Hyaluronic Acid Content of Effusions as a Diagnostic Aid for Malignant Mesothelioma

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ABSTRACT

A high-performance liquid chromatographic technique, using a size exclusion column (TSK-5000PW), has been developed for the quantification of hyaluronic acid (HA) in pleural and peritoneal effusions. Sample preparation requires only a 100-fold dilution of the exudate with phosphate buffer prior to analysis. Chromatographic conditions are: 0.05 M phosphate buffer (pH, 5.0) mobile phase at a flow rate of 1.0 ml/min, ultraviolet absorbance detection at 200 nm. The method resolves HA from all other glycosaminoglycans. The presence of HA is confirmed by the removal of the HA peak ( retention time, approx. 5.3 min) by incubation of a second sample aliquot with hyaluronidase. Effusions of 13 of 14 patients with confirmed malignant mesothelioma contained HA in the 0.3 to 11.1 mg/ml range. In only one case was no HA detected. None of the effusions from 56 control patients with various other primary tumors contained detectable HA, i.e., there were no false positives. An unidentified peak, not susceptible to hyaluronidase appeared in 11% (6 of 56) of the controls. A single mesothelioma case was correctly identified in a group of 10 coded samples. It is suggested that an effusion with an HA concentration >0.25 mg/ml, confirmed by hyaluronidase susceptibility, is an indication of the presence of malignant mesothelioma. The test is simple and rapid, and it is recommended that any effusion of uncertain etiology be screened for the presence of HA.

INTRODUCTION

Mesothelioma is a public health concern because of its association with occupational, environmental, and domestic exposure to asbestos. Such exposure is the major cause of the approximately 1000 cases of malignant mesothelioma in the United States each year (16). The overall prognosis for mesothelioma is very poor, with a median survival from diagnosis of only 7 to 13 months (10). A recent review of 4710 published cases summarizes the clinical aspects of the disease (19).

The pathological diagnosis of malignant mesothelioma of the pleura or peritoneum is based on consistent gross, microscopic, and histochemical findings. Differentiation from metastatic adenocarcinomas is based on the fact that the latter secrete mucins, whereas mesotheliomas secrete HA, which can be visualized by staining with Alcian blue or colloidal iron, with or without pretreatment with hyaluronidase (1, 3, 27). However, differentiation of mesotheliomas from metastatic tumors is often difficult, because of frequent problems in the interpretation of staining patterns (22).

Experimental Procedures

HA is a long-chain unbranched polymer composed of alternating (1 to 3) linked and (1 to 4) linked β-D-glucuronic acid and N-acetylg glucosamine residues. Chemical and biological properties of HA and the other GAGs have been extensively discussed (23). The presence of HA in effusions from patients with malignant mesothelioma was first reported 45 years ago (26). Subsequent work revealed that high concentration of HA in pleural and peritoneal exudates is frequently, but not always, associated with mesothelioma (7, 14, 18, 29, 33). In a study of 247 pleural fluids, HA concentrations >0.8 mg/ml were found only in the presence of mesothelioma, while lower levels occurred in other cancers or inflammation (30). Subsequent publications (2, 6, 12, 13, 17, 20, 21, 25) also concluded that high concentrations of HA were only found to be associated with mesothelioma, and that low HA levels were of limited diagnostic value. However, numerical values of “high” and “low” HA concentrations differed by as much as an order of magnitude. The reason for the relatively wide range of HA concentrations reported in pleural and peritoneal fluids may be that the analytical techniques were inadequate for the separation of HA from other GAGs present. Earlier studies used nondenaturing polyacrylamide gel electrophoresis (5, 34); sample preparation in these methods is tedious or difficult because of separating HA from other GAGs and difficulties in quantification.

The prior results constitute adequate evidence to justify further studies on the HA concentration of effusions as a potential marker for malignant mesothelioma. The present work describes a simple and rapid technique based on HPLC using a size exclusion column with an exclusion limit of approximately 7 × 10^6 daltons. In these columns, separation is based on the molecular size of the solutes (4, 31). This approach has the advantages of measuring HA as an intact, unhydrolyzed molecule without altering its biochemical integrity, and of providing complete separation from all other GAGs present.

