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TLC–LSIMS of neoglycolipids of glycosaminoglycan disaccharides and of oxymercuration cleavage products of heparin fragments that contain unsaturated uronic acid

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Abstract

Heparin and chondroitin sulfate disaccharides have been investigated by high-performance (HP) TLC and liquid secondary-ion mass spectrometry (LSIMS) after conversion to neoglycolipid derivatives by reductive-amination with an aminolipid (dihexadecyl phosphatidylethanolamine, DHPE). Mobility on HPTLC was largely determined by the number of sulfate groups present, but was also influenced by the position of sulfate, monosaccharide composition and linkage. The mass spectra acquired directly from the TLC plate provided quasimolecular and fragment ions from which composition, including sulfate content, and sequence information was obtained at high sensitivity.

Lipid DHPE conjugation and TLC–LSIMS were performed to analyse products of the oxymercuration reaction used to cleave unsaturated uronic acid (ΔUA) residues from glycosaminoglycan (GAG) fragments produced by enzymatic degradation with glycan lyases. Previously the identification of the product from ΔUA and the integrity of the remaining structures

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[1] Abbreviations: CI, chemical ionisation; DHPE, L-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine; GAG, glycosaminoglycan; HPTLC, high-performance TLC; LSIMS, liquid secondary-ion mass spectrometry; $NaBD_4$, sodium borodeuteride; $NaBH_3CN$, sodium cyanoborohydride; GalNAc, α-D-*N*-acetylgalactosamine; GlcNAc, α-D-*N*-acetylglucosamine; GlcN, α-D-glucosamine; IdoA, α-L-iduronic acid; ΔUA, 4,5-unsaturated uronic acid (4-deoxy-α-L-*threo*-hex-4-enopyranosyl uronic acid); 2S, 2-*O*-sulfate; 6S, 6-*O*-sulfate; and NS, *N*-sulfate.

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from oligosaccharides larger than disaccharide have not been made. Multiple and characteristic products of the cleaved ΔUA were detected and these can be used for identification of terminal ΔUA and its sulfate content. It was established with several disaccharides and a tetrasaccharide that glycosidic linkages and *O*- and *N*-sulfate groups are preserved in the remaining structures after removal of ΔUA. These results indicate that the oxymercuration reaction will be applicable to generating series of GAG fragments containing unmodified sequences for biological activity studies.

Keywords: Glycosaminoglycans; Oligosaccharides; Oxymercuration; Neoglycolipids

1. Introduction

Glycosaminoglycan (GAG) [1] polysaccharides interact with a range of proteins involved in cellular adhesion, motility and proliferation [1,2]. Binding to some proteins is thought to arise from relatively non-specific charge interactions while with others binding is to defined sequences, i.e. the pentasaccharide sequence required for activation of antithrombin III [3,4]. The study of such molecular interactions requires the generation of oligosaccharide fragments by either enzymatic or chemical depolymerisation of GAG chains. Structural characterisation of fragments used in activation and inhibition assays is then necessary to establish structure–function relationships.

Derivatisation is used extensively to increase detection sensitivity in chromatography and mass spectrometry of released glycoprotein oligosaccharides (e.g., [5–8]). However, it is not a common strategy for the analysis of oligosaccharide fragments derived from polysaccharide lyase digestion of GAG chains, probably due in part to the unsaturated uronic acid residues in these fragments [9] conferring UV absorption activity and thus a means of detection. An approach to the chromatographic separation and identification by liquid secondary-ion mass spectrometry (LSIMS) [8,10,11] of glycoprotein oligosaccharides is their conversion to neoglycolipids with phosphatidylethanolamine. These conjugates have also proved versatile probes to determine the binding activities of oligosaccharides in overlay experiments on TLC plates with reactive proteins [12,13].

As a step towards the structural analysis of GAG oligosaccharide chains and their use in binding activity studies, the present report describes the conversion of heparin and chondroitin sulfate disaccharides to neoglycolipid derivatives by reductive-amination with dihexadecyl phosphatidylethanolamine (DHPE) and subsequent characterisation by TLC–LSIMS [8,10].

We have also used this derivatisation approach to examine the oxymercuration reaction which was shown to cleave the unsaturated uronic acid (ΔUA) residue from a hyaluronic disaccharide produced by chondroitinase digestion [14]. Although the reaction has been used in several studies (e.g., [15,16]), the products have not been fully characterised, in particular the ΔUA residue or its substitution. Applying the oxymercuration reaction to several disaccharides and a hexasulfated tetrasaccharide, we have established the nature of cleavage products by their conversion to neoglycolipids and TLC–LSIMS analysis, and demonstrated the integrity of the remaining structures following removal of the ΔUA residue.

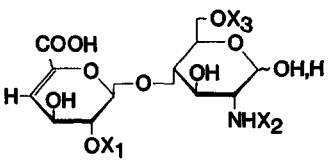
2. Experimental

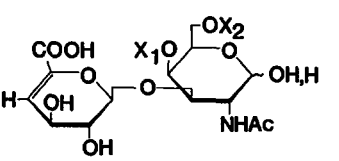
Materials.—Heparin and chondroitin sulfate disaccharides (for structures and designation see Table 1) were purchased from Sigma Chemical Co. (Poole, Dorset, UK). A heparin tetrasaccharide ΔUA(2S)α1-4GlcNS(6S)α1-4IdoA(2S)α1-4GlcNS(6S), (IX), was prepared from porcine intestinal mucosa and gave an NMR spectrum consistent with previously reported data [17]. DHPE (L-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine) was from Fluka Chemicals Ltd. (Glossop, Derbyshire, UK). Mercuric acetate and $NaBH_3CN$ were purchased from Aldrich (Gillingham, Dorset, UK), and primulin and $NaBD_4$ from Sigma. High performance TLC (HPTLC) plates (5 μm silica gel, aluminium-backed) were from Merck (Poole, Dorset, UK). All other chemicals and solvents used were of analytical grade.

