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Il Farmaco 56 (2001) 527–532

IL FARMACO

Mucopolysaccharides in aqueous solutions: effect of ionic strength on titration curves☆

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Abstract

We study the changes taking place in hyaluronic acid, chondroitin 4-sulfate (C4-S) and condroitin 6-sulfate (C6-S), at ionic strengths of 0.10, 0.15, and 0.20 in NaCl, in a neutralization process in aqueous solution. We apply the equation of Henderson–Hasselbalch modified for polyelectrolytes and evaluate the changes in the electrostatic free energy starting from the pK curves as a function of the dissociation degree. For a dissociation degree next to 0.4 corresponding to the –COOH group of the hyaluronic acid, we observed a change in the conformation of the three glycosaminoglycans studied. This conformational change takes place as a consequence of the break of intramolecular links and the beginning of the ionization process. The macromolecules in solution show a structure of random coil sufficiently expanded so that the interaction among the close ionizable groups is negligible. © 2001 Éditions scientifiques et médicales Elsevier SAS

Keywords: Hyaluronic acid; Chondroitin 4-sulfate; Chondroitin 6-sulfate; Henderson–Hasselbalch equation

1. Introduction

The structural properties of glycosaminoglycans in aqueous solutions can be characterized as a random-coil polyelectrolyte with some stiffness. The rigidity may be conferred by limited conformational freedom about the glycosidic linkages as well as intramolecular or intermolecular association of chain segments. These structures involve hydrogen bonds between adjacent sugar units, which depend on the spatial distribution of the residue of disaccharides [1]. Heatley and Scott [2] found the existence of two distinct stable configurations for hyaluronate depending on the environment: one for the water/dimethyl sulphoxide mixtures and another in aqueous solutions compatible with the existence of a water bridge between the uronate carboxylate and acetamido NH groups.

The behavior of all glycosaminoglycan chains can be attributed to its versatile conformation due to the negative charge. These molecules in solutions are typically polyanions and their hydrodynamic properties are influenced by the pH, the degree of hydration, the ionic strength, and the nature of counterions. The interactions depend not only on the chemical composition but also on the kind and spatial distribution of the ionic groups [3].

The conformational changes and the difference of structure in the polymer chain between hyaluronic acid (HA), chondroitin 4-sulfate (C4-S), and chondroitin 6-sulfate (C6-S) should be reflected in the values of the intrinsic viscosity of their solutions. This parameter depends not only on the dimensions of the polymer chain but also on the polymer–solvent interactions [4–7]. The different behavior of these macromolecules in solution influences their pharmaceutical and clinical applications. For example, the chondroitin sulfate is a Newtonian fluid that has a constant viscosity even with low shear rates. Because of this property, this compound should provide superior corneal lubrication compared to similar solutions of HA, which are pseudoplastic fluids, and transmit greater shear forces to the corneal epithelium with eyelid movement [8].

Park and Chakrabarti [9] observed a transition in the near-ultraviolet circular dichroism (CD) spectrum of HA upon lowering the pH and raising the fraction of

☆ XXIV International Congress of the Latin-Mediterranean Pharmaceutical Society, Assisi, Italy, 20–23 September 2000.

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0014-827X/01/$ - see front matter © 2001 Éditions scientifiques et médicales Elsevier SAS
PII: S0014-827X(01)01106-5

organic component in a mixed solvent. However, the transition was not observed in chondroitin sulfate solutions. Staskus and Johnson [10,11], by measuring the CD spectroscopy into the vacuum ultraviolet regions in the aqueous solutions of the polymer, have observed subtle changes as a function of pH in absorption and flow LD spectra for the $\pi-\pi^*$ transition region of HA. The ^{13}C NMR study [12] suggests that the addition of alkali to hyaluronate solutions produced changes in the chemical shifts and linewidth in the ^{13}C spectrum. In contrast, the chondroitin sulfate spectra are unaffected by alkali.

The quasi-elastic light scattering techniques [13] have been used for studying solutions of HA at several pH. Conformational changes were demonstrated between pH 2.5 and alkaline pH values. At pH 2.5 the glucuronic acid moiety is not dissociated and the structure contains some amount of double helical cross-links stabilized by hydrogen bonds. At alkaline pH the polymer is heavily charged (pK_a 3.21) and a configuration random coil extended is present. The chondroitin sulfate solutions show similar conformation changes as a function of the pH [14].

