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Isolation, purification and characterization of hyaluronan from human umbilical cord residues

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Abstract

The main objective of this paper is to discuss new procedures of the isolation of Hyaluronan. Hyaluronic acid can be obtained from human umbilical cord residual, which is obtained from other biopharmaceutical productions. The route involves treatment of human umbilical cord residuals with sodium chloride solution, followed by ammonium quaternary salt solution precipitation; the solid is re-suspended in calcium chloride solution in order to dissociate the hyaluronan ammonium quaternary salt complex followed by ethanol-induced precipitation to give a product. The product was purified four times by chloroform extraction, and characterized by chemical methods such as the Blumenkrantz and Asboe-Hansen uronic technique for uronic acid determination, Elson Morgan qualitative tests for hexosamines, intrinsic viscosity, ion-exchange chromatography, and ^{13}C NMR spectroscopy. The results showed that the product might be used in the formulation of ointment, lotion and cream for the treatment of skin diseases.

Keywords: Hyaluronic acid; Hyaluronan isolation; Residuals; Characterization

1. Introduction

In recent years, research has been conducted on the use of human placenta and the isolation of biological substances aimed at synthesizing medications. Hyaluronan (HA) is one of these biological substances that can be obtained from the human umbilical cord. HA is a member of the aminosugar and uronic acid containing carbohydrate polymers group known as glycosaminoglycans (GAGs) (Laurent, 1970; Meyer, 1958). The repeating -3)-β-D-glucopyranuronosyl-(1–4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1- disaccharide unit is the structure of this linear carbohydrate polymer of high molecular weight. Meyer and Palmer were first to isolate HA as a defined component of the intercellular matrix of connective tissue in the human placenta (Mahoney, Aplin, Calabro, & Hascall, 2001; Meyer & Palmer, 1958). The molecular configuration and structure of the disaccharide unit of sodium hyaluronate is represented in Fig. 1.

High molecular weight HA's have been found in various biological sources, such as rooster comb, umbilical cord, the zone of maturing chondrocyte (Sunwoo, Nakano, & Sim, 1998) and in bovine submaxillary glands (Devaraj & Bhavanandan, 1992). However, other animal tissues such as synovial fluid, vitreous and aqueous humor of the eye, and skin contain high molecular weight sodium hyaluronate (Balazs, 1984).

HA can be used to assist in the healing of wounds of the skin, and the treatment of periarthritis of the shoulders, as well in the viscoelastic fluid replacement surgery of the cornea (Meyer, 1958; Meyer & Palmer, 1958). HA can also be used as raw material for the preparation and synthesis of drugs, e.g. when combined with Diclofenac it is a powerful anti-inflammatory and analgesic, especially for the actinic

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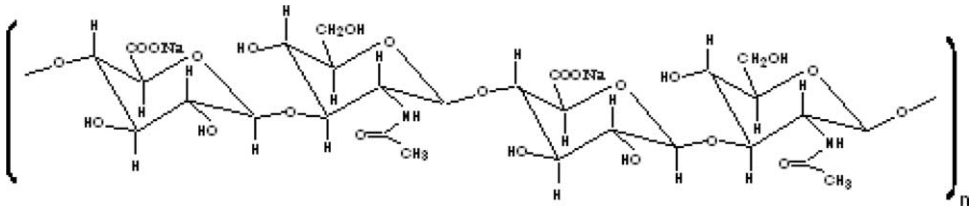

Fig. 1. Hyaluronan structure.

keratosis, carcinoma of basal cells, and osteoarthritic pain (Alam, Seed, & Willougby, 1995; Balazs, 1984; Brown, Marriot, & Martin, 1995; Casione, 1995; Orvisky et al., 1992). Furthermore, new biomaterials derived from HA for surgical application may be synthesized as alginate–HA complexes (Oerther et al., 2000; Oerther et al., 1999).

