AN IMPROVED METHOD FOR HYALURONIC ACID RADIOIODINATION

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SUMMARY

A simple procedure is described for preparing ^{125}I-labelling hyaluronan of high molecular weight. The reducing terminal group of hyaluronic acid was derivatized with tyramine through the formation of a Schiff base which was subsequently reduced with sodium cyanoborohydride.

By radioiodination of the aromatic ring, ^{125}I-labelled hyaluronic acid was obtained in high yield (40%) and high specific activity, 555 GBq/mmol (15 Ci/mmol).

Key-words:. *^{125}I, tyramine, glycuronans, hyaluronic acid, labelling*

INTRODUCTION

Hyaluronate is an ionic polysaccharide belonging to the general class of glycosaminoglycans. At physiological pH and ionic strength, each disaccharide unit [(D-glucuronic acid (1-3) N-acetyl- D-glucosamine (1-4)] bears one negative charge. High molecular weight hyaluronate (10^4-10^6 Daltons) plays an important role in the extracellular matrix of connective tissue and in affecting cellular behaviour. Indeed, many studies have suggested that hyaluronic acid interacts with other macromolecules in the extracellular matrix on the cell surface. The extent to which hyaluronate directly or indirectly interacts with cells and mediates subcellular events, leading to changes in cell metabolism and behaviour, may be fundamental to the understanding of developmental and reparative processes, such as wound healing.

A limiting factor for studying the metabolism of hyaluronate has been the unavailability of a suitable method for its labelling. Up to now, only a few biosynthetic or chemical methods for labelling hyaluronate have been described (1,2,3,4,5). Although the method proposed by Wilzbach (1) can produce tritium-labelled hyaluronic acid with high specific activity, 3.7 MBq/mg (0.1

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mCi/mg), most of the radioisotope is associated with the hydroxyl groups, this resulting in a loss of the isotope in aqueous solutions of the product.

Biosynthetic methods (2,4), using radioactive glucose or n-acetylglucosamine have been used to label high molecular weight native hyaluronate. However these labelling techniques are laborious and expensive and yield limited amounts of labelled product.

In previous work (6), we described the synthesis of ^{3}H-hyaluronic acid through a controlled periodate oxidation followed by reduction with NaB^3H_4. Dhal *et al.* synthesized labelled hyaluronic acid fragments by preparing their acetyl-^{125}I-tyrammine-cellobiosio derivatives (7).

Recently (8), we optimized a simple procedure for preparing ^{125}I labelled alginic acid. This method consists of the derivatization of alginic acid with tyramine through reductive amination of the carbonyl terminal and radioiodination of the aromatic ring introduced. High specific activity labelled alginate was obtained in high yield when the coupling reaction was performed at 60°C whereas no reaction occurred at 25°C. This appears to be due to the fact that at 60°C the polymeric structure is sufficiently disentangled to make the terminal aldehydic group of the polymer available to the tyramine. Unfortunately, the same labelling technique could not be applied to the hyaluronate due to its thermal lability.

The present paper describes a new labelling procedure suitable for preparing a radiolabelled derivative of hyaluronan without depolymerization of its structure.

EXPERIMENTAL PROCEDURE

Hyaluronic acid (PM=170.000) was purchased by Fidia Research Laboratories (Abano Terme, Italy). Sodium cyanoborohydride was obtained from Fluka (Buchs, Switzerland). Tyramine hydrochloride was obtained from Merck (Darmstadt, Germany). $Na^{125}I$ specific activity, 77.3 TBq/matom (2090 Ci/matom) was purchased from NEN Dupont (Bad Homburg, Germany).

Derivatization of hyaluronic acid with tyramine

20 mg (0.12 μmol) of hyaluronic acid was dissolved in 5 ml of an aqueous solution of 5% sodium acetate. Tyramine (17 mg, 98 μmol) was added and the mixture was stirred for 24 hours at room temperature. $NaCNBH_3$ (62.5 mg, 980 μmol) was added to the solution which was stirred for an additional hour (Fig.1). The homogeneous mixture obtained was dialysed for 24 hours at room temperature against 5% sodium acetate.

