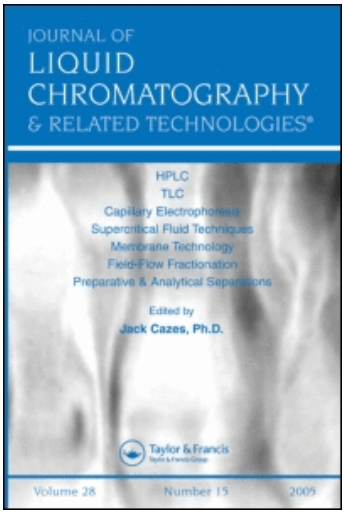

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Orviský, E. , Šoltés, L. , Chabrček, P. , Novák, I. , Kéry, V. , Stančíková, M. and Vinš, I.(1992) 'The Determination of Hyaluronan Molecular Weight Distribution by Means of High-Performance Size Exclusion Chromatography', Journal of Liquid Chromatography & Related Technologies, 15: 18, 3203 — 3218
To link to this Article: DOI: 10.1080/10826079208020879
URL: http://dx.doi.org/10.1080/10826079208020879

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(18), 3203-3218 (1992)

THE DETERMINATION OF HYALURONAN MOLECULAR WEIGHT DISTRIBUTION BY MEANS OF HIGH-PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY

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ABSTRACT

The high-performance liquid chromatographic (HPLC) method relevant to the size exclusion chromatographic mode (SEC) used for the distribution analysis of high-molecular-weight hyaluronans is described. The HPLC fillings used, to which was applied a phosphate buffer (50 mM, pH 7.8) effluent,

* Correspondence

consisted of a cross-linked hydroxyethylmethacrylate derivative gel (HEMA-BIO), which is commercially available, and a highly porous aminopropyl-silica sorbent (SG-10-6000-NH_2), which is not commercially available. For reliable hyaluronans a high-molecular-weight distribution analysis is necessary involving SEC chromatographic columns which must have an extremely high exclusion limit (SG-10-6000-NH_2). Calibration of these columns should be done using hyaluronate reference material.

INTRODUCTION

Hyaluronan (hyaluronate, hyaluronic acid) is a long chain unbranched endogenous polysaccharide consisting of repeating units of glucuronic acid and N-acetylglucosamine (cf.Figure 1). The molecular weight of hyaluronan isolated from the mammalian body ranges from $10^3$to 10^7Da. Various methods such as light scattering[1,2,3], sedimentation analysis[4,5], diffusion rate[6], viscometry[1,6,7], and HPLC[8,9,10] were applied to determine the molecular parameters of this important biopolymer. For HPLC analysis the above cited authors used hydrophilic organic polymer gels as fillings. Motohashi[9,11] used Shodex OHpak B-805 and B-806 column fillings, Beaty[10] used a Toyo Soda TSK G 6000 PW filling; Saari[12,13] used TSK 6000 PW and TSK 5000 PW; while Brown et al[14] used Bio-Gel SEC-60 XL and Waters Micro Bondagel/E-High A. Only satisfactory separation analyses for high-molecular-weight hyaluronan (M_w > 1x10^6 Da) were

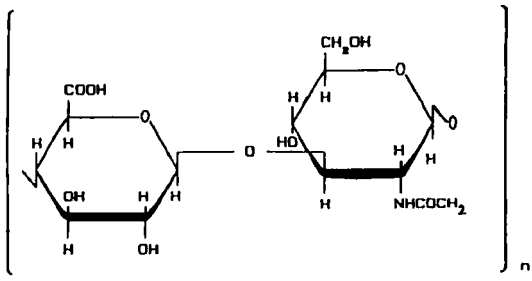

Figure 1. Chemical structure of hyaluronan.

reported on semi-soft gel by Saari and Konttinen[12].To date, the use of rigid gel has not been reported in the literature. Recently, a HPLC technique in the size exclusion chromatographic (SEC) mode was applied to determine the polymer concentration and molecular weight distribution of hyaluronan in synovial fluids[9,12,13].

