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The isolation and characterization of glycosaminoglycans in normal human serum

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Abstract: The optimization of conditions for the isolation and characterization of human serum glycosaminoglycans (GAG) is described, together with studies of the accuracy and reproducibility of the method. The principle of the method is proteolytic digestion of serum using papain followed by precipitation of GAGs from the digested sample with cetyl pyridinium chloride (CPC). The uronic acid level and electrophoretic separations can be obtained from a 5 ml serum sample. The mean CPC-precipitable uronic acid level in pooled normal serum was 10.8 mg l^{-1} serum. Using enzymatic and chemical analysis the major serum GAG was shown to be chondroitin sulphate (CS). Two distinct electrophoretic fractions were identified both consisting of CS but differing in their degree of sulphation. Dermatan sulphate, heparan sulphate and hyaluronic acid were not detected.

Keywords: *Chondroitin sulphate; glycosaminoglycan; human serum; proteolytic digestion.*

Introduction

Glycosaminoglycans are widely distributed in human tissues and are particularly abundant in the ground substance of connective tissue and cartilage. Glycosaminoglycans are linear heteropolysaccharides which are composed of disaccharide repeating subunits. These subunits contain a uronic acid component and an acetylated hexosamine (which may be sulphated). Total GAG concentration is generally expressed by the measurement of uronic acid. Urine GAG excretion has been studied in a variety of disease states including rheumatoid arthritis [1] and disseminated neoplasm [2]. Although urine studies have been of use, the GAGs found in urine represent only a small proportion of total GAG turnover in the body [3]. The measurement of serum GAGs would give a clearer idea of GAG metabolism in man and enable more meaningful studies to be carried out.

Several methods for the isolation and quantitation of GAGs in serum have been published [4, 5]. The volume of serum required by some methods is large (10 l) which makes the study of individual patients impossible [6]. The micromethods published vary in their sensitivity and specificity and the accurate identification of GAGs is not always

possible [7]. A micromethod developed by Warren and Manley [8] used laser nephelometry to measure the light scattering of CPC complexes in serum, before and after incubation with specific enzymes. This novel approach to the measurement of serum GAGs is entirely dependent on the specificity of enzymes for the characterization of serum GAGs and comparison with more conventional methodology, including the electrophoretic separation of isolated GAGs in different buffer systems, is desirable for confirmation. Such a method for the quantitative and qualitative measurement of serum GAGs applicable to the small volumes of serum normally available from individual patients in the Hospital Laboratory is described here.

Materials and Methods

Chemical and reagents

Normal pooled serum from 50 healthy male and female volunteers (age range 20–60 years) was divided into 5 ml aliquots and stored at −20°C for up to 12 weeks before assay. Papain, twice crystallized in 0.05 mol l^{-1} sodium acetate suspension, pH 4.5; neuraminidase, glucosamine, galactosamine and α-mannoheptitol (Perseitol) were obtained from the Sigma Chemical Co., Poole, UK. Chondroitinase ABC, heparitinase, *Streptomyces* hyaluronidase and reference samples of chondroitin 4-sulphate and hyaluronic acid were obtained from Miles Laboratories, Slough, UK. Sodium rhodizonate, ovine testicular hyaluronidase and cetyl pyridinium chloride (CPC) were obtained from BDH Chemicals Ltd., Poole, UK. Packing material for gas chromatography, 3% OV101 + 1% OV225 on Diatomite CQ 100–200 mesh, was obtained from Phase Separations Ltd., Queensferry, Clwyd, UK. Pyridine (A.R.) was refluxed over potassium hydroxide for 4 h and then redistilled. B.Pt 115–116°C.

Isolation of total serum glycosaminoglycans

Serum (5 ml) was mixed with cysteine hydrochloride (0.2 ml, 0.25 mol l^{-1}), disodium ethylenediaminetetra-acetic acid (EDTA) (0.2 ml, 0.25 mol l^{-1}), papain (0.1 ml containing approx. 40–100 units) and incubated for 24 h at 65°C. The digested sample was filtered through Whatman No. 42 filter paper and the precipitate washed with phosphate buffer (2.5 ml, pH 6.5, 0.1 mol l^{-1}). The clear filtered extract was transferred to visking tubing (8/32″) and dialysed against phosphate buffer (pH 6.5, 0.1 mol l^{-1}) containing merthiolate as a preservative for 48 h at 4°C. The phosphate buffer was renewed several times during dialysis.

