Analysis of Glycosaminoglycans in Urine by using Acridine Orange Fluorescence

By GREGORY P. DIAKUN, HAYDN E. EDWARDS, JOHN C. ALLEN and GLYN O. PHILLIPS
*School of Natural Sciences, North-East Wales Institute of Higher Education, Kelsterton College,
Connaught Quay, Deeside, Clwyd CH5 4BR, Wales, U.K.*

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The fluorescence technique described here utilizes the electrostatic interaction between the polyanionic sites of glycosaminoglycans and the cationic dye Acridine Orange to analyse urinary glycosaminoglycans from patients suffering from mucopolysaccharidoses. The basis of the titration is the decrease in the fluorescence of free Acridine Orange that occurs when it is bound to polyanions. The effect of the presence of possible interfering materials such as salt, proteins and trace materials in urine was evaluated. This fluorescence technique is technically simple.

Many techniques for the investigation and analysis of glycosaminoglycans in biological fluids have been developed. These include precipitation with cationic detergents, uronic acid assay, chromatography and the use of cationic dyes, which have been comprehensively reviewed and evaluated (Pennock, 1976). Scott & Newton (1975) have recovered varying fractions of the 'total' excreted urinary acid glycosaminoglycans by varying the reagent and the conditions under which a reagent is used. However (Whiteman, 1973*a,b*), some of these techniques are laborious, time-consuming and/or unreliable. The present paper describes an alternative technique, a fluorescence assay of glycosaminoglycans by using Acridine Orange.

In its free monomeric state, the cationic dye Acridine Orange exhibits a broad fluorescence with a maximum at approx. 530nm, and in its aggregated state a fluorescence with a maximum at approx. 630nm. Polyanions promote dye aggregation; this phenomenon gives rise to metachromatic and fluorescence changes. Such properties may be used to assay glycosaminoglycans in biological fluids. A similar technique has been utilized for the analysis of carrageenan (Cundall *et al.*, 1973). In the present paper the analysis of urinary glycosaminoglycans is described, including its use in the diagnosis and monitoring of mucopolysaccharidoses.

Experimental

Materials

Heparin, chondroitin sulphate, poly-L-lysine, poly-L-arginine, bovine serum albumin, creatinine and Methylene Blue were obtained from Sigma (London) Chemical Co., Kingston upon Thames,

Surrey, U.K.; hyaluronic acid was a gift from Professor E. A. Balazs, College of Physicians and Surgeons of Columbia University, New York, NY U.S.A. and CM-cellulose was obtained from Hercules Powder Inc., Wilmington, DE, U.S.A. All other reagents were analytical grade and solutions were prepared with distilled water.

Acridine Orange (Sigma) was purified by a method previously outlined (Lamm & Neville, 1965): an absorption coefficient (ϵ_{492}) of $5000\text{M}^{-1}\cdot\text{cm}^{-1}$ was obtained for a $20\mu\text{M}$ aqueous solution (Bradley & Wolf, 1959).

Urine samples (analysed within 24h) were obtained from patients with Hunter and Sanfilippo syndromes and from control subjects (children). The patients with Sanfilippo syndrome were managed by Dr. R. Stephens, Hospital for Sick Children, Great Ormond Street, London W.C.1, U.K. (first presented 14.7.72) and Dr. R. E. Pugh, Alder Hey Children's Hospital, Liverpool, U.K. (first presented 13.5.66). The patient with Hunter syndrome was managed by Dr. J. M. Semmens, Southlands Hospital, Shoreham-by-Sea, West Sussex, U.K. (first presented 11.9.76).

Methods

Fluorimetric measurements were made on a Perkin-Elmer MPF 43A spectrophotofluorimeter. An aqueous solution (20ml) of $10\mu\text{M}$ -Acridine Orange was pipetted into a beaker and two or three drops of aqueous $10\mu\text{M}$ -Methylene Blue added. Methylene Blue quenches the fluorescence of dimeric Acridine Orange and the dye-polyanion fluorescence at 630nm (Rowlands, 1974). The mixture was magnetically stirred, portions (2ml) were withdrawn into a fluorimeter cell (path length 1cm) and the fluorescence intensity measured (excitation 400nm;

emission 540nm). The cell's contents (2ml) were returned to the beaker, a known volume of aqueous glycosaminoglycan was added and the procedure repeated. The fluorescence of free Acridine Orange was taken as 100% and subsequent fluorescence intensity readings were related to this. The P/D ratio (polyanion/dye) could be calculated, since the concentrations of the dye and polyanion during the titration were known. A more accurate estimate of the end point was obtained by extrapolation (Fig. 1).

For the titration in the presence of salts the initial solution was a 1:1 (10 μ M) complex between Acridine Orange and heparin.

Glycosaminoglycans in urine samples were determined in a 20-fold dilution of the original urine, and

also after dialysis of urine samples against water for 24h. At the equivalence point of the titrations with urine, it was assumed that one dye molecule binds to each anionic site, and by knowing the concentration of dye at the equivalence point, the concentration of urinary glycosaminoglycans present can be calculated. This concentration is expressed in g/anionic site per litre.

