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PAPER

Disaccharide compositional analysis of chondroitin sulphate using WAX HILIC-MS with pre-column procainamide labelling; application to the placenta in pre-eclampsia†

Cite this: DOI: 10.1039/d3ay01578e

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Chondroitin sulphate (CS) and dermatan sulphate are linear negatively charged heteropolysaccharides. These glycosaminoglycans are involved in cellular signalling *via* binding to growth factors. CS is expressed in a range of tissue and biological fluids and is highly expressed in the placenta. There is evidence that decorin; a CS proteoglycan is significantly decreased in pre-eclampsia and fetal growth restriction. It is considered that GAG chain composition may influence cellular processes that are altered in pre-eclampsia. The goal of the present study was to develop an LC-MS method with precolumn procainamide labelling for the disaccharide compositional analysis of CS. The method was used to investigate whether the disaccharide composition of placenta-extracted CS is altered in pre-eclampsia. The study revealed differential disaccharide compositions of placental chondroitin sulphate disaccharide between pre-eclampsia and other pregnancy samples. This suggests that the method may have diagnostic potential for pregnancy disorders. Furthermore, the findings suggest that CS sulphation might play a significant role in maternal labour.

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Introduction

Chondroitin sulphate and dermatan sulphate are growth factor- and cytokine binding-glycosaminoglycans (negatively charged linear heteropolysaccharides) thus involved in cellular signalling.^{1,2} CS is present on cell surfaces and in great amounts in the extracellular matrix as vital components of proteoglycans (PG) where they help to maintain structural integrity.^{1,3} They are also present in biological fluids including urine,⁴ blood,⁵ CSF (cerebrospinal fluid)⁶ and are highly expressed in the placenta.⁷ Placental CSPGs are in the intervillous spaces, fibrous tissue and on cell surfaces to a lesser extent.⁷ Specifically, decorin, syndecan-1 (SDC-1), Integrin Beta-1 and biglycan have been identified in the placenta.^{8,9}

The placenta is central to the development of pre-eclampsia -, a serious pregnancy complication affecting 3–5% of all pregnancies. In normal placental development, invading extravillous trophoblasts (EVT) transform the high resistance decidua and myometrial uterine spiral arteries to low resistance blood vessels for adequate placental perfusion.¹⁰ This spiral

artery remodelling is incomplete in pre-eclampsia resulting in placental hypoperfusion and poor nutrient supply to the developing foetus.¹¹ Growth factors, the local cellular environment, oxygen tension and cytokines all influence the EVT invasion process.¹¹

Decorin is present in the wall of placental foetal blood vessels and the decidua.^{12,13} It is involved in endothelial cell development, angiogenesis and has been shown to have anti-proliferative, anti-migratory and anti-invasive effects on extravillous trophoblasts.¹⁴ There is evidence that decorin is significantly decreased in pre-eclampsia¹⁵ and foetal growth restriction.¹³ SDC-1, primarily a HSPG also carry CS chains; both chains cooperating in the binding to cytokines and fibroblast growth factor.¹⁶ SDC-1 is greatly expressed on the apical side of syncytiotrophoblasts⁹ and is vital for proper placental implantation and angiogenesis.¹⁷ However, there are contrasting reports of increased,¹⁸ unaltered¹⁵ and decreased^{19,20} levels of placental SDC-1 in pre-eclampsia. The full actions of specific growth factors are achieved *via* their binding to glycosaminoglycans (GAG), largely driven by interactions with the sulphate groups on the GAG chain. Therefore, it is likely that GAG chain composition independent of total amounts of GAGs may influence cellular processes which are altered in pre-eclampsia. In addition, hemodynamic modulation involving stiffness of maternal abdominal aorta, high resistance of small arteries and arterioles is also implicated in pre-eclampsia.²¹ It is possible

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1 that this modulation may be influenced by GAGs involved in
extracellular matrix alterations²² such as those on CSPGs.

5 CS chains comprise 40–100 differentially sulphated disac-
charide subunits of uronic acid (UA) $\beta(1 \rightarrow 3)$ linked to *N*-ace-
tylgalactosamine (GalNAc) resulting in structural
heterogeneity.^{23–25} The position two of the UA, four, and six of
the GalNAc can be sulphated. These yield non, mono, di, and
10 tri-sulphated disaccharides with the latter generally minimally
present.²⁵ The UA of CS is glucuronic acid (GlcA) which is
replaced by iduronic acid (IdoA) at varying degrees in dermatan
sulphate. Both chains are however usually present as co-
polymeric structures.²³