Cetyl pyridinium chloride (5 ml, 0.1% w/v) was added to the dialysed filtrate, mixed and allowed to stand overnight at room temperature (R.T.). After centrifugation and removal of the supernatant the precipitate was dissolved in sodium chloride (0.5 ml, 2 mol l^{-1}). The sample was centrifuged again and the salt solution transferred to ethanol (5 ml, 95% v/v). A further 0.5 ml sodium chloride (2 mol l^{-1}) was added to the original precipitate. After recentrifugation the salt solution was added to the ethanol. After standing overnight at R.T. the sample was centrifuged and the ethanol removed. The final GAG precipitate was washed with ethanol (1 ml, 95% v/v), dried at 65°C and dissolved in water (1 ml).

Uronic acid assay

The uronic acid content of the final extract (0.5 ml) was determined using the modified carbazole method of Bitter and Muir [9] and expressed as mg l^{-1} serum. The within assay

and between assay precision of the method was determined using a normal human serum pool. The accuracy of the method was assessed by recovery of standard CS and hyaluronic acid (HA) added to normal pooled serum and expressed as percentage uronic acid recovered.

Electrophoretic separation of serum GAGs

The extract (0.5 ml) was dried *in vacuo* over silica gel, redissolved in distilled water (100 μl) and dried again. The dried precipitate was dissolved in distilled water (25 μl). Ten microlitres was electrophoresed on cellulose acetate in veronal buffer (pH 9.2, 0.143 mol l^{-1}) at constant voltage (20 v/cm for 55 min) at 4°C [10]. Another 10 μl was electrophoresed on cellulose acetate in calcium acetate buffer (pH 5.0, 0.2 mol l^{-1}) at constant current (1 mA cm^{-1} for 2½ h) at 4°C [11]. The electrophoretic strips were stained in alcian blue (1% w/v in 2% acetic acid) and scanned in a Chromoscan (Joyce Loebl) using a 620 nm filter. Individual GAG fractions were expressed as a percentage of total alcian blue positive material.

Stability of serum GAGs under different storage conditions

Uronic acid and electrophoretic studies were carried out immediately on fresh pooled serum (5 ml) and compared with aliquots stored at R.T. for 7 days, 4°C for 7 and 14 days and −20°C for 12 weeks before assay.

Specific enzyme digestion of normal human serum GAGs

Glycosaminoglycan extracts (10 μl) were mixed with specific enzymes (5 μl) or saline (5 μl) and incubated for 16 h at 37°C. The solid enzymes were dissolved in physiological saline to give the following concentrations: chondroitinase ABC (0.2 mU μl^{-1} and 0.02 mU μl^{-1}), heparitinase (20 mU μl^{-1} and 2 mU μl^{-1}), *Streptomyces* hyaluronidase (0.001 TRU μl^{-1}), ovine testicular hyaluronidase (1 U μl^{-1}) and neuraminidase (0.041 mU μl^{-1}), After incubation 10 μl of enzyme digest was electrophoresed in both buffer systems.

Isolation of GAG fractions for chemical analysis

Pooled serum (95 ml) was divided into 5 ml samples and the total GAG isolated from each sample. The GAGs obtained from 95 ml pooled serum were dissolved in distilled water (0.5 ml). The total GAG sample was electrophoresed in 50 μl aliquots using veronal buffer and individual bands located by markers. The electrophoretic fractions were designated Fraction 1 (faster migrating band) and Fraction 2 (slower migrating band). Each fraction together with an appropriate blank area of cellulose acetate was eluted using sodium hydroxide (0.01 mol l^{-1}) for quantitative analysis. The individual fast and slow migrating fractions were pooled, dialysed against distilled water, concentrated and dried. Each GAG fraction was dissolved in distilled water (1.6 ml). Uronic acid assay of fractions and blanks was carried out as described above.

