The Quantitative Determination of Glycosaminoglycans in Urine with Alcian Blue 8GX

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1. The effect of $MgCl_2$ concentration on the interaction of Alcian Blue 8GX and glycosaminoglycans in the urine of patients with mucopolysaccharidosis was studied by using a new quantitative micro method for the measurement of Alcian Blue–glycosaminoglycan complexes. This provided a means of measuring the critical electrolyte concentrations of urinary glycosaminoglycans. 2. Theoretical considerations based on the preceding paper (Whiteman, 1973) and experimental evidence provided here show that Alcian Blue 8GX may be used for the direct quantitative determination of total urinary glycosaminoglycans. The method is simple, requires sample volumes of $50\mu l$ or less, and gives results comparable with those obtained by other more complicated methods.

Alcian Blue 8GX is a cationic dye that forms insoluble complexes with acid glycosaminoglycans under suitable conditions (Scott *et al.*, 1964).

In the preceding paper (Whiteman, 1973) a method was described for determining the Alcian Blue content of complexes formed from small quantities of glycosaminoglycan. The conditions required for maximum complex-formation with standard and urinary glycosaminoglycans were determined. The amount of complex formed was proportional to the amount of standard glycosaminoglycans in solution. A simple variation of the technique allowed a study of the critical electrolyte concentrations of glycosaminoglycans to be made.

The present report extends this method to the determination of the total glycosaminoglycan content of urine, a preliminary report of which has been given (Whiteman, 1972). The use of this method as a means of obtaining information on the nature of urinary glycosaminoglycans is also investigated.

Experimental

Materials

Cetylpyridinium chloride and Manoxol IB (dibutyl ester of sodium sulphosuccinic acid) were obtained from B.D.H. Chemicals Ltd., Poole, Dorset, U.K. Alcian Blue 8GX was a gift from I.C.I. Ltd., Blackley, Manchester, U.K. Sodium chondroitin 4-sulphate (reference standard) was a gift from Dr. M. B. Mathews, Department of Paediatrics, University of Chicago.

Human Tamm–Horsfall urinary glycoprotein was a kind gift from Dr. Wendy A. Ratcliffe, Department of Chemical Pathology, St. Mary's Hospital Medical

School, London, and was prepared as described by Fletcher *et al.* (1970).

Purified human serum albumin (fraction V) was supplied by Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., and yeast RNA and herring sperm DNA were from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.

All other reagents were A.R. grade.

Urine, preserved with merthiolate, was obtained from control subjects (children) and from patients with Hurler's and Sanfilippo's syndromes.

Methods

Determination of total urinary glycosaminoglycans by using Alcian Blue 8GX. The basic method is described in detail in the preceding paper (Whiteman, 1973). In the standard procedure, standard glycosaminoglycan solution ($20\mu l$ containing $1\text{--}10\mu g$ of glycosaminoglycan) or centrifuged urine ($20\mu l$ for mucopolysaccharidosis patients; $50\mu l$ for control subjects) was mixed with 1 ml of a reagent containing 0.05% (w/v) Alcian Blue 8GX and 50 mM $MgCl_2$ in 50 mM sodium acetate adjusted to pH 5.8 with acetic acid. After equilibration for 2 h at room temperature, the glycosaminoglycan–Alcian Blue complex was separated by centrifugation at $2000g$ for 15 min . After the precipitate had been washed with ethanol (2 ml) it was dissociated with 1 ml of 40% (w/v) Manoxol IB solution. The E_{620} of the resulting clear blue solution was measured in 1 cm microcuvettes and a Unicam SP.500 spectrophotometer. The glycosaminoglycan contents of urine samples were determined by reference to a calibration curve constructed by using chondroitin 4-sulphate as standard. Although the results could be conveniently expressed

as mg of glycosaminoglycan/litre of urine, they were converted into the equivalent uronic acid concentration for comparison with other methods; the sodium chondroitin 4-sulphate used in this study contained 34.1% (w/w) of uronic acid.

Variations in the standard procedure are indicated in the appropriate sections.

