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Note

A procedure for the analysis of glycosaminoglycan mixtures based on digestion by specific enzymes

George C. Slim [a], Richard H. Furneaux [a] and Selwyn C. Yorke [b]

[a] *Industrial Research Ltd., P.O. Box 31-310, Lower Hutt (New Zealand)*
[b] *New Zealand Pharmaceuticals Ltd., P.O. Box 1869, Palmerston North (New Zealand)*

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Glycosaminoglycans (GAGs) are classified on structural grounds into seven classes; hyaluronic acid, chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate, heparan sulphate, heparin and keratan sulphate. All are linear polymers of a repeating disaccharide unit composed of a 3- or 4-linked 2-amino-2-deoxyhexose residue and a 4-linked hexuronic acid moiety. The classes are differentiated on the basis of the stereochemistry and sulphation pattern of their disaccharide units (see Fig. 1). This gives rise to a group of compounds which are structurally and functionally diverse but have broadly similar physical properties, making their separation a complex problem[1]. GAGs are ubiquitous components of animal connective tissue and are almost invariably isolated as mixtures covalently bound to a core protein, with the exception of hyaluronic acid and heparin. They are known to have a wide range of biological activities[2] and as medical applications are found[3] the preparation of pure GAG fractions is imperative.

During work on the large scale fractionation of GAG mixtures it was found necessary to develop a means of analysis which would be relatively simple to perform and give the maximum of structural information, particularly with regard to the dermatan sulphate/chondroitin sulphate ratio. While there are a number of published methods for the analysis of GAG mixtures[1], none has proven entirely satisfactory. Electrophoresis[4,5] can separate the GAG polymers into their general classes but gives no indication of the disaccharide make up of the individual chains. High performance liquid chromatography[6] or capillary electrophoresis[7] after digestion of the polymer into fragments, on the other hand, gives the disaccharide composition but does not show which polymer of a mixture the disaccharides were part of. The same is true of ^{13}C NMR spectra[8] of complex GAG mixtures; generally they have too many signals to confidently assign and so are useful only for the analysis of mixtures of few components and for the structurally regular GAGs.

Distinguishing amongst dermatan sulphate, chondroitin 4-sulphate, and chondroitin 6-sulphate poses a particular problem. All three GAGs are synthesized in

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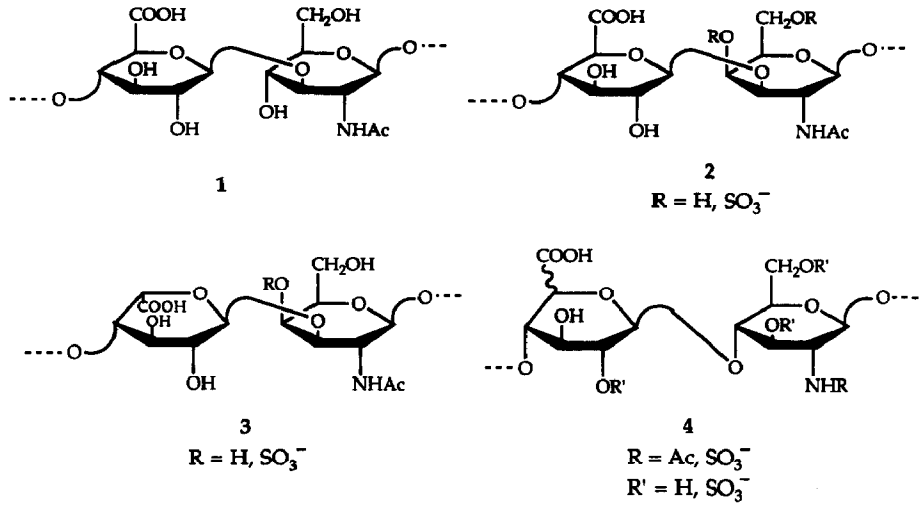

Fig. 1. Disaccharide units of important GAG structural types.

