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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; $n=3$).

99:1:0.1 (v/v)):B (acetonitrile/water/formic acid 98:2:0.1 (v/v)) 80:20 to A:B 20:80 in 20 min at the flow rate of 0.5 μL/min. The Q-TOF Micro was employed with a nano-Z-spray ion source. The source temperature was set at 60 °C, the desolvation temperature at 100 °C, the capillary voltage at 3.5 kV and the cone voltage at 42 V. Spectrum was elaborated by Mass Lynks (Waters) and the deconvoluted ESI mass spectrum of AT was obtained by using MaxEnt 1 software (Waters). The analysis revealed the presence of the main ATIII-α isoform and about 7% of ATIII-β isoform (55,655 Da) according to previous studies [10].

2.2.3. Spectrophotometric and spectrofluorimetric determinations

Actual concentration of AT was assessed using a UV–visible System HP 845× (Agilent Technologies) spectrophotometer by considering $A_{280}^{0.1\%}=0.65$ [11].

The study of interaction of AT with GAGs was also carried out by fluorescence spectroscopy [12] using a spectrofluorimeter FP-6200 Jasco (Jasco Europe, Cremella, LC, Italy). In our experiments, AT solution (2×10^{-7} M) in Tris buffer (0.2 M NaCl–0.05 M Tris–HCl, pH 7.5) was incubated with an active heparin sample dissolved in the same buffer at different concentration levels within 5×10^{-8}–1×10^{-5} M. Similarly AT solution was incubated with heparan sulfate solutions. The fluorescence spectra were recorded in the range 290–450 nm at the excitation wavelength of 280 nm; alterations in the fluorescence spectrum of AT (emission at 330 nm) induced by addition of GAGs were assumed as the response to be correlated to the strength of binding.

2.3. Solutions and ACE experiments

Neat ACE running buffer was an aqueous 20 mM sodium phosphate solution at pH 7.4; it was supplemented with the ligand at seven to ten concentration levels within the range 0.30–10.0×10^{-7} M (heparin samples) and 0.35–8.0×10^{-5} M (heparan sulfate). The applied sample consisted of a 20 mM sodium phosphate solution of the receptor AT (0.30 μM) and PAA (10.0 μM) used as the reference compound. The sample was electrophoresed in triplicate at each of the studied concentration levels and the migration time ratio of PAA to AT was assumed as the chemical response to be correlated to the ligand concentration. The binding constant estimation was based on the application of a nonlinear regression method (rectangular hyperbola) using the software package GraphPad Prism Version 4.03 (GraphPad Software Inc., CA, USA).

2.4. Methods for heparin characterization

The *in-vitro* anticoagulant activities of heparins were assayed according to the Heparin Sodium Monograph of USP 35. Briefly, the anticoagulant effect is due to the inhibition of activated blood coagulation factors, *i.e.*, factor Xa and factor IIa. Heparin binds to the plasma cofactor AT and the formed complex inhibits the activity of the coagulation factors Xa and IIa; the residual factors, not inhibited by complex formation, can be estimated by means of a spectrophotometric assay using a specific chromogenic substrate.

The molecular weight of the heparin samples was assessed by HP-SEC, using for separations two columns in series (TSK G3000SX.XL and G2000SW.XL) and a Broad Calibration method (“GPC” extension of Empower – Waters) for the calculation. The distribution table of the Broad Standard sample was assessed by a Triple Detector array.

The content of the tetrasaccharide ΔIIa-$\underline{\text{IIs}_{glu}}$, as a molar percentage, was obtained by SAX-HPLC, after complete enzymatic digestion of the sample using a mixture of Heparinases I, II and III [13].

3. Results and discussion

The binding interaction with AT is an important aspect of the anticoagulant mechanism of GAGs; AT binds heparin to a specific region which has been identified as a unique pentasaccharide that is considered to be responsible for nearly all of the energy of the interaction and it was found to contain a single binding site for AT [14,15]. The interaction of the pentasaccharide contained in heparin with AT produces a complex “heparin-AT” which inhibits factor Xa and factor IIa (thrombin).

Some commercial heparin batches from pig mucosa and some experimental batches of porcine, bovine and ovine mucosa were investigated for quantitative estimation of heparin-AT binding by means of ACE. The purpose of the study was to characterize heparin samples of different origin and to investigate the correlation between the binding constant and the heparin structure or its *in-vitro* biological activities.