Measurement of urinary glycosaminoglycan after the removal of Tamm-Horsfall glycoprotein. After equilibration with 0.5M-MgCl₂ at pH4.8 for 1 h at room temperature, over 80% of purified Tamm-Horsfall glycoprotein (50 µg) added to water (50 µl) or urine (50 µl) could be removed as a transparent gel by centrifugation at 2000g for 15 min. Precipitation of the glycoprotein was monitored by measuring the corresponding reduction in material chromophilic for Alcian Blue in the supernatant. The following procedure was therefore adopted as a means of measuring urinary glycosaminoglycan after the removal of Tamm-Horsfall glycoprotein.

Urine (0.2 ml) was mixed with 1M-MgCl₂ (0.2 ml) and allowed to equilibrate at room temperature for 1 h. After centrifugation at 2000g for 15 min, 0.1 ml of supernatant was mixed with water (0.1 ml) and 0.8 ml of a reagent containing Alcian Blue and sodium acetate buffer at a concentration 1.25 times that used in the standard reagent. The resulting Alcian Blue-glycosaminoglycan complex was then treated as in the standard procedure described above.

Determination of uronic acid. The Alcian Blue-precipitable uronic acid content of urine was determined by the method described in the preceding paper (Whiteman, 1973), apart from adjustments to the sample and reagent volumes to allow for differences in glycosaminoglycan concentration. Chondroitin 4-sulphate was used as a reference standard.

Cetylpyridinium chloride-precipitable uronic acid was determined by the method of Manley & Hawksworth (1966), including an autoanalyser modification of the carbazole reaction of Bitter & Muir (1962).

Characterization of urinary glycosaminoglycans by electrophoresis. Urine (1–2 ml) was mixed with 10 vol. of Alcian Blue reagent containing 50mM-MgCl₂ and equilibrated for 2 h at room temperature. After centrifugation, the complex was dissociated by vigorous shaking with 60 µl of 4M-NaCl-methanol (2:1, v/v). Free Alcian Blue was precipitated by the addition of 0.1M-Na₂CO₃ (20 µl) (J. E. Scott, personal communication) and water (60 µl). The mixture was left for 30 min at room temperature and then the Alcian Blue was removed by centrifugation. Glycosaminoglycans were precipitated from the clear supernatant (0.1 ml) by the addition of ethanol (0.4 ml). After centrifuging, the ethanolic supernatant was drained as completely as possible and the precipitate was taken up in water (20–40 µl).

Electrophoresis was carried out by the method of Wessler (1970) on Celagram cellulose acetate strips

(Shandon Southern Instruments Ltd., Camberley, Surrey, U.K.). Narrow bands of 1–2 µl of urinary glycosaminoglycan or reference standard glycosaminoglycan solutions (1 mg/ml) were applied. Electrophoresis proceeded for 3 h in 0.1M-barium acetate with an applied potential gradient of 7.5 V/cm. Strips were stained for 1 h with 0.05% (w/v) Alcian Blue containing 50mM-MgCl₂ in 50mM-sodium acetate buffer (pH5.8) and washed with a solution of 50mM-MgCl₂ in 50mM-sodium acetate buffer (pH5.8). Clear-blue bands on a white background were obtained.

Results

Interaction of Alcian Blue and urinary glycosaminoglycans

The effect of MgCl₂ concentrations on the formation of insoluble complexes between Alcian Blue 8GX and glycosaminoglycans in the urine of two patients with mucopolysaccharidosis is illustrated in Fig. 1. Maximum complex-formation occurred in the presence of 50mM-MgCl₂ both before and after dialysis

Fig. 1. Effect of MgCl₂ concentration on interaction of Alcian Blue and urinary glycosaminoglycans of patients with mucopolysaccharidosis

The ordinate shows the extinction of Alcian Blue released from Alcian Blue-glycosaminoglycan complexes, which were formed in different concentrations of MgCl₂. Reaction profiles are shown for urine samples both before and after dialysis against 1 litre of deionized water at 4°C. Urine of Hurler's syndrome: ●, before dialysis; ○, after dialysis (4 h). Urine of Sanfilippo's syndrome: ▲, before dialysis; △, after dialysis (4 h).