The yield of the reaction was about 30%, as evaluated by the absorbance of the dialysate at 280 nm.

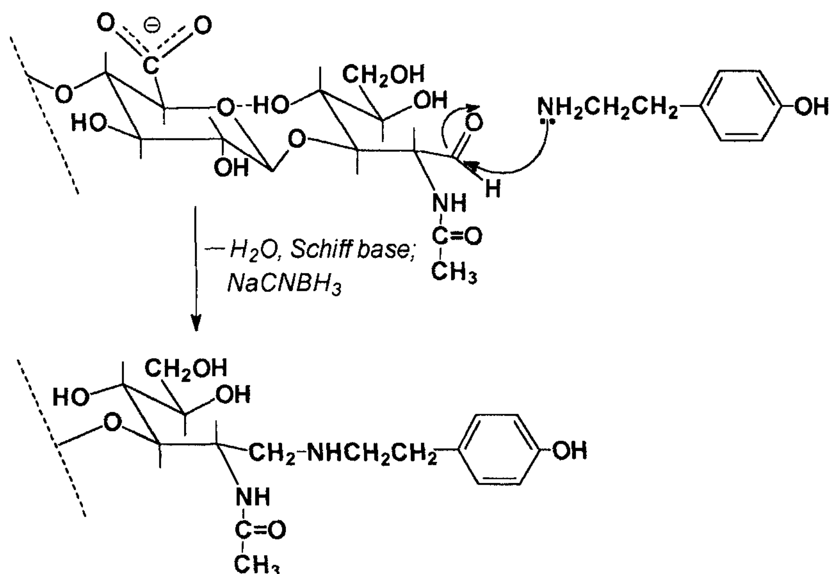

Fig.1 Scheme of the reaction

The final product was applied to a column (1x50 cm) packed with Sephacryl S-400 HR (Pharmacia LKB, Uppsala, Sweden) which was eluted with 10 mM PBS (pH 9.0) at a flow rate of 0.3 ml/min. The derivatized hyaluronic acid was eluted as a single peak.

The retention volume of hyaluronic acid is the same before and after derivatization, as judged by the elution pattern at 205 nm wavelength (Fig.2). This indicates that no fragmentation of the polymer occurs during the reactions needed for its labelling. Moreover, the chromatographic peaks detected at 205 and 280 nm exhibit the same maximum.

Radioiodination of the tyramine-hyaluronate adduct

Hyaluronic acid was coupled with tyramine and purified by dialysis as described above. Iodination was carried out with "carrier-free" $Na^{125}I$, in the presence of 1,3,4,6-tetrachloro-3,6-diphenylglycouril (Iodogen, Pierce Chemical Co., Rockford, IL, U.S.A). A 2 ml glass vial was coated with Iodogen by evaporating, with a gentle flow of nitrogen, 1 ml of a 10 μg/ml Iodogen solution in dichloromethane. The coated vial was repeatedly washed with 5% sodium acetate to remove any loose microscopic flakes of Iodogen. The dialysed solution of tyramine-hyarulonate adduct (1 ml) was transferred into the vial and 37 MBq (1 mCi) of $Na^{125}I$ was added under continuos stirring. The reaction was stirred for 15 min at room temperature and then dialysed at 4°C for 24 hours against 5% sodium acetate.

