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HYALURONIC ACID DETECTION FROM NATURAL EXTRACT BY DIODE ARRAY-CAPILLARY ELECTROPHORESIS METHODS

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Hyaluronic acid (HA) was separated by capillary electrophoresis (CE) under normal polarity in phosphate buffer pH 7.4. HA-derived monosaccharide obtained after hydrolysis with trifluoroacetic acid and derivatization with 4-aminobenzoic acid ethyl ester were separated by capillary electrophoresis under normal polarity in borate buffer pH 11. Both CE methods are simple and reliable for quantifying of HA in several natural extracts. The sensitivity of the methods (18.6±0.36 μg/mL detection limit for intact HA and 1.09 ±0.07 μg/mL for derivatized monosaccharide) is acceptable for an UV detection and to evaluate the content of HA in connective tissues extracts and, eventually, in cosmetic and pharmaceutical formulations.

INTRODUCTION

Hyaluronic acid (hyaluronan, HA) is an acidic linear non-sulfated glycosaminoglycan (GAG) formed from disaccharide units containing *N*-acetylglcosamine and glucuronic acid, generally found in animal tissues. Hyaluronan has molecular weight usually in the order of 10^5–10^7 Da[1] and has many biological roles, some requiring its presence in small quantities (*e.g.,* as a proteoglycan organizer in cartilage) whereas in others, it is the most important structural/functional entity (*e.g.,* its presence in vitreous, synovial fluid, ovarian follicles or skin)[2-5]. It is used as a diagnostic factor for many diseases such as tumours, rheumatoid arthritis and liver diseases, in ophthalmology and in skin care.[6-10] HA is highly hygroscopic, biocompatible and biodegradable biopolymer, very attractive for biomaterials fabrication. It is intensively used in cosmetics, surgery and drug delivery.[11-13]

Because it is expensive, it was replaced by chitosan or associated with chitosan and collagen. Recently, the interest for HA rose again being used intensively, especially in cosmetic formulation for its anti-aging properties (hydrating and radical scavenger properties).

The aim of this work is to evaluate the content of HA in some extracts from bovine or swine vitreous using capillary electrophoresis (CE) methods. Other analytical methods were former used, such carbazole/orcinol reaction for quantitative assay of glucuronic acid and high performance liquid chromatography (HPLC) with UV detection for quantification of derivatized N-acetylglucosamine.[14]

CE is an attractive separating technique for HA, or other GAGs because their negative charge assures the resolving power even in the presence of other contaminants and HA could be detected intact or hydrolyzed. CE also offers a high separation efficiency, short analysis time, low consumption of materials and automated and reproducibility of analysis.[15]

An attempt was made to find a cheaper and simple method regarding the equipment and experimental protocol, but, in the same time, with a

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good sensitivity and reproducibility for determination of HA from natural extracts or cosmetic formulations. Two methods were tested, one for intact HA which allows us to evaluate molecular mass, and other for hydrolyzed GAGs (or other polysaccharides). These methods are useful for a suitable chemical evaluation of some extracts and to utilize them in biomaterials fabrication (wound dressing, drug delivery etc). In addition, the second method could be used for monosaccharide content evaluation in other extracts (plants, yeasts etc).

RESULTS

Detection of intact HA

Using standard HA solution (conc. 20-800 μg/mL) the following calibration curve was obtained by CE separation of standard HA (rooster comb, Fig. 1):

$$A = 0.4459c - 8.2951;\ R^2 = 0.9975;$$
$$\text{LoD} = 18.6 \pm 0.36\ \mu g/mL$$

where A = peak area, c = concentration of HA, and LoD = detection limit; the limit of detection was defined as the concentration resulting from a signal to noise ratio of 3.

1. Detection of hydrolyzed HA

Table 1 summarises the main characteristics of the CE separation of ABEE derivatized carbohydrates and Fig. 2 contains the electropherogram illustrating the separation of five derivatized monosaccharide in 15 minutes. The derivatization reagent, ABEE is neutral at BGE pH and the excess of reagent is well separated from the derivatized monosaccharide peaks, which are very well differentiated (Fig. 2).

