Liquid-Chromatographic Determination of Hyaluronic Acid in Pleural and Ascitic Fluids

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A previously described method for determination of hyaluronic acid is modified for the analysis of pleural and ascitic fluids. After 20 µL of the acellular supernate is precipitated with ethanol, the precipitate is digested overnight with chondroitinases and chondroitin sulfatases. The content of hyaluronic acid-derived disaccharide is then analyzed by "high-performance" liquid chromatography. The high sensitivity (detection limit, <0.1 µg of hyaluronic acid per milliliter) and reproducibility (SD = 4% of the mean) enable accurate determination of hyaluronic acid, even in effusions from patients without signs of mesothelioma. The range of values obtained for pleural and ascitic fluids from patients without signs of this kind of tumor is the same as that found by using other techniques, being slightly higher in those patients showing signs of tissue destruction. The concentrations of hyaluronic acid were considerably increased in most, but not all, of the cases of mesothelioma.

Additional Keyphrases: mesothelioma • cancer • occupational hazards • glycosaminoglycans

Mesothelioma, the primary malignancy of mesothelial cells, may develop in the pleural or abdominal cavities after exposure to asbestos. Histologically, these tumors are composed of adenomatous or fibrous tissues, or both, sometimes displaying a mixed, "biphasic" growth pattern. These tumors often cause an increased formation of pleural or ascitic fluids, which may well be the first material presented for the clinical chemist/pathologist to examine. Distinguishing between this kind of tumor and carcinomas such as pulmonary adenocarcinomas is quite difficult. Therefore, detection of mesenchymal markers may help in establishing a diagnosis.

One such mesenchymal constituent is hyaluronic acid, which is present in the extracellular matrix of most connective tissues. Even though production of chondroitin sulfate has been described in one case of mesothelioma (1), hyaluronic acid is most commonly the major glycosaminoglycan (GAG) in this kind of tumor (2–6). Hyaluronic acid is probably secreted by the mesothelial cells, and mesotheliomas may produce hyaluronic acid in excessive amounts. The demonstration and quantitative determination of hyaluronic acid in tumor tissue (6) or in effusions (4, 5, 7, 8) has therefore been recommended as a diagnostic aid.

GAGs may be prepared from a tissue or from an effusion by proteolytic digestion and various kinds of precipitation reactions (4, 6–9). From the precipitates thus obtained hyaluronic acid may be isolated by ion-exchange chromatography (8). One can thus obtain well-purified hyaluronic acid preparation, but the procedure is somewhat laborious. For routine purposes a simpler procedure is often used: proteolytic digestion followed by precipitation reactions and subsequent electrophoresis (9). Even though the specificity of the latter procedure may be increased by an additional test for sensitivity to streptomycetes-derived hyaluronidase (EC 3.2.1.35), an enzyme specific for this GAG, this analysis is neither very sensitive nor very specific. In a similar approach described recently (10) the effect of hyaluronidase digestion on the high relative molecular mass (Mr) material was measured by "high-performance" liquid chromatography. However, this procedure does not completely exclude other hydrolytic enzymes, which may interfere by degrading other macromolecular compounds present.

Histochemical demonstration of the large excess of hyaluronic acid in mesotheliomas has also been attempted (11), exploiting the ability of the GAGs to bind cationic dyes such as Alcian blue, such that, by varying the electrolyte concentration or the pH, one may differentiate the nonsulfated hyaluronic acid from other acidic components such as the sulfated GAGs (12). Digestion with hyaluronidase in this procedure adds further information, but the complexity of the tissue and the possibilities for interaction with other endogenous substances render these types of reactions unreliable.

Recently, two specific methods for determination of hyaluronic acid have been published. One is based on the specific interaction between hyaluronic acid and the hyaluronic acid-binding region of cartilage proteoglycans. When used as a radioassay (13), this method can quantify extremely low amounts of hyaluronic acid, but the procedure is subject to various kinds of interference. The second method (14), on the other hand, is very insensitive to contaminants in biological systems. In it, treatment with chondroitinase is followed by isolation and liquid-chromatographic identification of the resulting 4,5-unsaturated disaccharides.

The aim of the present investigation was therefore to adapt this chromatographic method to the determination of hyaluronic acid in effusions. Because the specificity of the analysis might yield results different from those obtained by electrophoretic procedures, I also attempted to determine the range for "nonpathological" hyaluronic acid concentrations by analyzing a large number of ascitic and pleural fluids from patients with and without known mesothelioma.

