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Note

Analysis of hyaluronic acid in synovial fluid by reversed-phase liquid chromatography

PAOLA BRUN, ANNA DE GALATEO, ANDREA CAMPORESE, ROBERTA CORTIVO and GIOVANNI ABATANGELO*

Institute of Histology, University of Padova, 50 Via Falloppio, 35100 Padova (Italy)

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Hyaluronic acid (HA) is a large linear polysaccharide containing alternate residues of N-acetylglucosamine and glucuronic acid [1]. It is found predominantly in the extracellular matrix of the connective tissues, but also on the cell surface [2,3], and its relative molecular mass ranges from $5.0 \cdot 10^5$ to $8 \cdot 10^6$ [4].

HA forms the backbone of cartilage proteoglycan aggregates [5]. It has also been implicated in biological processes such as cell–cell interaction, cell–matrix adhesion, cell motility and cell differentiation, and it is the most characteristic component of synovial fluid. During the past two decades several studies have tried to establish a relationship between the amount, concentration and molecular mass of HA in the synovium and the severity of joint pathology [6,7]. In order to establish a correlation with clinical data, many efforts have been devoted to the isolation and characterization of HA in synovial fluid. However, considerable technical difficulties have arisen because HA is associated with large amounts of proteins and other glycosaminoglycans (GAGs).

Separation has been achieved using a number of different column chromatographic techniques [4,6,7]. However, these are generally time-consuming and require a large amount of starting material. Recently Motohashi et al. [8] have described a high-performance liquid chromatographic (HPLC) method for the separation and mass determination of HA in biological samples. In this case, the mass determination was reliable only with low-molecular-mass fractions (up to M_r $6 \cdot 10^5$) and the elution of GAGs was achieved after 20 min.

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With the aim of finding a rapid and sensitive method that would allow the separation of HA, we have investigated the use of reversed-phase HPLC.

EXPERIMENTAL

High-performance liquid chromatography

Separations were performed with a Beckman 344 HPLC instrument equipped with a Beckman 160 UV absorbance detector (fixed wavelength, 229 and 280 nm) and a Varian Model Vista 401 integrator. A Supelco (Bellefonte, PA, U.S.A.) Model Supelcosil, reversed-phase LC 318 column (50 mm×4.6 mm I.D., pore size 30 nm) protected by a Supelco LC 18 precolumn was used. The column was equilibrated with 20 m*M* NaH_2PO_4–150 m*M* NaCl (pH 6.5) (buffer A) and maintained at a flow-rate of 1.0 ml/min. Buffer B was acetonitrile.

Samples of 40 μl diluted to 500 μl in buffer A were injected into the column via the appropriate size sample loops. Separation of the sample components was carried out using a 50-min linear gradient of acetonitrile (from 0 to 60%) in buffer A at room temperature.

Elution profiles were monitored by measuring UV absorbances at 229 and 280 nm, and the detection signals were recorded at a chart speed of 2 mm/min.

Samples

Hyaluronic acid (HA), extracted and purified from rooster combs, was obtained by FIDIA Research Labs. (Abano Terme Italy) and was of three relative molecular masses: $1.67 \cdot 10^5$, $6.09 \cdot 10^5$ and $9.17 \cdot 10^5$.

Synovial fluid was obtained from the knees of healthy volunteer donors and from patients with knee arthropathy. The samples were centrifuged (1000 *g*, 20 min, twice) to remove cells or connective tissue debris, and supernatants were stored in aliquots in the presence of protease inhibitors at -20°C.

Examination of the concentration of the various materials.

Total and sulphated GAGs were determined spectrophotometrically using the method of Gold [9]. Concentrations of HA in solutions before and after HPLC analyses were determined by the carbazole method [10]. Protein was determined by the method of Lowry et al. [11] with bovine serum albumin as standard.