This paper is aimed at isolating, purifying and characterizing HA from human umbilical cord residues, which are obtained from other biopharmaceuticals productions such as the sunscreen commercialized by the Placental Histotherapy Center (Hollands, Barry, & Miyares, 2003). The isolation of HA from umbilical cord residues and the way the product performs are novel aspects in this paper never reported before.

2. Materials and methods

2.1. Reagents and others materials

Reagents for HA extraction: sodium chloride (Panreac, Barcelone, Spain), cetyltrimethylamonium bromide, calcium chloride, ethanol, acetone, chloroform, sodium monohydrogen phosphate (all Readel De Haen Aldrich, Germany). Reagents for product chemical characterization: hyaluronan from (Fluka Milwaukee, USA—this is the reference HA used in this work), sulfuric acid, sodium tetraborate, sodium monohydrogen phosphate heptahydrate, sodium dihydrogen phosphate monohydrate, D-glucuronic acid, polysaccharide standards of different molecular weight, sodium azide (Readel De Haen Aldrich, Germany), Blue Dextran (Pharmacia, Uppsala, Sweden), metahydroxibiphenyl and *N*,*N*-dimethylaminebenzaldehyde (Sigma, New York, USA); Sephacryl S 400 (Pharmacia, Uppsala, Sweden).

2.2. Analysis of raw material

The raw material used to manufacture the product (human umbilical cord residual) is duly tested to detect the possible presence of viruses, such as HIV, hepatitis B and hepatitis C, according to the standard approved by the State Center for Quality and Control of Drugs Center for Evaluation and Control of Drugs for the National System for Placenta Collection in Cuba and developed at the Placental Histotherapy Center (CECMED, 2002; WHO, 1994).

2.3. Isolation and purification

Human umbilical cord residuals (1 kg) were treated with 4 l of 0.2% sodium chloride solution and then filtered. The supernatant was treated with 300–600 ml of 1% cetyltrimethylamonium bromide (CTAB) solution and later centrifuged. The precipitate obtained was re-suspended by adding 0.9 M calcium chloride solution to dissociate the HA–CTAB complex; the suspension was treated with 25% v/v aqueous ethanol and the nucleic acids were precipitated and separated by centrifugation. The resulting suspension was deproteinized and defatted four times by chloroform solvent extraction (10% of the total volume). HA was precipitated by first adding 75% v/v aqueous ethanol and then acetone.

2.4. Characterization methods

2.4.1. Determination of uronic acids

The method of Blumenkrantz and Asboe-Hansen (Montreuil et al., 1986), based on the reaction of glucuronic acid contained in HA with meta-hydroxybiphenyl, was carried out. HA was treated in an ice bath with sulfuric acid in sodium tetraborate and heated to 100 °C. Then a 15% solution of meta-hydroxybiphenyl was added measuring the absorbance at 520 nm and comparing it versus glucuronic acid as reference standard.

2.4.2. Qualitative determination of N-acetylhexosamine

Elson Morgan's qualitative assay was used for the identification of *N*-acetylhexosamine present in GAGs, and mainly in HA (Carney, 1986). It is based on the reaction of the hexosamine fraction present in HA with *N*,*N*′-dimethylaminobenzaldehyde after hydrolytic liberation of the *N*-acetylhexosamine as hexosamine. For this purpose, samples were hydrolyzed in 8 M HCl for 4 h at 95 °C. The hydrolysate was adjusted to pH 10 by the addition of 4 M NaOH and converted to a known volume using distilled water. The resultant solution (250 μl) was mixed with 250 μl of acetylacetone reagent (formed by adding of 1 ml acetylacetone in 50 ml sodium carbonate 0.5 M and adding 250 μl of distilled water) and heating to 100 °C for 20 min. Add 1 ml of absolute ethanol, taking care to wash down all droplets of condensation into the bottom of the tube and *N*,*N*′-dimethylaminobenzaldehyde solution (250 μl, formed by dissolving 0.8 g recrystallized *N*,*N*′-dimethylaminobenzaldehyde in 30 ml absolute ethanol plus 30 ml

concentrated hydrochloric acid) heated to 65 °C for 10 min. The tubes are then cooled and shaken vigorously; the absorbance being measured at 530 nm.

