Urinary Glycosaminoglycan Excretion in Rheumatic Diseases

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We used Alcian Blue (AB) and dimethylmethylen blue (DMB) methods to measure glycosaminoglycan (GAG) excretion in the first morning urine specimens of patients with osteoarthritis (OA), ankylosing spondylitis (AS), and rheumatoid arthritis (RA) in different stages of disease. By the AB method, urinary GAG excretion in patients with RA was not different from healthy control subjects. However, the DMB method showed significant differences (in milligrams of GAG per gram of creatinine) for OA (median 25.4, range 14.3–44.0, P < 0.01, n = 23) and RA patients (median 33.0; range 10.0–147.6; P < 0.001, n = 63) in comparison with unaffected individuals (median 20.2; range 8.9–41.4, n = 38). We noted a significant difference in urinary GAG excretion between RA and OA patients (P < 0.01) and between RA and AS (P < 0.01) patients. The DMB method was further investigated by clinical decision analysis. The DMB method is simple and rapid and may be useful in diagnosing RA by distinguishing between RA and OA or AS.

Glycosaminoglycans (GAGs) are large-molecular-mass linear carbohydrate polymers composed of glucuronic or iduronic acid and N-acetylglycosamine or N-acetylgalactosamine units (1). 1 Chondroitin sulfate and heparan sulfate are derived from proteoglycans in which these GAGs are covalently linked at the reducing end to the hydroxyl group of serine residues (2). Proteoglycans containing GAG bound to hyaluronic acid form large aggregates that are enmeshed in a collagen network as the principal structural constituents of articular cartilage (3). Loss of proteoglycans is an early feature of matrix resorption (4, 5). A corresponding increase in GAGs has been reported in synovial fluid (6–13), serum (14, 15), and urine (16–19) of patients with rheumatic diseases.

Among the methods developed for measuring GAG concentrations in biological fluids, most are based on specific binding of GAG with cationic dyes, e.g., acridine orange, 1,9-dimethylmethylen blue (DMB), and Alcian Blue (AB), and subsequent spectrophotometric determination of GAG-dye complexes (20–23). More laborious and expensive but more specific methods were published recently: radioimmunoassay (24), polyacrylamide-gel separation (25), and monoclonal antibody-based enzyme-linked immunosorbent assay (26). Dye-based assays have been studied extensively regarding clinical diagnosis of mucopolysaccharidoses diseases (27–30). However, little attention has been directed to the relationship between the method for GAG determination and the diagnosis of rheumatic diseases.

Here, we compare two dye-based methods for the spectrophotometric measurement of GAG excretion in urine: AB (23) and DMB (27–30). We compare the methods with respect to identifying and classifying patients with rheumatoid arthritis (RA) in various stages of disease progression and activity, osteoarthritis (OA), and ankylosing spondylitis (AS). We also investigate the clinical validity of the DMB method.

Materials and Methods

Reagents. AB 8GX, sodium dodecyl sulfate, and DMB were purchased from Serva (FRG); chondroitin sulfate (66 kDa) was obtained from Koch/Light (UK); and other chemicals were pa. quality purchased from Lachema (Czechoslovakia).

Patients. GAG concentration was measured in first morning urine of hospitalized patients with, by ARA criteria (31), classical or definite RA (n = 63; mean age 46, SD 16, range 16–79 years), patients with AS (n = 14; mean age 44, SD 11, range 25–67 years), OA (n = 26; mean age 45, SD 10, range 24–66 years), and healthy control subjects (n = 38; mean age 47, SD 13, range 21–71 years). RA patients were divided into three groups by disease activity: low (L), moderate (M), and high (H) activity. Groups I, II, III, and IV of RA patients were created according to the criterion of degree of disease development (32). The following activity criteria were used: erythrocyte sedimentation rate, C-reactive protein, number of painful and swollen joints, and morning stiffness. RA patients were receiving nonsteroidal anti-inflammatory drugs (all patients), prednisone <10 mg/day (n = 16), and gold (Tauredon, Byk Gulden, Switzerland; n = 27). AS patients were diagnosed according to the New York criteria (33) and were treated only with nonsteroidal anti-inflammatory drugs. Most OA patients had primary generalized disease with no evidence of inflammatory joint disease. Ten of the 14 AS patients were taking no drugs, and 4 were taking only pure analgesics.

Urine specimens. The first morning urine was collected from each patient and healthy control subject and frozen without delay at −20 °C until assay.

DMB assay. The DMB assay was performed essentially according to the method of Whitley et al. (28). Briefly, the DMB dye solution was prepared by dissolving 16 mg of DMB, 3.04 g of glycine, 2.37 g of sodium chloride, and 0.5 mL of 0.1 mol/L hydrochloric acid in 1 L of redistilled water. The pH of the solution was adjusted to 3. For each assay, the patient's urine speci-
men was mixed with 1 mL of the dye solution at room temperature, and we measured absorbance at 525 nm without delay after mixing the sample and dye solution. The assay was calibrated vs a standard curve of chondroitin sulfate (5–100 mg/L). Urine samples were thawed, immediately divided (25, 50, and 100 μL), and diluted to a constant volume of 100 μL with buffered saline (per liter: 0.01 mol of phosphate and 0.15 mol of sodium chloride, pH 7.4). We plotted each measured absorbance vs urine dilution, and the intersection of the line with the abscissa was subtracted as background from the absorbance for each urine sample before we calculated the mean. The value reported for each urine sample was the mean of at least three measurements.