Hexosamine analysis

Samples (0.4 ml) of Fractions 1 and 2, blanks and CS (control) were mixed with 0.1 ml perseitol (internal standard). Concentrated HCl (0.5 ml) was added and samples hydrolysed for 2 h at 100°C. The hydrolysates were derivatised by the method of Murphy *et al.* [12] and the alditol acetates separated by gas chromatography. The hexosamines present in each fraction were identified by comparison with glucosamine and

galactosamine standards using relative retention times. Quantitation was carried out using peak height ratios and molar hexosamine: uronic acid ratios calculated.

Sulphate analysis

Samples (0.2 ml) of Fractions 1 and 2 and blanks were diluted with an equal volume of 6 M HCl then made up to a final volume of 0.5 ml with 3 M HCl. A chondroitin sulphate solution (200 mg l^{-1}) was treated similarly. Acid hydrolysis was carried out for 2 h at 100°C and HCl removed *in vacuo* over potassium hydroxide. The hydrolysed samples and CS control were dissolved in distilled water. Sulphate was determined using the method of Terho and Hartiala [13] and molar sulphate:uronic acid ratios calculated.

Results

Electrophoretic separation and measurement of individual GAG fractions

During the development of the present method for the isolation of GAGs from serum it was observed that two, sometimes three, alcian blue positive bands were obtained on electrophoresis of serum GAG extracts. The faster migrating band (Fraction 1) corresponded in electrophoretic mobility with chondroitin 4-sulphate. Scanning densitometry of various serum pools showed the relative percentages of alcian blue staining material in Fraction 1 (fast migrating band) and Fraction 2 (slower migrating band) to be approx. 55 and 45% respectively. Fraction 3 when present, was a faint band and accounted for only a small amount of the alcian blue staining material. The possibility that procedural artefacts may have been responsible for the production of positive staining bands was eliminated by carrying out the isolation and electrophoretic procedures on a blank sample using 5 ml of phosphate buffer in place of serum.

Precision and accuracy studies

The mean uronic acid value was 10.8 ± 0.7 mg l^{-1} serum with a relative standard deviation (RSD) of 6.7% for uronic acid determinations while the between assay RSD was 14.6%. The recovery of individual GAGs was 88.7% for CS and 78.9% for HA. The recovery of a mixture of CS and HA was 87%.

Stability of serum GAGs under different storage conditions

The uronic acid levels of sera kept under different conditions are shown in Table 1. Serum samples stored at R.T. for 7 days showed a decrease in uronic acid level whereas those kept at 4°C for up to 14 days showed little change. Samples stored at −20°C for 12

Table 1
CPC-precipitable uronic acid levels in sera kept under different storage conditions

Storage conditions	Serum uronic acid (mg l^{-1})
Fresh	11.6
R.T. for 7 days	8.6
4°C for 7 days	11.8
4°C for 14 days	11.3
−20°C for 12 weeks	10.9

weeks showed no significant decrease in uronic acid content. There was no obvious change in the electrophoretic separation of serum stored at R.T. and 4°C when compared with fresh serum (Fig. 1). The same electrophoretic pattern was obtained from frozen serum as in the other samples.

Specific enzyme digestion of normal serum GAGs

The results of incubation of serum extracts with different concentrations of chondroitinase and heparitinase are shown in Fig. 2. The control sample (serum extract + saline) showed the presence of two intense staining bands (Fractions 1 and 2)

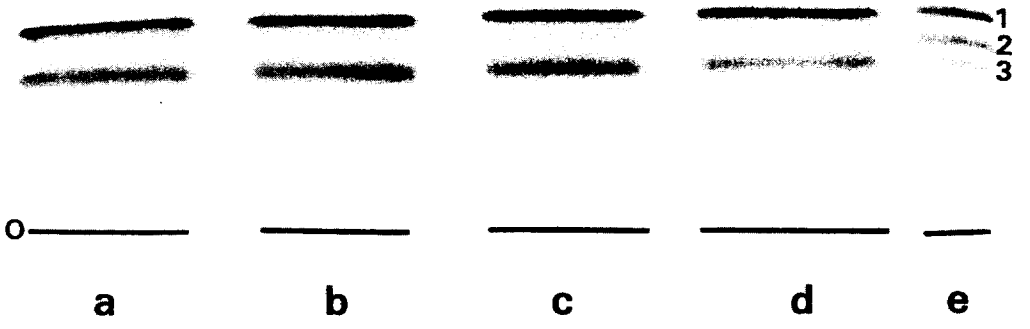

Figure 1
Electrophoresis in calcium acetate buffer of glycosaminoglycans isolated from sera stored under different conditions. (a) Fresh serum, (b) R.T. for 7 days, (c) 4°C for 7 days, (d) 4°C for 14 days, (e) standard containing chondroitin sulphate (1), dermatan sulphate (2) and heparan sulphate (3). O, origin.