of urine against water. The considerable loss of polyanionic material on dialysis is consistent with the presence of low-molecular-weight glycosaminoglycans in the urine of these patients. Electrophoresis confirmed that the patient with Sanfilippo's syndrome excreted predominantly heparan sulphate, whereas the patient with Hurler's syndrome excreted predominantly dermatan sulphate. Determination of the uronic acid precipitable by cetylpyridinium chloride and Alcian Blue indicated an excessive excretion of glycosaminoglycans in both patients.

The differences in the Alcian Blue reaction profiles (Fig. 1) appeared to be consistent with the known molecular-weight distributions of urinary glycosaminoglycans in these patients. Constantopoulos (1968), using gel filtration, showed that patients with Sanfilippo's syndrome excreted heparan sulphate with an average molecular weight of 2300, whereas the urine of patients with Hurler's syndrome exhibited a dermatan sulphate fraction of molecular weight approx. 9000 and a larger fraction containing both heparan sulphate and dermatan sulphate with an average molecular weight of 2300. The very low critical electrolyte concentration of most of the glycosaminoglycan excreted in our patient with Sanfilippo's syndrome and the more extended critical electrolyte concentration pattern found in our patient with Hurler's syndrome correlate well with the findings of Constantopoulos (1968).

Further evidence was provided by electrophoresis of a concentrate of urine from the patient with Sanfilippo's syndrome. Two bands were detected when strips were stained with Alcian Blue at different concentrations of MgCl_2 . One band corresponded to heparan sulphate with a critical electrolyte concentration between 0.1 and 0.2M- MgCl_2 and the other to chondroitin sulphate with a critical electrolyte concentration of 0.3–0.4M- MgCl_2 . The critical electrolyte concentration of chondroitin sulphate is also indicated by the small inflexion between 0.3 and 0.4M- MgCl_2 , shown in Fig. 1. Comparison of the reaction profiles for urine before and after dialysis shows a proportionately greater loss of material of low critical electrolyte concentration, which would be expected if the main factor causing differences in the critical electrolyte concentrations of polyanions in these specimens was a difference in the molecular-weight distribution of glycosaminoglycans. These findings suggest that the Alcian Blue method may provide useful information on the nature of the polyanions, in addition to its proposed use for the quantitative analysis of glycosaminoglycans (Whiteman, 1972). The method can be compared with the fractionation of cetylpyridinium chloride–glycosaminoglycan complexes in salt solutions, except that the amount of complex remaining is measured rather than the amount of glycosaminoglycan eluted from the complex. According to Scott (1970) greater repro-

ducibility of critical electrolyte concentration profiles is likely to occur in systems where equilibrium conditions are attained, as in the method presented here.

Equilibration of urinary glycosaminoglycans with Alcian Blue at room temperature

When polyanions in the urine of patients with Hurler's and Sanfilippo's syndromes were equilibrated with Alcian Blue, maximum complex-formation occurred well within the 2h equilibration period of the standard procedure (Fig. 2). It appeared therefore that maximum interaction of urinary glycosaminoglycans with Alcian Blue occurred in the presence of 50mM- MgCl_2 (pH 5.8) and could be measured by using the standard procedure previously described (Whiteman, 1973).

Effect of urine sample volume on complex-formation: standard recovery tests

When varying amounts of undialysed urine (from a normal infant), up to a total of 100 μl , were equilibrated with 1 ml of Alcian Blue reagent, proportionate increases in the amount of Alcian Blue–glycosaminoglycan complex formed were observed (Fig. 3). After dialysis, urine additions also produced a linear response but gave very slightly lower values (after allowing for volume changes). The apparent loss of a small amount of polyanion during dialysis was less than

Fig. 2. Rate of formation of Alcian Blue complexes with urinary glycosaminoglycan

Urine (20 μl) from patients with Hurler's syndrome (●) or Sanfilippo's syndrome (○) was equilibrated with Alcian Blue reagent (1 ml) containing 50mM- MgCl_2 according to the standard procedure. The ordinate shows the extinction of Alcian Blue released from complexes, which were formed after different periods of equilibration at room temperature.

