Determination of the presence of hyaluronic acid in preparations containing amino acids: The molecular weight characterization

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ABSTRACT

Several pharmaceutical preparations contain hyaluronic acid in the presence of a large variety of low molecular weight charged molecules like amino acids. In these mixtures, it is particularly difficult to determine the concentration and the molecular weight of the hyaluronic acid fragments. In fact, zwitterionic compounds in high concentration behave by masking the hyaluronic acid due to the electrostatic interactions between amino acids and hyaluronic acid. In such conditions the common colorimetric test of the hyaluronic acid determination appears ineffective and in the 1H NMR spectra the peaks of the polymer disappear completely. By a simple separation procedure the presence of hyaluronic acid was revealed by the DMB test and 1H NMR while its average molecular weight in the final product was determined by DOSY NMR spectroscopy alone. The latter determination is very important due to the healthy effects of some sizes of this polymer's fragments.

1. Introduction

Hyaluronic acid (HYA) is a linear polysaccharide composed of repeating units of D-glucuronic acid and D-N-acetylglucosamine, [-4]-beta-D-GlcpA-(1→3)-beta-D-GlcpNAc-(1→], extracted from a variety of animal tissues. Owing to its unique physico-chemical properties and relevant biological activities, hyaluronic acid has been used in a wide variety of products, such as food, biomedicine, biomaterials and cosmetics. Due to limited sources and the risk of viral infection the acid is now produced by optimized microbial fermentation, with high purification efficiency, low production costs and a low risk of cross-species viral infection (Izawa et al., 2011; Liu et al., 2010). The characterization of the molecular weight appears particularly important since fragments of hyaluronic acids with low-molecular-weight (LMW-HYA) reveal different biological activities (Stern et al., 2005; Forrester and Balazs, 1980; McKee et al., 1996). In fact average molecular weights in the range of 45.2–145 kDa fragments show pronounced free radical scavenging and antioxidant activities compared to those carried out by hyaluronic acid of very high MW (Ke et al., 2001). LMW-HYAs also behave as effective angiogenic factors (Cui et al., 2009) and can promote excisional wound healing through enhanced angiogenesis (Gao et al., 2006). Other evidence indicates that LMW-HYAs inhibit colorectal carcinoma growth by decreasing tumor cell proliferation and stimulating immune response (Alaiz et al., 2009). Their use has been also described as a new adjuvant candidate in the preparation of dendritic cell-based anticancer vaccines with potent immune-stimulatory properties (Alaiz et al., 2003). In fact for this reason it appears important to reduce the high-molecular weight hyaluronic acid in the LMW-HYA and many methods of degradation have been proposed (Stern et al., 2007) including the physical method (Gu et al., 2010; Kubo et al., 1993; Kwon et al., 2009; Miyazaki et al., 2001), the chemical approach (Rychly et al., 2006; Soltes et al., 2007; Yamazaki et al., 2005) and the enzymatic process (Chen et al., 2009; El-Safory et al., 2010; Alkaid et al., 2003; Lenormand et al., 2004).
2. Materials and methods

The AAA sample used for the analysis constituted of 0.1% HA as sodium salt, l-isoleucine, l-leucine, l-lysine, l-proline, l-valine, glycine, l-serine, l-alanine, l-cysteine, sodium bicarbonate placed in sterile 5 mL vials was kindly donated by the producer in Italy. DMAB, sodium tetraborate and HA at 13.50, and 208 kDa mean MW were obtained from Sigma Aldrich (USA). The Mini Dialysis Kit at 1000 Da cut-off was purchased from Amersham Biosciences (USA).

The p-dimethylaminobenzaldehyde (DMAB) assay is a UV–Vis test for oligosaccharides containing N-acetylglucosamine in the reducing end. In this work we used a slightly modified method as previously described by Reissig et al., 1955. For the assay two working solutions were used: tetraborate solution, prepared by dissolving 3 g of sodium tetraborate in 10 mL H₂O at pH 9, and then DMAB solution, prepared by dissolving 0.5 g DMAB in an acid mixture made up of 0.6 mL of HCl 12 N and 4.4 mL of glacial acetic acid. Both working solutions were prepared immediately before use. In order to perform the DMAB assay 0.1 mL of AAA sample was added to 0.1 mL of tetraborate solution in a glass tube vortexted, heated for 3 min and then placed in cold water. At this point 0.8 mL of DMAB solution was added and the sample was incubated for 30 min at 37 °C. The UV–Vis measurements were performed on a Perkin Elmer spectrophotometer Lambda Bio by transferring the sample in a quartz cuvette of 1 cm path-length and the absorbance was measured at λ = 544 nm.

The amino acid deprivation of the sample was achieved by precipitation of 5 mL of AAA sample with 100 mL methanol which was then removed by evaporation. The precipitate was solubilized in 5 mL of H₂O and dialyzed for 48 h using the mini-dialysis kit at 1000 Da cut-off for complete amino acid deprivation. During the dialysis the amino acid deprivation from the sample was assayed by 1H NMR spectroscopy following the decrease of specific resonances of amino acids until their complete absence and the consequent appearance of characteristic NMR HA resonances.