Preparation of DHPE derivatives.—DHPE conjugation was carried out with 10% water content in the reaction medium essentially as described [12]. Typically, DHPE solution (50 μL, 5 mg/mL, 1:1 MeOH–$CHCl_3$) was added to a disaccharide solution (50 nmol in H_2O) and the mixture allowed to dry under a stream of N_2 at below 40°C. The residue was resuspended in a mixture of H_2O (5 μL), 1:1 MeOH–$CHCl_3$ (45 μL), and freshly made methanolic $NaBH_3CN$ solution (10 mg/mL, 5 μL), and was then heated to 60°C for 18 h. After the reaction was completed, the mixture was dried under a stream of N_2 and redissolved in 25:25:8 $CHCl_3$–MeOH–H_2O (100 μL), and kept at −20°C as a stock solution.

TLC and TLC–LSIMS of DHPE derivatives.—Each of the DHPE conjugation mixtures (typically 1–2 nmol of starting oligosaccharides) was applied as a 5-mm band to an aluminium-backed HPTLC plate and developed in 60:35:8 $CHCl_3$–MeOH–H_2O for 8 cm. The bands were located under long wavelength UV light after spraying with primulin reagent (0.001% of primulin in 4:1 acetone–H_2O). The plate was primulin stained by an immersion procedure [12] when quantitative measurements were required

Table 1
Disaccharides used for DHPE conjugation study





I	ΔUA1-4GlcNAc	(X_1=X_3=H, X_2=Ac);	VIII	ΔUA1-3GalNAc	(X_1=X_2=H);
II	ΔUA(2S)1-4GlcNAc	(X_1=SO_3H, X_2=Ac, X_3=H);	IX	ΔUA1-3GalNAc(4S)	(X_1=SO_3H, X_2=H);
III	ΔUA1-4GlcNAc(6S)	(X_1=H, X_2=Ac, X_3=SO_3H);	X	ΔUA1-3GalNAc(6S)	(X_1=H, X_2=SO_3H).
IV	ΔUA(2S)1-4GlcNAc(6S)	(X_1=X_3=SO_3H, X_2=Ac);			
V	ΔUA1-4GlcNS	(X_1=X_3=H, X_2=SO_3H);			
VI	ΔUA(2S)1-4GlcNS	(X_1=X_2=SO_3H, X_3=H);			
VII	ΔUA(2S)1-4GlcNS(6S)	(X_1=X_2=X_3=SO_3H).			

by comparison of fluorescence intensities. For LSIMS analysis, each band was excised together with the aluminium backing to give a strip typically 1.5 × 5.5 mm and attached to the LSIMS probe tip by an electro-conducting adhesive [10]. Extraction solvent (25:25:8 $CHCl_3$–MeOH–H_2O) and matrix (2:2:1 diethanolamine–tetramethylurea–*m*-nitrobenzyl alcohol) were added to the surface of the silica gel prior to negative-ion LSIMS.

Oxymercuration treatment of heparin-derived oligosaccharides.—Oxymercuration was carried out essentially as described [14,18]. To a solution of heparin oligosaccharide (typically, 25 μg in 25 μL H_2O for disaccharides and 15 μg in 15 μL H_2O for the tetrasaccharide) was added an equal volume of mercuric reagent made from 20 mM mercuric acetate in 130 mM sodium acetate (pH 5.0). The mixture was kept at room temperature for 30 min and then immediately passed through a column of AG50W-X8 resin (H^+ form, 0.5 mL bed volume). The column was washed with H_2O (1.5 mL), and the combined eluate and washes were lyophilised.

Reduction and methylation of oxymercuration products.—The lyophilised oxymercuration product of III (25 μg) was dissolved in $NaBD_4$ (100 μL; 10 mg/mL 0.01 M NaOH) and kept at 6°C overnight. A few drops of 1:1 AcOH–H_2O were added to destroy the $NaBD_4$ and the solution was passed through an AG50W-X8 resin column (H^+ form, 0.5 mL bed volume). The combined eluate and washes were lyophilised and borate was removed by repeated co-evaporation with MeOH.

To investigate the effect of $NaBH_3CN$ on the ketone group in the cleavage product of ΔUA during DHPE conjugation, a separate experiment was carried out using $NaBH_3CN$ as reducing agent instead of $NaBD_4$. After removal of mercuric salt and lyophilisation the oxymercuration product of III was added to 1:1 $CHCl_3$–MeOH (100 μL), H_2O (10 μL), and methanolic $NaBH_3CN$ (3 μL, 10 mg/mL), and incubated at 60°C for 16 h. The mixture was dried under a stream of N_2 and redissolved in H_2O and treated in exactly the same way as described above for $NaBD_4$ reduction.

Methylation was carried out by the NaOH-CH_3I method [19]. Chloroform (1 mL) and (2 mL) were used to separate the permethylated oxymercuration product of the non-sulfated ΔUA residue from the sulfated glucosamine and excess reagents by extraction [13]. The $CHCl_3$ phase was washed three times with H_2O, dried, and the residue was redissolved in MeOH for analysis.

Conjugation of oxymercuration products to DHPE.—To the lyophilised product (e.g., 25 μg III) was added H_2O (5 μL), DHPE reagent (50 μL; 5 mg/mL 1:1 $CHCl_3$–EtOH), and freshly made ethanolic $NaBH_3CN$ solution (2 μL, 10 mg/mL), and the mixture was incubated at 60°C for 18 h. The solution was dried under a stream of N_2 and redissolved in 25:25:8 $CHCl_3$–MeOH–H_2O for analysis. For samples containing more sulfate groups or higher oligomers, H_2O (10%) was included in the reaction medium and EtOH was replaced by MeOH.