Uronic acid was determined after precipitation of glycosaminoglycans with cetylpyridinium chloride (Di Ferrante, 1967) by use of the modified carbazole test (Bitter & Muir, 1962). D-Glucuronolactone was used as a standard.

Creatinine was determined by the method of Kayser & Molitor (1956).

Results

Fig. 1 shows the effect of increasing concentrations of glycosaminoglycans on the monomeric fluorescence of Acridine Orange. With increasing polyanion concentration, the fluorescence of the free dye decreases and when all the sites of the glycosaminoglycans are occupied by the dye (P/D = 1) the equivalence point is reached. This point was determined by locating the intersection of the two linear sections of the curve.

The effect of increasing concentration of CaCl_2 on the electrostatic interaction between Acridine Orange and heparin is shown in Fig. 2 and a limiting salt concentration of 44.2 mM was obtained. Limiting salt concentration values of 480, 465 and 13.3 mM were obtained for NH_4Cl , NaCl and AlCl_3 respectively.

Titration curves were also performed with the addition of bovine serum albumin to both dye and polyanion. No effect was observed. However, poly-L-arginine and poly-L-lysine, at concentrations of 31.5 and 74.6 μ M respectively, completely disrupted the dye-

Fig. 1. Fluorimetric titration of glycosaminoglycans with Acridine Orange

The abscissa refers to the polyanion/dye (P/D) ratio and the ordinate to the fluorescence intensity of Acridine Orange (excitation wavelength 400nm and emission wavelength 540nm). Portions (0.5ml) of polyanion were added to 10 μ M-Acridine Orange (20ml) and after mixing thoroughly the fluorescence was noted: Δ , chondroitin sulphate; $\circ$, hyaluronic acid; $\blacksquare$, CM-cellulose.

Fig. 2. Effect of Ca^{2+} ions on a 1:1 heparin-Acridine Orange complex

Changes are shown in the fluorescence (excitation wavelength 400nm and emission wavelength 540nm) of 1:1 heparin-acridine orange complex (25 μ M) ($\bullet$) and Acridine Orange (25 μ M) ($\circ$).

Table 1. Total acid glycosaminoglycan component of urine specimens from normal subjects and patients suffering from mucopolysaccharidosis

(1) Glycosaminoglycan concentration expressed as equivalent/anionic site per litre of glycosaminoglycan from the end point of titration with cationic dye Acridine Orange; (2) calculated from end point by multiplying glycosaminoglycan concentration from (1) by heparin equivalent weight of 150 and expressed as mg of glycosaminoglycans/100ml of urine.

Date of birth (sex)	Syndrome	Glycosaminoglycan concn. from titration	Glycosaminoglycan concn. from titration after dialysis	Uronic acid	
				(mg/100ml)	(mg/mg of creatinine)
14.7.72 (female)	Sanfilippo	1.78 × 10 ⁻³ (1) 26.7 (2)	1.48 × 10 ⁻³ (1) 22.2 (2)	9.2	0.067
13.5.66 (female)	Sanfilippo	2.58 × 10 ⁻³ (1) 38.7 (2)	2.10 × 10 ⁻³ (1) 31.6 (2)	15.4	0.099
11.9.76 (male)	Hunter	5.48 × 10 ⁻⁴ (1) 8.2 (2)	5.33 × 10 ⁻⁴ (1) 8.0 (2)	1.3	0.118
Control 11.10.74 (female)		6.5 × 10 ⁻⁴ (1) 9.75 (2)	—	1.2	0.017
19.7.68 (male)		5 × 10 ⁻⁴ (1) 7.5 (2)	—	0.8	0.008

Fig. 3. Fluorimetric titration of urine
Urine from a patient with Sanfilippo syndrome, diluted 20-fold (●), and urine from same patient dialysed for 24h against water and diluted 20-fold (□) were titrated with Acridine Orange (10μM). For experimental details, see Fig. 1.

polyanion interaction. (The molarity of the poly-cations is based on the weight of repeating unit.)

Urinary components such as salts, proteins, bilirubin, urobilin, uroporphyrins, flavins and indole can affect the titrimetric assay of urinary glycosaminoglycans. Flavin fluorescence at between 400–600nm can be quenched by the addition of sodium dithionite (Beinert, 1960). This quencher at concentrations above 1mM had no effect on Acridine Orange fluorescence. Urine (20ml) of normal subjects containing sodium dithionite (10μM) was added to Acridine Orange (20ml of 10μM) and the resultant decrease in fluorescence was attributed only to dilution.

Table 1 and Fig. 3 show the data obtained for titration of single samples of urine from three patients with mucopolysaccharidosis and two control urines. The two types of mucopolysaccharidosis investigated were Hunter syndrome (MPS 11) and Sanfilippo syndrome (MPS 111), the former characterized by the excessive excretion of dermatan sulphate and heparan sulphate and the latter by the excessive excretion of heparan sulphate. The glycosaminoglycan concentrations in each urine sample were determined before and after dialysis. The urine samples were diluted 20-fold to obtain an equivalence point at a convenient concentration of 10μM-Acridine Orange (20ml).