2.4.3. Physicochemical methods of analysis

Determination of kinematic viscosity was achieved in a Scott Gerate Model capillary viscometer. The experimental sample was re-suspended in a 1% solution containing 8.166 g NaCl, 0.400 $Na_2PO_4 \cdot 7H_2O$ and 0.044 g $NaH_2PO_4 \cdot H_2O$, dissolved in demineralized water. The viscosity results are showed in $M^2\ S^{-1}$ (1×10^6 cSt (centistokes) = $1\ M^2\ S^{-1}$).

2.4.4. Gel filtration chromatography. Molecular weight determinations

HA molecular weight was measured using molecular chromatographic exclusion or gel filtration chromatography (Laurent, 1960; Motohashi & Mori, 1984; Orvisky et al., 1992; Sunwoo et al., 1998). A 32 cm × 1 cm column packed with Sephacryl S 400 was used. This column was equilibrated for 48 h using a buffer solution containing Na_2HPO_4 0.05 M, 0.1 M NaCl pH 7.5, and 0.02% w/v sodium azide, at a flow rate of 0.6 ml/min. A solution of Blue Dextran (2×10^6 Da MW) at 0.028 mg/ml was used to determine the void volume (V_o) of the column.

Polysaccharide standards of different molecular weights were used. The equation Log MW versus K_{av} was obtained by measuring the elution volume of each standard. The molecular weight for both samples being tested and the HA standard were calculated using the above described equation. Eluting fractions were detected by the sulfuric acid—anthrone technique measuring the absorbance at 620 nm (Trevelyan & Harrison, 1952).

2.4.5. Ion exchange chromatography

Anionic exchange Mono Q HR 5/5 column at a flow rate of 1 ml/min and a gradient formed by A: 50 mM NaH_2PO_4 buffer pH 7 and B: 1 M NaCl in 50 mM NaH_2PO_4 pH 7. The elution method was 0–100% B in 50 min. Aliquots (500 μl) of the standard and the experimental samples were injected at a concentration of 0.3 and 2.8 μg/ml, respectively, and detected at 229 nm.

2.4.6. Nucleotides and proteins contamination analysis by UV absorption

UV spectroscopy was used to estimate the level of nucleotides and proteins. This method of analysis was described by Balazs (1979), as a control for the final product. According to what was previously stated, the solution was prepared at 1% wt/v in 0.15 M NaCl. Absorbance was measured at 257 and 280 nm (Cintra 5 Unicam) in order to determine the level of nucleotides and protein, respectively; the absorbance levels for a 1 cm path length should be lower than 3 at 257 nm and lower than 2 at 280 nm.

2.4.7. Structural analysis

Analysis by ^{13}C NMR. The experimental HA obtained was dissolved at 10 mg/ml in deuterium dioxide (D_2O) at 57 °C (330 K). The ^{13}C NMR spectrum using an AC 250 F model (Bruker) was recorded, operating at 62.90 MHz and performing 140,000 scans with an acquisition time of 2.5 μs, a flit angle of 45°, broadband ^1H decoupling and no relaxation delay.

3. Results and discussion

Among the methods for the isolation of HA, the one recorded by Balazs (1979) is the most known and it is suitable for using human umbilical cord or rooster comb as a biological source. Although other methods have been reported (Sunwoo et al., 1998), in which the proteoglycans were extracted from the zone of maturing chondrocytes with 4 M guanidine-HCl, it was isolated following tissue mincing, ultrasonications, and lipid extraction, as well as extensive digestion with pronase and DNAse, and treatment with alkaline-borohydride and ethanol precipitation.