MATERIALS AND METHODS

Reagents. HA (Grade 1, human umbilical cord) and hyaluronidase (type VI-S from bovine testes and types II, III, and IV from ovine testes) were purchased from Sigma Chemical Co. (St. Louis, MO). Hyaluronidase from Streptomyces hyalurolyticus was obtained from Calbiochem-Behring (La Jolla, CA). High-purity chemicals and HPLC quality water were obtained from Fisher Scientific Co. (Springfield, NJ).

Phosphate Buffer. A 0.05 M phosphate buffer of pH 5.0 was used as the diluent for samples, for the preparation of calibration curves, and as the mobile phase. The buffer was prepared by mixing 250 ml of 0.2 M NaH₂PO₄ with 2 ml of 0.2 M Na₂HPO₄ and diluting to 1000 ml.

Patient Samples. Samples of pleural and peritoneal effusions were obtained from 14 patients with histologically proven malignant mesothelioma. Pericardial fluid was available from 3 of the 14 patients.
HA IN MESOTHELIOMA

Procedures and criteria for the diagnosis of malignant mesothelioma were as reported (10). There were 56 controls with effusions from a variety of cancers: 23 ovarian cancer, 14 lung cancer, 4 breast cancer, 4 pancreatic cancer, 3 gastrointestinal tract carcinomas, 2 benign tumors, and one each of hepatoma, endometrial cancer, uterine cancer, lymphoma, unknown primary cancer, and benign effusion. Aliquots of effusions were obtained when such fluids were removed from patients in the course of their management.

Samples were prepared for HPLC analysis by diluting 100-μl aliquots of effusions with 9.90 ml phosphate buffer. For samples that were to be treated with hyaluronidase the procedure was as follows. A 500-μl aliquot of fluid was incubated with 1000 units hyaluronidase (Type VI-S) in 100 μl buffer at 37°C for 18 h, and a 120-μl aliquot (the additional 20 μl accounts for the dilution caused by addition of enzyme) was diluted to 10 ml with buffer. Injection volume for HPLC analysis was 100 μl. Subsequent experiments showed that incubating effusates with 25 units of enzyme for 20 min yields the same results.

Calibration Lines. Solutions of 0, 0.25, 0.5, 1.0, 2.5, 5.0, 7.5, and 10.0 mg HA/ml buffer were prepared and 100-μl aliquots were diluted with 9.90 ml buffer to yield the same 1:100 dilution described for patient samples. To determine whether calibration standards prepared in buffer could be used for quantification of HA in patient samples, calibration lines obtained using standards prepared in buffer were compared with calibration lines obtained by the standard addition method using patient effusions. Calibration samples using effusions were made by taking 9.90 ml of fluid, which had been prediluted 100-fold with buffer, and adding 100-μl aliquots of HA standards of appropriate concentration.

Reproducibility. Intraday reproducibility for calibration samples was determined by analyzing buffer samples spiked with 0.5, 1.0, 2.5, and 5.0 mg HA/ml buffer; 8 replicates of each sample were analyzed, using the automatic sample injector. Interday reproducibility was determined by analyzing 8 samples in the 0- to 10.0-mg HA/ml buffer range on 4 consecutive days. The intraday and interday reproducibility of patient samples were tested using 4 samples from patients with malignant mesothelioma. HA concentrations were 0.6, 2.5, 2.6, and 11.1 mg HA/ml effusion. Two of these samples (2.6 and 11.1 mg/ml) were analyzed 5 times for intraday reproducibility. For interday reproducibility the same samples were analyzed (in duplicate) on 3 separate days, and 2 additional samples (2.5 and 0.6 mg/ml) were analyzed on 4 separate days within an 8-day period.

Incremental Sensitivity. Two effusions containing 0.9 and 2.4 mg HA/ml fluid were supplemented with 0.1 and 0.2 mg HA/ml, and the original and spiked samples were compared by analyzing each 6 times, using the automatic sample injector.

Stability of Patient Samples. To determine the stability of HA in effusions to freezing and thawing, and to stability in storage when diluted 100-fold, the following 4 types of samples were prepared and analyzed on 5 occasions over a 7-day period: (a) five 100-μl aliquots of the original effusion were frozen and thawed on every occasion; (b) a 1-ml aliquot of the original effusion was frozen and thawed on every occasion when a 100-μl aliquot (total of 5 freezing-thawing cycles) was taken out; (c) a 100-μl aliquot of the original effusion was diluted 100-fold and stored at 4°C with 100-μl aliquots analyzed on each occasion; (d) same as type c, but samples were stored at room temperature.