The potentiometric titration study of weak polyacids provides information about the conformational changes as a function of the ionization degree and is the usual approach for the interpretation of the thermodynamic behavior of polyelectrolyte solutions. Another advantage is that the potentiometric method is simple and non-destructive. However, little information about this technique applied to mucopolysaccharide solutions is found in the literature, in spite of an extensive study based on several conformational chain models development by Cleland [15–17]. In this work, we outline a potentiometric study to compare the aqueous solutions of HA, C4-S, and C6-S in a pH interval from 2.5 to 11, where the carboxyl groups are not dissociated and fully ionized, respectively. The conformation and the dimension of the linear chain of the polyelectrolytes in solution are studied at different ionic strengths and concentrations. We have used a simple graphic method for the calculation of the ionization degree starting from the equivalent points of the neutralization curve. The electrostatic interaction parameters are calculated for the conformational stages in the neutralization process, independent of the theoretical molecular model. The experimental conditions that produced the conformational changes are determined.

2. Materials

HA sodium salt grade III, from human umbilical cord (lot 25 F-0382), (C4-S) sodium salt, from whale cartilage (lot 104 F-0549), and (C6-S) sodium salt, from shark cartilage (lot 102 F-0418), were obtained from Sigma. Other reagents used were from Merck.

2.1. Sample preparations

We have prepared two kinds of mucopolysaccharide solutions, A and B. Solution A was acidified with 0.1 N HCl until pH 2.5 (–COOH group not dissociated). Solution B was alkalinized with 0.1 N NaOH until pH 11 (titrant solution). In both cases, the mucopolysaccharide concentration was 2×10^{-4}, 2×10^{-3}, and 2×10^{-2} (% w/v). The ionic strength (0.10, 0.15, and 0.20) was adjusted with NaCl in all samples.

3. Methods

3.1. Potentiometric titrations

The pH measurements were performed with a battery operating potentiometric pHmeter (Radiometer TTT8). A Radiometer (G202C) glass electrode and a (K 401) calomel electrode were used. Titrations were performed in a nitrogen atmosphere to maintain a CO_2-free atmosphere and thermostatically controlled at 25 and 37°C. Standardization was carried out stirring two kinds of buffers, pH 4 and 7 on the National Bureau of Standards scale. The initial charge was usually 5 ml of the sample for titration.

According to the polyelectrolyte theory, the titration curve of a weak polyacid in a salt solution, where no specific binding interferes with the dissociation of the acidic group and interactions along the chain are negligible, can be represented by the following equation:

$$\mathrm{pH} = \mathrm{p}K_0 + \log\frac{\alpha}{1-\alpha} + \frac{0.4343}{kT}\left(\frac{\partial \Delta G_e}{\partial Z}\right) \quad (1)$$

where K_0 is an intrinsic dissociation constant of the acidic group on the macromolecular chain for $\alpha = 0$, α is the dissociation degree of the group proportional to the number of protons dissociated (Z), $Z = -\alpha n$. For high values of α, $Z < |\alpha, n|$ because counterion effects cannot be neglected. ΔG_e represents the electrostatic free energy of the system, which can be related to the mean electrostatic potential around a polyion, by an extended Debye Hückel treatment for polyelectrolytes. In general, the polyelectrolyte correction factor ($\partial \Delta G_e / \partial Z$) will depend strongly on the charge of the polyion and the ionic strength (I) of the solution. If the polyelectrolyte undergoes, in the course of the titration, a conformational transition from a characteristic mean conformation to coil-like state, then we can apply Eq. (1) to each part of the titration curve. These states can be characterized by different K_0 and ΔG_e values.

The change of free energy of ionization depends on two parameters: the intrinsic standard free energy change, ΔG_0 (corresponding to the intrinsic association constant K_0), and the electrostatic free energy, ΔG_e. In our case, the variation of pH with α can be expressed

by a linear relationship, independent of the ionized polyelectrolyte form, through the so-called modified Hendersson–Hasselbalch (HH) equation:

$$\mathrm{pH} = \mathrm{p}K' + \eta \log\frac{\alpha}{1-\alpha} \quad (2)$$

where η takes into account the electrostatic interaction among charged groups and its value quantitatively indicates the deviation from the ideal behavior, that is, when the charged groups are independent of each other. The η value is sensitive to the molecular weight and the polydispersion of the samples.

