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ISOLATION AND CHARACTERISATION OF HIGH MOLECULAR WEIGHT [^{3}H]HYALURONIC ACID

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SUMMARY

A high-performance gel permeation chromatographic separation method was developed for the isolation and characterisation of high molecular weight [^{3}H]hyaluronic acid. The molecular characteristics of the labelled sample were M_w=3.92x10^{5} Da, M_w/M_n=1.55.

Key words: [^{3}H]Hyaluronic acid, high-performance gel permeation chromatography (HP GPC)

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Hyaluronic acid (HA) is an unbranched high molecular weight polysaccharide which consists of repeated disaccharide units of glucuronate β-1,3-N-acetylglucosamine linked together by β-1,4-bonds. To prepare radioactively labelled HA the label was introduced into the polymer chain by chemical reactions [1,2].

There are several chemical reactions which lead to the depolymerisation of HA/polysaccharides, e.g. hydrolysis in aqueous solutions at acid or alkaline pH [3,4], but also at neutral pH, which is much more pronounced at high temperatures [5,6]. These degradation reactions usually result in polymer with smaller molecular weight averages (M_w, M_v, M_n, ...) [1-6] and often in broadening of sample polymolecularity (M_w/M_n). We experimented with another simple way to prepare labelled hyaluronic acid by its alkylation with [^{3}H]methyl bromide (specific radioactivity 1.5 TBq.mmol^{-1}) in boiling liquid ammonia (at -33.5 ^oC) [7]. The reaction mixture, after evaporation of [^{3}H]CH_3Br and ammonia, was fractionated by a high-performance gel permeation chromatographic (HP GPC) separation method. The high molecular weight fraction was collected and an aliquot was reinjected to determine the molecular characteristics of [^{3}H]hyaluronic acid. Fig. 1 shows the radiochromatographic record of this sample. The molecular weight averages and sample polymolecularity are given in Table I.

To isolate a fraction of [^{3}H]HA with a small M_w/M_n value the same HP GPC arrangement was used and from the injected sample a narrow effluent fraction was analysed by its re-injection. The results of this step are represented in Fig. 1 and Table I.

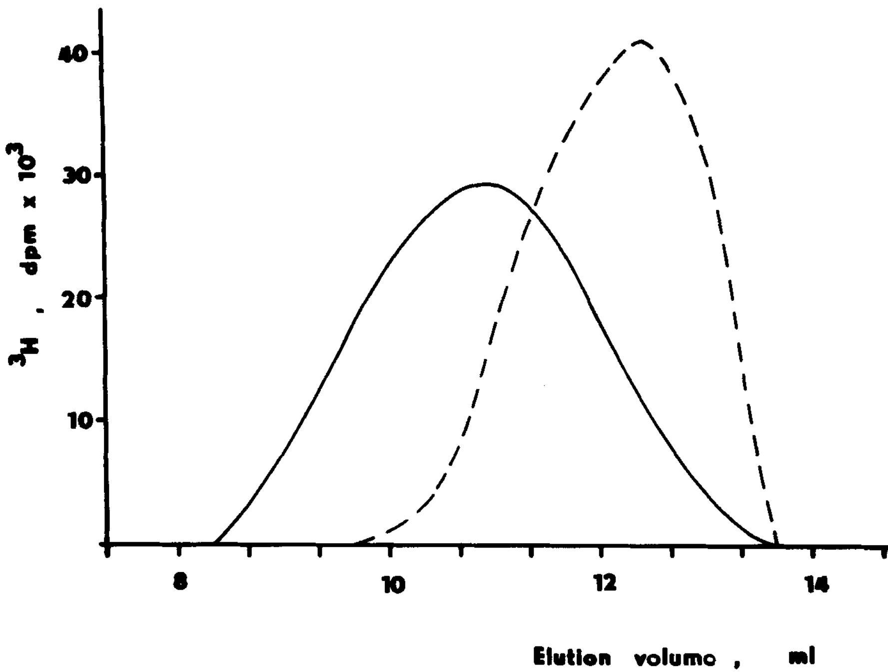

Fig. 1 Radiochromatograms of the [^{3}H]HA (full line) and its isolated fraction (dashed line).

Table I. Molecular characteristics of hyaluronate samples

Sample	M_w (Da)	M_w/M_n
[^{3}H]HA	3.92×10^5	1.55
[^{3}H]HA fraction	9.91×10^4	1.37
Unlabelled HA	6.49×10^5	1.44

Comparison of the obtained data with those for native, unlabelled HA evidently shows that:

a) Tritiation by the above procedure yielded [^{3}H]hyaluronic acid with molecular parameters close to the originally used HA polymer.

b) The high molecular weight [^{3}H]hyaluronic acid with M_w of 3.92×10^5 Da was structurally identical to the native un-

labelled HA, since the specific hyaluronidase enzyme depolymerised both biopolymers with the same kinetics.

c) The HP GPC method provides a powerful tool either for purification of the labelled polymer from tritiated low molecular weight by-products or for fractionation of the [^{3}H]HA to obtain a polysaccharide with the desired molecular characteristics (M_w, M_n, M_w/M_n...).

EXPERIMENTAL

Material

A sample of hyaluronic acid isolated from rooster combs was obtained from MOVIS, Holíč, Czechoslovakia. The [^{3}H]hyaluronic acid was kindly supplied by Dr. J. Filip, Institute for Research, Production and Application of Radioisotopes, Prague, Czechoslovakia.

Method

The high-performance gel permeation chromatographic system used comprises a high pressure pump (HPP 5001, Laboratorní přístroje, Prague, Czechoslovakia), an eight-port switching valve (Model PK 1, Vývojové dílny, Czechoslovak Academy of Sciences, Prague, Czechoslovakia), two stainless-steel columns (250 x 8 mm ID), connected in series, packed with Separon HEMA-S 1000 and Separon HEMA-S 300 (Tessek Ltd., Prague, Czechoslovakia; mean particle size 10 μm) and a differential refractometric detector (RIDK 102, Laboratorní přístroje, Prague, Czechoslovakia).

Chromatographic experiments were carried out at room temperature. The mobile phase was 0.1 M aqueous $NaNO_3$ solution. The flow rate of the eluent, "degassed" by purging it with helium was constant at 0.4 $ml.min^{-1}$. The HP GPC experimental setting was calibrated with hydroxyethylstarch reference materials.

The injected sample volume was 100 µl. Fractions of effluent were collected to determine ^3H radioactivity. Aliquots of 50 µl were mixed with 10 ml of Insta-Gel and measured by liquid scintillation spectrometer Packard 300 CD (Packard Instrument Inc., Downer Grove, IL, USA).

Enzymatic digestion of the hyaluronate samples was carried out by adding 48 IU of the hyaluronidase enzyme EC 3.2.1.35 per 1 mg of the biopolymer and by incubation at 37 °C in 0.067 M phosphate buffer pH 7.4. The molecular weight decrease of the sample with time was selected as indicator of depolymerization kinetics.

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