HPLC. The HPLC system used consisted of a Model U6K injector or a WISP Model 710B automatic sample injector, a Model 6000A pump, and a Model 730 data acquisition module, all from Waters Chromatography Division of Millipore, Inc. (Milford, MA), and a variable wavelength detector from DuPont Co. (Wilmington, DE). The column was a TSK 5000 PW size-exclusion column, 30 cm long x 7.5 mm inside diameter (Beckman Instruments, Berkeley, CA), protected by a guard column filled with 10/50 column packing material (Waters). The flow rate was 1.0 ml of the phosphate buffer/min. Compounds were detected by UV absorbance at 200 nm. The data acquisition module was set for quantification in the peak height mode.

RESULTS AND DISCUSSION

Specificity. We have investigated several available size exclusion columns under various experimental conditions. The best overall performance was obtained with the G5000 PW column. These columns are made of a porous, spherical semigel gel and have a hydrophobic surface functionality. Particle size is 17 ± 2 (SD) μm, theoretical plate number is 10,000/mm, and exclusion limit is 7 x 10⁶ daltons (dextran). Chart 1A shows a peak of 5 μg HA injected in 100 μl buffer. This corresponds to an initial sample concentration of 5 mg/ml, which is within the range found for mesothelioma patients. The intraday reproducibility of the retention time of HA (determined by the data system) was 5.29 ± 0.01 min (n = 7) for HA in buffer, and 5.29 ± 0.02 min (n = 7) for HA in an effusion. The retention time changed slightly from column to column within the 5.0- to 5.5-min range. The fact that the retention times of the HA used as the standard (from umbilical cord) and the HA found in the patient samples were identical does not necessarily mean that the molecular weights of the HA from both sources were the same, because differences within a narrow range would not be resolved by the column used. Similarly, it is possible that the HA peaks in both the calibration standards and in the effusions from patients with malignant mesothelioma may contain unresolved components, in accordance with the reported polydispersity of HA (23). This kind of polydispersity, if present at all, apparently does not influence the technique developed, as evidenced by the data given below. It is noted that none of the commercially available HA yielded a single peak; impurities amounting to a few percentage points were always present but were well resolved from the main HA peak.

Separation of HA from chondroitin sulfates and other GAGs was complete. In fact, GAGs eluted some 20 min after the HA, unresolved, and together with low-molecular-weight constituents of the biological fluids. However, because unresolved proteoglycans or other constituents with comparable molecular weights might still be present in the effusions, since sample preparation consisting of only a simple dilution, the identity of HA was confirmed in each case by determining its hyaluronidase suspe-
bility in a separate analysis. Chart 1B shows the analysis of a 100-μl injection from a 5-mg HA/ml sample after incubation with hyaluronidase and the usual 100-fold dilution. The HA peak has been completely eliminated. There was no need to remove the enzyme from the sample after incubation, because the peak corresponding to the enzyme has a much longer retention time (approximately 10 min) than HA does. Several commercially available hyaluronidase preparations were compared. These included hyaluronidase from S. hyalurolyticus, which is highly specific for HA (28), and from bovine and sheep testes. There was no apparent difference in the performance of these preparations for the present purpose. The final choice (type VI-S from bovine testes) was made on the basis of such convenience considerations as activity, availability, and cost.

Patient effusions were analyzed in 2 stages. First, in a rapid screening (30 min), it was determined if the fluid contained detectable HA. A peak observed at approximately 5.3 min was taken as an indication of the possible presence of HA. Samples with measureable HA concentrations were reanalyzed after hyaluronidase treatment to confirm the presence of HA.

In the analyses of clinical samples, the run was continued for 25 min after the elution of HA, to permit all other constituents of the fluids to elute from the column. It is noted that a very large peak always appeared at a retention time of 9.9 min. This peak, which is probably the sum of several unresolved components, could be eliminated by precipitation with perchloric acid (which also removed HA), but was not susceptible to digestion with trypsin or mixed chondroitinase lysates. The presence of this peak did not interfere with HA analysis, as long as appropriate dilutions were made.