The aim of the work here presented is to test commercially available columns filled with a semi-soft gel of the HEMA-BIO type and to compare the experimental results with those found by using an aminopropyl-modified highly porous silica gel.

MATERIALS AND METHODS

Materials and chemicals

Anhydrous sodium dihydrogen phosphate and anhydrous di-sodium hydrogen phosphate of analytical grade were purchased from Serva, Germany.

Calibration Standards

Dextran standards with various M_w ($2x10^6$, $5x10^5$, $2.5x10^5$ and $1.5x10^5$ Da) were purchased from TESSEK, Ltd., 110 01 Prague, CSFR.

Reference materials

Hyaluronan reference samples (M_w = $3x10^6$, $1.6x10^6$, $0.81x10^6$, $0.49x10^6$ and $0.39x10^6$ Da) were kindly provided by Dr. Ove Wik (Uppsala, Pharmacia, Sweden). Samples were diluted with an effluent used for SEC and injected into the HPSEC system at a concentration of 0.03 % (w/v).

Biological samples

Synovial effusions were taken from the knee joint of rheumatic patients under sterile conditions. The fluids were immediately centrifugated at 10000 g for 10 min at 8 °C in order to separate the cells. The fluids were subsequently frozen and kept at -30 °C until analysis. For HPSEC analysis the cleansed synovial fluid was diluted 10 times using an elution buffer, then filtered to remove debris, and injected into the HPSEC system.

Chromatography

The HPLC apparatus employed was the System Gold 126 Programmable Solvent Module with an 166 Programmable Detector Module adjusted at 206 nm, whose sensitivity was mostly at 0.1 AUFS (BECKMAN, USA). The injected sample volume was 20 µl. The elution buffer was 0.05 M sodium

hydrogen phosphate, with a pH value of 7.8. The flow rate was set at 0.5 ml/min. All chromatographic experiments were carried out at room temperature. The columns used were of stainless steel (8x250 mm I.D.). Each column was filled with HEMA-BIO 300, 10 μm, or HEMA-BIO 1000, 10 μm (TESSEK Ltd., Prague, CSFR-Denmark), or with the silica gel derivative SG-10-6000-NH_2, 9 μm (CS-Patent pending).

RESULTS AND DISCUSSION

In the present study we focused our attention on testing some SEC column fillings as to their suitability for hyaluronate separation. Two fillings, namely the semi-soft gel HEMA-BIO 300, 10 μm, and HEMA-BIO 1000, 10 μm, and one rigid silica gel derivative, coded SG-10-6000-NH_2, 9 μm, were tested. The column setting of each was primarily calibrated with dextran standards (cf. Figures 2-5). From the calibrations obtained we find a very poor suitability for HEMA-BIO 300 at the M_w range over (3-4)x10^5 Da for dextran standards. Hyaluronate reference materials are practically fully excluded (cf. Figure 2). The HEMA-BIO 1000, 10 μm, investigated indicates a very good separation selectivity for dextran standards (M_w > 2x10^6 Da). This column did not show satisfactory separation for used hyaluronate reference materials as can be seen in Figure 3. To increase the separation power, two HEMA-BIO 1000 columns

