phosphate, pH 7.4 buffer (the BGE) was chosen as the neat medium and the experiments were carried out in a highly hydrophilic poly(vinyl alcohol) coated capillary (effective length 8.5 cm). The applied sample, consisting in the receptor AT (0.30 μM) and phenylacetic acid (PAA; 10.0 μM) used as a reference compound, was electrophoresed at each of the studied concentration levels of the ligand (heparin samples, 0.30–10.0 $\times 10^{-7}$ M; heparan sulfate, 0.35–8.0 $\times 10^{-5}$ M) supplemented to the BGE. The migration time ratio of PAA to AT was assumed as the chemical response to be correlated to the ligand concentration and the binding constant estimation was based on the application of a nonlinear regression method (rectangular hyperbola). Under these conditions, a number of heparin samples were analyzed and their binding constants (Kd) were found within 14.2 and 56.1 nM (SD $\leq \pm 2.0$; $n=3$; coefficient of determination $r^2 \geq 0.96$). The good correlation of Kd values to the *in-vitro* activity (anti-factor Xa and anti-factor IIa), confirmed that the affinity for the target AT is an important feature of heparin samples and could be included among their physico-chemical characteristics.



## 1. Introduction

Heparin, a highly sulfated polydispersed glycosaminoglycan (GAG), is the most widespread clinical anticoagulant; it binds antithrombin III (AT), a member of serine proteinases superfamily, accelerating the antagonist effect on blood coagulation. In particular, heparin specifically recognizes AT in an electropositive region, by means of a unique pentasaccharide sequence that is present in about one-third of the heparin chains. The binding to this region of AT, defined as the pentasaccharide binding site, was found to be the primary responsible for the activation of the serine proteinase by inducing a conformational change leading to the formation of a high affinity complex [1,2]. Commercial heparin is mainly produced from pig mucosa; in some countries heparin from sheep and beef mucosa is available too.

Affinity capillary electrophoresis (ACE) offers the opportunity for quantitative estimation of the thermodynamics of molecular interactions under conditions that mimic the *in vivo* environment. This aspect makes ACE particularly attractive in the binding constant determination of receptor-to-ligand interaction where the nature and pH of the medium have to be consistent with biological conditions [3,4]. In fast binding interaction kinetics, ACE measurements are performed by analyzing the receptor (*i.e.*, AT) in the electrophoretic background electrolyte (BGE), supplemented with the ligand (*i.e.*, GAG), at various concentration levels. Under these conditions the change of the mobility of the receptor occurring as a consequence of the complexation, is correlated with the binding constant. The first report about the use of ACE to study the binding of AT to low-affinity heparins was published by Gunnarsson et al.; the analysis was performed at physiological pH and using a polyacrilamide-coated capillary to

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prevent the absorption of AT to the inner capillary wall [5]. The dissociation constant was calculated from the mobility of AT relative to that of a reference compound *versus* the concentration of heparin, by using the algorithm to solve the rectangular hyperbola, the model valid for 1:1 complexation. More recently, Varenne et al. [6] described the use of ACE for the determination of binding affinity of fucoidan, a bioactive polysaccharide, to AT. The same approach was also used in the determination of binding to AT of unfractionated heparin that exhibited dissociation constant in the order of $10^{-6}$–$10^{-7}$ M. The results obtained by the conventional ACE approach were then confirmed by the application of an alternative method, namely frontal analysis continuous CE [7,8]. The versatility of ACE in studying interactions involving antithrombin, was also exploited in the estimation of binding of small highly sulfated non-sugar molecules [9].

In the present study, 16 heparin samples from various origins (porcine, bovine and ovine mucosa) were characterized by evaluating biological and chemical attributes, namely: (i) anti-factor Xa (AXa) and anti-factor AIIa (AIIa) potencies, (ii) content of a tetrasaccharide (ΔIIa-$\underline{\text{IIs}_{\text{glu}}}$, *i.e.*, Δ-UA-GlcNAc,6S-GlcA-GlcNS,3S,6S) related to the specific pentasaccharide binding site, and (iii) molecular weight. In addition, AT binding affinity of the samples was estimated by an ACE method involving the use of a short (8.5 cm effective length) poly(vinyl alcohol)-coated capillary, which minimized the interactions of the protein with the capillary wall and provided short analysis time. An investigation on the correlation between binding constant, heparin structure (saccharide composition) and *in-vitro* biological activity was eventually carried out.

## 2. Experimental

### 2.1. Materials

Heparin samples from pig, sheep and beef mucosa and heparan sulfate from horse spleen, were from Opocrin S.p.A. (Corlo di Formigine, Modena, Italy); the synthetic analog of the pentasaccharide domain in heparin chains, (sodium fondaparinux; commercial name Arixtra) was from GlaxoSmithKline. In Table 1, the investigated heparin samples are reported together with their main attributes: anti-factor IIa and anti-factor Xa potencies, molecular weight as $M_w$ and $M_n$ and the % content of tetrasaccharide ΔIIa-$\underline{\text{IIs}_{\text{glu}}}$ (Δ-UA-GlcNAc,6S-GlcA-GlcNS,3S,6S) related to the specific pentasaccharide binding site.