For direct conjugation of the oxymercuration product of III, without removal of mercuric salt, the reaction solution was freeze-dried immediately after the reaction was stopped. To the residue was added H_2O (5 μL), 1:1 $CHCl_3$–EtOH (180 μL), DHPE solution (55 μL), and $NaBH_3CN$ solution (3 μL), and incubated at 60°C for 18 h.

LSIMS and GC–MS analysis of the reduced and methylated products.—LSIMS was carried out on a VG ZAB-2E instrument fitted with a caesium ion gun operated at 25

keV (for negative-ion detection) or 35 keV (for positive-ion detection) and an emission current of 0.5 μA. Full scan spectra were acquired at 30 s/decade using a VG Analytical 11-250J data system in the 'continuum' acquisition mode. For positive-ion LSIMS analysis of the reduced and methylated oxymercuration products, each sample was dissolved in MeOH and 1 μg was loaded onto the target precoated with thioglycerol matrix. GC–MS analysis was performed on a Jeol JMS-DX303 mass spectrometer using a BP-10 (25 m × 0.22 mm i.d. × 0.25 μm film) capillary column with helium as a carrier gas. The initial column temperature of 50°C was maintained for 0.5 min and then programmed to 130°C at 25°C/min, then at 5°C/min to 230°C. Full scan mass spectra were acquired at 70 eV electron energy and 300 μA, at a source temperature of 230°C. Ammonia was used as the reagent gas for chemical ionisation.

3. Results and discussion

Preparation and TLC separation of DHPE derivatives of heparin and chondroitin sulfate disaccharides.—Seven heparin and three chondroitin sulfate disaccharides (Table 1) with differing sulfation, composition, and glycosidic linkages were selected for conjugation to the amino-lipid DHPE as representing typical products from glycan lyase digestion of naturally occurring GAGs. The yields of disaccharide conjugates were estimated from the relative intensity of fluorescence of the derivatives on a TLC plate following staining with primulin reagent. These could only be estimated as extinction coefficients varied among the disaccharides. Expressing the relative conversion of non-sulfated disaccharides I and VIII (Table 1) to neoglycolipids as 100%, the yield for

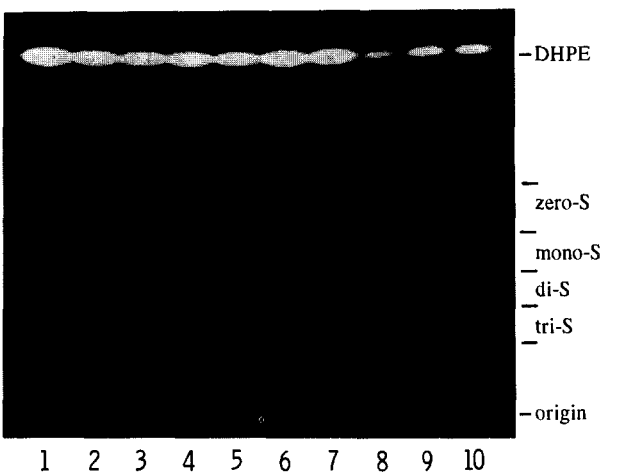


Fig. 1. TLC of the DHPE derivatives of GAG disaccharides. In order to produce a comparable level of fluorescence intensity of each derivative band the concentrations of disaccharides were adjusted (0.6-2.0 nmol disaccharide). The derivative bands shown are for disaccharides: lane 1, VII; lane 2, VI; lane 3, V; lane 4, IV; lane 5, III; lane 6, II; lane 7, I; lane 8, VIII; lane 9, IX; and lane 10, X. The regions marked zero-S, mono-S, di-, and tri-S indicate migration regions of zero-, mono-, di-, and tri-sulfated disaccharide-DHPEs, respectively.

X and IX was 50%, III was 25%, and VII, VI, II, and V were 15–20%. The differences in yield correlate with the number of sulfates in the disaccharide and probably reflect the reduced solubility of the more sulfated saccharides in the reaction solvent system. Increased water content aids in the dissolution of sulfated saccharides but does not favour the dehydration process of reductive-amination. Increased concentration of reducing agent and elevated temperature improve the conjugation efficiency but can also lead to boron adduct formation and sulfate loss.

As shown in Fig. 1, individual disaccharide–DHPE derivatives migrate into four regions (zero-, mono-, di-, and tri-SO_3H) with the number of sulfates playing the