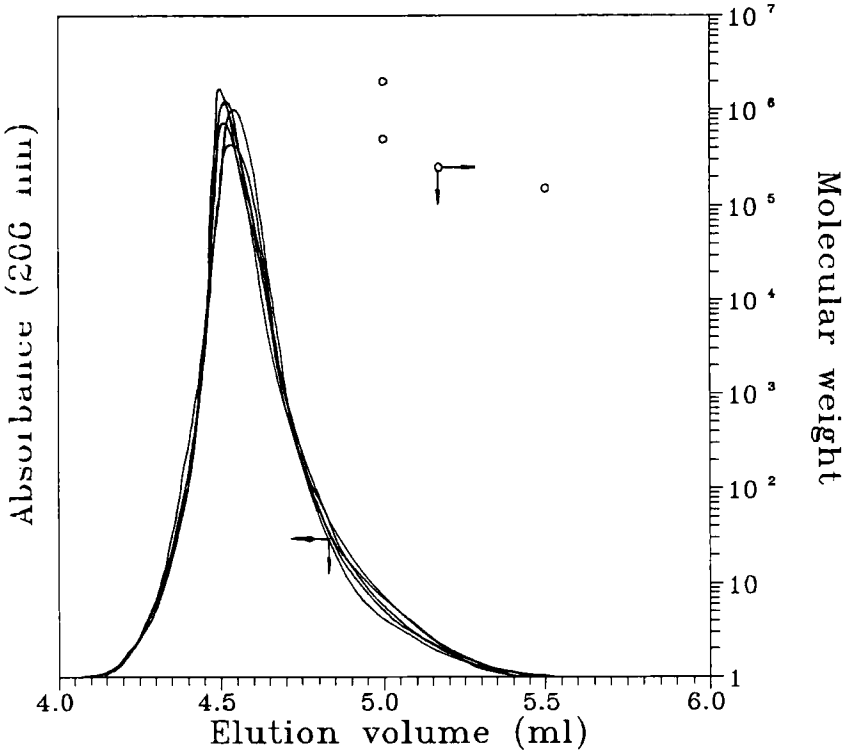

Figure 2. Calibration curve for HEMA-BIO 300, 10 µm, obtained with dextran standards ($2x10^6$, $5x10^5$, $2.5x10^5$ and $1.5x10^5$ Da)(o). Chromatographic curves represent real hyaluronan separation of reference material. The elution buffer was 50 mM sodium phosphate with a pH of 7.8, flow rate 0.5 ml/min, UV detection adjusted at 206 nm, sensitivity 0.1 AUFS. Injection volume 20 µl, concentration of samples 0.03 % (w/v).

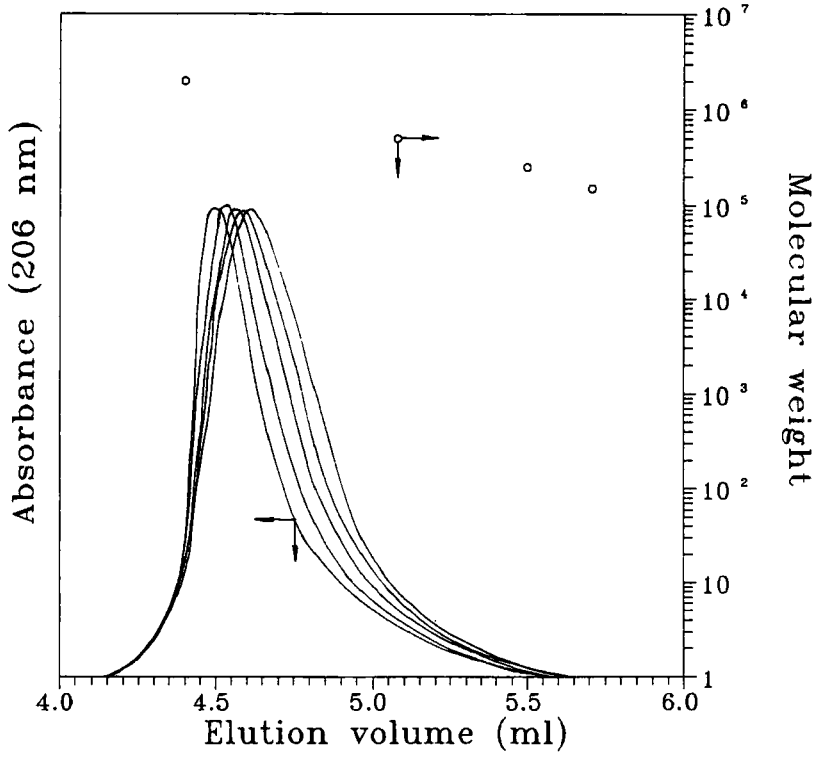

Figure 3. Calibration curve for HEMA-BIO 1000, 10 µm, obtained with dextran standards ($2x10^6$, $5x10^5$, $2.5x10^5$ and $1.5x10^5$ Da)(o). Chromatographic curves represents real hyaluronan separation of reference material. HPSEC conditions were the same as in Figure 2.