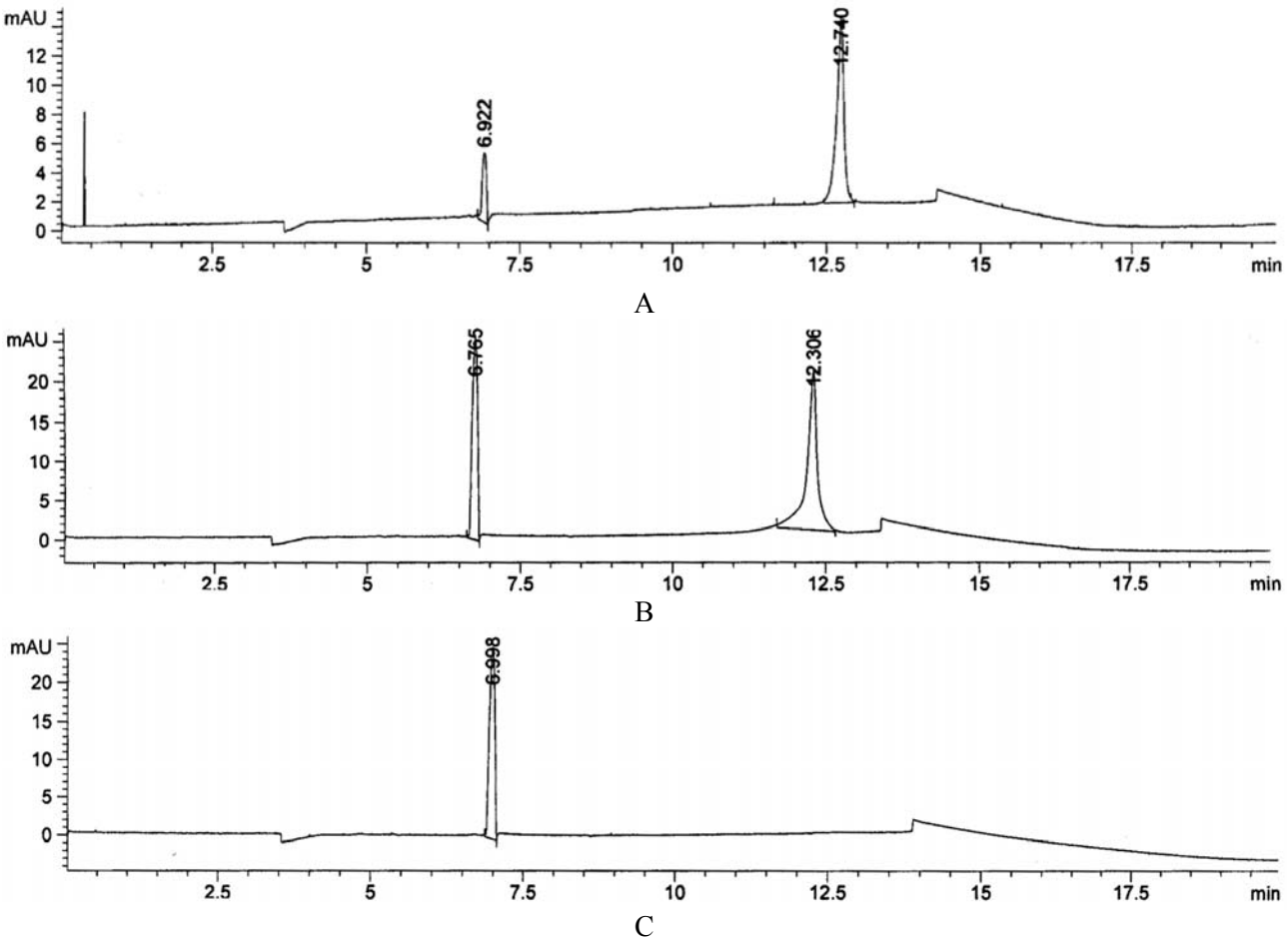

Fig. 1 – Electropherogram for standard HA, 300 μg/mL (A) and 800 μg/mL (B) and for HA sample (our extract from bovine vitreous) (C).