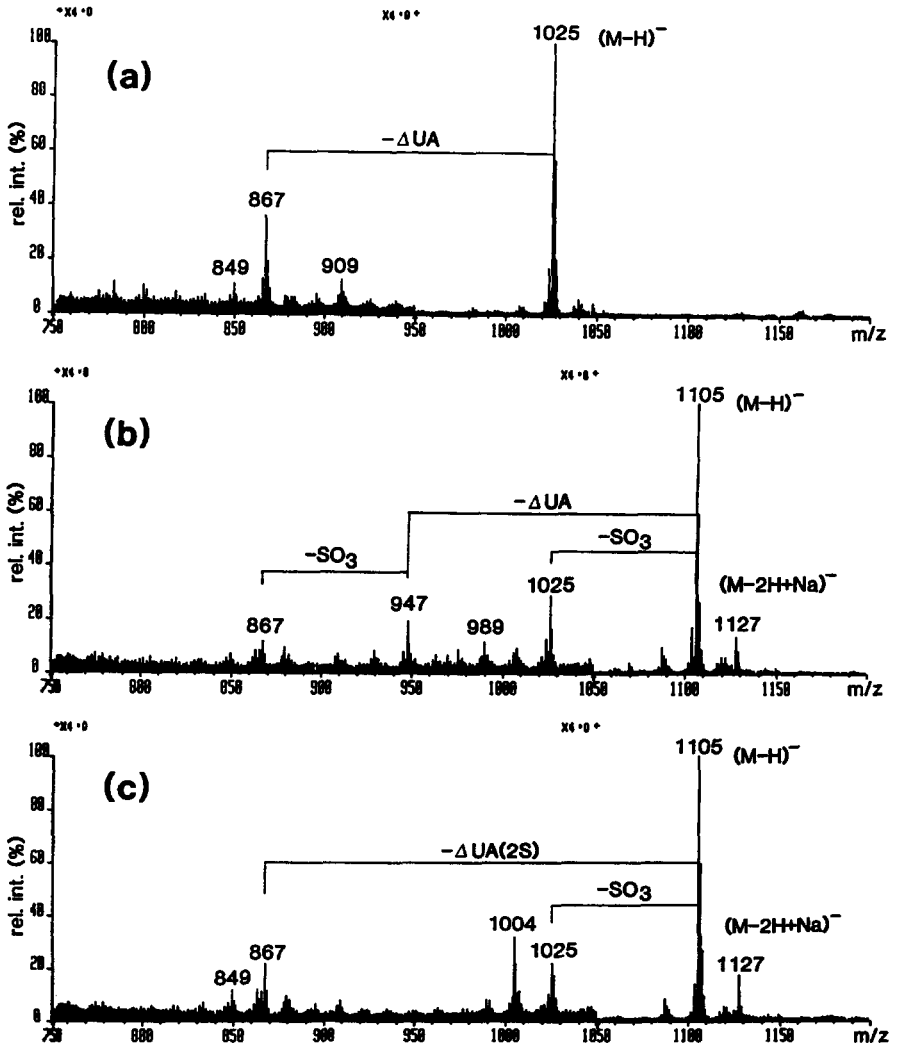


Fig. 2. Negative-ion LSI mass spectra of DHPE derivatives of disaccharides: (a) I; (b) III; (c) II; (d) VI; and (e) VII.

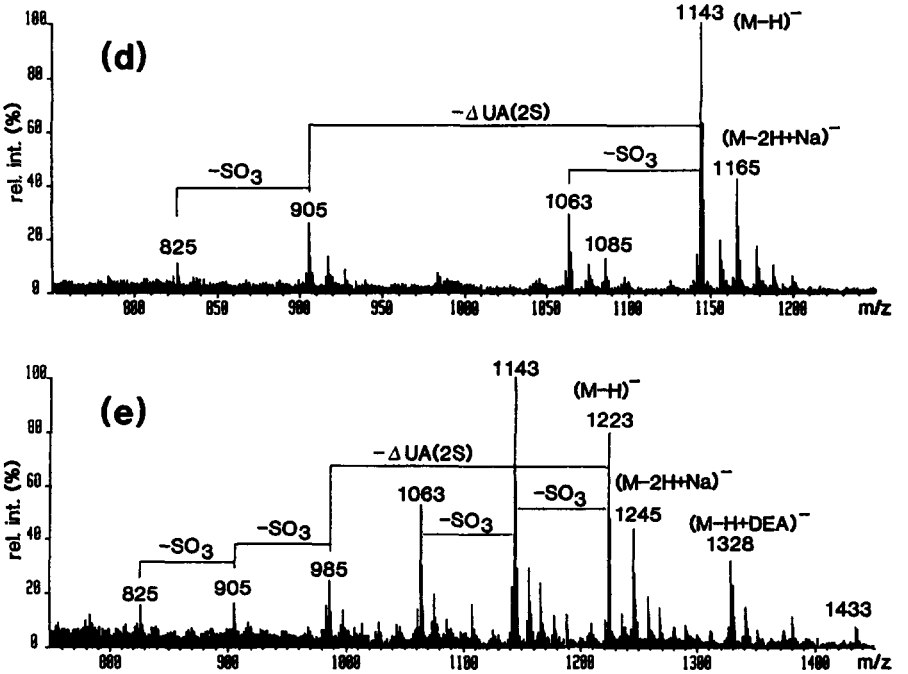


Fig. 2 (continued).

dominant role in mobility. Non-sulfated I-DHPE and VIII-DHPE have the fastest migration but clearly resolve (Fig. 1, lanes 7 and 8) and reflect the difference in isomeric composition of -4GlcNAc and -3GalNAc residues, respectively. The five mono-sulfated disaccharides chromatograph in the next region and show mobility differences based on sulfate position, monosaccharide composition and linkage. *N*-Sulfated V (lane 3) does not resolve from II (lane 6), but is well separated from III (lane 5) which differs by having a 6-*O*-sulfate in the glucosamine residue. Similarly, in the region of disulfated derivatives, IV (lane 4) with a 6-*O*-sulfated glucosamine residue runs slower than the 2-*N*-sulfated VI (lane 2). The slower migration of the 6-*O*-sulfated analogue is also apparent in the chondroitin sulfate series where mono-sulfated IX-DHPE (lane 9) and X-DHPE (lane 10) are clearly resolved. The slowest migration is exhibited by the trisulfated VII-DHPE. The 6-*O*-sulfate when present in the hexosamine residue retards migration compared with isomeric structures. Of the heparin disaccharides tested only monosulfated V and II were not resolved although these are readily differentiated from their mass spectra (see below).