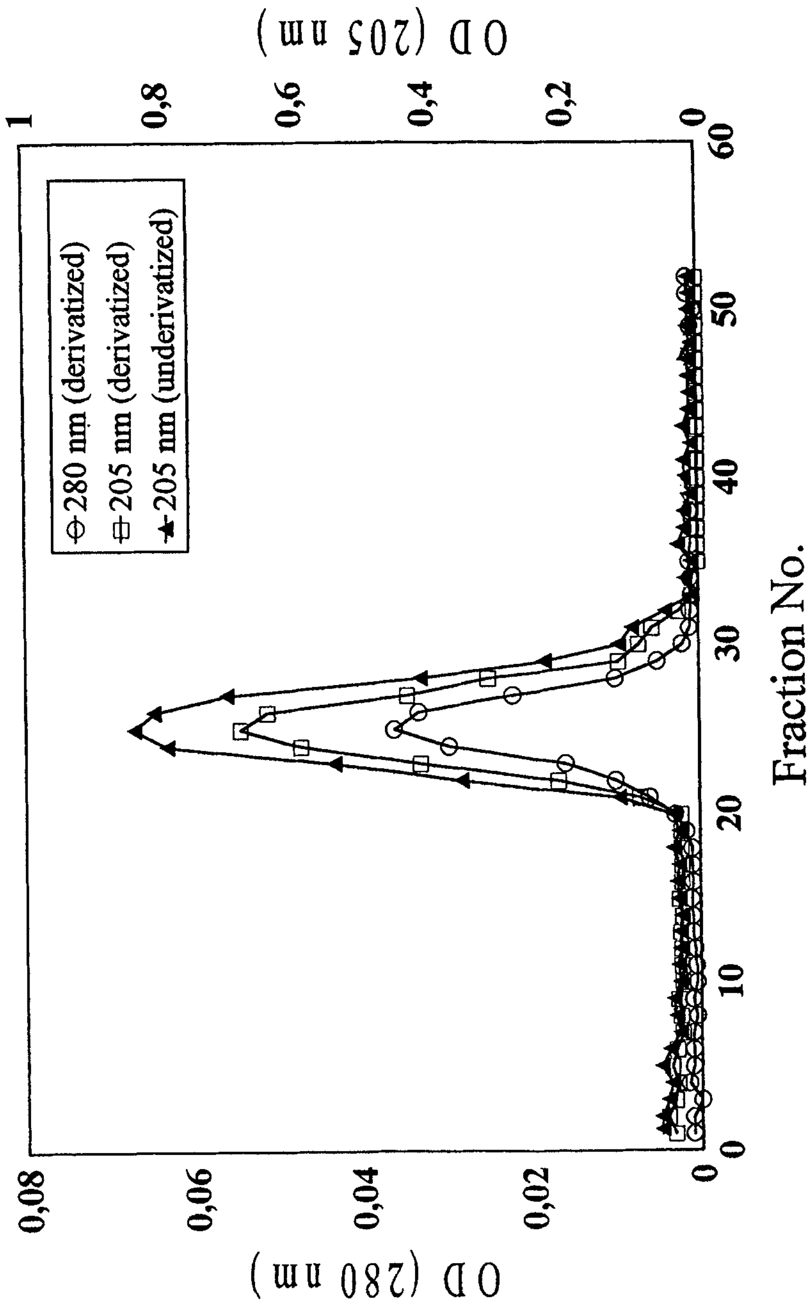

Fig. 2 Comparison of elution patterns of derivatized and underivatized hyaluronic acid.

The specific radioactivity of the product was 555 GBq/mmol (15 Ci/mmol) as assessed by solid phase scintillation counting of a small aliquot of a solution of known concentration. The yield of the labelling procedure was 40%.

The homogeneity and the radiochemical purity of the iodine-labelled derivatives were assessed by thin layer chromatography analysis on silicic acid plates (Merck, Darmstadt, Germany), using a solution of ethyl-acetate/iso-propanol/water (35:43:22 v/v) as developing mixture. The distribution of the radioactivity on the plates was examined by radiochromatoscanning, using a Bioscan System 200 instrument (Canberra Packard, U.S.A). More than 90% of the radioactivity was found on the iodinated hyaluronan spot.

CONCLUSION

This paper describes an easy procedure for preparing a radiolabelled derivative of high molecular weight hyaluronan without depolymerization. The first step of the derivatization procedure involves the reaction with tyramine of the free carbonyl of the glycuronan molecule giving the Schiff base adduct. This reaction is accomplished by using AcONa (5% p/v) as reaction medium. The salt addition, increasing ionic strength of the solution, causes the disentangling of the hyaluronate stiffened molecule, offering a better attack of the tyramine molecule.

Due to the simplicity of the procedure described and to its good yield, this method can be recommended for labelling with high efficiency both glycuronan and high molecular weight polysaccharides with a free aldehydic group .

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