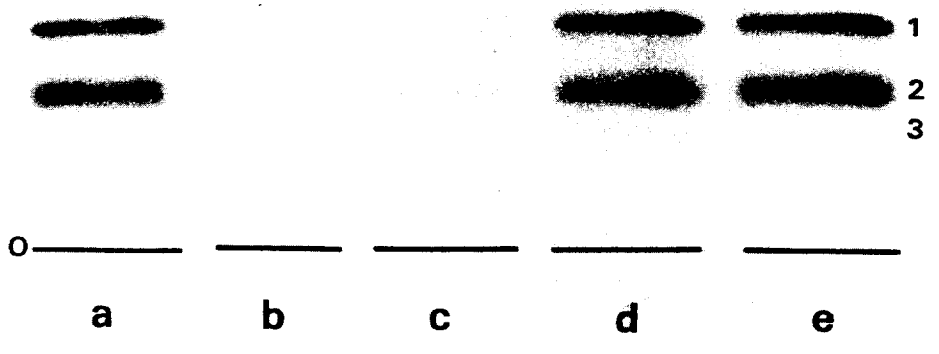

Figure 2
Electrophoresis in calcium acetate buffer of papain digested serum after incubation with the enzymes chondroitinase and heparitinase. (1) Fraction 1, (2) Fraction 2 and (3) Fraction 3. (a) Control, (b) serum extract + chondroitinase (0.2 mU μl^{-1}), (c) serum extract + chondroitinase (0.02 mU μl^{-1}, (d) serum extract + heparitinase (20 mU μl^{-1}) and (e) serum extract + heparitinase (2 mU μl^{-1}). O, origin.

and a third faint band (Fraction 3) which migrated more slowly. At a chondroitinase concentration of 0.2 mU μl^{-1} Fractions 1 and 2 disappeared but Fraction 3 remained. At the lower enzyme concentration, 0.02 mU μl^{-1} Fractions 1 and 2 were only partially digested. These results confirmed the presence of CS as the major serum GAG. Incubation of serum extracts with heparitinase showed no significant decrease in the intensity of Fractions 1 and 2, therefore the presence of heparan sulphate (HS) was not indicated. Fractions 1 and 2 were completely digested by ovine testicular hyaluronidase, confirming the absence of dermatan sulphate. Fraction 3 was not digested by heparitinase. The possibility of HA being present in serum extracts was investigated using *Streptomyces* hyaluronidase. Electrophoresis of control and hyaluronidase digested sample in veronal buffer did not indicate the presence of HA. Fraction 3 disappeared on digestion with neuraminidase.

Chemical analysis of normal human serum GAGs

The results of hexosamine and sulphate analysis are shown in Table 2. There was no significant interference from blank samples in either the hexosamine or uronic acid measurements. The sulphate values were corrected for the small values obtained with the blank samples. The hexosamine in Fraction 1 was identified as galactosamine and a molar hexosamine:uronic acid ratio of 0.86 was obtained. In Fraction 2 the major hexosamine was also galactosamine and a molar ratio of 0.82 was calculated. A small amount of glucosamine, about 2% of the total hexosamine, was identified in Fraction 2 which implied the presence of GAGs other than CS. Galactosamine was identified as the hexosamine in the CS control and a molar ratio of 0.91 was obtained. The sulphate:uronic acid ratios for Fractions 1 and 2 and CS were 1.45, 0.26 and 0.84 respectively.