AB assay. The AB assay was performed according to the method of Whiteman (23). AB solution was always freshly prepared by dissolving 50 mg of AB in 0.05 mol/L sodium acetate buffer, containing 0.05 mol of magnesium chloride per liter (pH 5.8). For each assay, we mixed 100 μL of the sample with 2 mL of AB solution and let this equilibrate at room temperature for 2 h. After centrifuging the mixture at 2000 × g for 10 min, we washed the precipitate twice with 2 mL of ethanol, resuspended it in 2 mL of 50 g/L sodium dodecyl sulfate reagent and measured the absorbance at 620 nm. Absorbance values were compared with the standard curve for chondroitin sulfate (5–100 mg/L) for calibration. We used 50- and 100-μL urine samples for GAG measurement.

Statistics. We compared median values for urinary GAG excretion of healthy control subjects and patients by the Mann–Whitney U-test; hypotheses with P ≤0.05 were considered statistically significant. We used methods of clinical decision analysis for calculating the receiver-operated characteristic (ROC) curves, sensitivities (true positive rate: the proportion of patients with the disease who have a positive test result) and specificities (true negative rate: the proportion of healthy control subjects who have a negative test result) (34).

Results

Comparison of methods. The calibration curve for GAG when we use the AB method is a straight line (r = 0.990), but with the DMB method it is a nonlinear curve (Figure 1). This agrees with observations of another author (CB Whitley, personal communication). The DMB calibration curve can be approximated with two linear segments that each show large degrees of linearity (r = 0.998 and r = 0.992). Distinguishing the two linear parts of the DMB method in range of chondroitin sulfate concentrations used necessitated two correlations with the AB method: r = 0.995 and r = 0.985, respectively. The total correlation (r = 0.965) of both methods of calibration was also good. However, there was no correlation between methods (r = 0.202) for GAG concentrations in the urine of 38 healthy control subjects. Correlation was even less for GAG concentrations of 63 RA patients (r = 0.089). Therefore, the results for GAG excretion in urine obtained by these methods should be judged by each method individually.

Urinary GAG excretion in rheumatic diseases. Table 1 summarizes urinary GAG excretion in patients and healthy control subjects. Median values for GAG excretion measured by the AB method were approximately twofold greater than by the DMB method. Despite the greater GAG excretion, there were no significant differences between patients and control subjects by the AB method. In contrast, median GAG excretion measured by the DMB method was significantly greater for the patients than for the control subjects. Therefore, further work was focused on GAG excretion measured by DMB.

The urinary excretion measured by the DMB method for AS patients (Table 1, Figure 2) vs healthy control subjects as well as for AS patients vs OA patients was statistically nonsignificant. However, significant differences were observed between the RA patients and control subjects (P <0.001), patients with OA and RA (P <0.001), and the AS and RA patients (P <0.01). Effect of stage of disease. Comparison of urinary excretion in groups of patients with different degrees of RA development according to the criteria (32) are shown in Table 2 and Figure 3. Differences in GAG excretion between control subjects and RA patients were statistically significant. However, differences between individual groups of RA patients were not significant.

![Figure 1. Calibration curves for the DMB (●) and AB (○) methods, read at 525 and 620 nm, respectively](image)

| Table 1. GAG Concentrations (g/mol creatinine) in First Morning Urine Specimens Measured by the AB and DMB Methods |
| --- | --- | --- | --- |
| Diagnosis | Control subjects (n = 38) | RA (n = 63) | OA (n = 38) | AS (n = 14) |
| **AB method** | | | | |
| Median | 5.37 | 6.74 | 6.49 | 4.75 |
| **DMB method** | | | | |
| Median | 2.29 | 3.73* | 2.87* | 2.40 |
| Range | 1.01–4.68 | 1.13–16.70 | 1.62–4.98 | 1.35–4.56 |

* P <0.001 vs control subjects.  
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ent, comparison of groups of RA patients having moderate or high degrees of activity with control subjects revealed very significant differences.

Clinical validity of DMB method. Because of the overlap between the categories in the scatter diagrams, we used clinical decision analysis methods to obtain more-objective information about the clinical validity of the DMB method. We focused our attention on clinical uses of the DMB method: distinguishing between healthy control subjects and RA, OA, and AS patients and distinguishing between the individual diseases.

First, we calculated sensitivities and specificities for GAG urinary excretion in RA, OA, and AS patients by using the mean plus slope (2.58 + 0.97 g of GAG per mole of creatinine) for GAG concentration in control subjects as the decision value. The specificity (0.842) and sensitivities (0.556 for RA, 0.566 for OA, and 0.357 for AS, respectively) suggest that the test is relatively highly specific but is less sensitive for diagnosing the patients. Similarly, we obtained sensitivities of 0.476 and 0.476 and specificities of 0.783 and 0.786, respectively, when the urinary GAG excretion was compared for RA and AS patients. The decision values of the mean plus slope for GAG urinary excretion were (3.03 + 0.77) g/mol creatinine for OA and (2.69 + 1.03) g/mol creatinine for AS.