TLC–LSIMS of DHPE derivatives.—Five representative negative-ion spectra of heparin disaccharide-DHPE derivatives are shown in Fig. 2. Each of the derivatives gives a spectrum with an intense quasimolecular $[M - H]^-$ ion. Sodium adduct ions $[M - 2H + Na]^-$ appear in all the sulfate-containing disaccharides as do adduct ions with diethanolamine (DEA) matrix, particularly with trisulfated VII-DHPE (Fig. 2e). The latter contributes to a distribution of ion current in the spectra of heavily sulfated

oligosaccharides and hence lower individual ion intensities. In some instances ions of 12 Da higher than the quasimolecular ions are present from boron related adducts (e.g., Fig. 2d).

Fragment ions which retain the charge on the reducing terminal bearing the DHPE group simplify interpretation of spectra. There are two principal series, one from de-sulfation and the other from glycosidic-bond cleavage. The ions from loss of sulfate (-80 Da) dominate the fragmentation, particularly when two or more sulfate groups are present (Figs. 2d and 2e). In such cases consecutive losses of sulfate by multiple cleavages involving *O*- and *N*-sulfate linkages (e.g., m/z 1063, Fig. 2e) arise due to the lability of sulfate groups. In the spectrum of the trisulfated VII-DHPE the greater intensity of ion m/z 1143 (Fig. 2e), from the loss of one sulfate (-80 Da), reflects the greater chance of sulfate loss.

Fragment ions from glycosidic-bond cleavage are generally weaker than those from de-sulfation but are clearly recognisable and allow the sequence to be assigned. The major fragment ion at m/z 867 in the spectrum of I-DHPE (Fig. 2a) from loss of ΔUA (-158 Da) from the $[M-H]^-$ corresponds to a Y-cleavage (glycosidic-bond cleavage with a hydrogen transfer to give a 'reducing' terminal fragment ion; for nomenclature see [20]) with elimination of the ΔUA moiety. The same Y-cleavage ion in the spectrum of III-DHPE (m/z 947, Fig. 2b) is consistent with the ΔUA-GlcNAc(6S)-DHPE sequence. The losses of 238 Da from the $[M-H]^-$ ions of neoglycolipids of II, VI, and VII, confirm the presence of the sulfate in the ΔUA residue. The Y-ion at m/z 867 in the spectrum of II-DHPE (Fig. 2c) distinguishes this disaccharide from its isomeric structure III (Fig. 2b, Y-ion: m/z 947) and confirms the ΔUA(2S)-GlcNAc-DHPE sequence. Similarly, the losses of ΔUA(2S) residues from VI-DHPE and VII-DHPE (Figs. 2d and 2e) result in ions m/z 905 and 985, respectively, and showing their reducing terminal monosaccharides. Unlike neoglycolipids of neutral and sialylated oligosaccharides, no distinctive ^{1,5}X- and Z-cleavage ions are observed [8,12].

In the spectra of the di- and tri-sulfated disaccharides VI and VII, respectively, the de-sulfation process is highly favourable such that consecutive cleavages of a glycosidic-bond and sulfate linkage occur. These multiple cleavages give ions at 80 Da lower than the corresponding Y-ions, such as m/z 905 and 825 in VII-DHPE (Fig. 2e) which indicate the two sulfates in the m/z 985 ion.

Additional ions from ring fragmentation also arise from the non- and mono-sulfated disaccharides. In the spectra of I-DHPE and III-DHPE (Figs. 2a and 2b), m/z 909 and 989 derive from ^{0,2}X-cleavages with losses of 116 Da from $[M-H]^-$ ions, respectively, giving additional structural information but not sufficient to assign sulfate position. This might be obtained by collision induced dissociation MS/MS where extensive ring cleavages have been observed in free disaccharides [22]. The origin of the prominent ion m/z 1004 in the spectrum of 2-*O*-sulfated II-DHPE (Fig. 2c) is unclear.

A major advantage of the DHPE neoglycolipids for analysis of GAG disaccharides is the high sensitivity of detection of their LSI mass spectra even when acquired directly from the HPTLC plate. The amounts of neoglycolipids required to give composition and sequence information differ depending on structure. Spectra from neoglycolipids of the non- and mono-sulfated GAG disaccharides generally give clear sequence information from ~ 500 pmol starting sugar although intense quasimolecular ions are generated

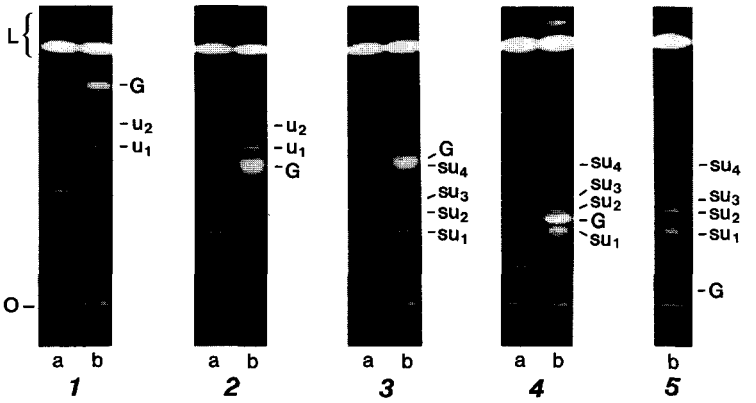


Fig. 3. TLC of the DHPE derivatives of disaccharides I, III, VI, and VII (lanes 1a–4a, respectively), and their reaction products from the oxymercuration reaction (lanes 1b–4b, respectively). The products of the reaction from tetrasaccharide XI as DHPE conjugates, are in lane 5b. G indicates bands containing the remaining structure after removal of ΔUA; U_1 and U_2, products of ΔUA; SU_1–SU_4, products of sulfated-ΔUA; O, origin and L, lipid reagent-related bands.

from only 10–50 pmol sugar. The lowest LSIMS sensitivity resulted from neoglycolipids of disaccharides with the greatest number of sulfate groups due to both reduced surface activity in the liquid matrix and to the distribution of ion current among the several adduct ions and desulfated fragment ions.