Lyophilized human Antithrombin (AT) 25–30 IU, containing sodium chloride 11 mg, having molecular weight 57,875, was from Chromogenix Instrumentation Laboratory (Milan, Italy); phenylacetic acid (PAA) was from Sigma–Aldrich (Milan, Italy). Sodium chloride, sodium phosphate, sodium hydroxide, Tris(hydroxymethyl)aminomethan (Tris) and all the other chemicals were form Carlo Erba Reagenti (Milan, Italy). Water used for the preparation of sample solutions and buffers, was purified by a Milli-RX apparatus (Millipore, Milford, MA, USA).

### 2.2. Instrumentation

#### 2.2.1. Capillary electrophoresis

ACE experiments were carried out using an Agilent $^{3D}$CE instrument (Agilent Technologies, Waldbronn, Germany), equipped with a diode array detector set at 200 nm and temperature control system set at 25 °C. Data were collected on a personal computer using the software integration system Chemstation Agilent Rev. A. 09. 01.

Capillaries permanently coated (PVA-coated) with highly hydrophilic poly(vinyl alcohol) were provided by Agilent Technologies; the internal diameter of the capillary was 50 μm and the total length was 64.5 cm. In the described ACE application the electrophoretic runs were performed by using the short-end of the capillary (effective length 8.5 cm). At the beginning of each day the capillary was rinsed for 10 min with water and in-between runs it was flushed sequentially with water (5 min) and running buffer (4 min). Hydrodynamic injections of the samples were performed at 50 mbar for 2 s and electrophoretic runs were carried out by application of 20 kV (detection at the anodic end of the capillary).

#### 2.2.2. Liquid chromatography–mass spectrometry

Characterization of AT was performed by LC (Agilent 1100) coupled with a nano-ESI-QTOF (Micromass, Waters, Manchester, UK). In particular, AT standard solution (0.5 mg/mL in water) was injected (1 μL) onto a C8 chromatographic column (Agilent Zorbax 300SB, 50 mm × 75 μm id, 3.5 μm particle size) and eluted using a linear gradient from A (water/acetonitrile/formic acid

**Table 1**
Characteristics of the studied heparin samples.

| Sample | Source | Batch | $M_w$[a] | $M_n$[b] | AIIaU/mg[c] | AXaU/mg[d] | %ΔIIa-$\underline{\text{IIs}_{\text{glu}}}$[e] | Kd (nM)[f] |
|---|---|---|---|---|---|---|---|---|
| 1 | Porcine | Commercial | 19,900 | 16,050 | 166 | 172 | 1.7 | 49.4 |
| 2 | Porcine | Commercial | 20,000 | 16,400 | 190 | 191 | 1.8 | 39.1 |
| 3 | Porcine | Commercial | 18,900 | 15,800 | 188 | 199 | 2.3 | 34.1 |
| 4 | Porcine | Commercial | 18,100 | 15,700 | 206 | 204 | 2.0 | 30.7 |
| 5 | Porcine | Commercial | 18,300 | 15,550 | 206 | 205 | 2.4 | 35.6 |
| 6 | Porcine | Commercial | 18,500 | 14,700 | 199 | 194 | 2.7 | 44.2 |
| 7 | Porcine | Commercial | 19,550 | 15,650 | 191 | 185 | 2.5 | 39.5 |
| 8 | Porcine | Experimental | 25,000 | 21,000 | 273 | 246 | 3.5 | 14.2 |
| 9 | Ovine | Experimental | 15,750 | 12,550 | 199 | 170 | 1.6 | 34.0 |
| 10 | Ovine | Experimental | 17,000 | 13,550 | 173 | 162 | 1.7 | 42.1 |
| 11 | Ovine | Experimental | 16,750 | 13,400 | 211 | 198 | 1.9 | 33.5 |
| 12 | Ovine | Experimental | 16,900 | 13,550 | 209 | 191 | 1.8 | 28.2 |
| 13 | Bovine | Experimental | 18,900 | 15,650 | 111 | 121 | 0.5 | 50.8 |
| 14 | Bovine | Experimental | 19,300 | 16,000 | 104 | 121 | 0.4 | 54.3 |
| 15 | Bovine | Experimental | 21,850 | 17,450 | 110 | 129 | 0.5 | 56.1 |
| 16 | Bovine | Experimental | 23,700 | 18,650 | 129 | 147 | 0.4 | 51.6 |

[a] $M_w$ is weight-average molecular weight.
[b] $M_n$ is number-average molecular weight.
[c] AIIa U/mg is the anti-factor IIa activity.
[d] AXa U/mg is the anti-factor Xa activity.
[e] % ΔIIa-$\underline{\text{IIs}_{\text{glu}}}$ is the molar percentage of tetrasaccharide Δ-UA-GlcNAc,6S-GlcA-GlcNS,3S,6S (RSD% < 4.3); $n=3$.
[f] Kd is the binding constant (SD ≤ ± 2.0; $n=3$).