NMR experiments were performed on a Bruker Avance instrument operating at 700.13 MHz. All the samples were prepared by dissolving them in H₂O containing 10% D₂O for the lock signal and transferred on 5 mm NMR tubes. In order to avoid the water signal at 4.7 ppm the 1H NMR experiments were acquired using the zgpgr pulse sequence with 32 K and 64 scans at 298 K.

The DOSY calibration curve of HA at different MW was performed in order to correlate the MW of the HA molecules with their Diffusion coefficient. For the lowest limit of the curve the Diffusion coefficient of water molecules (MW = 18 Da) was taken into consideration. For this three HA samples were purchased from Sigma Aldrich with a mean MW of respectively 13 kDa, 50 kDa, and 208 kDa. HA reference samples were dissolved in water and 10% D₂O at 0.1% w/v concentration transferred in 5 mm NMR tubes; for each of them the DOSY spectra at 298 K were acquired. The log Diffusion coefficient (logD) was then correlated to the log MW of the HA molecules giving an R² of 0.99. All the DOSY spectra were performed by using the zgpgrpr2s pulse sequence in order to suppress the water signal at 4.7 ppm. During the DOSY experiment 32 mono dimensional spectra were obtained with 64 scans in a linear increasing gradient varying from 5% to 95% with a Δt of 70 ms and a Δ of 2 ms. The spectra were then analyzed by using the DOSY module implemented in Bruker software TOPSPIN 3.1.

3. Results

As already reported the presence of amino acids and other molecules resulted both in the complete absence of reactivity in the DMAB test and the absence of the HA resonance in the NMR spectrum. In fact in the NMR spectrum in Fig. 1 only the resonances of the amino acids contained in the sample appear while the resonances due to the HA are not visible. In fact it is well known that the HA resonances are located at around 5.2 ppm and between 3.9 and 3.2 ppm in the 1H NMR spectrum with a well-defined envelope (Scott et al., 1984). Evidently the high concentration of amino acids with respect to HA may mask the peaks of HA: moreover, intermolecular interactions which HA is able to establish with amino acids change to a great extent the tumbling of the macro-molecule while the NMR resonances broaden as to be undetectable. It is important to mention that the original AAA sample studied was prepared with HA (MW of 1.7 Mda) at a concentration of about 0.1% w/v with the addition of amounts of amino acids such as: l-isoleucine, l-leucine, l-lysine chlorohydrate, l-proline, l-valine, glycine, l-serine, l-alanine, l-cysteine and sodium bicarbonate. This solution was autoclaved for the sterilization with cycles of 20 min at 121 °C and a pressure of 2 bar. The final product was inserted in disposable vials for direct use as a pharmaceutical preparation.

3.1. The analysis of hyaluronic acid by colorimetric DMAB method

To reveal the HA in the AAA sample, the DMAB assay was performed and the development of a red colored product due to the reaction of the terminal N-acetylglucosamine unit of HA with the reagent DMAB (Hynes and Ferretti, 1994) followed spectropho-
rometrically. In this case the test was unsuccessful, indicating the presence of negligible amounts of free HYA in the preparation, very far from the declared concentration of 0.1% w/v of HYA in the original product.

Because the presence of HYA in the AAA product was warranted, a purification procedure for the amino acid deprivation of samples was adopted as reported in Section 2 (The scheme is in Fig. 2). Moreover one of the vials of AAA used in the study was exposed to ultrasound for 6 h in order to investigate the effects of ultrasound on MW distribution (see below). The samples have been labeled as Amino acid Deprived and Sonicated (ADS) and Amino acid Deprived (AD), respectively.

The results of the DMAB test of the samples obtained are reported in Fig. 3. The absorbance due to the color developed by the reaction is clearly visible. As expected the color, developed in samples at a known mean MW of 13, 50, and 208 kDa (samples b, c, and d in Fig. 3), increases upon decreasing the MW. In fact the reaction involves the reducing terminal of the chain of HYA which, at the same monomer molarity, is more concentrated in low MW samples. Both microdialyzed samples (e and f in Fig. 3) showed a very low absorbance, which increased significantly after many steps of purification from amino acids (f-ADS and b-AD in Fig. 3). Moreover, the sonicated sample (f-ADS in Fig. 3) presented the highest value of absorbance. In fact the longer sonication time leads to a more fragmented HYA. In the NMR spectra appears also evident that in the purified samples the resonances of HYA at around 5.2 ppm and between 3.9 and 3.2 ppm are clearly visible (see Fig. 4a and c). The comparison of NMR spectra of ADS and AD reported in Fig. 4 shows a slight difference between sonicated (Fig. 4d) and non-sonicated (Fig. 4h) in their resonances line width. This is in line with the fragmentation induced by sonication on the AADS sample which decreases the line width according to the lower molecular weight of the fragments. These results obtained after many steps of purification confirm that a large number of amino acid molecules strongly bind to the HYA polymer interfering in the DMAB test and increasing the molecular tumbling and the internal flexibility to broaden the NMR resonances beyond detection.

