Characterization of an extract obtained from human umbilical cord rich in glucosaminoglycans

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Palabras clave: extracto biológico humano, sal sódica de ácido hialurónico, glucosaminoglicanos, cordón umbilical humano.
Key words: human biological extract, hyaluronic acid sodium salt, glucosaminoglycans, human umbilical cord.

RESUMEN. El presente trabajo tuvo como objetivo la caracterización de un extracto obtenido a partir de cordón umbilical humano, el cual puede estar compuesto por glucosaminoglicanos y principalmente sal sódica de ácido hialurónico. Este ácido resulta muy interesante debido a sus múltiples aplicaciones en diferentes áreas, principalmente como una posible materia prima para la obtención de diferentes productos farmacéuticos. El extracto correspondiente fue caracterizado por métodos químicos como la determinación de ácidos urónicos por el método de Blumer, azúcares reductores por la técnica de Bradfor y la prueba de Elson Morgan. Sin embargo, la cromatografía líquida y las técnicas químico-físicas fueron usadas para comparar la sal sódica del ácido hialurónico con un patrón de referencia debido a que estos métodos son los más usados como técnicas de caracterización de glucosaminoglicanos. La determinación del peso molecular por técnicas de exclusión molecular usando gel de Sephacryl S400 con un peso molecular promedio de 500 a 800 kDa, el cual puede ser comparado con la viscosidad cinemática por viscosimetría capilar, la medición a 254 y 280 nm por espectrofotometría de absorción para la determinación de las concentraciones de nucleótidos y proteínas respectivamente, la cromatografía líquida de alta presión (HPLC) en fase reversa usando columnas LC 318 con dos sistemas de gradiente y la cromatografía líquida a baja presión por intercambio iónico en columna Mono Q HR 5/5 con un sistema de gradiente salino. Los resultados demostraron que el extracto contiene sal sódica de ácido hialurónico y también pequeñas cantidades de péptidos sin estar presentes otros GAG. Este producto puede ser empleado como sustancia de referencia en diferentes formas farmacéuticas.

ABSTRACT. The objective of the present paper was the characterization of an extract obtained from human umbilical cord which may be composed by glucosaminoglycans (GAG) and mainly hyaluronic acid sodium salt. This acid is interesting due to its multiple applications in different areas, mainly as a possible raw material for the production of different pharmaceutical products.. Thus, the extract was characterized by chemical methods as uronic acid's determination by the Blumer methods, reductive sugar by the 2,4-dinitrophenols, protein by the Bradfor techniques and the Elson Morgan test. However, the liquid chromatography and physicochemical techniques were used in order to compare the hyaluronic sodium salt present with a reference sponsor, because those methods are the most used as characterization techniques of glucosaminoglycans. The molecular weight determination was obtained by exclusion molecular test using Sephacryl S 400 gel with an media molecular weight around of 500-800 kDa which may be compared to the kinematics viscosity in centistokes measured by a capillary viscometer. Protein and nucleotide determination were done by an absorption spectrophotometry reading at 280 and 254 nm . The results showed that the extract contains hyaluronic acid sodium salt and also small quantities of peptides and other GAG were not present. This product may be used as a reference substance in different pharmaceutical dosage forms.

INTRODUCTION

At the present time, the obtainment of products from natural products is very important all over the world. The human umbilical cord contains an important group of substances, among them, the heteropolysaccharides, present there with a very high biological potential. The hyaluronic acid sodium salt (HA) is one of those heteropolysaccharides and it is a member of a family including aminosaccharides known as glucosaminoglicans (GAG).[1-4] Hyaluronic acid is synthesized in the fibroblasts' membranes, cells of the vascular smooth muscle, epithelial cells, endothelial cells, etc.[5,6] However, it is known that those monopolysaccharides can be isolated from other sources, such as the pleural fluid, synovial liquid and cock's comb.[4]

The hyaluronic acid is interesting due to its multiple applications in different areas, such as Ophthalmology, where a protection of the surface of the tissue is necessary.[1,7-10] In Orthopedics, it is an artificial substitute of the synovial liquid and is successfully used for the periarthritis of the shoulder and the osteoarthritis of the knee.[7,11,12]

The HA is also interesting as a cosmetic for the care of the nails, the skin and the hair. It does not cause any greasy or astringent sensation, and its effect is felt because of the softness and freshness it causes.[7,13]

The hyaluronic acid can be also used as a raw material for preparing other drugs through synthesis,

and in this way, it is known that diclofenac, when combined with HA is a powerful anti-inflammatory and analgesic, specially for the actinic keratosis, the carcinoma of the basal cells and the osteoarthritic pain.[14,15,17,26]

Therefore, the aim of this paper was the characterization of the GAG present in an extract of human umbilical cord and as a raw material for the production of different pharmaceutical products.