		Predominant positions sulphated
1	Hyaluronic acid	None
2	Chondroitin 4-sulphate	Hexosamine C-4
2	Chondroitin 6-sulphate	Hexosamine C-6
3	Dermatan sulphate	Hexosamine C-4
4	Heparin and heparan sulphate	Very variable

nature by a common polymerization of glucuronic acid and *N*-acetylgalactosamine. The resulting regular polymer is enzymatically modified by sulphation at various positions and by epimerisation of glucuronic acid at C-5, which gives the iduronic acid residues characteristic of dermatan sulphate. These modifications are, in general, incomplete and give rise to inhomogeneities within each polymer chain. In particular, dermatan sulphate chains contain regions bearing glucuronic acid residues that are usually characteristic of chondroitin sulphate[9] so that there is no clear dividing line between chondroitin sulphate and dermatan sulphate. The ratio of iduronic acid to glucuronic acid in a polymer is important because iduronic acid is conformationally more flexible than glucuronic acid[10] allowing the polymer to adopt a wider variety of conformations in solution and therefore to have different physical properties. The increased flexibility accounts for the greater cation binding properties of dermatan sulphate over the chondroitin sulphates and its more general biological activity[11].

The present paper describes a method of analysis of GAG mixtures utilizing selective enzymes to digest specific components of GAG mixtures[12] which is rapid, robust, and suitable for use in an industrial setting. The use of enzymes gives a selectivity for the different GAG species which is not available by any chemical means. In conjunction with electrophoresis and NMR the method allows the

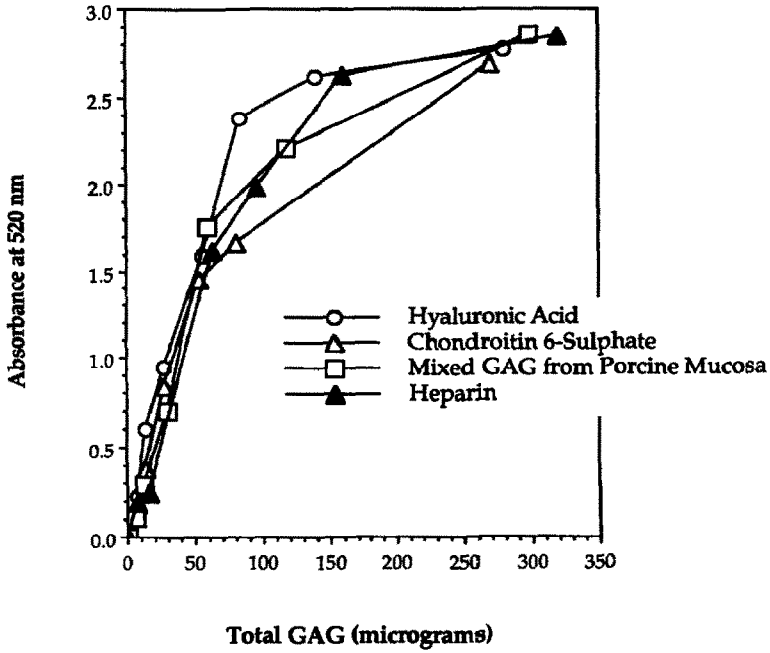

Fig. 2. Response curves for colorimetric assay with different GAGs.

determination of chondroitin sulphate-like regions within dermatan sulphate polymers and, to some extent, their distribution.

To analyze a GAG mixture by this method, samples are first separately digested with a number of commercially available specific GAG enzymes. Any undigested GAG is precipitated and collected by centrifugation. The content of uronic acid is determined by the method of Blumenkrantz and Asboe-Hansen[13]. This particular colorimetric assay was chosen because the original authors had demonstrated that it had the same response for chondroitin 4-sulphate, chondroitin 6-sulphate, and dermatan sulphate. It was found that hyaluronic acid, heparin, and the GAG from porcine intestinal mucosa also give the same response (Fig. 2). The total GAG concentration of the test solution was chosen to be at the top of the linear part of the absorbance vs. concentration response curve so as to give the assay maximum sensitivity.