Quantification. Quantification was accomplished with the aid of calibration lines. These were obtained by dissolving known amounts of HA in water, followed by appropriate dilutions with the mobile phase to yield samples with HA concentrations in the 0- to 10-mg/ml range. The peak heights obtained were plotted against concentration. Regression analysis of the calibration lines gave intercepts very close to the origin, and correlation coefficients >0.99. The slopes of the lines showed excellent intraday reproducibility; however, numerical values of the slope exhibited a gradual decrease concomitant with the aging of the UV lamp (lifetime, approximately 500 h) and the cleanliness of the mirrors in the detector. No internal standard was used for the assay because of the difficulty in obtaining a suitable biopolymer with appropriate solubility and chromatographic characteristics. The clean chromatographic peak and good interday reproducibility of HA measurements (see below) ensure the accuracy of the method. The technique of standard addition may be used if confirmation of quantification is needed. The intercept with the X axis of the line derived from standard addition to the patient sample gives the concentration of HA in the exudate as illustrated in Chart 2.

To prove that calibration curves prepared in the mobile phase were valid for the quantification of HA in biological fluids, a comparison was made with diluted effusions, using the standard addition technique. The lines obtained in both cases (Chart 2) were parallel, with the difference between them representing the HA concentration in the patient sample. When such lines were obtained using samples which did not contain HA, the 2 lines were coincident.

The lower limit of quantification was 0.25 mg HA/ml effusion. The lower limit of detection was 0.1 mg HA/ml fluid. These values were obtained with no sample preparation, other than the 100-fold dilution with buffer described. The limit of detection of pure HA was 100 ng.

Reproducibility. The intraday reproducibility of the calibration samples (4 samples with different HA concentrations, each analyzed 8 times) gave relative standard deviations in the 4 to 9% range. The interday reproducibility (8 samples with different HA concentrations, each analyzed on 4 separate days) gave relative standard deviations in the 2 to 16% range, with the higher values occurring at the lower concentrations. The interday reproducibility of patient samples was determined by analyzing 2 samples with concentrations of 11.1 and 2.6 mg HA/ml exudate, 5 times on a single day. The relative standard deviations (coefficient of variation) were 4 and 6%, respectively. The interday reproducibility of patient samples was determined by analyzing (in duplicate) the same 2 samples on 3 separate days, and 2 additional samples (2.5 and 0.6 mg HA/ml HA/ml) on 4 separate days. The relative standard deviations were in the 6 to 12% range. It is seen that reproducibility is essentially the same for both calibration and patient samples and, as expected, interday reproducibility is somewhat better than interday reproducibility. These values are considered adequate for the purpose of the present work.

Incremental Sensitivity. The incremental sensitivity of the method was determined by adding 0.1 and 0.2 mg HA/ml effusion, and analyzing the samples 6 times each. Data were evaluated by the Wilcoxon rank sum test. For the sample containing 2.4 mg HA/ml, the probability of detecting a 0.2-mg HA/ml increment was >99%. In the sample containing 0.9 mg HA/ml, an increment of 0.1 mg HA/ml was detected, with a probability of 93%, and an increment of 0.2 mg HA/ml with a probability of >99%.

Stability of Patient Samples. The set of experiments designed to determine the stability of HA in effusions over a 7-day period revealed that the HA concentration of the samples remained stable throughout 5 freeze-thaw cycles [see type b in "Materials and Methods"] and also after 100-fold dilution, as long as sample concentrations were coincident.
storage was at 4°C [see type c in “Materials and Methods”]. All HA concentrations obtained were within ±13% of those of the controls [see type a in “Materials and Methods”]. When 100-fold diluted effusions were stored at room temperature, the HA began to degrade after 2 days, as evidenced by the appearance of 2 partially resolved chromatographic peaks, rendering quantification inaccurate and inappropriate.

**Patient Samples.** Chart 3 illustrates chromatograms obtained from 3 samples. Chart 3A shows a sample from a patient with malignant mesothelioma; incubation with hyaluronidase removed the entire peak, indicating that it consisted solely of HA. The HA concentration in this sample was 2.4 mg/ml. Chart 3B shows a sample from a control patient (ovarian cancer). Here no peak was detected, and therefore there was no need to incubate the sample with hyaluronidase. Chart 3C is a sample from a patient with lung cancer. This is one of the few occasions (11%) when a peak was detected in a control. Incubation with hyaluronidase revealed that this peak was not HA; the HA concentration of this sample was <0.1 mg/ml. The nature of the compound(s) comprising this peak is unknown; the peak could not be removed by chondroitin ABC lyase or protease.