Characterisation of oxymercuration products by TLC–LSIMS of their DHPE derivatives and by reduction / methylation—Neoglycolipid formation and TLC–LSIMS was also used to investigate the oxymercuration reaction to remove the terminal unsaturated uronic acid residue. The products of oxymercuration of disaccharides I, III, VI, VII, and a tetrasaccharide fragment of heparin, ΔUA(2S)α1-4GlcNS(6S)α1-4IdoA(2S)α1-4GlcNS(6S) (XI), were conjugated to DHPE, separated by HPTLC (Fig. 3, lanes b) and analysed by TLC–LSIMS. No derivative bands corresponding to the starting disaccharides (lanes a) were detected in the product lanes at the concentrations loaded on the TLC plate, indicating that the reaction was essentially complete within the reaction time of 30 min. A distinct pattern of products was apparent that clearly defined the terminal ΔUA and whether it contained sulfate.

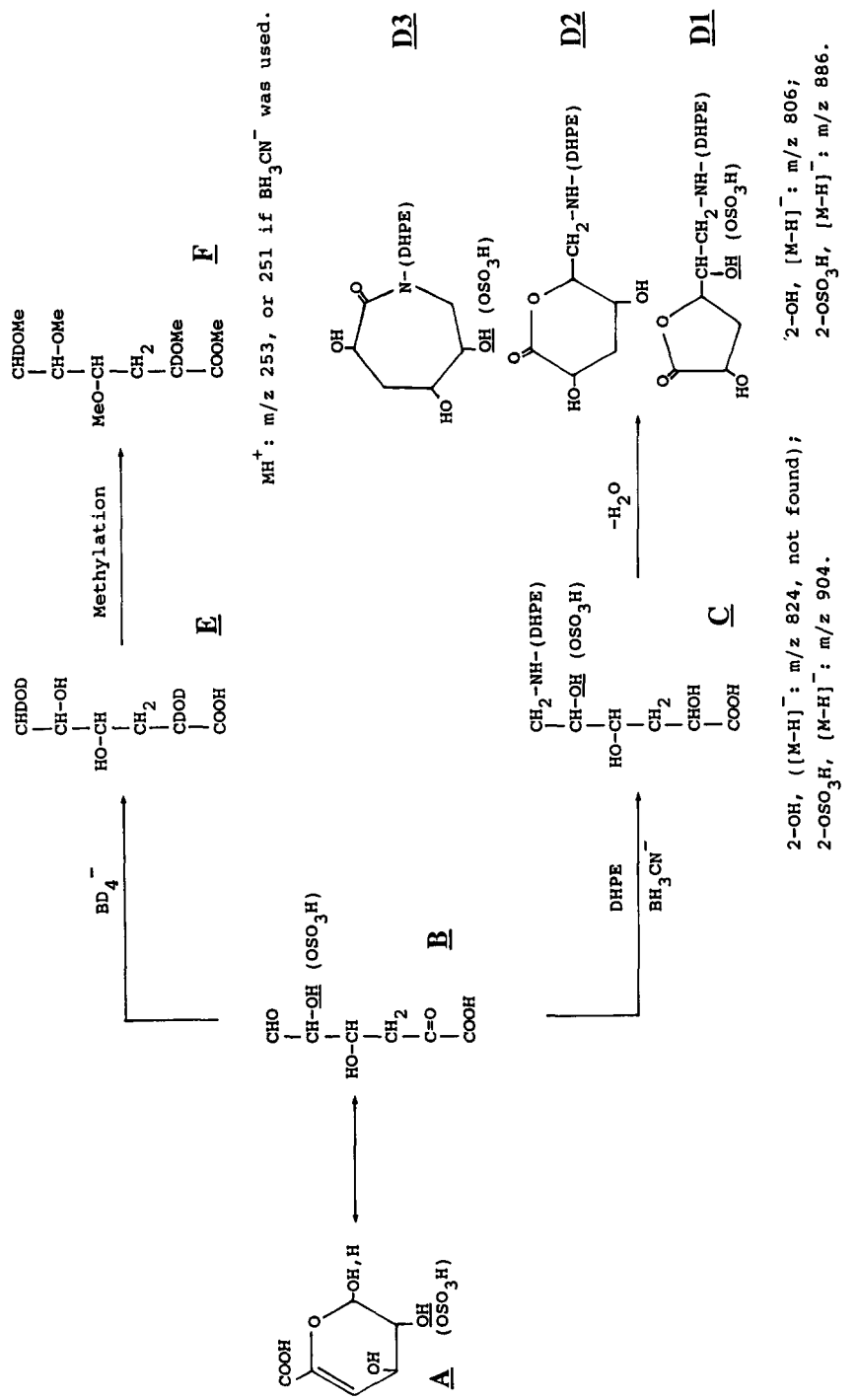
The bands marked G (glucosamine) in each of the product lanes (Fig. 3) contained the expected intact reducing terminal structures formed by removal of the ΔUA residue from each oligosaccharide. LSI spectra confirmed their identities as GlcNAc (m/z 867) from I, GlcNAc(6S) (m/z 947) from III, GlcNS (m/z 905) from VI, the GlcNS(6S) (m/z 985) from VII, and trisaccharide GlcNS(6S)-IdoA(2S)-GlcNS(6S) (m/z 1562) from XI (Table 2). Previously, GlcNAc was detected from among the cleavage products of oxymercuration of a hyaluronic disaccharide only by comparison with a standard on paper electrophoresis and chromatography [14].