3.1. Development and optimization of ACE conditions: Selection of capillary and BGE

The interaction of a full-length heparin sample with AT was reported to show a $k_{off}\sim0.2\ s^{-1}$[1], thus describing a sufficiently rapid kinetics for application of dynamic-equilibrium ACE. In this format of ACE, the mobility of the receptor AT, electrophoresed in a BGE containing the ligand (the investigated GAG), is correlated to the ligand concentration and binding constant. Actually, because of the short binding relaxation time, the free analyte AT is in equilibrium with the ligand, in the time scale of conventional electrophoretic runs. In this condition, the peak appearance time of AT is assumed as the response to be correlated to the 1:1 binding [3,4]. An important assumption for reliable ACE experiments is that interactions with capillary wall have no influence on the receptor–ligand equilibrium, thus, in order to limit the adsorption of AT, a PVA-coated capillary was used.

GAGs–AT equilibrium has been described as a polyelectrolyte–protein interaction that involves both ionic and nonionic forces [1]. The electrostatic contribution to the complexation is significant and the estimation of the binding constant should be carried out under biological conditions. However, the ionic strength of physiological solution ($I=0.15$ M) appears to be rather high to perform electrophoretic experiments, thus in order to limit joule heating and according to previous studies [5–7,16] a 20 mM sodium phosphate, pH 7.4 buffer ($I=0.05$ M) was chosen as the neat medium for ACE binding experiments. At the constant voltage of 20 kV and capillary cartridge temperature of 25 °C, the anodic migration of AT using the short – end of the separating capillary (effective length 8.5 cm), occurred in 8.75 min (inter-day RSD%=0.71; $n=9$) and the measured electric current was about 20 μA. The reproducibility of migration time is a prerequisite in ACE studies, however, for providing more reliable description of AT migration, the mobility ratio of AT *versus* a non-interacting specie was used [5,17–19]. In the present study, PAA was selected as a reference compound by assuming that it does not interact with both GAGs and AT. In the used PVA-coated capillary, the EOF is strongly suppressed thus the migration time ratio PAA/AT was calculated at each of the tested GAG concentration levels, as the chemical response to be correlated to the concentration of the ligand for binding constant estimation.

3.2. Application of ACE to the determination of AT binding to heparan sulfate (HS)

In ACE experiments, the concentrations of both ligand and analyte are strictly related to each other and have to be carefully selected. A major factor to be considered in the choice of ligand

concentration range is the magnitude of the binding constant to be determined; in particular the additive concentration should cover the central to upper portion of the binding isotherm (fraction of the complexed analyte, above 0.5) [3,4,20]. Preliminary experiments were performed using, as a ligand, a sample of heparan sulfate (HS). HS is a GAG with a structure very similar to that of heparin, but not containing, or containing very little amounts of the pentasaccharide sequence responsible of the specific interaction with AT [21]. In particular, the selected HS sample proved to not contain detectable amount of pentasaccharide sequence (data not shown). HS was thus added to the BGE within the concentration range $0.35–8.0 \times 10^{-5}$ M.

In the performed experiments, the concentration of the receptor AT should be sufficiently small (*i.e.*, 10–100-fold lower) compared to the ligand in order to carry out the electrophoretic runs under linear isotherm conditions [3,19]. An AT solution at 0.30 μM concentration was used as the sample. Although relatively high concentration levels of HS were added to the BGE, the background signal was limited also using UV detection at 200 nm, and an adequate response for the peak of the receptor was achieved.

The mobility of AT in neat buffer was found to be -5.16×10^{-5} $cm^2\,s^{-1}\,V^{-1}$, whereas that of HS was -3.11×10^{-4} $cm^2\,s^{-1}\,V^{-1}$, thus under the described conditions (suppressed EOF) the HS-AT complex has to show higher mobility (lower migration time) than free AT. In Fig. 1 are reported the typical electropherograms obtained for AT analysis at different concentrations of HS supplemented to the BGE. The AT peak appearance time decreases at increasing concentration of the ligand, indicating the interaction between HS–AT. The migration time of the reference compound PAA was found to increase from 2.18 to 3.14 min within the investigated HS concentration range, as a consequence of the buffer viscosity change. This observation supports the use of relative mobility (migration time ratio PAA/AT) instead of the net mobility measured for the solute [22]. The fast analysis time allowed for the treatment of several data points for each experimental set and the determined relative mobility values (mr) could be correlated to the corresponding concentrations of the ligand by the general algorithm for monovalent binding (rectangular hyperbola) [5]. Linearization approaches are often applied, however we preferred to use nonlinear regression method for direct estimation of binding constant (Kd) as recommended in particular when data are collected outside ideal conditions of the additive concentration range [23]. The binding constant obtained for the considered HS sample was found to be 4.07 ± 0.68 μM with good determination coefficient ($r^2 \geq 0.85$).