Materials and Methods

Materials

Reagents: High-M, hyaluronic acid ("Healon") was obtained from Pharmacia, Uppsala, Sweden. Chondroitinase AC (EC 4.2.2.5) and ABC (EC 4.2.2.4) and chondroitin-4-sulfatase (EC 3.1.6.9) and chondroitin-6-sulfatase (EC 3.1.6.10) were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals were of reagent grade.

Solutions: Hyaluronic acid was precipitated by adding an ethanol/acetate solution, which was prepared by mixing 19 volumes of 99.5% ethanol with one volume of 250 g/L aqueous sodium acetate. The digestion mixture was 3 g/L Tris buffer (pH 7.5) containing each of the above enzymes at

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a final concentration of 100 U/L. The chromatographic mobile phase was a phosphate buffer prepared by dissolving 9 mmol of NaH₂PO₄ in 800 mL of water, adjusting the pH to 2.5 with H₂PO₄, and finally adding water to a total volume of 3 L.

Patients' samples: Over a two-year period 292 samples of pleural fluids from 222 patients and 104 samples of ascitic fluids from 96 patients were collected consecutively from the Department of Pathology, Huddinge University Hospital, Huddinge, Sweden, and from the Department of Clinical Cytology, Sabbatsbergs Hospital, Stockholm, Sweden. All samples had been sent to the laboratories for cytological examination. Some of the samples were preserved with ethanol (250 mL/L, final concentration), but most samples were freshly obtained. The diagnosis of malignant mesothelioma of the pleura could be established within six months in 10 cases (25 samples) and a corresponding abdominal tumor in one case (three samples).

The cytological smears were prepared according to routine procedures from pellets obtained by centrifugation at 3000 × g for 10 min. A 1-mL aliquot from each supernate was stored at -20 °C until analysis. For correlating biochemical and cytological findings, all the material from Huddinge University Hospital was re-screened to record signs of reactive mesothelial cell changes ("mesotheliosis"), inflammation, and malignancy, as well as the presence of significant amounts of blood.

Procedures

Assays. The first 155 pleural fluids and 48 ascitic fluids were analyzed for their total GAG content as well as for hyaluronic acid. Sufficient material for both these analyses was obtained by diluting, in 1.5-mL Microfuge Tubes (Beckman Instruments), 470 μL of fresh supernate (630 μL of supernate from the ethanol-preserved samples) with enough ethanol to make a final concentration of 300 mL/L. Any precipitate formed, which could contain a small proportion of the total hyaluronic acid, was removed by centrifugation in a Beckman Microfuge (estimated centrifugal force 10 000 × g) for 5 min. The hyaluronic acid in the supernate was precipitated by mixing with four volumes of the ethanol/acetate solution, then recentrifuging for another 5 min. The pelleted precipitate was incubated overnight at 37 °C with 400 μL of the digestion mixture. I used 10 μL of this digest for the chromatographic determination of hyaluronic acid, the rest for the colorimetric determination of total GAG, estimated as uronic acids by an automated version (15) of the carbazole reaction (16).

The remaining 133 pleural fluids and 58 ascitic fluids were analyzed only for hyaluronic acid. For this analysis I mixed 20 μL of the cell-free supernate with 80 μL of the ethanol/acetate solution, centrifuged, and digested the pellet with 100 μL of the digestion mixture; 20 μL of the digest was used for chromatography.

Calibration lines: To a sample containing 12 μg of uronic acid per milliliter I added known amounts of highly concentrated hyaluronic acid, to give final concentrations of uronic acid as great as 1300 μg/mL. I used 20 μL of the sample for the analysis as above, previous experiments having demonstrated a virtually complete and linear analytical recovery of purified solutions of hyaluronic acid after digestion and chromatography (14).

Reproducibility: I assessed intra-day reproducibility by using eight samples from patients with known mesothelio-

mas (hyaluronic acid concentrations: 49.6–226 μg of uronic acid per milliliter of effusion) and eight further samples from cases where this diagnosis had not yet been established (uronic acid, 4.66–55.7 μg/mL). From each sample I assayed six 20-μL aliquots. The interday reproducibility was tested by repeating the quadruplicate analysis of a sample (uronic acid concentration 19 μg/mL) for five consecutive days. The inter- and intraday variability obtained was assessed by use of an analysis-of-variance test.