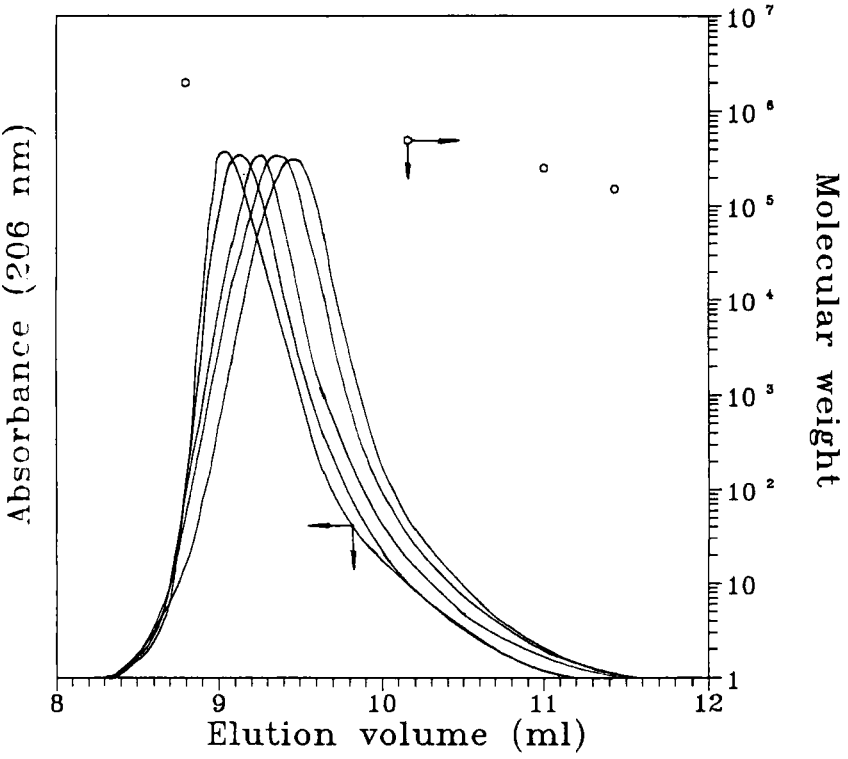

Figure 4. Calibration curve for two HEMA-BIO 1000, 10 µm, in series obtained with dextran standards ($2x10^6$, $5x10^5$, $2.5x10^5$ and $1.5x10^5$ Da)(o). Chromatographic curves represents real hyaluronan separation of reference material. HPSEC conditions were the same as in Figure 2.