MATERIALS AND METHODS

Analysis of the raw material

The raw material used for preparing the extract (human umbilical cord) and after that, for its characterization, is duly tested in order to detect the possible presence of some virus, such as HIV, hepatitis B and hepatitis C, according to the standards approved by the Center for Evaluation and Control of Drugs (CECMED) for the National System for Placenta Collection.[18-20]

Isolation and characterization

A kilogram of umbilical cord, previously washed with A cold ethanol of commercial quality produced in Cuba, was taken and later centrifuged. Those cords were treated with 4 L of sodium chloride solution 0.2 % Panreac pharmaceutical quality and then filtrated. The supernatant obtained is treated with 600 mL of a cetylmethylamonium bromide 1 % quality pa. Readel de Haen and once deproteinized and degreased, then the mucopolysaccharides are precipitated with acetone quality pa also Raedel De Haen.

Characterization methods

Colorimetric techniques

Determination of proteins. The Bradford[21] technique was used and BSA pa V factor of Kohn Readel D Haen was used as a calibration pattern at different concentrations to draw the calibration curve by developing color and reading against a target with water at 612 nm .

Determination of uronic acids

This determination was carried out by using the Blumerck and Ashoe[22] technique based on the reaction of the metahydroxyphenyl with the uronic acids present in the polysaccharides.

Determination of reductive sugars

This determination was carried out by using the 2,4-dinitrophenol (2,4-DNF) technique that allowed the detection of the presence of reductive sugars.[23]

Qualitative determination of *N*-acetylhexosamines

This technique known as Elson Morgan's qualitative assay is used for the identification of *N*-acetylhexosamines present in GAG and mainly the HA.[24]

Chemical physical techniques. Determination of viscosity

It was carried out by using a capillary viscometer Schott Gerate Gerate Model from Germany. The sample tested is resuspended 1 % by using a solution containing 8.166 1 g NaCl (Readel De Haen) quality, 0.400 3 g $Na_2HPO_4 \cdot 7H_2O$ and 0.044 55 g $NaH_2PO_4 \cdot H_2O$, both from the same commercial firm and similar quality solved in demineralized water.

Gel filtration chromatography. Determination of the molecular weight

The AH molecular weight was determined by using molecular exclusion chromatography, filtration type through gel,[25-27] A (32X1) cm column, packed with Sephacryl S 400 (Pharmacia) was used. This column was balanced for 48 h by using a buffer pH 7.5 containing Na_2HPO_4 pa (Readel De Haen) at a concentration of 0.05 mol/L, in 0.1 mol/L NaCl and 0.02 % sodium azide from the same commercial firm at a 0.6 mL/min flow. The column was balanced with Dextran Blue, $2 \cdot 10^6$ molecular weight (Pharmacia) at 0.028 mg/mL to assess the dead volume (Vo). Moreover, polysaccharide standards of different molecular weights (Readel de Haen) were used in order to find a correlation equation between the MW log of each standard and the Kav constant obtained for each standard to find the molecular weight both for the sample being tested and the H A standard. Those standards are eluted from the column and detected at different retention times at 620 nm after the color development by the sulphuric anthrone technique.[28]

Reversed phase chromatography

A Pharmacia HPLC equipment with a variable wave length detection system and a data processing method for calculating the retention times and the area under the curve. The chromatographic conditions were the following:

Flow 1 mL / min .

Paper speed 0.2 cm/min .

Detection interval 0.01 AUFS.

Detector output 1 000 mV .

Wave length (λ) 229 nm .

Concentration of the sample 2.5 μg/μL .

Injected volume 100 μL .

Elution method: Gradient formed 0-60 % B in 50 min 60 % B for 10 min and 100 - 0 % B in 10 min .

Type of column Supercosil LC 318.

The system was formed by a gradient with a buffer A NaH_2PO_4 20 mmol/L in NaCl 150 mmol/L pH 6.5 and an acetonitrile organic modifier B Raedel De Haen quality. This technique is used for separating polysaccharides from the peptides present in the synovial fluid.[29]

Ion exchange chromatography

Ion exchange chromatography is usually used to separate HA from other polysaccharides that could be present and to identify them mainly because of the difference in ionic force among HA and other possible polysaccharides like SCh and Sh, etc. For this test it was used anionic exchange liquid chromatography by using a high-resolution monodispersed pre-packed column (5X50) mm Mono Q HR 5/5 at a flow of 1 mL/min and a gradient formed by a phosphate A buffer 50 mmol/L pH 7 and a B buffer formed by A + NaCl solution (mol/L). Five hundred microliters of the standard and the experimental sample were injected at a concentration of 0.3 and 2.8 μg/μL, respectively.[30] The eluted fractions of the column were detected at 229 nm .