Fig. 3. Complex-formation between Alcian Blue and urinary glycosaminoglycan

The ordinate shows the extinction of Alcian Blue released from complexes, which were formed with different volumes of urine. The effect of dialysis in 1 litre of deionized water (4°C) is also shown. Urine of normal infant: ●, before dialysis; ○, after dialysis (4h).

that observed in the patients with mucopolysaccharidoses (i.e. Hurler's and Sanfilippo's syndromes). This may be due to the presence in normal urine of material of higher molecular weight. It is relevant that Constantopoulos (1968) found glycosaminoglycans with an average molecular weight of 8000 in normal urine. The possibility that equilibrium conditions would be significantly altered in the presence of urine was avoided by using a maximum sample volume of 50 μl of urine to 1 ml of Alcian Blue working reagent. In practice, 50 μl samples gave satisfactory results for normal urine samples, whereas only 10 or 20 μl was required for urine from patients with mucopolysaccharidosis. Good recoveries of standard chondroitin 4-sulphate were obtained in the presence of urine from patients with Hurler's syndrome. The amount of complex formed under these conditions was a simple function of the total amount of polyanion present in the system (Fig. 4).

Interaction of serum and plasma polyanions with Alcian Blue

Table 1 summarizes the interaction of serum, plasma and purified serum albumin with Alcian Blue at different concentrations of MgCl₂. Large amounts of complex (some of which was insoluble in the Man-

Fig. 4. Recovery of chondroitin 4-sulphate-Alcian Blue complex in the presence of urine of a patient with Hurler's syndrome

Alcian Blue-chondroitin 4-sulphate complexes were formed both in the presence and absence of urine (5 μl) from a patient with Hurler's syndrome. The standard procedure (see under 'Methods') was used and the ordinate shows the extinction of Alcian Blue released from the complexes. ●, Chondroitin 4-sulphate only; ○, chondroitin 4-sulphate plus urine (undialysed; 5 μl) from patient.

oxol reagent) were formed when serum or plasma was equilibrated with Alcian Blue reagent containing no added MgCl₂. In the presence of 50 mM-MgCl₂ only a trace of insoluble complex remained and the complex dissociated by the Manoxol reagent produced relatively little colour when the standard procedure was used. These results suggest that the interaction of Alcian Blue with serum or plasma proteins is largely prevented by the presence of 50 mM-MgCl₂, and that the complexes obtained at 50 mM- or higher concentration of MgCl₂ are due mainly to serum or plasma glycosaminoglycans. Interpolation of results obtained by this method at 50 mM-MgCl₂ gives a value of 45 μg of glycosaminoglycan/ml for normal plasma.

Purified human serum albumin (fraction V) produced insignificant amounts of precipitate when in complex with Alcian Blue in the presence of 50 mM-MgCl₂. With the standard procedure recoveries were close to 100% when known amounts of chondroitin 4-sulphate were added to a solution of purified human serum albumin (10 mg/ml). Thus it would appear that albumin does not block the reaction of Alcian Blue with glycosaminoglycan in the presence of 50 mM-MgCl₂ at pH 5.8.

Table 1. *Effect of MgCl₂ concentration on the interaction of plasma and serum polyanions*

Portions (20 μ l) of serum, plasma (with EDTA as anticoagulant) or albumin solution (10mg/ml; human fraction V, Sigma) was equilibrated with Alcian Blue reagent containing different concentrations of MgCl₂ as described under 'Methods'. —, Not measured.

Concn. of MgCl ₂ (mm)	Alcian Blue content (E_{620} —blank)		
	Serum	Plasma	Albumin
0	0.487*	0.570*	0.027
10	0.231*	0.293*	0.027
20	0.161*	0.211*	—
40	0.092	0.113	—
50	0.087	0.075	0.008
60	0.062	0.062	—
80	0.048	0.046	—
100	0.039	0.041	—
200	0.038	0.038	—

* Large proportions of these complexes were not dissociated by the Manoxol IB reagent.