Discussion

A rare genetic defect in the synthesis and activity of lysosomal enzymes essential for the degradation of connective-tissue glycosaminoglycans results in disorders known as Mucopolysaccharidoses, characterized by excessive excretion of glycosaminoglycans

(Table 1). The urinary glycosaminoglycans profile can be directly related to the type of mucopolysaccharidosis (McKusick, 1972).

The glycosaminoglycan assay described here is based on the decrease in monomer fluorescence of purified Acridine Orange that occurs when it binds electrostatically to the anionic sites of the glycosaminoglycan. An Acridine Orange concentration of $10\mu\text{M}$ was used, since above this value the concentration of dimeric dye and the resultant novel fluorescence increases at the expense of the monomer fluorescence.

Addition of salt to a 1:1 dye–polyanion complex increases the fluorescence intensity as a result of dye displacement, the concentration of salt required completely to disrupt dye–polyanion interaction being termed ‘limiting salt concentration’ (Jooyandeh *et al.*, 1974; Edwards *et al.*, 1977). Limiting salt concentration can be directly related to the electropositivity of the cation, and the presence of salt in urine at certain concentrations can affect the titrimetric assay of glycosaminoglycans. Salts can also influence the fluorescence of free Acridine Orange. The initial sharp decrease in the free dye fluorescence (Fig. 2) as cations were added is due to salt-promoted dimer formation, which increased in the order $\text{Na}^+ \geq \text{NH}_4^+ > \text{Ca}^{2+} > \text{Al}^{3+}$, in agreement with previous findings (Moulik *et al.*, 1976).

Proteins can effect the interaction between dye and polyanion by (a) masking the anionic sites as a result of electrostatic interaction and/or (b) direct association between the dye and the anionic charges of the protein. Some dyes could also associate with the hydrophobic regions of some proteins, although the polar nature of Acridine Orange does not indicate that this is a major factor in this system. Furthermore, no difference was observed on addition of bovine serum albumin during the titration. However, poly-L-lysine and poly-L-arginine displaced Acridine Orange from the dye–heparin complex and the degree of displacement can be related to the variation in the cationic charge distribution of these two polypeptides. This strong interaction between glycosaminoglycans and polycations does not have a parallel in urinary proteins. Whiteman (1973b) has observed that serum albumin does not block the reaction of the cationic dye Alcian Blue with glycosaminoglycans in the presence of 50mM-MgCl_2 at pH 5.8. His data also indicate that the removal of the Tamm–Horsfall glycoprotein, low concentrations of which are present in urine, does not significantly alter the concentration of urinary glycosaminoglycans as determined by Alcian Blue and uronic acid assays.

Other trace components in urine, such as bilirubin, flavins, indole, uroporphyrins etc., can interfere with the titration of polyanions by using Acridine Orange, by (a) interacting with the polyanion of Acridine Orange and/or (b) by quenching or masking the dye

fluorescence. Their fluorescence yield is low compared with that of $10\mu\text{M}$ -Acridine Orange and the results show that, at the concentrations present in urine of normal subjects, no quenching of Acridine Orange fluorescence was observed.

The sharp and steady decrease in the relative fluorescence intensity during the titration of the dialysed and non-dialysed urine indicated that salts were not present in concentrations that would interfere with the titration. Therefore, the difference between dialysed and non-dialysed samples is due solely to variation in glycosaminoglycan concentrations. Patients with Sanfilippo syndrome showed an apparent decrease in urinary glycosaminoglycans of 17% and 18.5%, and the patient with Hunter syndrome a decrease of 2.5%. This indicates the presence of lower-molecular-weight glycosaminoglycans in the urine of patients with Sanfilippo syndrome. The low concentrations of glycosaminoglycans present in the patient with Hunter syndrome is due to the age of the subject. However, when the data are expressed in terms of the creatinine excretion, the expected increase over results for normal subjects was observed (Table 1). Uronic acid assays support the titrimetric data.

In the present study the concentration of glycosaminoglycans from the titration is expressed in equivalents/anionic site per litre (i.e. the unit weight of glycosaminoglycan per anionic site). For comparison, these concentrations have also been expressed in Table 1 in mg/100ml of urine, based on heparin equivalent weight of 150 (calculated by dividing the molecular weight of a heparin tetramer repeating unit by the number of anionic sites on the unit). Since the type and amount of glycosaminoglycans varies with the urine sample the value of expressing concentration in the latter way is only limited.

Although mucopolysaccharidoses are clinically diagnosed at an early age, the biochemical tests for urinary glycosaminoglycans are important to assess the enzymic deficiency and changes in glycosaminoglycan excretion after treatment such as fibroblast transplants (Dean *et al.*, 1976). The analytical technique described here is simple, sensitive and reproducible.

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