Analytical recovery and incremental sensitivity: From each of the eight non-mesothelioma cases just mentioned, six 20-μL aliquots were supplemented with 20 μL of a hyaluronic acid solution containing 90 μg of uronic acid per milliliter before precipitation with 160 μL of the ethanol/acetate solution. To estimate the recovery, I compared results with those for six 20-μL aliquots of the hyaluronic acid solution digested with 80 μL of the digestion mixture but omitting the ethanol-precipitation step. Similarly, I studied assay sensitivity with test samples to which hyaluronic acid had been added in known increments.

Chromatography: The phosphate mobile phase was pumped with an LDC minipump (LDC/Milton Roy, Riviera Beach, FL) at a flow rate of 0.9 mL/min. Samples were injected with an Altex 210 injector (20-μL loop) onto a 250 × 4.6 mm (i.d.) column of Hypersil-APS (Shandon Southern Products Ltd., Runcorn, U.K.) protected by a 30 × 4.6 mm (i.d.) precolumn packed with the same material. The absorbance of the effluent was monitored with an LDC Spectromonitor III at 231 nm and recorded at both 0.02 and 0.1 A full-scale.

Results and Discussion

Analytical Variables

The hyaluronic acid-derived disaccharides were well separated from other ultraviolet-absorbing compounds in the fluids studied (Figure 1). In assays of crude preparations such as these, considerable amounts of material will, of course, elute with the elution front, but this didn't seem to affect the elution of the GAG-derived disaccharides. Thus the hyaluronic acid-derived disaccharide showed baseline separation from nonsulfated chondroitin-derived disaccha-

Fig. 1. Separation of disaccharides obtained after digestion of two pleural fluids with chondroitinase and sulfatase, each fluid containing approximately 20 μg of hyaluronate-derived glucuronic acid

The two chromatograms were obtained with the same column, when new (a) and 18 months later, after about 1000 runs (b). To keep the separations sharp in the old column, the phosphate concentration of the mobile phase was decreased to 5 mmol/L.

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ride. Both sugars had a tendency to elute in split peaks, probably because of partial separation of the anomeric forms. To avoid late-eluting "ghost peaks" from the sulfated chondroitin sulfate disaccharides, I included sulfatases in the digestion mixture. Consequently all chondroitin sulfate-derived disaccharides were eluted in the peak corresponding to nonsulfated chondroitin, from which the chondroitin sulfate content of the effusion could be calculated. The present procedure therefore enables the positive identification and determination in these effusions of compounds specifically derived from hyaluronic acid and those from chondroitin sulfate. With the possible exception of the assay based on interaction with cartilage proteoglycans (13), this specificity is matched by no other method.

To slow the deterioration of the columns, I attempted to reduce the amounts of contaminants injected with the sample by precipitating hyaluronic acid with ethanol before the digestion with chondroitinase. Precipitation of hyaluronic acid from a pleural or an ascitic fluid with ethanol at a final concentration of 600 to 700 mL/L will, however, be accompanied by a heavy co-precipitation of other compounds. For example, precipitation of 400-μL aliquots of the effusion in Microfuge Tubes yielded pellets in which it was sometimes impossible to digest more than half of the hyaluronic acid present. To overcome these difficulties, I used a two-step procedure when assessing larger volumes of sample. Ordinarily, hyaluronic acid is soluble in 300 mL/L aqueous ethanol, but in the present method, a little of this GAG was sometimes lost in the initial step. Precipitating 20-μL aliquots formed pellets that were never too large for complete digestion of hyaluronic acid, and with such samples the precipitation could be performed in a single step.

Assay of the smaller (20-μL) aliquots of the eight supplemented test samples yielded analytical recoveries of 97% (SD 3%). These results reflect the losses during the entire preparation procedure, most of which appear to occur during the ethanol-purification step. The coefficients of variation (CVs) for the intraday recovery experiments were 2.0 to 6.7% (average 4.1%) for the non-mesothelioma samples, 0.3 to 6.4% range (average 2.0%) for the hyaluronic-acid-supplemented samples, and 1.4 to 2.8% (average 2.2%) for the samples from mesothelioma patients. When hyaluronic acid standards were digested without previous ethanol precipitation, the corresponding CV was <1%. Thus it seems as if most of the variability is incurred in this step. When the interday reproducibility was tested, the CV for the five daily quadruplicate determinations was 6.1%, and analysis of variance could not verify that this figure was larger than that for the intraday variability ($F_{4,13} = 2.63$). These errors are fully acceptable for the purpose of the present study.

