Journal of Chromatography, 566 (1991) 9–18
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5782

Assay of synovial fluid hyaluronic acid using high-performance liquid chromatography of hyaluronidase digests

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(First received October 15th, 1990; revised manuscript received November 29th, 1990)

ABSTRACT

A high-performance liquid chromatographic method for the determination of hyaluronic acid levels in synovial fluids has been developed. The hyaluronidase sample digests, containing an internal standard (benzoic acid), were separated on a reversed-phase octadecylsilyl column eluted with 0.01 *M* tetrabutylammonium phosphate–acetonitrile (83:17, v/v) at pH 7.35. The determination was made on 1:10 diluted samples, by using a calibration curve from 50 to 500 μg/ml of human umbilical cord hyaluronic acid. For validation, the synovial fluids were simultaneously analysed by this method and a radiometric method: a high correlation was found between the two (correlation coefficient 0.94). The proposed method can be used to determine specifically the high hyaluronic acid levels of synovial fluids without interferences from other glycosaminoglycans or non-steroidal anti-inflammatory drug treatment.

INTRODUCTION

Hyaluronic acid (HA) is a linear polysaccharide made up of alternating D-glucuronic acid and 2-acetamido-2-deoxy-D-glucose. It is a major component of the extracellular matrix of connective tissue and is present mainly in umbilical

0378-4347/91/$03.50

cord, vitreous aqueous humour and synovial fluid, conferring high viscosity and properties of exclusion [1]. This glycosaminoglycan is synthesized by fibroblasts and is degraded by a specific hydrolase, testicular or lysosomal hyaluronidase, in tetrasaccharides (T) and hexasaccharides (H) [2]. The measurement of HA in tissues and body fluids (plasma, synovial fluid and urine) is useful in the diagnosis of inflammatory arthropathies [3], hepatic [4] and renal disorders [5], systemic sclerosis [6] and cancers [7].

HA has been assayed by various methods, the oldest being colorimetry using a modification of the carbazole method for detecting hexuronic acid [8,9]. However, this method was not specific, because other compounds such as neutral sugars, generate colour in this assay. Methods such as enzyme-linked immunosorbent assay (ELISA) [10–12] and radioassay [13,14] are sensitive and fast for the measurement of serum HA levels. However, in order to evaluate HA in synovial fluid, a specific method is necessary because the levels are very high, and large amounts of macromolecules such as other glycosaminoglycans might interfere. Different authors have used a high-performance liquid chromatographic (HPLC) method requiring enzymic digestion of HA by hydrolases, such as chondroitinase [15–17] or hyaluronidase [18–20]. Recently, Brun *et al.* [21] described an assessment of HA in synovial fluid without enzymic digestion, but the HA was eluted in front of the solvent.

This paper describes a rapid, specific method for measuring HA in pathological synovial fluid from patients with arthritic diseases, based on the separation of hyaluronidase digestion products of HA by reversed-phase HPLC and the use of an internal standard. We chose hyaluronidase because of its high specificity for HA, as shown by its inability to hydrolyse other glycosaminoglycans, such as chondroitin sulphates [19].

EXPERIMENTAL

Reagents

Hyaluronic acid from human umbilical cord (grade I) and *Streptomyces* hyaluronidase (EC 3.2.1.3.5; type IX) were from Sigma (La Verpillière, France). Tetrabutylammonium phosphate (Pic A reagent) was from Waters Assoc. (St. Quentin en Yvelines, France). Acetonitrile and methanol were from SDS (Villeurbanne, France). Phosphoric acid, benzoic acid (BA), sodium phosphate ($Na_3PO_4 \cdot 12H_2O$), and sodium acetate were from Merck (Nogent sur Marne, France). All reagents for chromatography were HPLC grade. Hyaluronate assay kits (HA test 50) were obtained from Pharmacia Diagnostica (St. Quentin en Yvelines, France).

