TRITIUM LABELLED HYALURONIC ACID DERIVATIVE

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SUMMARY

High molecular weight hyaluronic acid derivative was prepared in a radioactive form, labelled with tritium.

The mucopolysaccharide was oxidized under mild conditions by sodium periodate. After purification the oxidation product was reduced with $[^3H]NaBH_4$. The tritium labelled hyaluronic acid derivative was obtained with a high specific radioactivity (0.15 mCi/mg) and was found to have similar physical and chemical characteristics (high molecular weight, infrared spectrum and electrophoretical behaviour) to the parent compound. The radiochemical purity of labelled hyaluronic acid derivative was found to be greater than 95%.

INTRODUCTION

Hyaluronic acid is an unbranched polysaccharide consisting of regularly alternating beta-D-glucuronic acid and 2-acetamido-2-deoxy-beta-D-glucose (N-acetylglucosamine) residues.

Several methods have been published for labelling hyaluronic acid, but a still unsolved problem is the preparation of high molecular weight and high specific activity hyaluronic acid.

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Tritium labelling by the Wilzbach technique (1) gave rise to hyaluronic acid with a high specific activity (0.1 mCi/mg), but much of the tritium was associated with hydroxyl groups leading to undesiderable radiolysis with consequent depolymerization of the molecule (2,3). The preparation of N-[^{3}H]acetyl-hyaluronic acid (4) seems to be a quite complex method and very little information is available on the stability of labelled hyaluronic acid. Oligosaccharides from hyaluronic acid have been recently labelled by preparing ^{125}I-derivatives (5).

Biosynthetic methods, in which hyaluronic acid is biosynthesized from a radioactive precursor (glucose or N-acetylglucosamine) added to cell or tissue cultures, while providing a high molecular weight product, does not generally appear to be suitable in terms of either significant radiochemical yield or of specific radioactivity (6,7,8,9). A method providing high molecular weight [^{3}H]hyaluronic acid with a high specific radioactivity (10) has been described, but it does not seem to satisfy the requisites of low cost and easy applicability, due to the requirement of appropriate cell cultures.

The purpose of this work is to set up a new procedure for radioactive labelling of hyaluronic acid derivative fulfilling the following requirements: a) easy applicability and low cost; b) no significant modifications to the molecular weight; c) high specific radioactivity; d) good yield.

The approach used consists of a controlled periodate oxidation of hyaluronic acid, followed by reduction with [^{3}H]-$NaBH_4$.

Periodate has generally been used to oxidize vicinal hydroxyl groups in carbohydrates to aldehydes (11,12), which may be reduced by $NaBH_4$. As hyaluronic acid is a molecule consisting of regularly alternating glucuronic acid and N-acetylglucosamine, the only carbohydrates suitable to be oxidized by periodate are the glucuronic acid moieties.

This study describes the best conditions for a mild oxidation of hyaluronic acid by periodate, followed by reduction of the product with tritiated $NaBH_4$.

EXPERIMENTAL

Hyaluronic acid (sodium salt) used for this study was kindly supplied by Fidia Research Laboratories (Italy). Sodium [^{3}H]-borohydride (5 Ci/mmol) was purchased from Amersham (England).

Periodate oxidation

The optimal conditions (time and temperature of the reaction; stoichiometry of the reagents) for the oxidation of hyaluronic acid in several glucuronic acid moieties were determined in preliminary experiments. The course of the reaction was assessed by following the disappearance of periodate in function of time by recording the decrease of optical density at 223 nm.

The final procedure was as follows: 1 ml of a 1 M sodium periodate solution was added under vigorous stirring to a 100 ml aqueous solution of high molecular weight (MW=500,000) hyaluronic acid (sodium salt, 74 mg/100 ml), previously heated at 80° C. After 90 sec the reaction was stopped by cooling to 4°C

and adding an excess of glycerol (1 ml).

Oxidized hyaluronic acid was purified from low molecular weight reaction products by ultrafiltration at a pressure of 2 Kg/cm^2 using an Amicon ultrafiltration cell model 12, equipped with an Amicon Diaflo XM 300 membrane. Molecules with a molecular weight smaller than 300,000 daltons were not retained. Unfiltered high molecular weight oxidized hyaluronic acid was recovered by several washings with phosphate buffer 10 mM, pH=7.4 of the upper section of the filtration cell, dialyzed overnight against the same buffer and then against distilled water and finally liophylized (final recovery: 63 mg).