The pK_a determination has been carried out with two very simple methods. Starting from the derived curves, the equivalence point is determined. This point assumed the complete ionization to $\alpha = 1$. At pH 2.5 corresponding to the not dissociated –COOH group, $\alpha = 0$, and in the neutralization curves an ionization degree corresponds to each pH. The values obtained are similar to those calculated starting from the expressions for the degree of neutralization α_n:

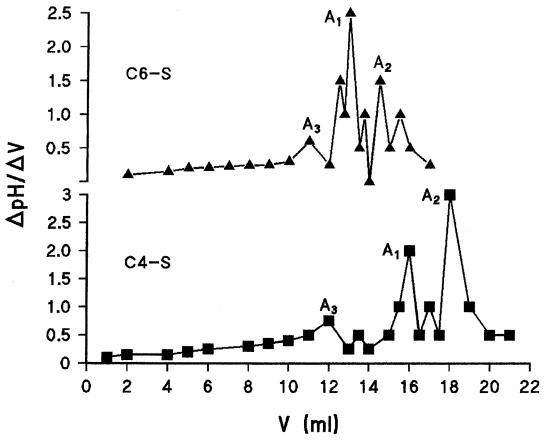

Fig. 1. Titration curves for C4-S and C6-S at concentrations 2×10^{-4} (% w/v) and ionic strength 0.15.

Table 1
Equivalence point value corresponding to the $\Delta\mathrm{pH}/\Delta V = f(V)$ curves, for hyaluronic acid, C-4S, C-6S, and pK values for several ionic strengths and substrate concentrations

C (% w/v)	I	AH		C4-S		C6-S	
		E.p.	pK_a	E.p.	pK_a	E.p.	pK_a
2×10^{-4}	0.10	5.3	2.9				
	0.15	5.0	2.9	5.81		5.19	3.3
	0.20	5.2	2.9				
2×10^{-3}	0.10	5.2	3.0	5.32	3.3	5.07	3.3
	0.15	5.3	3.1	5.60	3.2	5.39	3.2
	0.20	5.2	3.0	5.58	3.0		3.9
2×10^{-2}	0.10	6.07	3.0				
	0.15	6.01	3.1				
	0.20	5.35	3.0				

$$\alpha = \alpha_n + \frac{H^+}{y} \qquad \alpha_n = \frac{M_b V_b}{M_a V_0} \qquad y = \frac{M_a V_0}{V_0 + V_b}$$

where y is the dilution factor, V_0 and M_a are the volume and the initial concentration of acid, V_b and M_b are the volume and concentration of the added base. The concentrations of both acid and alkaline solution are the same.

4. Results and discussion

We assumed for the HA macromolecules only one ionizable site: the –COOH group on the glucuronic acid residues. The difference between chondroitin sulfate acid and HA consists in the *N*-acetylhexosamine residue, which is galactosamine rather than glucosamine, and also there is a sulfate group, which is ionized in the pH range of the titrations. The ester group for each residue of disaccharide is in position four or six of the ring of galactose. C4-S present this group in the axial position and C6-S in the equatorial position.

The curves derived show the complexity of the neutralization process in these compounds. The curve obtained for HA indicates the existence of two ionizable groups. The former (A_1) corresponds to the –COOH of glucuronic acid of the repetitive unit of the disaccharide, common in all the glycosaminoglycans, and the latter to the one that is the object of this study. In the curves for both chondroitin sulfate (Fig. 1), a bigger polydispersion of peaks was observed. In these compounds, the ionization of the sulfate group appears overlapped by the ionization of the carboxyl group of the glucuronic acid. The curves corresponding to C6-S present greater complexity than the curves obtained for C4-S due to the differences in the configuration among them (the esterification of the sulfate group is in position four for C4-S and in position six for C6-S). In C6-S, the steric hindrance is smaller and the sulfate groups are more accessible to the aqueous medium. The ionization process is thus favored. This fact is in agreement with the highest values of viscosity that are obtained for this compound [7].

We do not observe variations of the ionic strength in comparison with mucopolysaccharides like heparine in which the neutralization curves are very sensitive to the changes of ionic strength [18]. The pK_a values for the chondroitin sulfate are higher than for HA due to the presence of sulfate groups in these compounds (Table 1). The presence of these negative groups determines a greater attraction toward H^+ groups. These values are in agreement with those obtained by Cleland [16] and Mathews [14].