Sample collection

Samples of synovial fluid, obtained by puncture from the knee joints of 25 patients (18–65 years old) with arthritic diseases, were immediately transferred to

sterile tubes. The samples were centrifuged at 1000 *g* for 10 min and were stored at −20°C until analysis.

HPLC apparatus

The chromatograph (from Waters Assoc.) consisted of a Model 590 solvent-delivery system, a sample injection valve fitted with a 50-μl loop, a Lambda Max Model 481 variable-wavelength UV detector and a Model 740 data module. The column was a reversed-phase Radial Pak™ C_{18} cartridge (10 cm × 8 mm I.D., 4-μm particle size) enclosed in a radial compression module and coupled to a Guard Pak™ C_{18} pre-column (Waters Assoc.)

Enzymic digestion

HA was dissolved in 0.05 *M* sodium phosphate buffer (pH 5) (1 mg/ml), and the internal standard (BA) was dissolved in methanol (1 mg/ml). Working solutions of the internal standard were obtained daily by dilution (1:30 v/v) in 0.02 *M* sodium acetate buffer (pH 6).

Various amounts of HA solution (5–50 μl) or synovial fluid (5–10 μl) were added to 200 μl of sodium acetate buffer (pH 6), and 20 μl of *Streptomyces* hyaluronidase (0.1 TRU/μl in 0.02 *M* sodium acetate buffer, pH 6) (1 TRU = 1 turbidity reducing unit = 1 I.U.) were added. Enzymic digestion over 48 h was studied at 4, 20 and 35°C. At the end of the digestion, 20 μl of internal standard working solution were added to each enzymatic digestion. These mixtures were stored at −20°C until injection into the HPLC column.

Identification of tetrasaccharide and hexasaccharide from HA

A 1-ml volume of the solution of HA (1 mg/ml) was digested for three days at 35°C in the presence of 1 ml of sodium acetate buffer and 20 TRU of *Streptomyces* hyaluronidase. The digestion products were separated by HPLC as described below. The hydrolysis products tetrasaccharide and hexasaccharide were identified in the eluted fractions by measurement of uronic acid using the carbazole method [8].

HPLC procedure

Elution was carried out isocratically at a flow-rate of 1.3 ml/min with 0.01 *M* tetrabutylammonium phosphate–acetonitrile (83:17, v/v) with the pH adjusted to 7.35 with phosphoric acid. The eluate was monitored at 232 nm.

Radioassay procedure

The Pharmacia HA test is a radiometric assay based on the use of specific HA-binding proteins (HABP) isolated from bovine cartilage and labelled with ^{125}I. The synovial fluid samples were usually diluted 5000-fold in 0.02 *M* sodium acetate buffer (pH 6.0). The samples were incubated with [^{125}I]HABP for at least 60 min. Then the free [^{125}I]HABP was incubated with HA-Sepharose particles for

45 min. After centrifugation, the radioactivity of the particles was measured with an LKB 1270 automatic gamma counter. The counts (B) for standards and unknowns were expressed as a percentage of the mean counts of the zero standard (B_0). The percentage values for the standards were plotted against the HA concentration on a linear–log regression, and the concentrations of the unknown samples were read from the standard curve.

Statistical analysis

The values obtained by the two methods were compared using a paired Student's t-test, and the relationship between two variables was studied by linear regression analysis, using the Statview SE+ graphics computer program (Abacus Concepts, Berkeley, CA, U.S.A.; 1988) on a Macintosh Plus microcomputer.