The separation of derivatized glucuronic acid and N-acetyl glucosamine from hydrolyzed HA, standard (Sigma) and from our extracts, are given in figures 3 and 4. Several artefact peaks appeared in the vicinity of N-acetyl glucosamine, probably due to degradation products. The recovery rates of standard HA are between 89 and 92%, and the quantification of HA in our extracts based on calibration curves of glucuronic acid and N-acetyl glucosamine show HA content between 72 and 75%.

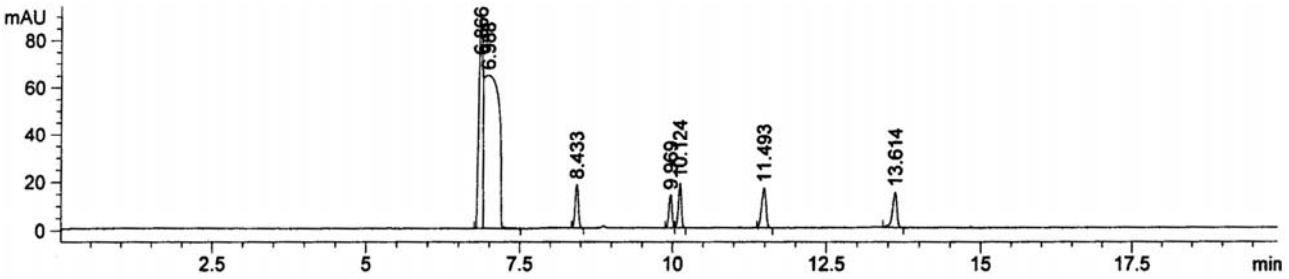

Fig. 2 – Electropherogram of mixed derivatized standard monosaccharides; 6.966 – derivatization reagent excces; 8.433-2-deoxy D-ribose; 9.969-N-acetyl D-glucosamine; 10.124-D-glucose; 11.493-D-galactose; 13.614-D-glucuronic acid.

Table 1

The main characteristics of the ABEE derivatization method

Compound	**Calibration curve equation**	**R^2**	**LoD (μg/mL)**	**LoQ (μg/mL)**
N-acetyl D-glucosamine	*A = 2.1371c – 1.7583*	0.9959	0.82 ±0.11	2.74 ±0.21
D-glucose	*A = 3.1372c – 2.7872*	0.9953	0.89 ±0.12	2.96 ±0.17
D-galactose	*A = 3.8645c – 3.2642*	0.9954	0.84 ±0.05	2.81 ±0.09
D-glucuronic acid	*A = 3.6804c – 4.0266*	0.9976	1.09 ±0.07	3.64 ±0.12
2-deoxy D-ribose (IS)	*A = 2.8494c – 2.3391*	0.9959	0.82 ±0.02	2.73 ±0.23

LoD=detection limit; LoQ= quantification limit (signal/noise ratio of 3)

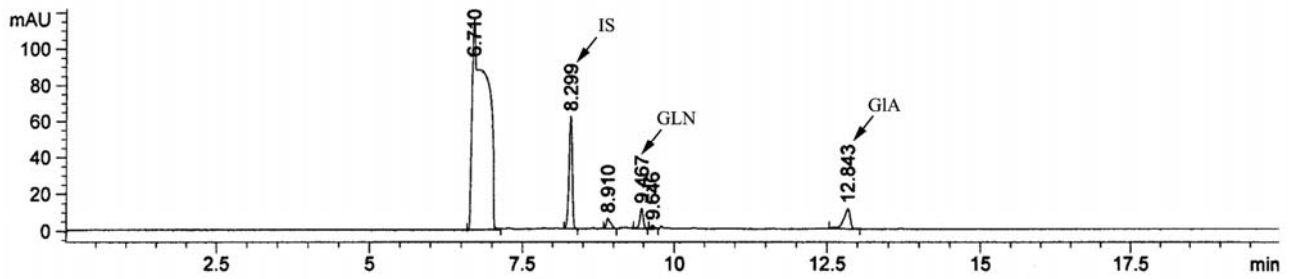

Fig. 3 – Electropherogram of standard hydrolyzed HA (rooster comb); 6.710 –derivatization reagent excces; 8.299-2-deoxy D-ribose; 9.467-N-acetyl D-glucosamine (NAc-GLN); 12.843-D-glucuronic acid (GlA).