Interaction of Alcian Blue with Tamm-Horsfall urinary glycoprotein

The effect of MgCl₂ concentrations on the interaction of Alcian Blue and Tamm-Horsfall urinary glycoprotein is shown in Table 2. Maximum interaction occurred in the presence of 50mm-MgCl₂ and the critical electrolyte concentration pattern resembled that obtained with polycarboxylates. This polyanionic effect is presumably due at least partly to the presence of sialic acid, although Fletcher *et al.* (1970) found only 4.4% (w/w) of sialic acid in Tamm-Horsfall glycoprotein. Colour development (E_{620}) in the standard procedure with 50mm-MgCl₂ was about one-fifth of that obtained by using the same weight of chondroitin 4-sulphate. The low concentration of Tamm-Horsfall glycoprotein present in normal subject's urine and its relatively low uptake of Alcian Blue make it unlikely that its presence contributes significantly to the values ascribed to total urinary glycosaminoglycans by using the present method. However, a method of removing Tamm-Horsfall glycoprotein before the addition of Alcian Blue is described in the Experimental section. It removed about 80% of Tamm-Horsfall glycoprotein (chromophilic for Alcian Blue) added to water or urine. Analyses of urine samples by the Alcian Blue method, with and without prior removal of Tamm-Horsfall glycoprotein, are illustrated in Table 3.

Other substances reacting with Alcian Blue

DNA and RNA form complexes with Alcian Blue under the conditions of the standard procedure but give lower extinction values than does an equal weight of chondroitin 4-sulphate (Table 4). It is most un-

Table 2. *Effect of MgCl₂ concentration on the interaction of Alcian Blue and Tamm-Horsfall urinary glycoprotein*

Tamm-Horsfall glycoprotein (20 μ l of a solution containing 1 mg/ml) was mixed with Alcian Blue reagent (1 ml) containing different concentrations of MgCl₂, and the amount of complex formed was measured by using the standard procedure.

Concn. of MgCl ₂ (mm)	Alcian Blue content (E_{620} —blank)
0	0.254
25	0.374
50	0.374
100	0.201
200	0.092
300	0.055
400	0.055
500	0.056
600	0.016
800	0.012

likely that these substances would appear in centrifuged urine in concentrations sufficient to alter values ascribed to glycosaminoglycans, measured by using the Alcian Blue method. The results described in Table 4 show that uronic acids and hexose 6-sulphates do not react with Alcian Blue under standard conditions. Many other substances apart from those in Table 4 were investigated, including other amino acids, carboxylic acids, bile salts and acids and steroid conjugates; none formed complexes with Alcian Blue by using the standard procedure.

Table 3. Comparison of different procedures for the determination of urinary glycosaminoglycans

The total glycosaminoglycan content of five urine specimens (two control subjects; three patients with mucopolysaccharidosis) was determined by using the various procedures described under 'Methods'.

Subject	Glycosaminoglycan measured by ...		Alcian Blue content of complex (mg of uronic acid/100ml of urine)		Uronic acid content of complex (mg/100ml of urine)		Main electrophoretic fractions
	Creatinine (mg/100ml of urine)	Total glycosaminoglycan	After removal of Tamm-Horsfall glycoprotein	Alcian Blue complex	Cetylpyridinium complex		
Mucopolysaccharidosis: type I* (Hurler's syndrome, 1 year)	9	4.9	4.9	4.7	4.6	Dermatan sulphate	
Mucopolysaccharidosis: type I* (Hurler's syndrome, 5 years)	44	15.8	15.4	15.7	15.6	Dermatan sulphate	
Mucopolysaccharidosis: type III* (Sanfilippo's syndrome, 5 years)	92	14.4	14.8	16.9	13.1	Heparan sulphate, Chondroitin sulphate	
Control: child (10 years)	172	2.1	2.1	2.0	2.3	Chondroitin sulphate	
Control: child (5 years)	107	4.0	3.8	3.2	3.0	Chondroitin sulphate	

* Mucopolysaccharidosis types refer to the classification of McKusick *et al.* (1965).