Table 2
Hexosamine and sulphate content of GAG fractions isolated from normal human serum

Sample	Hexosamine present	Uronic acid (μ mol l^{-1})	Hexosamine (μ mol l^{-1})	Sulphate (μ mol l^{-1})	Molar ratio Hexosamine: Uronic acid	Molar ratio Sulphate: Uronic acid
Fraction 1	Galactosamine	151.8	130.4	220.3	0.86	1.45
Fraction 2	Galactosamine	495.2	395.3	130.2	0.82	0.26
Fraction 2	Glucosamine		9.2	—	—	—
CS	Galactosamine	179.4	162.5	—	0.91	—
CS	—	96.9	—	81.3	—	0.84

Discussion

The use of papain digestion and CPC-precipitation for the isolation of GAGs from serum was found to be a practical and reliable technique which could be used in routine Hospital Laboratories. Although a method using proteolytic digestion has been reported previously [14], insufficient GAG was isolated by this method for quantitative analysis and only electrophoresis was carried out. Lipid extraction and buffering of serum during digestion were found to be unnecessary. Dialysis was found to be necessary after papain digestion as small molecular weight fragments interfered with CPC-precipitation. The most suitable buffer for this purpose was 0.1 mol l^{-1} phosphate buffer which appeared to

enhance the CPC-precipitation reaction. It has been shown that certain divalent anions are capable of stabilizing polyanion–quaternary ammonium complexes [15].

Electrophoretic separation was carried out in two buffer systems. Calcium acetate was chosen because of its ability to separate chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate. Separation in this buffer system is thought to occur because of differences in the backbone structure of polysaccharides and the degree of sulphation on galactosamine residues [11, 16]. Therefore over and undersulphated GAGs can be separated using calcium acetate. Unfortunately HA and HS co-migrate in calcium acetate so an alternative buffer system, Michaelis' veronal, which would separate these GAGs was also used.

A criticism of previous methods for the isolation of serum GAGs has been the lack of precision and accuracy studies [7]. The precision and accuracy reported here were felt to be acceptable in view of the extensive manual techniques involved in the isolation procedure. In view of these results it was decided that the method could be used as a routine assay. Investigation of storage conditions was important for an assay which was to be used routinely. Serum GAGs were shown to be stable at −20°C for up to 12 weeks.

The major component of Fractions 1 and 2 was CS. Fraction 3 was neuraminidase labile and PAS positive suggesting a glycoprotein. Investigation of enzyme specificity showed that chondroitinase ABC digested HA at high concentrations so optimum enzyme concentrations were used to avoid any cross reaction. The differing mobilities of the two fractions was accounted for by different degrees of sulphation. Although the existence of different structural forms of CS in plasma has been recognised the reports of their individual chemical composition have varied [5, 17]. As the two forms of CS could not be analysed using enzyme digestion alone, a detailed chemical analysis was carried out.

Chemical analysis showed that serum contained both over and undersulphated CS together with a small amount of glucosamine-containing GAG. On the basis of hexosamine and uronic acid analysis the undersulphated CS accounted for 77% of total serum GAG content which was in agreement with Juvani *et al.* [18] who reported a value of 80%. The structure of undersulphated CS in plasma has been studied in more detail by examination of the unsaturated disaccharides produced after digestion with chondroitinase [5, 14]. In this way both non-sulphated and sulphated disaccharides have been identified. The presence of oversulphated CS in human plasma was first reported by Murata *et al.* [17] who identified a disulphated disaccharide subunit from plasma CS.

The possibility that plasma contained HS and HA has been suggested previously [4, 6]. A recent study by Warren and Manley [19] using the technique of laser nephelometry suggests that HS may be present in normal sera at approximately 10% of total serum GAGs. Using the present method glucosamine accounted for only 2% of the total hexosamine content. It would appear that a technique such as laser nephelometry may be more sensitive at detecting small amounts of GAGs other than CS. However, the method described here is able to distinguish between the two forms of CS and measures their relative proportions which laser nephelometry cannot.

The origin of GAGs in human serum and plasma is not completely understood at the present time. Serum GAGs may originate from leucocytes which have been shown to contain chondroitin 4-sulphate [20]. Other potential sources of serum GAGs are connective and vascular tissue whose turnover could result in GAGs passing into the circulation. The knowledge to be gained from the study of serum GAGs in normal individuals and in patients with known disease states would help to advance the

understanding of GAG metabolism in man. The use of the method presented in this paper would assist such studies.

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