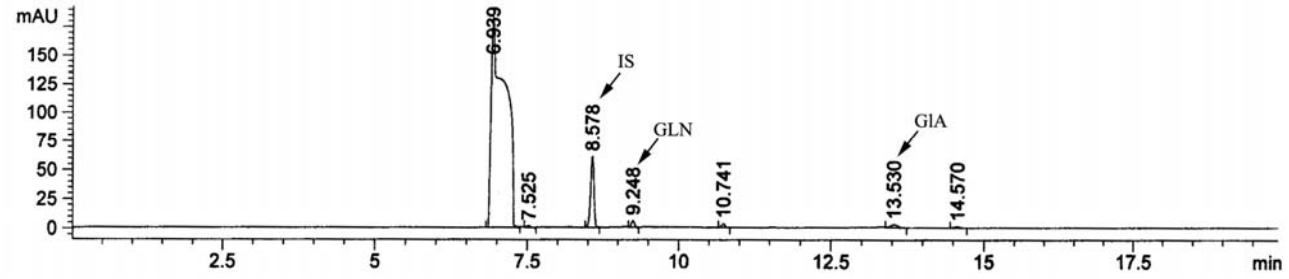

Fig. 4 – Electropherogram of hydrolyzed HA sample (our extract from bovine vitreous).

DISCUSSION

The electropherograms for intact standard HA (rooster comb, Figs. 1A and 1B) are very interesting because two peaks were obtained, the first at 6.7-6.9 minutes and the second at 12.3-12.7 minutes. Other research works in similar conditions[16-17] obtained a single peak for HA. In our case, the first peak appears only at concentrations higher than 100μg/mL HA, and the second is representative and appear for the beginning (from 20 μg/mL). The similar results were obtained with HA from umbilical cords.

Our samples present a single peak, at any solution concentration, at 6.8-6.9 minutes (Fig. 1C). These results confirm our previous studies[14] regarding the molecular mass of HA extracted by us from bovine or swine vitreous. Comercial hyaluronic acid (Sigma) has a major fraction with high molecular mass (not mentioned, but probably around of 10^6 Da) and a smaller fraction with medium molecular mass (probably around $2x10^5$ Da). Our extracted HA has only the fraction with medium molecular mass. The quantification of HA in our extracts based on calibration curve show a content between 75 and 78 % in all the samples.

The results are appropriate because they reveal a high content of HA in our extracts, and the medium molecular mass is suitable for its use in biomaterials fabrication (wound dressing hydrogels or other cosmetic formulations). [18-19]

The second method is more complicated because implies derivatization procedure but has the advantage to be suitable for other glycosaminoglycans or polysaccharides. Capillary electrophoresis with DAD detection is not a very sensitive procedure, but for our purposes is very suitable, rapid (~ 15 minutes for a run), very low consumption of reagents (order of mL) and reliable. For this stage of our needs, the methods did not need validation.

EXPERIMENTAL

HA (from rooster comb and from human umbilical cords), benzocaine (ABEE/4-aminobenzoic acid ethyl ester), D-glucuronic acid and N-acetyl D-glucosamine were purchased from Sigma, reagent grade di-potassium hydrogen phosphate and potassium di-hydrogen phosphate were supplied from Riedel-de Haën, sodium cyanoborohydride (CBH) from Aldrich and D-glucose, D-galactose and 2-deoxy D-ribose were supplied from Merck.