It was initially envisaged that the undigested GAG polymer would be collected by dialysis but this did not give reproducible results. Precipitation with 75% aqueous methanol saturated with sodium chloride, however, proved both rapid and reproducible. Under the conditions used a standard solution of chondroitin 4-sulphate disaccharide was not precipitated nor were the products of hyaluronidase digestion of hyaluronic acid, typically tetramers and hexamers[14] (data not shown).

A flow chart of the procedure is shown in Fig. 3.

The results of digestion of GAG mixtures by chondroitinase ABC, which digests hyaluronic acid, chondroitin 4-sulphate, chondroitin 6-sulphate, and dermatan

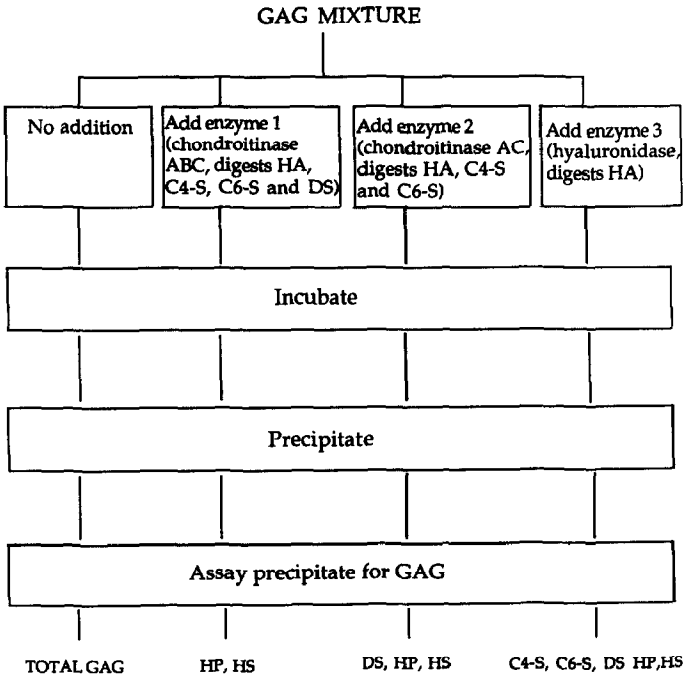

Fig. 3. Flow chart for the analysis of GAG mixtures containing hyaluronic acid (HA), chondroitin 4-sulphate (C4-S), chondroitin 6-sulphate (C6-S), dermatan sulphate (DS), heparin (HP), and heparan sulphate (HS).

sulphate; chondroitinase AC, which digests hyaluronic acid, chondroitin 4-sulphate, and chondroitin 6-sulphate; and microbial hyaluronidase which digests hyaluronic acid alone are shown in Table I. Methanol precipitation of a control solution of the GAG mixture under test which has not been enzyme treated gives the total GAG content of the sample, avoiding the need to remove degraded GAG by dialysis and minimizing the effects of interference in the colorimetric test by contaminants. The Blumenkrantz–Asboe-Hansen test is, however, susceptible to interference from neutral sugars and glycoproteins so this assay is only suitable for reasonably pure GAG samples. The amount of hyaluronic acid is given directly

TABLE I

Digestion of GAG mixtures by specific enzymes

Enzyme	Percent of mixture digested (expected value in parentheses)		
	I [a]	II [a]	III [b]
Chondroitinase ABC	92 (100)	97 (100)	59
Chondroitinase AC	87 (85)	91 (90)	35
Hyaluronidase	29 (32)	4 (0)	5

[a] Mixture of standard compounds; expected values derived from the known proportions of components.
[b] Mixed GAG from porcine mucosa.