The chromatographic procedure used is very sensitive for the detection of hyaluronic acid. On the basis of differentiation from baseline noise, the detection limit would be better than 0.1 μg of uronic acid per milliliter of effusion, even though the digestion step dilutes the sample fivefold before injection. Even when one takes into account the variability of the preparation procedure, this detection limit is still better than 0.5 μg/mL. Similarly, the experiments indicate a 95% probability of detecting a 0.5 μg/mL increment in a sample containing 5.0 μg of uronic acid per milliliter.

The standard curve obtained for uronic acid-supplemented test samples (Figure 2) is linear over a very wide interval; regression analysis of nonlogarithmic coordinates shows correlation coefficients exceeding 0.9999 and intercepts that are very close to the origin. In routine work in my labora-

![Fig. 2. Standard curve obtained with a sample to which various amounts of hyaluronic acid were added. Apart from the highest extreme, all values show a close linear correlation to peak height. Bars indicate ± 1 SD of triplicate determinations](image-url)

tory the use of one defined disaccharide standard suffices for the quantification.

**Clinical Correlations**

Table 1 details the distribution of hyaluronic acid contents in the pleural and ascitic fluids studied. Those in nonmesothelioma cases contained 10.0 ± 8.6 and 6.2 ± 4.0 μg of uronic acid per milliliter (mean ± SD), respectively, indicating great variability in concentrations of hyaluronic acid. Most nonmesothelioma samples contain less than 25 μg of hyaluronic-acid-derived uronic acid per milliliter. Theoretically this corresponds to 50 μg of hyaluronic acid per milliliter; in practice, however, to prepare a corresponding standard it would be necessary to dissolve 75–100 μg of a lyophilized sodium hyaluronate preparation in 1 mL, and the exact hyaluronic acid content would be determined colorimetrically. A few single samples from patients in whom mesothelioma has not yet been found showed uronic acid concentrations in the 25–75 μg/mL range, the peak value so far being 70 μg/mL. Some of these values were obtained from patients with empyema or secondary malignancy; however, the follow-up time in some of these cases has been too short to exclude the presence of an occult mesothelioma.

Four of the fluid samples obtained from patients with mesothelioma showed hyaluronic acid concentrations (uronic acid) <25 μg/mL. Among these, three samples were taken

| Table 1. Distribution of Hyaluronic Acid (HA) Concentrations in Pleural and Ascitic Fluids |
|-----------------------------------------------|---------|---------|---------|
| HA content * | Pleural fluids | Ascitic fluids | Total |
| <25 | 3/259 a | 1/101 | 4/360 (1) e |
| 25–49 | 4/11 | 0/1 | 4/12 (30) |
| 50–74 | 5/9 | 1/1 | 6/10 (60) |
| 75–99 | 2/2 | 1/1 | 3/3 (100) |
| ≥100 | 11/11 | — | 11/11 (100) |

*a Measured as uronic acid, μg/mL of sample. b No. of patients with mesothelioma/total no. of patients. c Numbers in parentheses are percent incidence of mesothelioma.
from patients from whom earlier effusions had contained high amounts of hyaluronic acid, whereas the remaining "negative" sample represented the only specimen obtained from that patient. In 10 of the mesothelioma samples values were in the borderline region of 25–75 μg/mL; the remaining 14 all had hyaluronic acid concentrations (uronic acid) exceeding 75 μg/mL, the peak value being 817 μg/mL. Because hyaluronic acid is secreted from the mesothelial cells, one might expect that in some mesotheliomas the ability to produce this GAG would be lost. In only one of the 11 mesothelioma patients could increased amounts of hyaluronic acid not be demonstrated; in four of them the highest values were in the borderline interval, and in the remaining six patients at least one exudate contained more than 75 μg of hyaluronic-acid-derived uronic acid per milliliter.

These concentrations Accord well with the results given by Harington et al. (4), where 30 μg of uronic acid per milliliter delineates the "nonpathological" values. In a later study by Friman et al. (8), considerably higher concentrations were demonstrated. In that study, however, hyaluronic acid was isolated as the fraction eluted with 0.15–0.5 mol/L NaCl solution from an AG1 × 2 ion-exchange column; this fraction may also have contained sulfated low-Mr GAGs and acidic glycoprotein oligosaccharides, both of which could interfere with the analyses and give erroneously high values.

To assess the inter- and intra-individual variation, I collated the results for repeatedly taken "normal" samples. The average difference in uronic acid concentrations in two determinations of the same sample was 4.3 μg/mL for pleural fluids and 2.1 μg/mL for ascitic fluids. Even though the number of sample determinations was not sufficient for an adequate statistical analysis of variance, these results indicate considerable variations, both interindividually and intersample—intra-individually.