The remaining bands in the product lanes were tentatively deduced to arise from the liberated ΔUA residue from the identical patterns of the doublet bands marked U_1 and U_2 (uronic) from the non-sulfated ΔUA of I and III, and the quadruplet bands marked

Table 2
Mass spectral data from oxymercuration products

	$[M-H]^-$ of TLC bands of DHPE derivatives							$NaBD_4$-reduced and methylated		$NaBH_3CN$-reduced and methylated	
	U_1	U_2	SU_1	SU_2	SU_3	SU_4	G	MH^+, LSIMS;	MH^+, CIMS	MH^+, LSIMS;	MH^+, CIMS
II	806	806					947	253	253	251	251
IV	806	806					867				
III			904	886	886	886	905				
I			904	886	886	886	985				
XI			904	886	886	886	1562 [a]				

[a] Observed as a Na and boron adduct ion at m/z 1618, $[M-3H+2Na+BH]^-$.


A
B
C
D1
D2
D3
E
F
DHPE
BH_3CN^-
$-H_2O$
BD_4^-
Methylation
MH^+: m/z 253, or 251 if BH_3CN^- was used.
2-OH, $([M-H]^-$: m/z 824, not found);
2-OSO_3H, $[M-H]^-$: m/z 904.
2-OH, $[M-H]^-$: m/z 806;
2-OSO_3H, $[M-H]^-$: m/z 886.

Scheme 1.

SU_1, SU_2, SU_3, and SU_4 (sulfated-uronic) from sulfated ΔUA of VI, VII, and XI (Fig. 3). Bands U_1 and U_2 (lanes 1b and 2b) each gave an $[M - H]^-$ ion at m/z 806 (Table 2) while bands SU_2, SU_3, and SU_4 (lanes 3b–5b) each had the same $[M - H]^-$ ion at m/z 886, 80 Da higher corresponding to the sulfate group. The SU_1 band showed an $[M - H]^-$ at m/z 904, 18 Da higher than the other three SU bands. This was postulated to be the DHPE derivative (Scheme 1, **C**), of sulfated and reduced 'keto acid' (**B**), the non-sulfated analogue of which was proposed as the main product from the ΔUA residue of a hyaluronic disaccharide, although evidence was limited to colour reaction, together with a second unknown component observed on Sephadex chromatography [14]. By dehydration **C** may form several isomeric lactones (e.g., **D1** and **D2**, Scheme 1) and possibly an amide (**D3**), which would explain the multiple products with the same molecular masses. However, **D2** would not be formed in the presence of a sulfate group at the 2-position. Either lactonisation or amide formation is likely to occur between the carboxyl group and hydroxyl or amino groups, particularly when hemiacetal ring

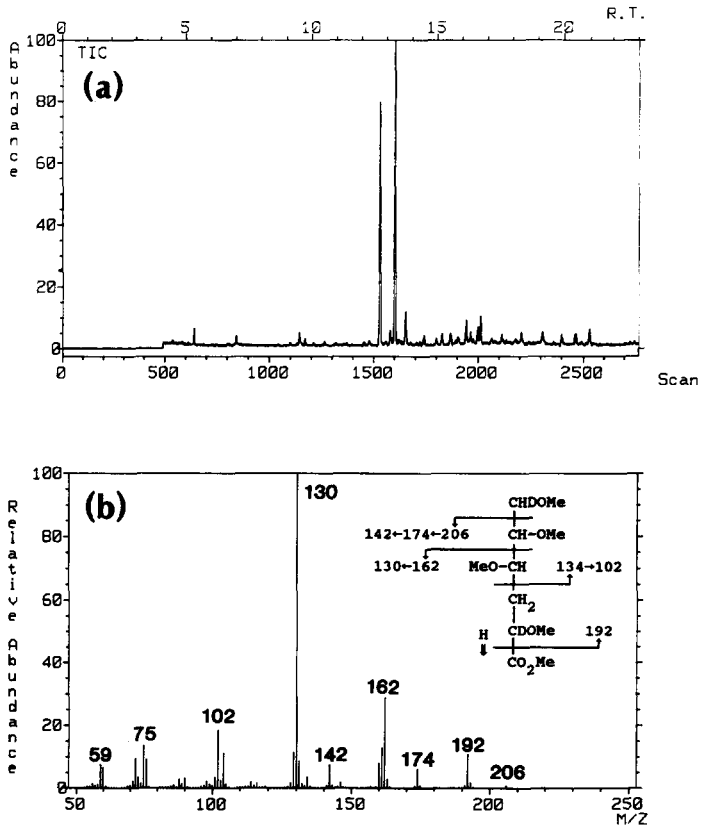


Fig. 4. GC–MS analysis of the reduced and methylated products from the ΔUA residue liberated from disaccharide III by oxymercuration. The total ion chromatogram (a) obtained by EI shows two peaks with almost identical mass spectra (b). Fragmentation (inset) was consistent with structure **F**.

formation is not possible due to loss of the aldehyde group to reductive amination. Reduction of the 5-keto function in **B** would give rise to two stereo isomers and contribute to the complexity of products. No TLC band was found corresponding to a double-conjugation product of both aldehyde and keto groups to DHPE.

Confirmation of the oxymercuration products of the ΔUA residue was sought by LSIMS and GC–MS analysis following their reduction and methylation. III was selected as being representative. Following removal of mercuric salt by ion-exchange, the mixture was reduced with $NaBD_4$ and methylated. Chloroform extraction was used to fractionate non-sulfated ΔUA products from the sulfated GlcNAc [13]. Both LSIMS and CIMS analyses of the residue showed a MH^+ ion at m/z 253 (Table 2) in agreement with the mass of a fully methylated 4-deoxy-alditol acid (Scheme 1, **F**) arising from reduction of both ketone and aldehyde groups of the possible keto acid (**B**). GC–MS analysis of this product revealed two components (Fig. 4a) each giving an almost identical EI mass spectrum with fragment ions consistent with two isomeric reduced and methylated keto acids (**F**).