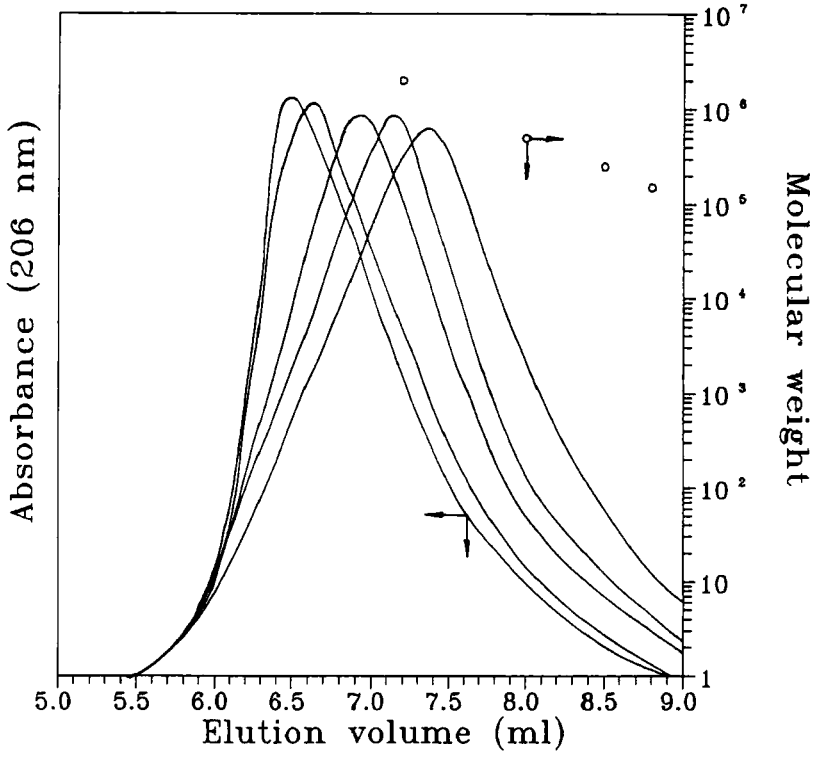

Figure 5. Calibration curve for SG-10-6000-NH_2, 9 µm, obtained with dextran standards ($2x10^6$, $5x10^5$, $2.5x10^5$ and $1.5x10^5$ Da)(o). Chromatographic curves represent real hyaluronan separation of reference material. HPSEC conditions were the same as in Figure 2.

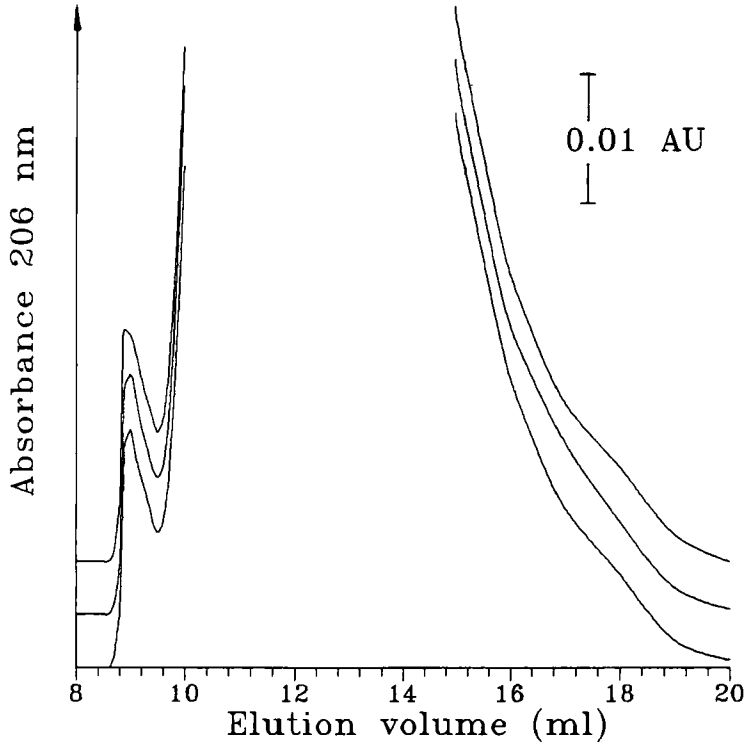

Figure 6a. HPSEC chromatographic curves in the triplicate run of synovial effusion separated on two columns of HEMA-BIO 1000 in series. A typical non-inflammed synovial effusion was taken from an osteoarthritic patient (100 cells in µl). Synovial effusion was injected according to the procedure described in the section Materials and Methods. HPSEC conditions were the same as in Figure 2.