Tritium labelling of a hyaluronic acid derivative

100 mCi of $[^3H]NaBH_4$ (0.5 mg) were added to 50 mg of oxidized hyaluronic acid solubilized in 50 ml of phosphate buffer 10 mM, pH=7.4. The vial containing the radioactive material was washed with 0.5 ml of the same buffer and the washing added to the mixture. The solution was continuously stirred at room temperature for 20 min and then 50 mg of unlabelled $NaBH_4$ were added in order to achieve complete reduction. The mixture was maintained at room temperature for an additional 20 min. At the end of the reaction, the solution containing the labelled hyaluronic acid derivative was dialyzed overnight under a constant flow of distilled water, in order to eliminate any excess of tritium, and finally dried several times under vacuum, to eliminate tritiated water. The recovered product (47.2 mg) was solubilized in 10 ml of 10 mM phosphate buffer, pH=7.4, sterilized by Amicon Millex filtration and stored at 0-4°C.

Analytical methods

The homogeneity of the labelled hyaluronic acid derivative was determined by paper electrophoresis, under the condition specified below. The radiochemical purity of the labelled hyaluronic acid derivative was determined by automatic radioscanning of the electrophoretical strips using a Berthold TLC Linear Analizer mod 282, equipped with an Apple II Europlus data system. The specific radioactivity of radiochemically pure hyaluronic acid derivative was determined by assaying radioactivity in a liquid scintillation counter (Packard 460D) and by evaluating the hyaluronic acid derivative content by means of the colorimetric method of Britter and Muir (13).

Paper electrophoresis was performed on Elvi apparatus, with Gelman Sepraphore III cellulose acetate 8x3 cm stripes, sodium barbital buffer 0.025 M, pH=8.8, at 300 V (1.5 mA/strip) for 45 min. Alcian Blue 1 g/l in HCl 0.01 M was the staining solution and HCl 0.01 M the destaining solution.

Infrared spectra of the hyaluronic acid derivative were carried out by a Perkin-Elmer infrared spectrophotometer mod 398 using the method of solid suspension in KBr. Data were processed by a Perkin-Elmer data station mod. 3600.

RESULTS

The course of the oxidative reaction of hyaluronic acid by periodate, at a final concentration of 10 mM was monitored, as described in the experimental section, by recording the decrease of optical density at 223 nm, at different temperatures and times, as shown in tab. 1.

TAB 1 – Time course of oxidation reaction at different temperatures. Values are expressed as relative optical density x 100

	60"	90"	2'	3'	4'	5'	10'	15'
4°C	100	100	100	100	100	100	100	100
25°C	100	100	100	100	100	100	100	100
50°C	98	98	97	97	96	95	94	93
80°C	91	88	87	85	83	80	68	55

The optimal temperature allowing significant consumption of periodate was found to be 80°C. Under these experimental conditions, an "over-oxidation" of hyaluronic acid with consequent formation of low (smaller than 300,000 MW) molecular weight fragments was observed (12). Tab. 2 presents the recovery of high molecular weight hyaluronic acid derivative (MW higher than 300,000) after periodate oxidation at 80°C for increasing time.

TAB 2 – Percentage of high molecular weight hyaluronic acid derivative recovered after oxidation at 80°C for increasing time.

0'	1'	90"	120"	3'	4'	5'	10'	15'
100%	90%	85%	80%	65%	45%	25%	8%	5%

On the basis of these preliminary experiments we evaluated the optimal conditions for hyaluronic acid oxidation, with consequent preservation of integrity of the original molecule to

be as follows: temperature: 80°C; time of the reaction: 90 sec.

The presence of aldheydic groups due to oxidative treatment was shown by infrared spectroscopy. In fact, amplified differential spectra between native and oxidized hyaluronic acid showed in the latter one, a characteristic peak at 1735 cm^{-1}.

By the described labelling procedure, 7 mCi of a tritiated hyaluronic acid derivative, with a specific activity of 0.15 mCi/mg were obtained. The radiolabelled hyaluronic acid derivative displayed an electrophoretical behaviour identical to that of the original compound. More than 96% of the radioactivity present in the fraction was found, as assessed by radiochromatoscanning, to be bound to the electrophoretic spot corresponding to hyaluronic acid, indicating a radiochemical purity of more than 96%.

The infrared spectra of native and labelled hyaluronic acid were found to be pratically identical, revealing that no significant structural changes had occurred in the course of the radiolabelling procedure.

In order to evaluate the amount of radioactivity introduced in the molecule by the reduction of terminal aldehydic group, a sample of native hyaluronic acid was processed in the same way, as reported in labelling procedure, but omitting the periodate oxidation. A very low amount of radioactivity (0.001 mCi/mg) was found to be bound to the polysaccharide molecule.

DISCUSSION

This paper describes a new and easy method of obtaining a labelled hyaluronic acid derivative with a high specific

radioactivity.

Highly tritiated hyaluronic acid derivative (0.15 mCi/mg) in a good yield, with minimal structural modification from the parent compound can be prepared with no need of sophisticated apparatus and a specifically equipped laboratory, as is necessary in the case of biosynthetic methods, which require the availability of cell cultures.

In conclusion, this method, due to its good efficiency, easy applicability and low cost seams to satisfy the requirements necessary to obtain a highly tritiated hyaluronic acid derivative to be employed in biological and pharmacological studies.

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