RESULTS AND DISCUSSION

HPLC method

The separation of the two oligosaccharides (T and H) and the internal standard (BA) was satisfactory: average retention times were 4.5, 9.9 and 11.7 min, respectively (Fig. 1). The variation of these times was low under our experimental

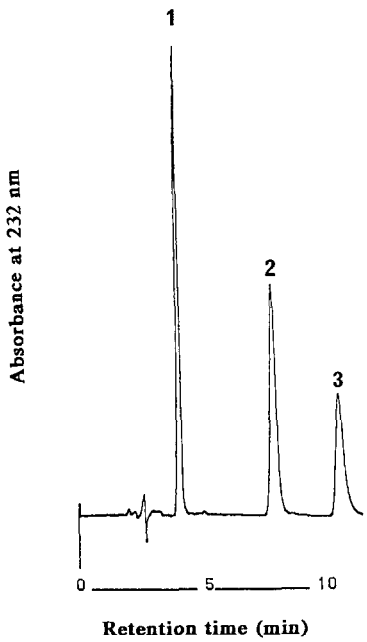

Fig. 1. Chromatogram of enzymic digestion products of HA in the presence of the internal standard (benzoic acid). Conditions: isocratic elution; flow-rate, 1.3 ml/min; C_{18} column; eluent, acetonitrile–Pic A reagent (17:83, v/v) at pH 7.35. Peaks: 1 = tetrasaccharide; 2 = hexasaccharide; 3 = internal standard.

conditions: the coefficients of variation (C.V.) calculated after ten successive days were 1% (T), 2% (H) and 2.8% (BA).

The present method was derived from the method proposed by Chun *et al.* [19]. The major improvements are the use of an internal standard, BA, and a calibration assay with HA itself, specifically hydrolysed into two oligosaccharides, T and H, in each assay series.

The quantification was based on the ratios of the peak height of T or H to that of BA. Linear regression analysis showed a good linearity between these ratios and HA concentrations from 50 to 500 μg/ml ($T/BA = 0.126 + 7.685 \cdot 10^{-3}$ [HA], $r = 0.998$; $H/BA = -4.133 \cdot 10^{-3} + 2.669 \cdot 10^{-3}$ [HA], $r = 0.997$). In addition, the ratio of the sum of the T and H peak heights to the BA peak height [(T + H)/BA] plotted *versus* HA concentration, showed a high correlation: $(T + H)/BA = 0.124 + 1.035 \cdot 10^{-2}$ [HA], $r = 0.999$.

Other HPLC assays [15–21] have been based on colorimetric methods to quantify the degradation products, using standard T and H solutions previously prepared for calibration, and assuming a proportional production of T and H, *i.e.* a constant ratio T/H under the enzymic conditions used. However, our experiments showed that the peak-height ratio T/H is not constant for different HA concentrations (Fig. 2), or for various durations and temperatures of hydrolysis (Fig. 3). Moreover, T/H differed between indivuals (Table I).

The reproducibility of this method was evaluated from five measurements of two calibration points: 100 and 400 μg/ml. The C.V. were 6.77% (102.05 ± 6.91 μg/ml) and 2.56% (408.94 ± 10.46 μg/ml), respectively. The precision, calculated as the difference between the mean detected values and theoretical values, was 2.05% for 100 μg/ml and 2.23% for 400 μg/ml.

No interferences were observed with the following non-steroidal anti-inflammatory drugs: diclofenac, diflunisal, etodolac, fenbufen, fenoprofen, flurbipro-

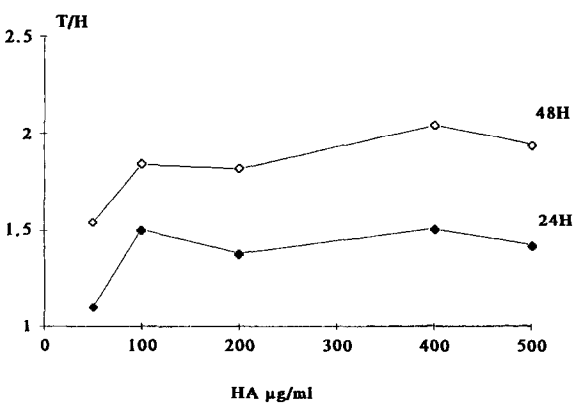

Fig. 2. Comparison of peak-height ratios T/H after hydrolysis of various concentrations of HA (μg/ml) for 24 and 48 h.