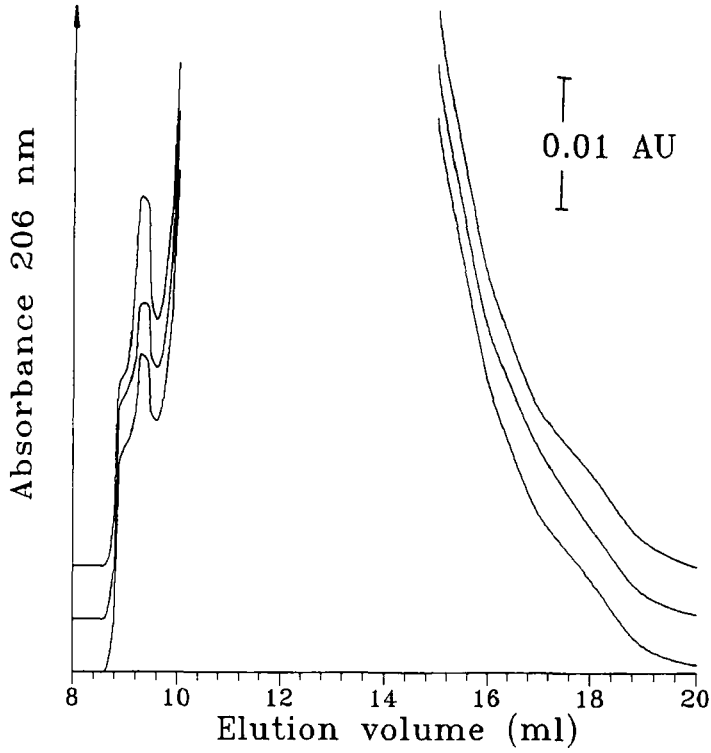

Figure 6b. HPSEC chromatographic curves in triplicate run of synovial effusion separated on two columns of HEMA-BIO 1000 in series. A typical inflammed synovial effusion was obtained (33 000 cells in µl) from a patient with active rheumatoid arthritis. Synovial effusion was injected according to the procedure described in the section Materials and Methods. HPSEC conditions were the same as in Figure 2.

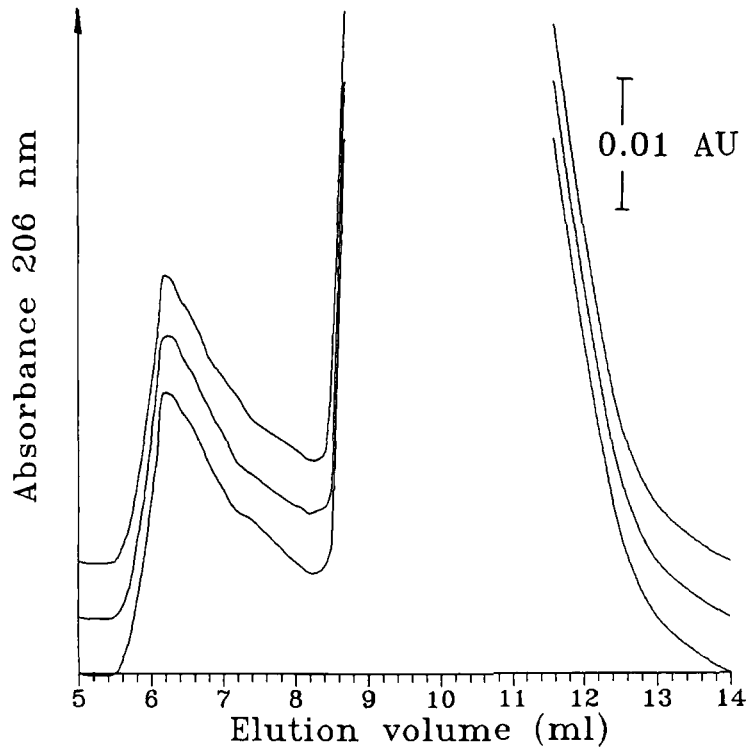

Figure 7a. HPSEC chromatographic curves in triplicate run of synovial effusion separated on the SG-10-6000-NH_2 column. The same non-inflammed osteoartritic synovial effusion was taken as in Fig.6a (100 cells in µl). HPSEC conditions were the same as in Figure 2.

were used (cf. Figure 4) in the series. Such an experimental arrangement offers excellent separation and high selectivity for all high molecular weight dextran standards. The calibration curve obtained is similar to that published by Motohashi[9] with respect to Shodex OHpak B-806 and B-805. The