<table>
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<th>Table 3. Comparison of Urinary GAG Excretion (g/mol creatinine) in Patients with Different Amounts of RA Disease Activity</th>
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<td>Diagnosis</td>
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*P < 0.0003 vs control subjects.
*P < 0.0001 vs control subjects.
ROC curves, which reflect true- and false-positive rates for each individual group of patients compared with the control subjects at different decision values, are shown in Figure 5. Although >60% of RA and OA patients were diagnosed correctly at decision values >2.26 and 2.82 g of GAG per mole of creatinine, respectively, the method loses its validity in diagnosing AS patients. At the same decision values, results for ~20% of control subjects are falsely positive.

The ROC curves may also help resolve questions about the validity of the DMB method for distinguishing RA from OA and AS. The ROC curves calculated for RA compared with OA and AS are very close to the ROC curve for RA compared with the control subjects (Figure 6).

**Discussion**

Despite relatively good correlation between the calibration curves for the AB and DMB methods, their use for measuring urinary GAG excretion led to different results. The lack of correlation of the GAG concentrations obtained by these methods suggests that it would be difficult to compare results obtained by different authors. The relevance of urinary GAG excretion, determined by the AB method, to disease activity was described by Mbuyi et al. (18): In 42 RA patients, correlation of urinary GAG excretion with disease activity was statistically significant in 24-h urine specimens (P <0.02) but not in 2-h specimens.

The differences and lack of correlation between the AB and DMB methods for measuring GAG excretion in urine specimens have two possible explanations: First, because the AB method is based on precipitation, other oligomers/polymers, possibly of noncarbohydrate origin, may coprecipitate with the AB and interfere with the assay. Second, the AB procedure is dependent on both precipitation and resolution; because the precipitation of GAGs by positively charged agents depends on molecular mass, this method is less efficient for low-Mₚ GAG. According to Chuck et al. (19), the ratio of low- to high-Mₚ GAGs is greater in RA patients than in control subjects. Some of these low-Mₚ GAGs in RA could have been missed by the AB procedure.

The effect of treatment on urinary GAG excretion was later studied by the AB method by Chuck et al. (19); they noted no difference in total 24-h urinary GAG excretion in RA patients who had received only nonsteroidal anti-inflammatory and analgesic drugs. Our results are in complete agreement with these observations. When the AB method was used, we found no difference in the first morning urine between RA patients who were treated with nonsteroidal anti-inflammatory drugs and the healthy control subjects.

We used the methods of Draper et al. (27), Whitley et al. (28, 29), and DeJong et al. (30) for measuring GAG excretion in untreated urine by spectrophotometric determination of DMB–GAG complexes. These authors pointed out the disadvantages of the AB method, which include the instability of AB dye reagent. The AB method is also much more laborious and potentially flawed by the precipitation of other substances that may react with AB and cause GAG concentrations to be approximately double those found with the DMB method.

Urinary GAG excretion in RA patients increases only slightly in the first two stages of disease development and then increases rapidly up to twice the stage I value by stage IV. However, the method is not useful for distinguishing between the individual stages of RA development. At stage I, GAG urinary excretion is already different from that of healthy control subjects.

Differences in urinary excretion of GAGs at different disease activities of RA patients are not statistically significant. With the DMB method, it is possible to distinguish only moderate and high disease activity.
It was also shown that drugs, including antibiotics, at physiologically important concentrations have little if any influence on the DMB assay (28). The role of pharmacological treatment was not broached in our study.

In other DMB-based studies, urinary excretion was highly age dependent in healthy individuals (29). Our mean values were similar, and we noted a similar range of GAG excretion for healthy control subjects.

Clinical decision analysis of the DMB-measured GAG urinary excretion showed an application of the method for clinically distinguishing RA and OA patients from healthy control subjects as well as distinguishing RA patients from OA and AS patients. Analysis by ROC curves follows well the statistical significance of individual groups of patients. Analysis with ROC curves allows estimation of the probability of correct diagnosis at different concentrations of GAGs in urine.

We found the DMB method to be more convenient than AB and also more sensitive for differentiating the various rheumatological diseases. In addition, compared with our AB results, our DMB results agree better with GAG measurement by methods that measure sugars specific for GAG in RA patients (35, 36). Distinguishing RA and OA patients from the healthy control subjects as well as the RA patients from the OA and AS patients may be helpful in the clinical evaluation of such patients. Longitudinal evaluation of the urinary GAG excretion of individual RA patients during the progression and treatment of disease is warranted. In addition, the DMB method should be compared with a more-specific method, e.g., monoclonal antibody assay (26) or radioimmunoassay (24).

In summary, we demonstrate that measuring urinary GAG excretion in the first morning urine specimens by the simple and rapid DMB method may be useful in understanding the pathophysiology of rheumatic diseases. The DMB method could aid in distinguishing between RA and OA or AS diagnosis when further verified by more-specific assays.

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References