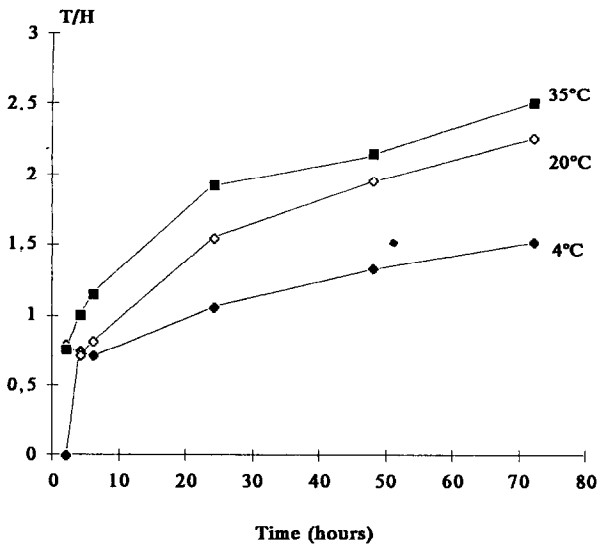

Fig. 3. Effect of temperature and time on the formation of the oligosaccharides T and H during enzymic digestion of HA (500 μg/ml) for 2, 4, 6, 24, 48 and 72 h, expressed as the ratio T/H of the peak heights obtained by HPLC.

fen, ibuprofen, indomethacin, ketoprofen, naproxen, niflumic acid, piroxicam, pirprofen, sulindac, tenoxicam, tiaprofenic acid.

Optimization of enzymic digestion

A standard solution of HA (500 μg/ml) was incubated with hyaluronidase for 48 h at three temperatures (4, 20 and 35°C) and the products were assayed by the present HPLC method. The largest amounts of the two oligosaccharides were obtained at 20°C: the levels obtained at 4 and 35°C were 30.2 and 64.5%, respectively, of the (T + H)/BA value at 20°C. This result was confirmed using four concentrations of HA (50, 100, 200 and 400 μg/ml) digested at 20 and 35°C (Fig. 4). The enzymic degradation was dependent on the temperature and duration of hydrolysis and was maximal in incubations at 20°C for 48 h. At higher temperatures, the enzymic degradation of HA was reduced and was not complete after 48 h of hydrolysis. These results differed from those obtained previously [22]; this discrepancy could be explained by major changes in experimental conditions (pH, substrate and enzyme concentrations). In Table II, we compare the results of hydrolysis with hyaluronidase stored (A) as usual at 4°C before used in digestion of HA and (B) at 35°C for 24 h before use. The two differently stored batches of enzymes produced different initial amounts of the hydrolysis products (Table II), suggesting thermal denaturation of the enzyme at 35°C rather than reduced enzymic activity at this higher temperature. However, denaturation was reversible,

TABLE I

HA LEVELS IN PATHOLOGICAL HUMAN SYNOVIAL FLUIDS AS DETERMINED BY A RADIOASSAY AND THE PRESENT HPLC METHOD

The concentrations evaluated by HPLC were calculated using calibration by T/BA, H/BA and (T + H)/BA.

Sample No.	HPLC method				Radioassay
	Ratio T/H	[HA] (mg/ml)			[HA] (mg/ml)
		T/BA	H/BA	(T+H)/BA	
1	2.56	1.157	1.286	1.194	1.441
2	2.03	0.681	0.684	0.682	0.741
3	2.00	0.317	0.222	0.287	0.229
4	2.12	0.894	0.938	0.906	0.999
5	2.53	0.480	0.399	0.457	0.451
6	2.33	0.405	0.304	0.376	0.247
7	2.11	0.267	0.209	0.251	0.197
8	2.32	1.079	0.981	1.052	0.984
9	2.32	0.631	0.589	0.619	0.593
10	2.35	1.242	1.118	1.209	1.168
11	2.46	1.341	1.114	1.280	1.194
12	2.27	0.770	0.662	0.739	0.799
13	2.44	1.273	1.114	1.231	1.189
14	2.61	1.321	1.082	1.252	1.310
15	2.38	1.463	1.257	1.409	1.056
16	2.27	0.330	0.304	0.322	0.309
17	2.42	0.794	0.630	0.748	0.677
18	2.40	0.646	0.470	0.597	0.696
19	2.50	0.581	0.494	0.556	0.498
20	2.25	0.411	0.247	0.365	0.323
21	2.50	0.794	0.630	0.748	0.680
22	2.59	0.621	0.406	0.561	0.397
23	2.61	1.228	1.013	1.168	1.313
24	2.43	1.252	1.108	1.212	0.996
25	2.70	1.252	1.013	1.185	1.465