Our extraction method of partially purified-HA, essentially that of Danishefsky, 1966[20] consists in extraction of HA from bovine or swine vitreous with sodium chloride solution, and precipitation with cetylpiridinium chloride and ethanol.[14]

CE detection of intact HA

An Agilent CE system with diode array detector (190-600 nm) was used. Fused-silica capillaries HPCE standard, 50 μm id, 72 cm length were obtained from Agilent Technologies. The capillaries were preconditioned for 15 min with 1M sodium hydroxide before first run. The separations conditions were: +20 kV voltages, 30°C capillary temperature, 15 min migration time and the detection set to 193 and 195 nm. The preconditioning conditions were: before each run, flush the capillary for 7 minutes with 0.1 N sodium hydroxide, water for 3 minutes, and background electrolyte (BGE for 7 minutes. After each run the capillary was flushed for 7 minutes with background electrolyte (BGE).

The background electrolytes used were: 40 mM di-sodium hydrogen phosphate, 40 mM sodium dodecyl sulphate and 10 mM sodium tetraborate, pH 9[16] and 20 mM reagent grade di-potassium hydrogen phosphate and potassium di-hydrogen phosphate, pH 7.4.[17]

The second procedure, with 20 mM phosphate buffer pH 7.4 turned out to be most suitable with regard to peak shape and analysis time and was used in further experiments. All buffer solutions were filtered through membrane filters of 0.45 μm.

HA extracts from bovine vitreous humour[14] and standard HA solutions were prepared by dissolving 1 mg/mL HA in sodium chloride 0.4 M. All the experiments were made in triplicates.

CE detection of hydrolyzed HA

The same Agilent CE system and fused-silica capillaries were used. Carbohydrates derivatized with 4-aminobenzoic acid ethyl ester were separated by CE with an alkaline borate BGE.[21] Briefly, the derivatization procedure is as folows: just before use, dissolve 1 mg CBH in 100 μL of 10% (w/v) ABEE disolved in 10% acetic acid in methanol; 49 μL of this solution is mixed with 1 μL sugar standard (100 mg/mL in water) and heat for 15-20 min at 80°C. After completion of the reaction methanol is added to the total volume of 2 mL.

Background electrolyte used (after many trials) was 200 mM borate buffer, pH 11. The separations conditions were: +25 kV voltages, 30°C capillary temperature, 20 min migration time and the detection set to 306 nm. The preconditioning conditions were: before each run, flush the capillary for 7 minutes with 0.1 N sodium hydroxide, water for 3 minutes, and BGE for 7 minutes. After each run the capillary was flushed for 7 minutes with BGE. The resulting peak areas are average values from three consecutive electropherograms.

Commercials HA and our HA extracts were hydrolyzed with 3M trifluoroacetic acid (TFA) (20 mg HA in 2 mL 3 M TFA) for 6 hours at 100°C and derivatized as above. An internal standard (IS, 2-deoxy-D-ribose) was added in each sample.

CONCLUSIONS

Two simple and reliable CE methods to determine of HA from extracts were applied and

optimized according to our experimental conditions. The sensitivity of the methods (18.6±0.36 μg/mL detection limit for intact HA and 1.09±0.07 μg/mL for derivatized monosaccharide) is in the range for an UV detection and appropriate for actual and further purposes, to evaluate the content of HA in other connective tissues extracts and, eventually in cosmetic and pharmaceutical formulations. In our opinion, the method for determination of intact HA is more convenient than HPLC or CE methods which involve derivatization procedures, because is very simple, rapid and economical. In addition, some information about the hydrodynamic volume of the molecule is obtaining, this fact being important for further utilization of the extracted HA. Recently, an elaborated agarose-gel electrophoresis method was reported for molecular mass calculation of HA.[22, 23]

The second CE method is appropriate in the case that other glycosaminoglycans are present beside HA in the extracts (e.g. chondroitin sulphate, data not shown) and could be applied to a large range of polysaccharides extracted from natural sources. Both methods proved a HA content in our natural extracts between 72 and 78%.

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