For the qualitative and quantitative determination of the quantity of HA (%), a method was used that takes into account the area under the curve of every peak (Aj) and the total area of the ΣAi according to the expression:

$$HA = (Ai / \Sigma Ai) \cdot 100 \quad (\%)$$

compared to a similar chromatogram by using the same standard above mentioned.

Absorbance analysis at UV Light

Spectroscopy is used to show the number of present nucleotides and proteins. The solution used for measurement was prepared at 1 % in a NaCl solution 0.15 mol/L . The absorbance is measured at 257 nm where the levels of nucleotides in solution, whose value reported in the literature should be < 3.[7]

The solution absorbance measured at 230 nm is used to assess the concentration of proteins, mainly

associated to aromatic amino acids. Those levels should be < 2.[7]

RESULTS AND DISCUSSION

The values obtained in triplicate from the analysis of the experimental sample (Table 1), regarding proteins, uronic acids, reductive sugars, viscosity, the PM and the UV analysis at 280 and 257 nm are shown, both for the sample tested and the HA standard.

According to the results obtained (Table 1), the reductive sugars have an average value of 3 % and an average content of uronic acids of 59.3 ppm whose presence and quantity are an important indicator characterizing those types of polysaccharides.

Protein concentrations found by the Bradfor method equal to 0 and absorbance values at 280 nm (< 1) are shown in Table 1. This was not contradictory because the colorimetric method used (Bradfor Technique) is not sensitive enough to detect the small quantities left after the product has been deproteinized. The assay at 280 and 257 was established by Balazs[7] in 1993 as a control of the finished product.

The positive results obtained from the Elson Morgan test are an indicator of the presence of *N*-acetylhexosamines in this type of polysaccharide.

With regards to the viscosity analyses, the molecular weight and the UV analysis, values very similar between the sample obtained and the reference standard are observed. The molecular weight of the experimental sample (500 000) and the observance values obtained are into the reported interval.[7]

According to the bibliography, HA 1 % in a of physiological buffer solution should behave as a viscoelastic fluid to be able to be used in Ophthalmology and Orthopedics. In the case discussed, the isolated product in the laboratory behaves very similarly to the standard with values > 1 000 cSt .

In both system chromatograms (Fig. 1), the polysaccharides elute at the beginning with low retention times and small peaks appear due to the presence of peptides not sensitive to the Bradfor technique, but yes at 229 like one observes with the same retention time for the standard A and the product obtained in the laboratory (Table 2).

The results observed in the standard are not unusual, because as it is known that the most part of the commercial hyaluronic acid known through he information obtained from different bibliographic sources, like the Fluka or the Sigma catalogues can contain a peptide concentration of < 5 %.

In the case of the ion exchange chromatography (Fig. 2) the peaks related to the same former peptides and a peak characteristic of the hyaluronic acid sodium salt can be seen and no other peak is seen.

The retention times appear reported according to the order of appearance (Table 3). In this table, the retention times for the tested sample and the standard, and the areas under the curve of each peak and the sum of all the areas are shown.

Table 1. Analytic results corresponding to the experimental sample and the standard hyaluronic acid.

Assay	Sample in assay	Standard hyaluronic acid
Reductive sugars (%)	3	3
Uronic acid (ppm)	60.5	58.0
Proteins (%)	0	0
Viscosity (cSt)	> 1 000	> 1 000
UV analysis		
289 nm	< 1 (0.190)	< 2 (0.198)
257 nm	< 1 (0.243)	< 3 (0.250)
Molecular weight (Dalton)	500 000	750 000

Table 2. Results of the reversed-phase chromatography. Comparison of standard with the sample tested.