Table 4. Comparison of complex-formation of various substances with Alcian Blue

Samples (20 μ l) of solutions containing 1 mg of the substance/ml were made to form complexes with Alcian Blue containing 50 mM-MgCl₂ by using the standard procedure. —, No reaction.

Substance	E_{620} — blank
Sodium chondroitin 4-sulphate	1.85
Tamm-Horsfall glycoprotein	0.374
DNA (herring sperm)	0.388
RNA (yeast)	0.603
Glucuronic acid	—
Galacturonic acid	—
Glucose 6-sulphate	—
Galactose 6-sulphate	—
Muramic acid	—
N-Acetylneuraminic acid	—
Chondrosine	—
L-Cysteine (5 mg/ml)	—
GSH (5 mg/ml)	—

Comparative analysis of urinary glycosaminoglycans by the Alcian Blue technique and uronic acid determinations

The results of the determination of urinary glycosaminoglycans by Alcian Blue binding and from the uronic acid contents of Alcian Blue- and cetylpyridinium chloride-precipitable complexes are compared in Table 3. There was good correlation between the three methods despite differences in the concentration of the urine specimens (as measured by the urinary creatinine concentration) and in the type of glycosaminoglycan present.

A series of duplicate determinations on ten urine samples gave an average coefficient of variation of $\pm 2.7\%$.

General Discussion

A simple highly reproducible micro method for the determination of total urinary glycosaminoglycans has been described. It has several advantages over other methods. Apart from the initial centrifugation of a small quantity of urine no further preparation of the sample is required. The whole procedure can be carried out in a small disposable plastic tube, no heating is required and the reagents used are neither noxious nor dangerous. Although a 2 h period of equilibration and two 15 min periods of centrifugation are advised, the actual time involved in manipulative procedures is about 15 min and large numbers of samples can be analysed in one batch, a feat difficult to achieve by using the carbazole reaction for uronic acids.

The Alcian Blue technique measures glycosaminoglycans by virtue of their polyanionic nature rather than the constituent groups in the backbone chain. In this respect it provides a new parameter of glycosaminoglycan analysis and may be a useful complement to other methods. No single chemical determination is entirely satisfactory, owing to the variable constituents of different glycosaminoglycans. Uronic acid measurement by the modified carbazole reaction depends not only on the amount of uronic acid present but also partly on the type of uronic acid. The keratan sulphates contain little or no uronic acid. The Alcian Blue method has been shown to be relatively specific under the conditions prescribed. The main theoretical objection to the method is the reaction given by nucleic acids, but in practice this is unlikely to be of importance in the measurement of urinary glycosaminoglycans. The reaction given by Tamm-Horsfall urinary glycoprotein with Alcian Blue is interesting and suggests that parts of the molecule present anionic charges in a manner comparable to the glycosaminoglycans. Apart from the method for the removal of Tamm-Horsfall glycoprotein described in the Experimental section of the present paper, it may be possible to remove both this glycoprotein and nucleic acids by using an ultrafiltration technique, since the molecular weights of these substances are much higher than are the molecular weights of urinary glycosaminoglycans.

A previous report (Whiteman, 1973) indicated how the Alcian Blue method described here may also be used to obtain qualitative information about standard glycosaminoglycans in solution by a study of critical electrolyte concentrations. The reaction profiles of urinary glycosaminoglycans in patients with mucopolysaccharidosis correlate well with the

known molecular-weight distributions of these substances in the urine of such patients. The method may also be extended on a larger scale to allow fractionation of glycosaminoglycans, thus obviating the use of ion-exchange columns. An example of how glycosaminoglycans may be isolated by using this system was indicated in the Experimental section in the preparation of urinary glycosaminoglycans for electrophoresis.

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