This paper describes a source for the isolation of HA sodium salt from a sub-product generated of the human umbilical cord treated with organic solvent, never so far reported. The procedure employed was simpler and has several advantages compared with other known methods. For instance, many steps are needed to develop Balazs's method (1979). In the first stage of the Balazs method, the raw material is washed with water until the blood is removed from tissue and then crushed and stored. In the second stage, the tissue is treated with 20 volumes of water and one volume of chloroform, stirred for 24 h and filtered of in order to extract HA and other polysaccharides. In our methodology, the first Balazs stage is not needed, because raw material remaining from other productions was used, as well as 0.2% w/v aqueous sodium chloride solution was used as the main solvent for HA extraction. This concentration of sodium chloride solution is more effective when extracting polar compounds, including HA.

This method may also be compared with that of Danishevsky and Bella (1966), since a 0.2% sodium chloride solution was used, similarly, as solvent to extract HA from raw material, but in our case human umbilical cord residual was used as biological source and 0.9 M calcium chloride solution to dissociate the HA–CTAB complex only. These aspects have not been reported before.

In the next step of our process, nucleic acids and nucleotides were eliminated by precipitation with 25% v/v aqueous ethanol in a ratio volume of 1/3 v/v. The absorbance ratio A_{260}/A_{280} of the above solution before and after ethanol addition changed from 2.5 to 0.8, suggesting major nucleic acid and nucleotide elimination. Later on, the amount of ethanol is increased to 3 volumes per volume of solution to precipitate HA in raw form.

Table 1
Analytical results of the experimental sample assay and HA standard

Assay	Experimental sample	HA standard
Uronic acid (ppm)	60.5	58.0
N-acetylhexosamine	Positive	Positive
Viscosity (cSt)	>1000 cSt (>0.011693 $M^2\ S^{-1}$)	>1000 cSt (>0.016636 $M^2\ S^{-1}$)
UV analysis		
257 nm	0.120	0.118
280 nm	0.093	2.0
Molecular weight	500,000	750,000

The elimination of nucleic acid and nucleotides had not been solved by previous isolation methods.

Uronic acid presence and quantity are important indicators characterizing this type of carbohydrate polymer. The average content of uronic acid was 60.5 and 58.0 ppm for experimental sample isolated from human umbilical cord residues and reference sample from Fluka, respectively. The Elson Morgan's analysis was also carried out as a qualitative test for the presence of *N*-acetylhexosamine in the HA preparation. Table 1 shows the five-fold replicated analytical results obtained for the experimental sample assayed and the reference standard.

The level of protein was determined [after clarification (the samples at 1% w/v were centrifuged at 11,000*g* RCF (Relative Centrifugal Field), where necessary; when the samples had been stored in crystalline form for a week or more, then the solutions prepared clarification was necessary, but when the solution was prepared just after the purification, the clarification was not necessary] by measuring the absorbance at 280 nm (0.093) equivalent to a protein concentration of 1×10^{-4} g/ml for the experimental sample (see Table 1). This compares favorably with the absorbance value at 280 nm (2.0 at 1 cm path length) for a reference HA standard (Fluka material as described in Section 2 above) having a protein concentration of 2.2×10^{-3} g/ml.

Similarly, the level of nucleic acid and nucleotides were determined by measuring the absorbance value at 257 nm (0.120 at 1 cm path length) equivalent to a nucleic acid and nucleotides concentration of 6 μg/ml for the experimental sample. This also compares favorably with the absorbance value at 257 nm (0.118 at 1 cm path length) for a reference HA standard having a nucleic acid and nucleotides concentration of 5.9 μg/ml; the absorbance value measured is also significantly lower than 3.0, the value originally established by Balazs (1979) as an HA quality control and as being an upper limit above which the nucleic acid contamination is unacceptably high. In the Balazs experimental samples, the measured absorbance values are around $A_{257}=0.243$.