and after 24 h of hydrolysis at 20°C the results (A *versus* B) of hydrolysis on the two differently stored enzymes were not significantly different ($p > 0.16$).

HPLC validation

The HPLC method was validated by comparing the results with those obtained by radiometric assay using 25 pathological synovial fluids. The results calculated using a calibration with T/BA and H/BA ratios obtained by HPLC were significantly different from each other ($p < 0.0001$) and from the results

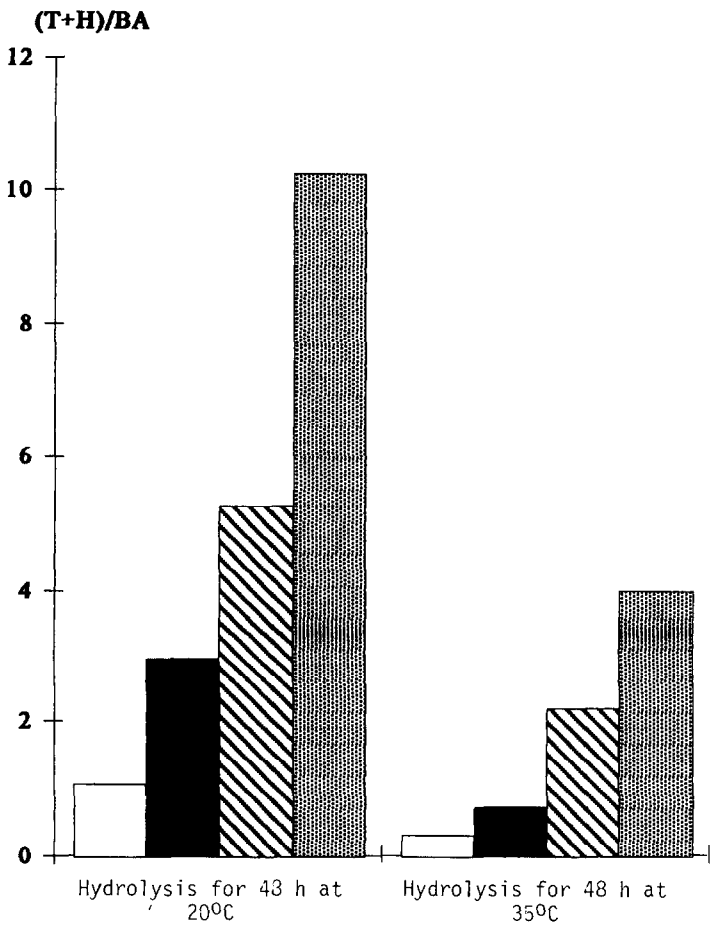

Fig. 4. Effect of temperature on the enzymic digestion of various concentrations of HA (50, 100, 200 or 400 μg/ml) for 48 h. Formation of two oligosaccharides at 20 and 35°C using the internal standard benzoic acid. The HPLC results are expressed as the ratio (T + H)/BA: (□) 50 μg/ml; (■) 100 μg/ml; (▨) 200 μg/ml; (▩) 400 μg/ml.

calculated with calibration by (T + H)/BA ratio ($p<0.0001$). Only the results obtained with calibration by (T + H)/BA ratios were not significantly different from the results derived from the radiometric method ($p>0.50$) (Table I).