TABLE II

Results of analysis of GAG mixtures

GAG	Percent of mixture digested (expected value in parentheses)		
	I [a]	II [a]	III [b]
Chondroitin sulphates	58 (52)	87 (90)	30 (12)
Dermatan sulphate	5 (16)	6 (10)	24 (48)
Hyaluronic acid	29 (32)	4 (0)	5 (0)
Remainder	8 (0)	3 (0)	41 (37)

[a] Mixture of standard compounds; expected values derived from the known proportions of components.
[b] Mixed GAG from porcine mucosa; expected values from electrophoresis.

from the amount digested by microbial hyaluronidase. Subtraction of this from the amount digested by chondroitinase ABC gives the combined amount of chondroitins plus dermatan sulphate and the remainder (i.e., the undigested portion) is the amount of heparin and heparan sulphate. Since it was known that the porcine intestinal mucosa did not contain keratan sulphate no attempt was made to broaden the scope of the assay to test for it. The amount of dermatan sulphate in a mixture is determined from the difference between the amount of the GAG mixture digested by chondroitinase ABC and that digested by chondroitinase AC. The results of this analysis are shown in Table II.

While the analysis method described gives the composition of mixtures of standard compounds (mixtures I and II) with reasonable accuracy, the results for the digestion of GAGs from porcine mucosa (mixture III) differ substantially from

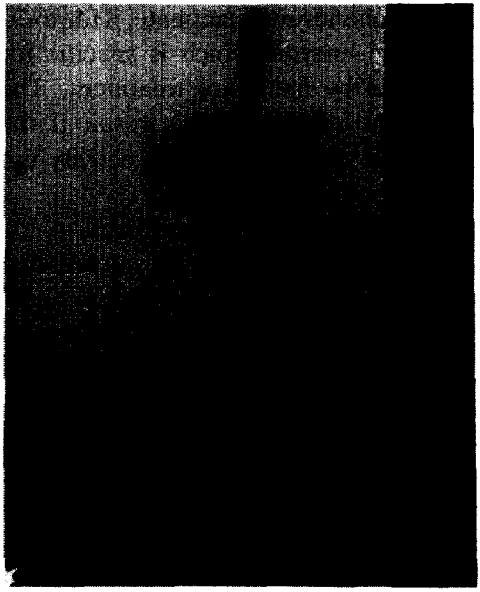

Fig. 4. Electrophoretogram of GAGs from porcine mucosa compared with standard compounds.

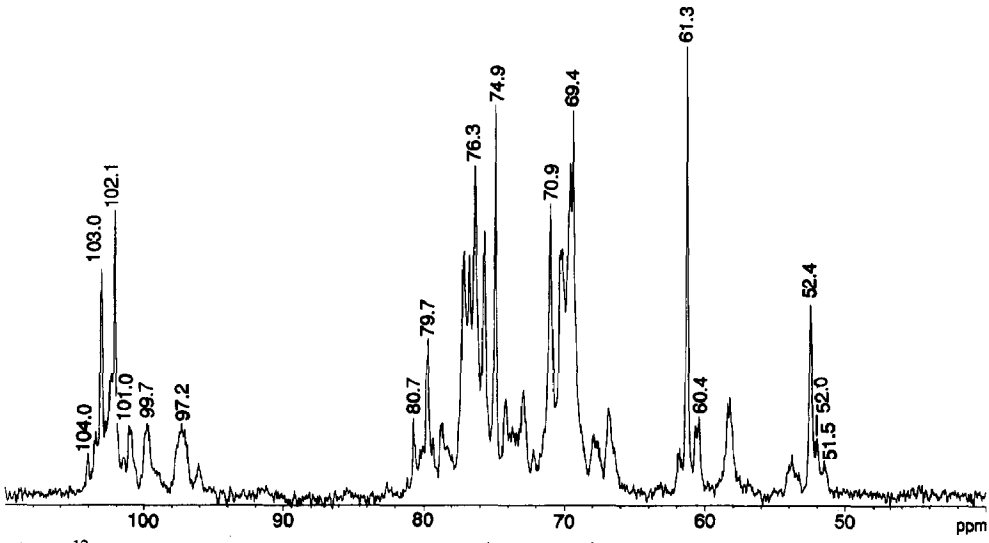

Fig. 5. ^{13}C NMR of GAGs from porcine mucosa (6990 scans).