With regard to the viscosity, the molecular weight and the UV analysis, very similar values between experimental sample and reference standard were obtained. According to the literature (Balazs, 1979), 1% HA w/v solution in a physiological buffer should behave as a visco-elastic fluid

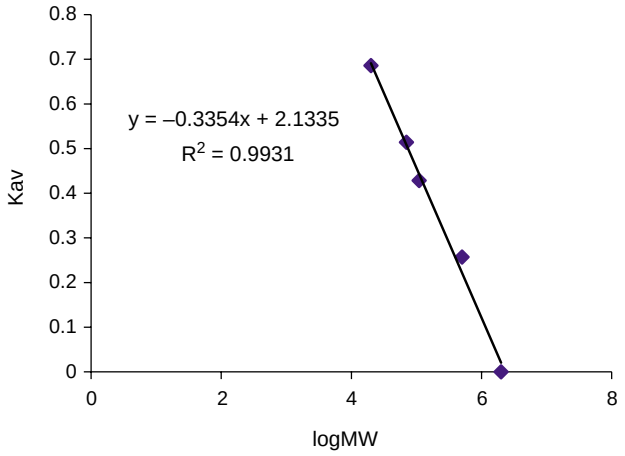

Fig. 2. Calibration curve of K_{av} versus Log MW of standard polysaccharides of known molecular weight.

which can be used in ophthalmology and orthopedics. Our product behaves very similarly to the standard with values >1000 cSt (centistokes); 0.011693 $M^2\ S^{-1}$ for the experimental sample and 0.016636 $M^2\ S^{-1}$ for the HA reference standard.

Size exclusion chromatography has been used for the analysis of molecular weight (MW) by other researchers, and thus (Karlsoon & Bergman, 2003) have shown the use of this method in the determination of the distribution of molecular masses of sodium hyaluronate preparation. Fig. 2 shows the K_{av} versus log MW graphic, where $K_{av}=(V_e-V_o)/(V_t-V_o)$, where V_e, V_o and V_t are elution volume, column void volume and total bed volume, respectively. In this case, the column void volume (V_o)= elution volume for Blue Dextran 2000. The final equation from the experimental data was $K_{av}=2.13354-0.335351$ Log MW. The statistical analysis showed an R^2 of 99.3069%, a correlation coefficient $R=-0.996529$, mean absolute error 0.016 and standard error of 0.025. The average molecular weight of the experimental sample (500,000 Da) and the reference standard (750,000 Da) were determined using the above described equation.

In the case of the ion exchange chromatography (Fig. 3), the major peak at 20.49 min obtained for

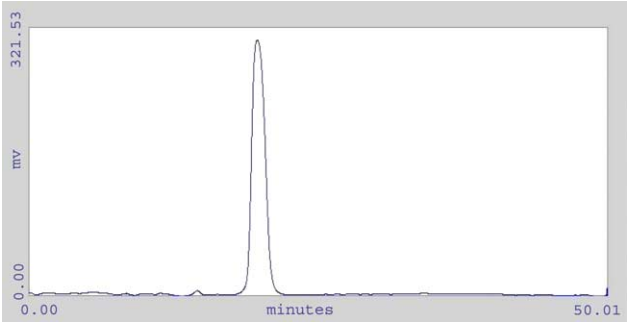

Fig. 3. Anion exchange chromatography of HA purified from umbilical cord residues on a Mono Q HR 5/5 column.