3.2. The characterization of molecular weight of hyaluronic acid by DOSY NMR

The characterization of the molecular weight of HYA samples has been also reported by other authors using other techniques such as electrophoresis on SDS-PAGE and agarose gel (Bhilo et al., 2011); gel filtration chromatography (Yeung and Marecal, 1999) dynamic light scattering (Maleki et al., 2007). Other crometric methods such as carbazole determination of uronic acid (Bitter and Muir, 1962), high performance capillary electrophoresis (Plätzer et al., 1999), and, to a lesser extent, HPLC has also been used for quantification. Our approach is based on DOSY-NMR technique, an attractive non-destructive method making it possible to determine a sample's MW by measuring its diffusion coefficient, using small quantities; the technique is relatively fast and accurate when compared to the upper mentioned methods. The DOSY-NMR technique allowed us to characterize the MW of the HYA polymer in the AAA samples. DOSY-NMR is a powerful tool for structural studies of macromolecules and their interactions making it possible to measure translational diffusion of dissolved molecules. In fact the value of Diffusion coefficient provides direct information on translational dynamics, including intermolecular interactions (Brand et al., 2005; Cohen et al., 2005), aggregation and conformational changes (Kvam et al., 1992; Johnson, 1999). Moreover, DOSY in the second dimension makes it possible to measure Diffusion coefficient without a physical separation of mixture components (Coltun et al., 1990; Evans et al., 1972). In our case this approach allowed us to determine the MW of the HYA fragments provided that a calibration curve had been measured. The result of the calibration curve is reported in Fig. 5 where in the log-log plot the molecular Diffusion coefficients are reported as a function of the MW of the polymers studied. (The polymers of 15, 30, 200 kDa have been used as standards). To avoid meaningless the mean interval the diffusion coefficient measured by the resonance of

\[ \text{Abs} (\lambda = 540 \text{ nm}) \]

\[ a, b, c, d, e, f, g, h \]

\[ 0.00, 0.25, 0.50, 0.75, 1.00 \]

\[ 0, 1, 2, 3, 4, 5, 6 \]

\[ (g) \quad \text{1H NMR spectrum before amino acid deprivation} \]

\[ (h) \quad \text{1H NMR spectrum after deprivation of amino acids without sonication} \]

\[ (f) \quad \text{1H NMR spectrum after deprivation of amino acids and sonication as reported in Section 2} \]

\[ \text{and the procedure of Fig. 2.} \]

\[ \text{Fig. 4. NMR spectra of the AAA sample} \]

\[ \text{(g) 1H NMR spectrum before amino acid deprivation and} \]

\[ \text{(h) 1H NMR spectrum after deprivation of amino acids without sonication and} \]

\[ \text{(f) 1H NMR spectrum after deprivation of amino acids and sonication as reported in Section 2} \]

\[ \text{and the procedure of Fig. 2.} \]
water molecule has been included due to high linearity behavior of that value. In fact in the calibration curve including water Diffusion coefficient the linearity correlation coefficient was $R^2 = 0.99$. Strictly the hydrodynamic radius should be reported in abscissa instead of MWS, but at a first approximation one can consider the correspondence between the two values. The results of the DOSY experiments performed on the sample AD (left panel) and ADS (right panel) are reported in Fig.6 where the resonances of the two samples are easily distinguishable at different MWS.

3.3. The effects of sonication on molecular weights of HYA

The DOSY spectra shown in Fig. 6 indicated that the ultrasound treatments led to a fragmentation of the hyaluronic acid in the preparation of AAA samples. In fact in the two samples the average MW of hyaluronic acid appears to be significantly different. In particular in the AD sample, the MW was estimated at about 8000–11000 Da. On the ADS sample the MW was estimated at about 270–500 Da, demonstrating that in our case the sonication produces a fragmentation of HYA polymer. This approach may be considered as complementary with the methods already established for the determination of MW of hyaluronic acid. The results reported here are a demonstration of utility of the DOSY NMR technique. Further studies for a complete validation of this technique with results obtained by other techniques is planned in the near future.

4. Discussion and conclusion

In conclusion the procedure of purification and verification by DMAB test and by NMR spectroscopy of HYA present in commercial preparations for human use, such as AAA has proved successful. It must be concluded that the presence of large amounts of amino acids is able to completely mask the colorimetric test and to fail the determination of the hyaluronic acid by NMR spectroscopy. The purification procedure adopted led to a complete characterization by $^1$H NMR of the HYA present in the samples, removing the interference of other compounds. Moreover the DOSY-NMR spectroscopy allowed us to determine the molecular weight of the HYA fragments present in the product. Furthermore, the sonication in the conditions adopted led to a further increase in fragmentation of the HYA visible from the DOSY spectra. Thus, the sonication of AAA improves the fragmentation process and may find a broad application for obtaining LMW-HYA. Indeed, fragments have been studied and were found to actively stimulate many types of cells and to facilitate the clearance of debris or infectious agents (Cothup et al., 1990; Francis et al., 1972; Maharjan et al., 2011).

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References


Fig. 6. The DOSY NMR spectra of the AD sample (left) and ADS sample (right). The arrows are for visual guidance.