the results obtained by electrophoresis[15] (Fig. 4). The two methods agree to within experimental error in determining the [chondroitin 4-sulphate, chondroitin 6-sulphate, dermatan sulphate]:[heparan sulphate-like substance]:[hyaluronic acid] ratio but differ significantly in their measurement of the [chondroitin 4-sulphate, chondroitin 6-sulphate]:[dermatan sulphate] ratio. The reason for this divergence is that if a polymer is substantially dermatan sulphate but contains some chondroitin sulphate-like regions it will have a different mobility on electrophoresis than a polymer which is wholly or substantially chondroitin sulphate. However, both polymers will be sufficiently degraded by an enzyme which is specific for chondroitin sulphate so as not to be precipitated by methanol treatment. The enzyme digestion result could also differ from the electrophoresis result if the chondroitinase AC enzyme used had some residual chondroitinase B activity but this activity was not seen in the digestion of standard compounds.

To obtain an independent analysis of the porcine mucosa constituents a ^{13}C NMR spectrum was run (Fig. 5). The complex nature of the mixture meant that not all of the components could be resolved. The spectrum is dominated by peaks from dermatan sulphate[16] with other assignable peaks for chondroitin 4-sulphate and chondroitin 6-sulphate[17].

Integration of ^{13}C NMR spectra is not generally applicable because the relaxation time of the nucleus as well as its abundance has an effect on peak area. In this case, however, it is reasonable to assume that the C-2 aminohexose carbons of chondroitin 4-sulphate, chondroitin 6-sulphate, and dermatan sulphate will be in a similar magnetic environment and therefore have similar relaxation times. Integration of the peaks for chondroitin 4-sulphate at 52.0 ppm, chondroitin 6-sulphate at 51.5 ppm, and dermatan sulphate at 52.4 ppm gave a ratio of 10:15:75, respec-

tively, or 25 : 75 total [chondroitin sulphate] : [dermatan sulphate]. This is very close to the 20 : 80 total chondroitin sulphate : dermatan sulphate ratio given by electrophoresis. The similarity of the two results suggests that the dermatan sulphate polymers contain only a low level of chondroitin sulphate disaccharide residues.

^{13}C NMR is clearly a good way to determine the relative levels of chondroitin 4-sulphate, chondroitin 6-sulphate, and dermatan sulphate without recourse to digestion and analysis of the disaccharides by HPLC. Electrophoresis is useful to determine the amounts of different intact GAG polymers but gives little information as to their make-up, particularly with regard to chondroitin sulphate and dermatan sulphate mixed polymer chains.

The combination of electrophoresis and selective enzyme digestion gives more information, however. From the differences in the results of the two methods shown in the Tables I and II we can say that the dermatan sulphate polymer contains some chondroitin sulphate disaccharide residues. ^{13}C NMR gives the absolute ratio of the three different disaccharide units and we can combine all this information to give the amount of dermatan sulphate polymer which contains chondroitin sulphate-like disaccharides (in the case of the porcine mucosa $\sim$ 15%). The only information needed to fully characterize the polymer is the spacing of the chondroitin sulphate regions. This could be obtained by specific enzyme digestion and measuring the size of the resulting fragments. The full characterization of GAG polymers in this way has not been accomplished yet and is required if a deeper understanding of their structure and function is to be reached.