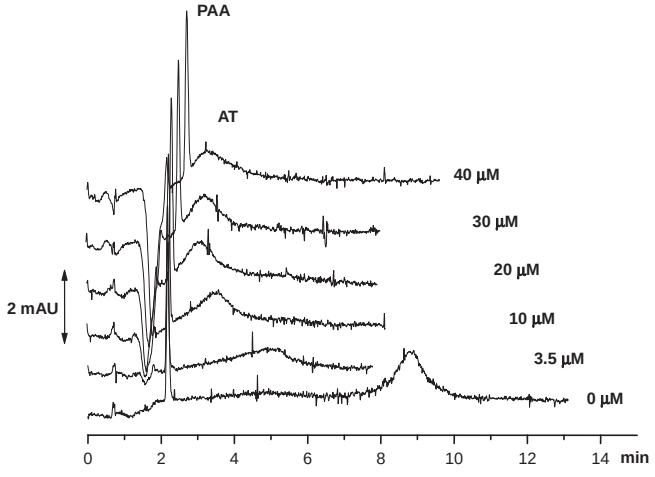

Fig. 1. Electropherograms showing interaction of antithrombin (AT, 0.30 μM) with heparan sulfate (HS) in the presence of phenylacetic acid (PAA, 10 μM) as the marker. BGE: 20 mM sodium phosphate solution at pH 7.4 containing different concentrations of HS as reported in the figure. Conditions: PVA-coated capillary (50 μm i.d.; 8.5 cm effective length; 64.5 cm total length); voltage 20 kV; injection at 50 mbar × 2 s; detection UV at 200 nm.

3.3. Application of ACE to the determination of AT binding to heparin samples from different sources and origin

This study was mainly aimed at finding a suitable tool for bridging biological activities with chemical characteristics of different heparin samples, thus the general conditions optimized for the determination of the binding constant of HS were applied to the analysis of heparin samples. Heparin is expected to bind AT with lower dissociation constants compared to that obtained for HS, being the latter devoid of the specific sulfated pentasaccharide sequence that has been reported as the major responsible for the interaction of GAGs to the characteristic binding site of the protein. This consideration led us to carry out the binding study of heparin–AT interaction in a concentration range of heparin that is one to two orders lower than that was previously chosen for HS. Under these conditions, the evaluation of the effect of the concentration of AT on the mobility ratio in the whole heparin concentration range was considered. The mobility ratio variations found when two AT samples at concentrations of 0.30 and 1.0 μM were injected, were within 5–10%, suggesting that in order to obtain reliable data, the accurate preparation of the AT sample solution is necessary. On the other hand, a reconstituted aqueous solution of AT (1 IU/mL corresponding to about 0.16 mg/mL) is reported to be stable for 3 months at −20 °C (Chromogenix, Milan, Italy), thus to carry out binding experiments, AT working samples were prepared each day at exactly 0.30 μM by proper dilution of a single stock solution of AT stored at −20 °C.

The mobility of heparin in neat BGE was found to be -3.61×10^{-4} $cm^2\,s^{-1}\,V^{-1}$, thus very similar to that of HS; accordingly, the complexation heparin–AT should lead to increased mobility at increased ligand concentrations. The electropherograms obtained for AT analysis at different concentrations of a heparin sample from pig mucosa are shown in Fig. 2. As expected the migration time of AT decreased at increased heparin concentration. Differently to that observed in experiments involving HS, the migration time of the reference compound PAA was found not affected by addition of increasing concentrations of the ligand because the viscosity change in case of BGE containing heparin was very limited. However, the use of mobility ratio instead of AT net mobility was still used for determination of binding constants. Under the described conditions, 16 heparin samples and the synthetic analog of the pentasaccharide (fondaparinux) were analyzed and the binding constants