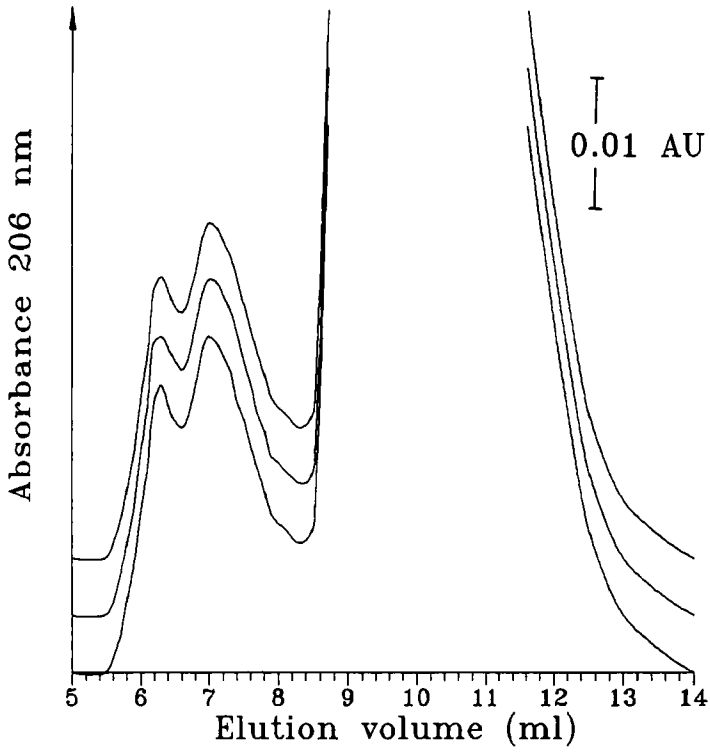

Figure 7b. HPSEC chromatographic curves in triplicate run of synovial effusion separated on the SG-10-6000-NH_2 column. The same typical inflammed synovial effusion as in Fig.6b (33 000 cells in µl) obtained from a patient with active rheumatoid arthritis was applied. HPSEC conditions were the same as in Figure 2.

results presented in Figure 4 for the hyaluronate reference materials indicate as well slight improvement in the separation power of the HEMA-BIO 1000 column couple.

In comparison to results shown in Figure 4, results *from* Figure 5 document a significant improvement regarding the

separation of hyaluronate reference materials when using highly porous aminopropyl-modified silica gel. The results obtained clearly indicate that highly porous aminopropyl-modified silica gel (SG-10-6000-NH_2) should be the column of choice when making a hyaluronate separation analysis.

Hyaluronate represents a most characteristic macromolecular component of synovial fluids and gives them their typical rheological properties. Several studies have aimed to show a connection between the properties of hyaluronan in the synovial effusion and the severity of joint affections. The applicability of both column settings for analysis of hyaluronate in synovial effusion is exemplified by a triplicate run of two different synovial effusions (Figures 6-7a,b). Two different synovial effusions were selected for comparison: first, a typical non-inflammed synovial effusion was taken from an osteoarthritic patient (100 cells in µl)(Figures 6a, 7a); and second, a typically inflammed sample (33 000 cells in µl) was obtained from a patient with active rheumatoid arthritis (Figures 6b, 7b). The figures are typical HPSEC chromatograms of synovial effusions. The first peak represents hyaluronan and the second one proteins[13,15]. It can clearly be seen, that the SG-10-6000-NH_2 column is suitable for hyaluronan molecular weight distribution determination.

CONCLUSIONS

The present study shows that in order to obtain a reliable distributional analysis it is essential that high-molecular-weight hyaluronate by HPSEC columns with extremely high exclusion limits in the order of 10^7-10^8 Da for nonpolar saccharides or globular proteins should be used, and that calibration by hyaluronate reference material should be done.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the kindnes of Dr.Ove Wik for the hyaluronate reference materials.

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Received: March 25, 1992
Accepted: April 27, 1992