EXPERIMENTAL

General methods.—Powdered acetone extract of porcine mucosa was provided by New Zealand Pharmaceuticals Ltd. Chondroitinase ABC (EC 4.2.2.4), chondroitinase AC 1 Flavo (EC 4.2.2.5), and hyaluronidase from *Streptomyces hyalurolyticus* (EC 4.2.2.1) were purchased from Seikagaku Corporation (Japan). Chondroitin 4-sulphate (70%, remainder chondroitin 6-sulphate, from bovine trachea), chondroitin 6-sulphate (90%, remainder chondroitin 4-sulphate, from shark cartilage), dermatan sulphate (85%, remainder chondroitin sulphates A and C, from bovine mucosa), hyaluronic acid, heparin, and chondroitin 6-sulphate disaccharide were all purchased from Sigma Chemical Co. The stated composition of the GAG preparations was checked by NMR and used in the calculations of the concentration of standard mixtures. *m*-Hydroxydiphenyl (3-phenyl-phenol, 90%) and sodium tetraborate (ACS reagent) were purchased from Aldrich Chemical Co. All remaining reagents were Analar quality or better. Water was glass distilled. UV spectra were recorded on a Hewlett–Packard 8451A diode array spectrophotometer in quartz cuvettes. ^{13}C NMR spectra of 6% GAG solutions in D_2O were recorded at 65°C on a Varian Unity 500 operating at 125.7 MHz, with a pulse width of 90° and an acquisition time of 1.26 s. Electrophoresis was performed by a modification of the procedure of Cappelletti et al[4].

Reagent and buffer solutions.—GAG solutions were made up to ~3 mg/mL in water. Chondroitinase ABC and chondroitinase AC were used as 5 U/mL solutions and hyaluronidase as a 500 TRU/mL[18] solution. Buffer solution I was 0.25 M Tris · HCl (pH 8.0). Buffer solution II was 0.25 M Tris · HCl (pH 7.3). Buffer solution III was 0.25 M sodium acetate. Buffer solution IV was 0.02 M sodium acetate adjusted to pH 6.0 with dilute acetic acid then made up to 0.15 M NaCl by adding solid NaCl. The precipitant consisted of 75% aq MeOH saturated with NaCl. The H_2SO_4 reagent was made by dissolving sodium tetraborate decahydrate (0.477 g) in 98% H_2SO_4 (100 mL) to give a concentration of 0.0125 M. MHDP solution was made up by dissolving *m*-hydroxydiphenyl (0.15 g) in 0.25% NaOH solution (100 mL).

Digestion of GAG samples.—The following solutions were mixed, in triplicate, for each enzyme: 20 μL GAG sample solution; 20 μL buffer (for chondroitinase ABC, 10 μL each of I and III, for chondroitinase AC, 10 μL each of II and III, and for hyaluronidase, 20 μL IV); 10 μL enzyme solution (chondroitinase ABC, 50 mU, chondroitinase AC, 50 mU, hyaluronidase, 5 TRU).

For each GAG solution and enzyme under test an "enzyme minus" control was prepared from the same solutions with distilled water replacing the enzyme. Both the "enzyme plus" digest and the "enzyme minus" control were incubated at 37°C for 2 h (chondroitinase ABC and chondroitinase AC) or overnight (hyaluronidase). Undigested GAGs were precipitated by the addition of 500 μL precipitant buffer and collected as a pellet by centrifugation at 13000 rpm for 10 min. The supernatant was decanted and the pellet washed with a further 500 μL of precipitant buffer before being spun for a further 10 min. The supernatant was once again decanted and the precipitate redissolved in 125 μL of water.

Uronic acid assay[13].—The sample solution (125 μL) was cooled in ice–water and 1 mL H_2SO_4 reagent, which was kept at 4°C, was added, the tube was vortexed and heated in a boiling water bath (or heating block at 100°C) for 5 min, then cooled in ice–water for a further 5 min. Then 20 μL of MHDP solution was added and, after vortexing, the absorbance of the pink solution was measured at 520 nm. The absorbances were referenced to a blank made up with 125 μL water replacing the digest or control solution.

The amount digested was calculated by subtraction of the absorbance at 520 nm of the Blumenkrantz–Asboe-Hansen test solution for the tube with active enzyme from that of the tube with heat-treated enzyme. The result was converted into a percent of the absorbance of the heat treated sample and averaged over at least three repeat determinations.

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