The reduction of a keto group by $NaBH_3CN$ during the conjugation was unexpected but confirmed by an additional experiment. The oxymercuration products of III were incubated with $NaBH_3CN$ under identical conditions used for DHPE conjugation. Following methylation the products were shown by LSIMS and GC–MS to be identical to those from BH_4^- reduction, except for the 2 mass unit decrease (Table 2 and Scheme 1, **F**), indicating both ketone and aldehyde groups had been reduced.

It has been suggested previously that the keto acid is unstable to acid treatment [14,18,21] and was lost during paper chromatography. Parallel experiments excluding the AG50 cation-exchange separation step gave almost identical results from both DHPE and reduced-methylated derivatives, and no other major products were found. This indicated that, contrary to the earlier report [21], the acidic nature of the strong cation-exchange resin did not influence the products formed.

4. Conclusions

The combined use of HPTLC and in situ LSIMS analysis has been shown to be a sensitive and reliable method for separation and structural identification of the GAG disaccharides as DHPE derivatives, and possibly can serve as an alternative approach to disaccharide-compositional analysis of fragments isolated from biological polysaccharides.

Oxymercuration has been successfully used for the removal of ΔUA residues in heparin disaccharides and a tetrasaccharide with investigation of the reaction being facilitated by TLC–LSIMS analysis of the reaction products after conversion to DHPE derivatives. The multiple but characteristic products of the cleaved ΔUA allow microscale identification of the terminal ΔUA residues of GAG fragments produced by glycan lyase treatment. This has assisted in the assignment of the ΔUA residue in a ^1H NMR spectroscopic study [23] of several heparin fragments in which variation in proton chemical shifts made assignment uncertain. The integrity of the remaining structures after removal of ΔUA, including glycosidic linkages and *O*- and *N*-sulfate groups,

indicates that oxymercuration will be applicable to the preparation of unmodified GAG fragments for biological function studies.

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References

[1] L. Kjellén and U. Lindahl, *Annu. Rev. Biochem.*, 60 (1991) 443–475.
[2] T.E. Hardingham and A.J. Fosang, *FASEB J.*, 6 (1992) 861–870.
[3] U. Lindahl, G. Backstrom, and L. Thunberg, *J. Biol. Chem.*, 258 (1983) 9826–9830.
[4] D.H. Atha, A.W. Stephens, and R.D. Rosenberg, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984), 1030–1034.
[5] S. Hase, T. Ikenaka, and Y. Matsushima, *Biochem. Biophys. Res. Commun.*, 85 (1978) 257–263.
[6] A. Dell, *Adv. Carbohydr. Chem. Biochem.*, 45 (1987) 19–72.
[7] J.W. Webb, K. Jiang, B.L. Gillece Castro, A.L. Tarentino, T.H. Plummer, J.C. Byrd, S.J. Fisher, and A.L. Burlingame, *Anal. Biochem.*, 169 (1988) 337–349.
[8] A.M. Lawson, W. Chai, G.C. Cashmore, M.S. Stoll, E.F. Hounsell, and T. Feizi, *Carbohydr. Res.*, 200 (1990) 47–57.
[9] B. Casu, in D.A. Lane, and U. Lindahl (Eds.), *Heparin*, Arnold, London, 1989, pp 25–49.
[10] W. Chai, G.C. Cashmore, R.A. Carruthers, M.S. Stoll, and A.M. Lawson, *Biol. Mass. Spectrom.*, 20 (1991) 169–178.
[11] W. Chai, G.C. Cashmore, M.S. Stoll, S.J. Gaskell R.S. Orkiszewski, and A.M. Lawson, *Biol. Mass. Spectrom.*, 20 (1991) 313–323.
[12] T. Feizi, M.S. Stoll, C.-T. Yuen, W. Chai, and A.M. Lawson, *Methods Enzymol.*, 230 (1994) 484–519.
[13] C.-T.Yuen, A.M. Lawson, W. Chai, M. Larkin, M.S. Stoll, A.C. Stuart, F.X. Sullivan, T.G. Ahern, and T. Feizi, *Biochemistry*, 31 (1992) 9126–9131.
[14] U. Ludwigs, A. Elgavish, J.D. Esko, E. Meezan, and L. Roden, *Biochem. J.*, 245 (1987) 795–804.
[15] L.-Å. Fransson, B. Havsmark, and I. Silverberg, *Biochem. J.*, 269 (1990) 381–388.
[16] G. David, X.M. Bai, B. Van der Schuern, J.-J. Cassiman, and H. Van den Berghe, *J. Cell Biol.*, 119 (1992) 961–964.
[17] Z.M. Merchant, Y.S. Kim, K.G. Rice, and R.J. Linhardt, *Biochem. J.*, 229 (1985) 369–377.
[18] M. Kusche, U. Lindahl, L. Enerback, and L. Roden, *Biochem. J.*, 253 (1988) 885–893.
[19] I. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.
[20] B. Domon and C.E. Costello, *Glycoconj. J.*, 5 (1988) 397–409.
[21] N.J. Palleroni and M. Doudoroff, *J. Biol. Chem.*, (1956) 499–508.
[22] D.J. Lamb, H.M. Wang, L.M. Mallis, and R.J. Linhardt, *J. Am. Soc. Mass Spectrom.*, 3 (1992) 797–803.
[23] W. Chai, E.F. Hounsell, C.J. Bauer, and A.M. Lawson, *Carbohydr. Res.*, 269 (1995) 139–156