Moreover, there was a high correlation between the levels of HA in the same samples as determined by these two independent methods: [HA](radioassay) = $-0.101 + 1.072$ [HA](HPLC), $r = 0.94$ (Fig. 5).

In addition, for five samples containing high levels of HA, we have studied two different dilutions (1:10 and 1:20) (Table III). Results showed that the values obtained with the higher dilution were not significantly different from the others ($p = 0.383$ by Student's paired t-test). These data suggest that our method could

TABLE II

COMPARISON OF ENZYMIC DIGESTION PRODUCTS OF HA

Values are for (A) a hyaluronidase stored at +4°C before hydrolysis, and (B) a hyaluronidase heated at 35°C for 24 h before hydrolysis. The results are expressed as the peak-height ratios of tetrasaccharide plus hexasaccharide to internal standard [(T + H)/BA], for various amounts of HA, digested during 2, 4, 6, 24 and 48 h (p is the probability of the paired Student's t-test).

[HA] (μg/ml)	2 h		4 h		6 h		24 h		48 h	
	A	B	A	B	A	B	A	B	A	B
50	0.151	0.111	0.313	0.242	0.353	0.325	0.557	0.537	0.567	0.677
100	0.250	0.140	0.800	0.380	0.641	0.678	1.053	1.109	1.212	0.984
200	0.304	0.225	1.139	1.145	1.189	0.702	1.947	1.773	2.246	2.242
400	0.259	0.221	1.728	1.469	1.886	1.693	4.667	4.217	4.235	3.933
500	0.259	0.240	1.896	1.776	2.136	1.587	4.454	2.875	5.301	4.774
p	0.025		0.084		0.108		0.221		0.166	

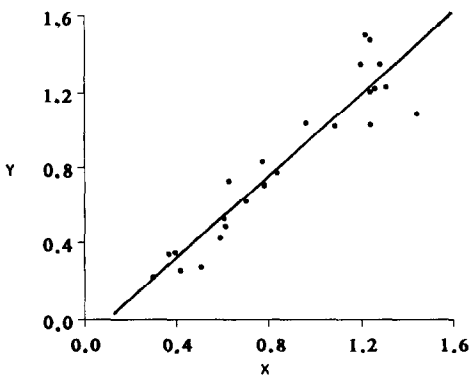

Fig. 5. Correlation curve of HA levels (mg/ml) quantified by radioassay kit (y-axis) and HPLC method (x-axis) using the same samples.

TABLE III

COMPARISON OF THE HA LEVELS OBTAINED BY HPLC METHOD AFTER TWO DIFFERENT DILUTIONS (1:10 AND 1:20)

Sample	Concentration (mg/ml)	
	1:10	1:20
1	1.209	1.194
2	1.280	1.390
3	1.231	1.444
4	1.252	1.264
5	1.409	1.336

be used for synovial fluids with HA concentrations requiring higher dilution without modification of the enzymic digestion or the analysis conditions.

CONCLUSION

Our method enables a reliable assay of HA in pathological synovial fluids with very high concentrations of this glycosaminoglycan. It is an easy and rapid assay, requiring less dilution of samples than the radiometric method [13,14] or the new ELISA methods [11,12]. The main improvement over other published HPLC assays is the calibration with HA itself hydrolysed in each assay series and the use of an internal standard [15–21].

ACKNOWLEDGEMENTS

The authors thank Mrs. S. Leveque for excellent technical assistance, and Mr. Moncelon and Mr. De Ceaurriz from INRS for receiving us in their Research Department. The following laboratories supplied drugs as pure substances: Boots Dacour (Courbevoie), Cassenne (Paris), Ciba-Geigy (Rueil Malmaison), Lederlé (Rungis), Lilly France (Saint-Cloud), Merck Sharp & Dohme-Chibret (Paris), Pfizer (Orsay), Roche (Neuilly-sur-Seine), Roussel Uclaf (Paris), Specia-Rhône Poulenc (Paris), Syntex (Puteau), Upsa (Rueil Malmaison), Wyeth-Byla (Paris).

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