In the curves of pH versus log $\alpha/(1-\alpha)$ for the modified HH equation, between two and three linear tracts of different slopes were obtained. The different

Table 2
pH values and dissociation degree (α) corresponding to transition point of the Hendersson–Hasselbalch curves for different concentrations and ionic strengths

C (% w/v)	I	AH				C4-S				C6-S			
		T_1	α_1	T_2	α_2	T_1	α_1	T_2	α_2	T_1	α_1	T_2	α_2
2×10^{-4}	0.10	3.0	0.61										
	0.15	3.0	0.58			3.0	0.71						
	0.20	3.0	0.66										
2×10^{-3}	0.10	2.6	0.28	3.3	0.70	3.3	0.50			3.2	0.50		
	0.15	2.8	0.28	3.7	0.80	2.8	0.30	4.5	0.96	3.0	0.33	4.0	0.80
	0.20	2.8	0.39	3.7	0.78	2.8	0.40	4.0	0.92	2.8	0.20	3.8	0.91
2×10^{-2}	0.10	2.6	0.16	3.6	0.76								
	0.15	2.7	0.40	4.0	0.85								
	0.20	2.6	0.20	3.7	0.90								

Table 3
The slope values (n) of different segments on Hendersson–Hasselbalch equation

C (% w/v)	I	AH			C4-S			C6-S		
		n_1	n_2	n_3	n_1	n_2	n_3	n_1	n_2	n_3
2×10^{-4}	0.10	0.40	1.07							
	0.15	0.39	0.94		0.29	0.98	1.55	0.43	0.98	1.64
	0.20	0.40	1.05							
2×10^{-3}	0.10	0.30	0.67	0.98	0.46	0.98		0.44	0.94	
	0.15	0.31	0.75	1.27	0.28	0.98	1.94	0.41	0.95	1.27
	0.20	0.22	0.85	1.00	0.40	0.98	1.50	0.23	0.81	2.53
2×10^{-2}	0.10	0.23	0.96	1.77						
	0.15	0.18	1.17	2.12						
	0.20	0.19	0.80	1.31						

segments correspond to the different conformational forms adopted by the substrate in the course of neutralization and the slope is a measure of the electrostatic interactions of the system. In Table 2 the pH values and the ionization degrees corresponding to the conformational transition are indicated. In conditions of weak ionic strength and diluted solutions only, a point transition was observed at around $\alpha = 0.6$ and pH 3.0, for the three studied substrates: HA, C4-S, and C6-S. For the rest of the experimental conditions, two points of conformational change were obtained: the first one for α next to 0.3 and the second for α between 0.8 and 0.9. In these systems, the first point would be related to the rupture/formation of hydrogen bonding, which facilitates the ionization process. For the chondroitin sulfates the values of the dissociation degrees corresponding to the former point are higher than for HA. The formation of hydrogen bonding in these compounds is weakened by the steric hindrance and the electrostatic interactions that take place because of the ester group in position four (C4-S) or six (C6-S) of the hexosamine ring. The conformation changes from the compact random coil to the expanded random coil can be considered. The latter point appears for very high dissociation degrees, therefore the density of negative charge of these solutions is high, and the repulsion effects are important. For diluted solutions and low ionic strength, the ionization process is easier, and the conformational transition to the extended random coil was only observed.

In Table 3 the slope values (n) of the different segments corresponding to the HH equation are shown. Three types of values corresponding to the different conformational states of the macromolecule are obtained. In the first part of the curve, for all compounds studied and under all the experimental conditions, the value is $n < 1$. These values are commonly related to the formation of micelles [19] and in our case they indicate the formation of new intermolecular connections with the solvent to facilitate the ionization process, thus reflecting the equilibrium among the rigid and flexible forms. The second segment is characterized by a slope $n = 1$, and the HH equation is verified. Therefore the configuration is sufficiently expanded so that the distances among charges are large enough to consider the ionizable groups independently. In the last segment

$n > 1$, this value reflects that the system presents a high density of negative charge. Therefore the electrostatic repulsion and the formation of counterion layers are favored. The addition of NaCl influences this state and we obtained smaller n values when the ionic strength was higher. An effect of charge screening takes place and the electrostatic field of the carboxyl group of glycosamine chains decrease with the addition of electrolyte [20].