Sample	Retention time		
	1	2	3
Standard (A)	2	27.0	27.5
Tested (B)	2	27.3	29.4

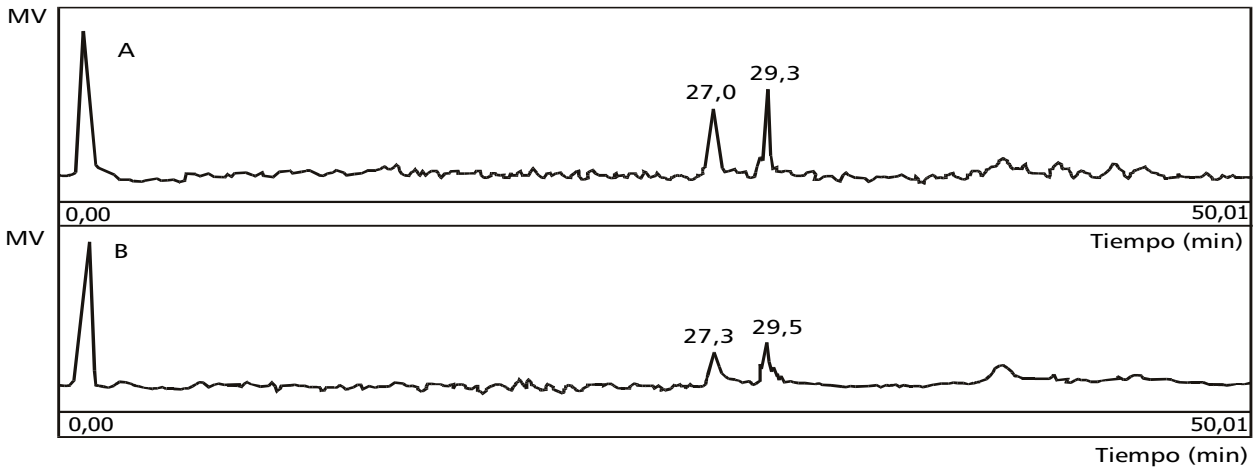

***Fig. 1**. System of solvent in reversed phase. Buffer 1 NaH_2PO_4 20 mmol/L in NaCl 150 mmol/L pH 6.5 and acetonitrile organic modifier.*

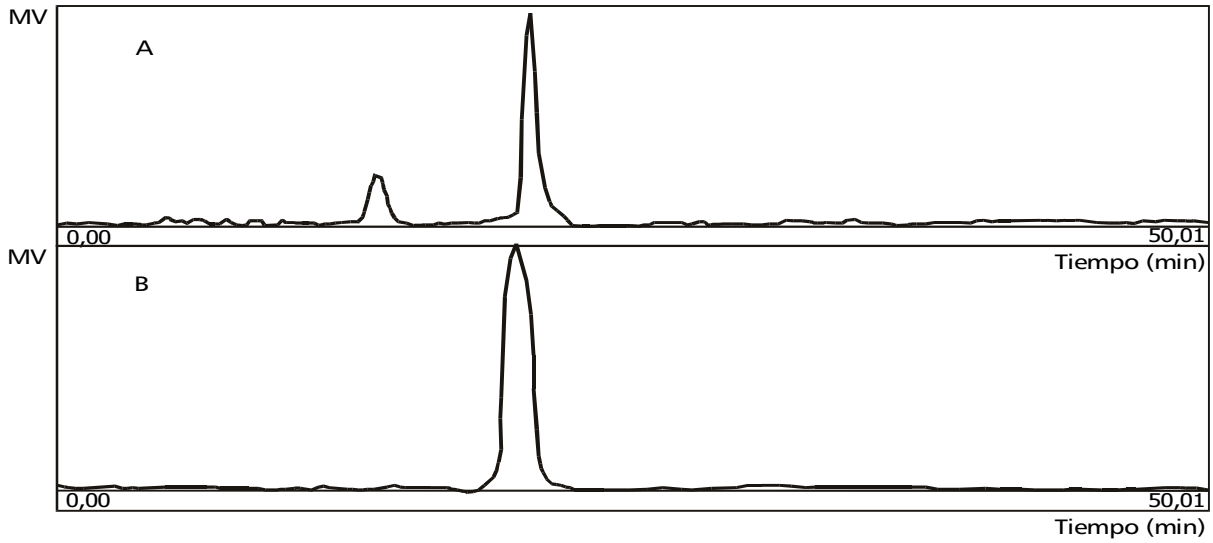

***Fig. 2**. System of gradient by ionic exchange Buffer 1: Phosphate at 50 mmol/L pH 7 and buffer 2: NaCl solution 1 mol/L .*

Table 3. Results of ion exchange chromatography.

Sample	Retention time		Area		ΣA	HA
	1	2	1	2		(%)
Tested	14.53	19.70	2.41	384.13	386.5	99.38
Standard	13.92	20.44	19.96	72.15	92.1	78.34

Both for the standard and the tested sample obtained in the laboratory, a peak appeared 14 min that must be associated to peptides and a high intensity peak associated to the hyaluronic acid sodium salt around 20 min .

Considering the areas of the peaks integrated and the total area, the conclusion is reached that HA % in the standard is 78 and the one of the tested sample 99 and no peak was found from other polysaccharides, at least with this elution conditions.

This ion exchange technique can be interesting as a routine control for HA analysis in extracts of umbilical cord used as drugs or cosmetics. That is why it is suggested that their validation must be properly assessed.

CONCLUSIONS

The ion exchange technique can be used as a control technique for those products after their validation is performed.

If the experimental sample obtained in the laboratory is compared to the standard sample it can be used as internal standard for the production of drugs or cosmetics by using this product as active principle.

The product obtained in the laboratory has a molecular weight (500 000) found into the interval of the molecular weights reported for this type of compound.

The reversed-phase chromatography allows to foresee the presence of peptides that cannot be detected by the Bradfor colorimetric method.

The purpose of this paper is reached, at least with the used techniques, although the authors suggest to continue working with this aim.

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