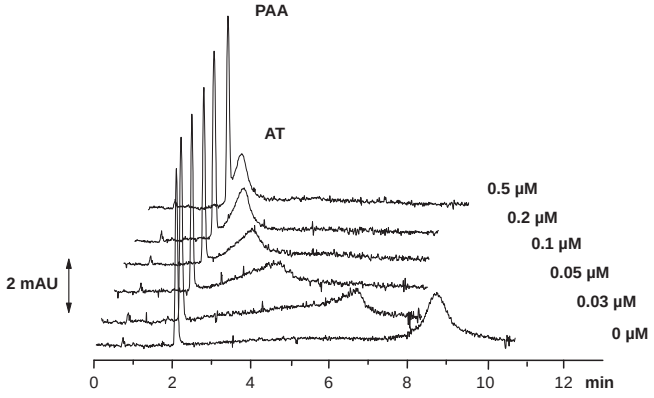

Fig. 2. Electropherograms showing interaction of antithrombin (AT, 0.30 μM) with heparin (porcine, sample 1) at the concentrations reported in the figure. For conditions and symbols see Fig. 1.

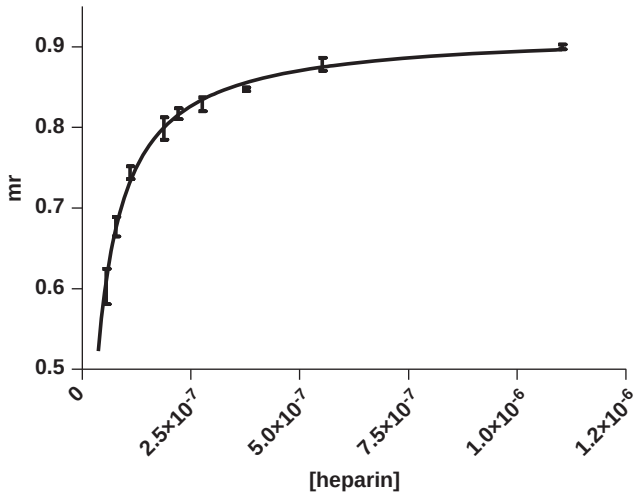

Fig. 3. Graph showing the mobility ratio (mr) variation as a function of heparin concentration (ovine, number 12). The data (average of three independent experiments), fitted to a 1:1 binding site hyperbola, provided a determination coefficient $r^2=0.973$.

were estimated; the results are reported in the Table 1. In Fig. 3 is reported a representative graph showing the mobility ratio variation as a function of heparin concentration (ovine sample number 12). Determination coefficients higher than $r^2 \geq 0.96$ were obtained and the Kd values could be determined with adequate precision ($SD \leq \pm 2.0$; $n=3$).

3.4. Fluorescence spectroscopy

Fluorescence spectroscopy is conventionally used for determination of binding AT–heparin, based on the enhancement of fluorescence of tryptophan residue of AT induced by heparin interaction [12]. In our experiments, AT solution (2×10^{-7} M) in Tris–HCl buffer, was incubated with a porcine heparin sample (sample 3) in the concentration range 5×10^{-8}–1×10^{-5} M. The maximal enhancement in fluorescence (excitation at 280 nm) corresponded to an approximately 40% increase in the intrinsic fluorescence of AT. This effect was obtained using heparin at the concentration level in the order of 10^{-6} M. A similar behavior was observed for HS only when it was used at concentrations 100-fold higher with respect to that of active heparin (data not shown). This result suggests that: (i) the behavior of HS and heparin in promoting spectral transition are similar, and (ii) Kd of HS is expected to be two-order higher than that of active heparins, according to the results obtained by ACE. A complete study on the Kd estimation of HS by fluorescence could not be carried out because solutions at concentration above 10^{-4} M (HS) became slightly turbid and caused disturbances in fluorescence measurements. This consideration highlights the advantages of ACE that in addition requires for a lower amount of reagents than fluorescence spectroscopy. On the other hand, the latter approach allows for Kd estimation to be carried out in physiological conditions ($I=0.15$ M) that might not be easily fulfilled in CE because of joule heating. To this regard it has to be underlined that binding of GAGs to AT is dependent on ionic strength; a comparison of Kd data by the present ACE method with those by fluorescence spectroscopy obtained under similar ionic strength conditions, suggests a substantial agreement [15]. In particular, although ACE approach revealed an overestimation of the binding, the obtained ACE Kd data were found to be of the same magnitude of those obtained by the conventional method.