RESULTS AND DISCUSSION

As shown in Fig. 1, HA is well separated from bovine serum albumin on the reversed-phase column. In fact, HA is eluted within the void volume of the column, whereas the protein was retarded and was eluted after 38 min, corresponding to a concentration of 30% acetonitrile. The same elution profile was obtained when the individual macromolecules were separately chromato-

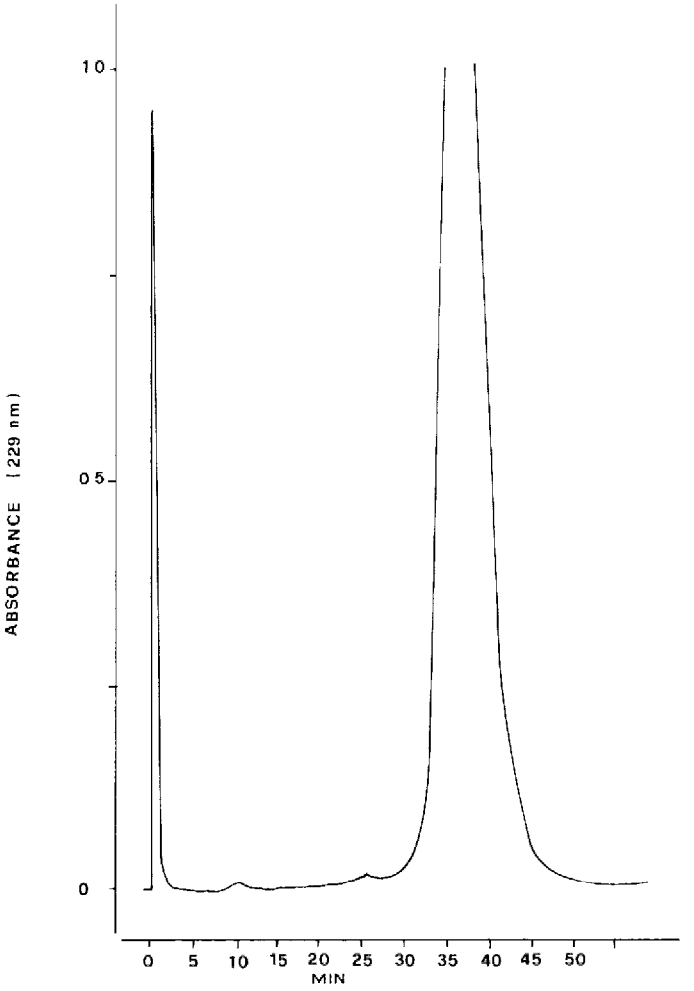

Fig 1. Reversed-phase chromatography of HA and bovine serum albumin (BSA); 100 μg of HA and 1 mg of BSA per 40 μl of the starting buffer were chromatographed as described in Experimental. The peak at 0.6 min is hyaluronate, as confirmed by the carbazole reaction; the peak at 38 min is BSA, as confirmed by the Lowry reaction.

graphed on the column (data not shown). No differences were seen using either HA standards of different molecular masses or other GAG standards, such as chondroitin sulphates A and C, which also eluted in the void volume with HA (data not shown).

Fig. 2 shows the chromatographic separation of the synovial fluid components. The peaks were collected and analysed by the carbazole and Lowry methods. The uronic-positive material was confined to the first peak and the protein moiety appeared after 42 min. Proteins were absent in the first peak,