The plot $pK = f(\alpha)$ allows us to determine the intrinsic pK (pK_0) (Table 4). This value is calculated by extrapolation for $\alpha = 0$ of the linear segment in which the theoretical curve agrees with the experimental one. These curves (Fig. 2) allow us to evaluate the variation of the electrostatic energy, ΔG_e, for the three states of the system given by the HH equation. The change of electrostatic free energy is represented by the surface between the two pK curves, the experimental one and the theoretical one. Firstly, these curves have a value of $\Delta G_e > 0$ (experimental surface above the theoretical curve S_1), which corresponds to the new unstable conformation due to the rupture of the intramolecular hydrogen bonding between the carboxyl and acetamido groups of its structure and to the formation of new intermolecular bonding with the aqueous medium to facilitate the ionization process. The intramolecular bonds are responsible for the rigidity presented by the chains of HA and chondroitin sulfate. The loss of rigidity of these structures is the cause of the conformational transition from a compact random coil with some stiff segments to flexible random coil. Mathews [14] considers that instead of a true conformational change between stiff and flexible states, there exists a quick equilibrium between them and for this reason the properties of these macromolecules in solution are sometimes difficult to explain. Secondly, for $0.4 \leq \alpha \geq 0.9$, the theoretical and experimental curves are coincident. This conformational stage is characterized by $\Delta G_e = 0$ and the change of the free energy corresponds to the constant of intrinsic ionization, K_0, which indicates that the configuration of the system is random coil sufficiently expanded to neglect the electrostatic interactions among the groups. For values of $\alpha \geq 0.9$ (S_2) a small surface exists with a value of $\Delta G_e > 0$ due to the electrostatic repulsions for the high density of negative charge and the formation of counterion layers is favored. We do not observe differences among the behavior of the three glycosaminoglycans studied.

Table 4
Intrinsic pK (pK_0) calculated by extrapolation of $pK = f(\alpha)$ curves

C (% w/v)	I	pK_0		
		AH	C4-S	C6-S
2×10^{-4}	0.10	3.02		
	0.15	3.10	3.32	3.34
	0.20	3.10		
2×10^{-3}	0.10	3.10	3.32	2.94
	0.15	3.20	3.30	3.24
	0.20	3.10	3.06	2.95
2×10^{-2}	0.10	3.10		
	0.15	3.00		
	0.20	3.08		

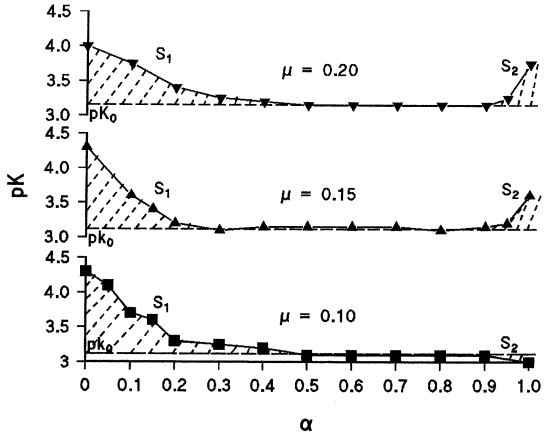

Fig. 2. pK versus ionization degree curves for C4-S at concentration 2×10^{-4} (% w/v) and at different ionic strengths.

5. Conclusions

For the three glycosaminoglycans, we determined that the conformational change from compact ramdom coil to extended random coil takes place for an ionization degree around $\alpha = 0.4$, which is independent of the ionic strength. For values of $\alpha > 0.9$ the new form undergoes expansion as its increasing negative charge and extent of conformation varies with the ionic strength, as expected by a flexible polyelectrolyte ion. The increase of the ionic strength decreases the effects of electric field, and the formation of counterion layers is favored.

The conformational changes take place in two stages. The former is characterized by the rupture of the intramolecular links of the hydrogen bond; the molecule loses its rigidity and the quick equilibrium within rigid and flexible states can coexist (for α values between 0.0 and 0.3). In the latter stage, the expansion of the random coil takes place as a consequence of the electrostatic repulsion (for α values between 0.3 and 0.4). The conformation of expanded random coil is stable in solution and the change of free energy of the ionization process corresponds to the constant of intrinsic ionization. Theoretical macromolecular models with different charge distributions are applicable only to the first stage of the ionization process.

Acknowledgements

This paper was supported by Dirección General de Investigación PGC2000-2246-E.

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