3.5. Correlation of binding data with in-vitro anti-factor Xa, anti-factor IIa and content of tetrasaccharide

The heparin samples considered in the present study were from both commercial and experimental batches of different origin (porcine, ovine and bovine mucosa). Among them it was included an experimental batch of heparin (sample 8) obtained from porcine mucosa, fractionated to increase its anticoagulant potency. Thus, the variety of considered samples allowed the study to be addressed in probing heparins whose *in-vitro* activity ranged in a large span of potency (104–273 IU/mg and 121–246 IU/mg for AIIa and AXa, respectively). Interestingly, the estimated binding constants showed a good correlation with the *in-vitro* activity; in particular as it can be seen in Fig. 4a, a determination coefficient $r^2=0.889$ was found between anti-factor IIa activity and Kd and a similar relationship was observed for anti-factor Xa activity, even though with a lower determination coefficient ($r^2=0.803$). Pentasaccharide (fondaparinux), whose affinity to AT estimated by the present method was found to be 4.40 nM, and HS were not considered for correlation because their potency cannot be assessed by the heparin USP assays.

The good correlations found can be considered as a confirmation of the major role of the affinity between heparin and AT in determining the *in-vitro* activity, with no regards to the sample origin. Eventually the lack of a clear relationship between the contents of the tetrasaccharide ΔIIa-$\underline{\text{IIs}_{glu}}$ and the AT-binding affinity (Fig. 4b), is in agreement with previous studies, thus

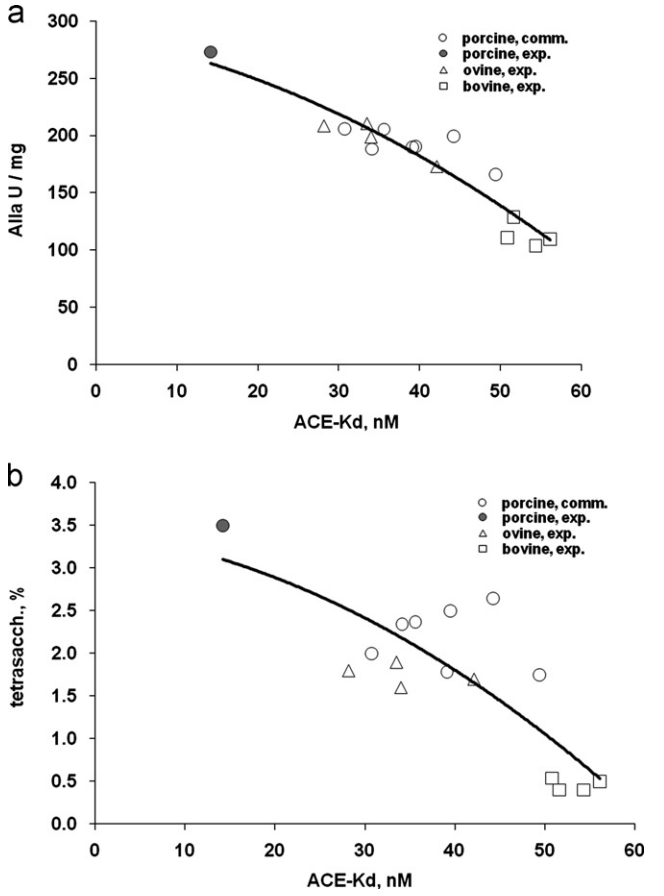

Fig. 4. (a) Correlation ($r^2=0.889$) of anticoagulant activity (AIIa) of heparin samples with dissociation constant (Kd). (b) Correlation ($r^2=0.657$) of the molar percentage of tetrasaccharide ΔIIa-$\underline{\text{IIs}_{glu}}$ of heparin samples with dissociation constant (Kd).

confirming that other structures, adjacent to the tetrasaccharide, could be involved at different degrees in interaction with AT [24].

4. Conclusion

The present study, carried out on a number of heparin samples from different sources, revealed that binding constant (Kd) of the interaction of antithrombin with heparin could be related at good extent to the anticoagulant *in-vitro* activity. The importance of including Kd among the physicochemical attributes of heparin accounts for the necessity of reliable methods to probe heparin affinity to AT. To this regard ACE, after a careful optimization of the relevant experimental parameters, showed to be a valuable and convenient approach allowing fast analyses and small sample consumption thus suitable for high/medium throughput screening.

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