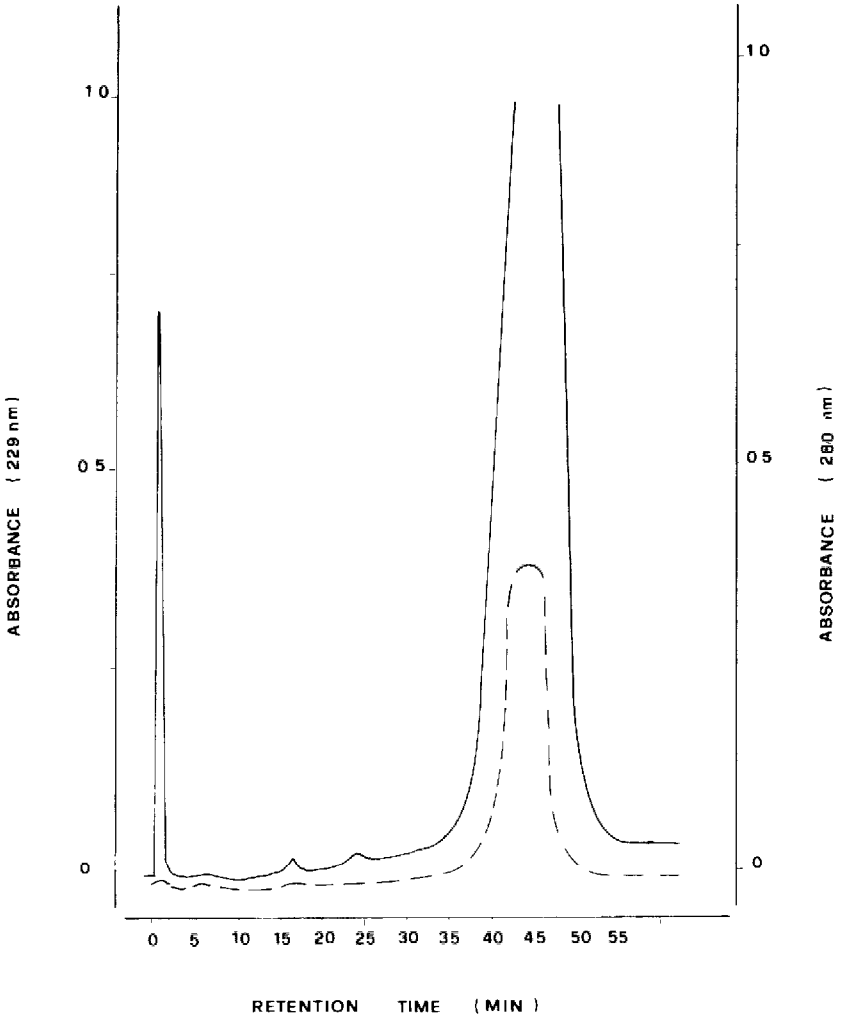

Fig 2 HPLC profile of synovial fluid; 40 μl of synovial fluid obtained from a patient with knee arthropathy were injected. The first peak is carbazole-positive material and the second one is the protein peak. The absorbance was measured at 229 nm (solid line) and at 280 nm (dashed line).

where the carbazole gave a positive reaction, thus confirming that the material was GAG in the nature. The majority of the GAG material is presumably hyaluronate. In fact, our analyses of synovial fluid specimens with the method of Gold [9] suggested that the sulphated GAGs are present only in concentrations from 2 to 6% (data not shown). Other authors [12–14] who used different techniques reached the same conclusions.

It is known that the large excess of proteins in biological samples makes the determination of uronic acid by the carbazole method unreliable. Reversed-

phase HPLC is currently used to separate proteins by virtue of their different hydrophobicities. The hydrophobic functional groups are bound to the stationary phase, and organic solvents must be used to elute the proteins. We have demonstrated that with this chromatographic method it is possible to separate GAGs from the protein moiety of the synovial fluid and to determine their exact concentration avoiding the interference of the associated proteins. The advantages of this method are the small amounts needed for the separation and determination of GAGs in the synovial liquid and the speed of the fractionation. GAGs present in synovial fluid were eluted in less than 2 min, which could be an important advantage for routine analyses. Another method based on HPLC analysis has been recently described by Motohashi et al. [8]. In this case, however, the dimensions of the columns (500 mm×8 mm I.D.) represents a limitation for standard laboratory equipment, whereas our method is adaptable for routine analyses.

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