When discussing this variability, one must consider the heterogeneity in pathogenetic mechanisms. On the basis of rescreening results (Table 2), low hyaluronic acid concentrations could be demonstrated in cases where the fluid had a low cell count and in cases that exhibited signs of mesothelial cell proliferation only, without simultaneous dominance of inflammatory cells. Among fluids containing malignant cells or large amounts of granulocytes, lymphocytes, or erythrocytes, higher hyaluronic acid contents were more common. This accords well with the results of Friman et al. (8), who found increased hyaluronic acid concentrations in samples from patients with pleural effusion. During tissue destruction, therefore, hyaluronic acid may leak into the exudate in serous cavities from the surrounding connective tissues, or perhaps from the mesothelial cells proper, whereas in pure transudates hyaluronic acid seems to be excluded. This idea also agrees with the very large inter-individual variability found in the mesothelioma patients from whom repeated samples were obtained. In these cases there was often considerably less hyaluronic acid in samples obtained a few weeks later than in the first sample. Apparently, the tumor cannot synthesize the hyaluronic acid at the same rate as the fluid volume is refilling, and the concentration of the hyaluronic acid increases later.

For the diagnosis of mesothelioma, Friman et al. (8) recommended determining both the absolute and relative concentrations of hyaluronic acid. In the series reported here, however, the correlation between these two was high (Table 3); a similar covariance was seen in the mesothelioma group. When the total concentration of this polysaccharide was inconclusive, I could not find that any further diagnostic gains were made by determining the relative amounts.

The clinical significance of this simple analysis for detecting mesothelioma is perhaps best reflected by comparison with the cytological reports. In the present series cytological examination of the 28 mesothelioma specimens disclosed malignant cells in only seven samples and cells suspicious for malignancy in another five samples. No suspicion of malignancy was obtained for the other 16 samples, and so cytology failed to assist in diagnosis of malignancy in five of the 11 mesothelioma patients. Because increased concentrations of hyaluronic acid were seen more often than were malignant cells, the determination of this GAG could be one of the more important tests to establish this difficult diagnosis.

As estimated from the number of cases detected in the two hospitals during the previous five years, four patients in this population would be expected to have mesothelioma. The number found was considerably higher, however. Although this might reflect a truly increased incidence, it seems more likely that the condition often has been misdiagnosed earlier, perhaps as a metastatic adenocarcinoma. For further evaluation of the clinical importance of this determination, the method is now in regular use at the Huddinge University Hospital, where it is performed on all ascitic and pleural fluids sent for cytological examination. A more complete evaluation, however, will have to wait for further mesothelioma cases to be collected and for an adequate follow-up time of suspect material.

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Table 2. Hyaluronic Acid Content in Nonmesothelioma Samples

<table>
<thead>
<tr>
<th>Morphological characteristic</th>
<th>No. of samples</th>
<th>HA content (μg of UA per mL, mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignant cells present</td>
<td>24</td>
<td>6.3 ± 3.4</td>
</tr>
<tr>
<td>Inflammatory cells in large numbers</td>
<td>52</td>
<td>7.2 ± 5.2</td>
</tr>
<tr>
<td>Erythrocytes in large number</td>
<td>33</td>
<td>7.5 ± 4.9</td>
</tr>
<tr>
<td>Reactive mesothelial cell changes</td>
<td>25</td>
<td>4.9 ± 3.6</td>
</tr>
<tr>
<td>Low cell count</td>
<td>10</td>
<td>3.4 ± 2.5</td>
</tr>
</tbody>
</table>

*Based on cytological findings at rescreening.

Table 3. Correlation between Hyaluronic Acid (HA) Concentration and Its Relative Proportion of the Total Glycosaminoglycan (GAG) Content

<table>
<thead>
<tr>
<th>Concentration of HA, μg of UA per mL</th>
<th>Relative proportion of HA (% of total GAG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–19</td>
<td>1/171</td>
</tr>
<tr>
<td>20–29</td>
<td>2/11</td>
</tr>
<tr>
<td>25–74</td>
<td>0/1</td>
</tr>
<tr>
<td>Total</td>
<td>3/183</td>
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</tbody>
</table>

*No. of specimens from patients with mesothelioma/total no. of pleural and ascitic fluids. Grand total no. of specimens: 203 (155 pleural fluids, 48 ascitic fluids), collected from 152 patients.

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