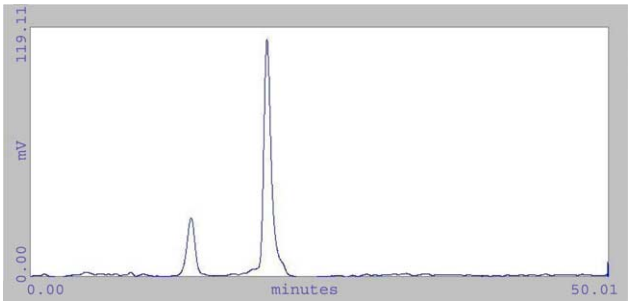

Fig. 4. Anion exchange chromatography of HA used as reference standard on Mono Q HR 5/5 column.

the experimental sample corresponds to HA sodium salt and the small peak [first (left to right)] of lower retention time (14.18 min), with strong absorbance at 280 nm, was assigned to contaminating peptides. In Fig. 4 (obtained for the reference standard), the retention times were similar to the experimental sample; in this case, the major peak at 20.72 min corresponds to HA sodium salt and the small peak of lower retention time (14.15 min) assigned to contaminating peptides. Gel filtration chromatography on Sephadex G 25 SF was used to confirm the presence of peptides corresponding to the lower retention time fraction in the ion exchange chromatographic process, measurement being made at 214 nm. This fraction's retention time was similar to that of β-insulin used as known standard (results not shown).

Table 2 shows the retention time average (X) according to appearance order for the sample assayed and the standard; the areas under each peak and the sum of all areas as well as standard deviation (SD) and the variation coefficients (VC) of each peak were shown. Both, the standard and the tested sample were analyzed, a peak appeared at 14 min, which could be associated to peptides and a high intensity peak at around 20 min associated to the HA sodium salt. Considering the total area of peaks integrated and the area of each peak, it was found that HA% in the reference preparation was 78 and the tested sample 99, at least under the elution conditions used.

Table 2
Results of ion exchange chromatography

Sample	Rt(1)	Rt(2)	$A1$	$A2$	ΣA	HA%
Experimental	14.18	20.49	2.41	384.13	386.5	99.38
SD	0.22	0.35	0.18	2.65	2.38	0.20
VC (%)	0.18	1.79	7.71	0.69	0.62	0.20
Standard	14.15	20.72	19.95	71.67	92.00	
SD	0.25	0.5	0.011	0.57	0.57	78.21
VC (%)	1.79	2.44	0.06	0.8	0.62	0.16
						0.21

Experimental, HA purified from umbilical cord residuals; Standard, commercial reference. Rt(1), Rt(2), retention times; Areas $A1$ and $A2$; ΣA, sum areas; HA%, hyaluronan percent; SD, standard deviation; VC, variation coefficient.

Table 3
^{13}C NMR analysis of HA purified from human umbilical cord residual and standard

Signals	Chemicals shifts (ppm)	
	Experimental	Reference (Scott & Heatley, 1999)
Carboxylate	173.4	174
Acetamide	174.5	175
Anomeric carbons	100.1	100
	102.7	104
Methyl groups	22.1	22.0
C2 (ring carbon atom)	53.9	54.5
C6 (ring carbon atom)	60.4	60.5

3.1. Structural analysis

3.1.1. ^{13}C NMR spectrum discussion

The main signals of the experimental HA are shown in Table 3. These signals can be compared with those reported before (Scott & Heatley, 1999), in which the ^{13}C NMR spectrum of HA was recorded at 30 °C from a product solution of 10 mg/ml in deuterium dioxide containing 0.29 M NaCl and 0.05 M sodium phosphate buffer (pH 7.4). The signals shown corresponded exactly with those reported previously by Scott and Heatley (1999).

Two carbonyl group resonances (C=O) from the carboxylate and acetamide group at 173.4 and 144.5 ppm were found, respectively. Two anomeric carbon signals appeared at 100.1 and 102.7 ppm, and the acetamide (CH_3) carbon appearing at 22.1 ppm. The signals at 53.9 and 60.4 ppm were assigned to the C2 and C6 (CH_2OH) carbons of the glucosamine residue. Other signals can be assigned to structural carbons present in the HA. All the resonances described above confirmed the presence and purity of HA in the final product.

In the present paper, a novel method for the isolation of HA from human umbilical cord residues, never before reported in the literature, as well as its purification and characterization by chemical and physicochemical methods have been presented.

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