![Chart 3](chart3.png)

**Table 1**: HA concentrations obtained from mesothelioma samples with and without hyaluronidase treatment.

<table>
<thead>
<tr>
<th>Case</th>
<th>HA (mg/ml)</th>
<th>HA after hyaluronidase (mg/ml)</th>
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<tr>
<td>1</td>
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<tr>
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<tr>
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</tr>
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<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
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</tr>
<tr>
<td>14</td>
<td>0</td>
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</tr>
</tbody>
</table>

*Data are averages from replicate analyses.

Table 1 gives the quantitative results on all mesothelioma patients. Effusion from 13 of 14 patients (93%) with confirmed malignant mesothelioma contained HA in the 0.3- to 11.1-mg/ml concentration range. In all 13 cases (100%), the peak was completely removed by hyaluronidase, indicating that it consisted solely of HA. There was one false negative. In the case of this patient, both peritoneal and pleural effusions (and also pericardial fluid) were available, but HA was not detected in any of them. It is noted that in 2 mesothelioma cases where HA was readily found in the pleural effusions, pericardial fluid samples were also obtained, but these did not contain detectable HA.

Among the control samples, 50 of 56 (89%) exhibited no HA peak at all (HA < 0.1 mg/ml). There were 6 of 56 (11%) controls (4 lung cancers, 1 ovarian cancer, and 1 pancreatic cancer) which contained an unidentified peak, with approximately the same retention time as HA, which was not hyaluronidase susceptible. The fact that no HA was found in the control samples contradicts previous work (6, 12, 17, 20, 25) which reported the occasional finding of small quantities of HA in effusions from patients with cancers other than mesothelioma. An explanation may be found by considering the major difference between the previous and present analytical techniques, namely, that the present HPLC method quantifies HA in its intact form and fully resolved from other GAGs. The technique is highly specific, because no false positives were encountered in 56 effusions.

Based on these results, the diagnosis of malignant mesothelioma is strongly suggested when the HA concentration of a pleural or peritoneal effusion is >0.25 mg/ml. Using this criterion, a single sample from a patient with confirmed malignant mesothelioma was correctly identified in a panel of 10 coded samples submitted for evaluation. This sample contained 2.4 mg HA/ml, and 100% of the peak was hyaluronidase susceptible. No other sample contained detectable HA.

**HA as a Marker for Mesothelioma.** Malignant transformations of normal cells often result in the synthesis and subsequent secretion of abnormal amounts of endogenous constituents and/or abnormal biochemical substances. A number of such tumor markers have been proposed for various cancers (11, 24). Although HA may not be found in all cases of malignant mesothelioma, it should be of considerable help in the differential diagnosis of malignant mesothelioma from adenocarcinoma, a difficult challenge, even for experienced pathologists (15). The usefulness of quantification of HA in differentiating tumor from reactive mesothelium is currently unknown. Results may also be inconclusive when low HA concentrations are found.

A pleural or peritoneal effusion is often the first sign of mesothelioma, and may be present for several months before diagnosis (32). For example (8), 25% of patients with malignant mesothelioma had a delay of diagnosis for over 6 months. These patients are often misdiagnosed as having tuberculosis, lupus erythematosus, or rheumatoid arthritis. Cytological examination of the fluid obtained by thoracentesis is often negative (9). Pleural needle biopsy is often difficult in view of the thickened pleura and has been reported to be negative, and thus misleading, in 45 of 57 (79%) cases of mesothelioma (9). It is believed that the simple technique proposed herein could be used to assay all effusions of uncertain etiology. If positive for HA, this would alert physicians to the high probability of mesothelioma, leading them to undertake the invasive procedures (thoracotomy, thoracos-
copy, laparotomy) necessary for pathological confirmation of the diagnosis of mesothelioma. This would greatly help in making a diagnosis at a much earlier stage than is now often the case. Earlier diagnosis might make surgical resection and chemotherapy more effective in the management of malignant mesothelioma.

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