15 For disaccharide compositional analysis, GAGs are generally
liberated from de-fatted tissues using proteolytic enzymes such
as papain²⁴ or actinase E,²⁶ extracted using anion exchanger
diethylaminoethyl (DEAE)²⁷ or quaternary ammonium
columns^{28,29} and purified using alcohol precipitation,²⁸ molec-
20 ular weight cut-off filters³⁰ or desalting columns.²⁷ The extracted
GAG are then often depolymerised into constituent disaccha-
rides using specific GAG lyases³¹ or alternatively, by hydrazin-
olysis followed by nitrous acid deaminative cleavage.^{32,33} These
disaccharides can then be analysed using high-performance
25 liquid chromatography (HPLC) with mass spectrometry (MS)
with or without derivatisation. Analysis of native GAG disac-
charides avoids the extra sample derivatisation steps. However,
anomeric separation is a significant complication.^{34,35} In addi-
tion, SAX-HPLC which is often used here is incompatible with
MS due to the high salt composition of mobile phase. Deriva-
30 tisation with hydrophobic fluorophores have significant
advantages of improving RP LC separation,²⁹ increasing MS
sensitivity,³⁶ offering fluorescent detection³⁷ and avoiding
anomeric LC separation.³⁴ In the current study derivatisation of
GAG disaccharides with procainamide offered several benefits.
35 These advantages include the facilitation of HILIC separation,
utilisation of solvents more compatible with MS and the
detection of derivatives using ESI MS in the positive ion mode.
We have previously shown that procainamide derivatisation
also significantly increases GAG disaccharide MS sensitivity
40 relative to AMAC derivatisation.³⁸

Mixed mode chromatography such as WAX-HILIC offers
unique selectivity for the separation of polar and charged ana-
lytes because it offers multiple retention modes.³⁹ Here,
45 hydrophilicity is combined with electrostatic interaction reten-
tion mechanisms. This is particularly useful for the separation
of GAG disaccharides where more than one sulphation posi-
tional isomer is present in addition to differentially sulphated
disaccharides. To the best of our knowledge, WAX-HILIC-MS
50 analysis of procainamide-labelled CS disaccharide has not
been previously reported.

The aim of this study was to develop a novel WAX-HILIC-MS
method for the disaccharide compositional analysis of CS.
Three HILIC columns with different chemistries were tested for
55 the separation of eight procainamide-labelled disaccharides.
Upon optimisation of separation, we applied this method to
investigate if the disaccharide composition of placenta-
extracted CS is altered in pre-eclampsia.

Experimental

Materials

Chondroitin sulphate (sodium salt from bovine cartilage),
hyaluronic acid (sodium salt from rooster comb), chon-
5 droitinase ABC (from *Proteus vulgaris*), actinase (from *Strepto-*
myces griseus), procainamide hydrochloride, sodium
cyanoborohydride, CHAPS, molecular weight cut off filters (3
kDa and 10 kDa, Millipore), 0.22 μm Millex-GP filters (Milli-
10 pore), anion exchange spin columns (Q Mini H, Sartorius) and
Hank's balanced salt solution were purchased from Sigma-
Aldrich (Poole, U.K.). Chondroitin sulphate unsaturated disac-
charides (0S_{CS}, 2S_{CS}, 4S_{CS}, 6S_{CS}, 2S6S_{CS}, 2S4S_{CS}, 4S6S_{CS}, TriS_{CS})
and disaccharide internal standard ($\Delta\text{UA}_2\text{S} - \text{GlcNCOEt-6S}$)
15 were purchased from Iduron (Manchester, U.K.). LC-MS grade
dimethyl sulphoxide (DMSO), acetic acid, Tris-HCl, calcium
chloride, sodium chloride, sodium acetate, urea, chloroform,
methanol, UPLC water, HPLC grade acetonitrile (ACN) and
ammonium formate were purchased from Fisher Scientific
20 (Loughborough, U.K.). Lyophilisation was carried out using
a Scan Vac CoolSafe 55-4 freeze dryer (Labogene, Lynge,
Denmark).

Procainamide labelling of CS disaccharides

25 Procainamide labelling of disaccharides was carried out as
previously reported³⁸ with a slight modification. Samples con-
taining CS disaccharides (with or without the disaccharide
internal standard) were lyophilised and re-constituted in 20 μL
of 0.4 M procainamide prepared in a mixture of DMSO and
30 acetic acid (7:3, v/v). They were subsequently incubated at
room temperature for 15 min and 20 μL of 1 M sodium cyano-
borohydride was added. The mixture was vortexed and then
incubated at 45 °C for 4 h. This was subsequently diluted in 60
 μL of 80% ACN and an aliquot (2 μL) was injected onto the UPLC
35 column.

HILIC-UPLC-UV-MS/FLR conditions

40 A Waters ACQUITY H-Class UPLC system was used coupled to
an SQD2 or fluorescence (FLR) detector with an in-line diode
array detector (DAD). To achieve optimum separation of
procainamide-labelled CS disaccharides, an ACQUITY Glycan
BEH Amide (1.7 μm ; Waters, Ireland, U.K.), ACE HILIC-B (3 μm ;
45 HiChrom, Reading, UK), and GlycanPac AXH-1 (1.7 μm ; Thermo
Fisher Scientific, Loughborough, UK) HILIC based 2.1 \times
150 mm columns were tested. The columns were maintained at
55 °C and a flow rate of 0.35 mL min⁻¹ was used. Solvent A, B
and C were ammonium formate (pH 4.4), ACN and water
50 respectively. For the detection of procainamide labelled disac-
charides, a wavelength of 299 nm was set on the DAD and an
excitation and emission wavelength of 330 and 380 nm
respectively was used on the FLR.

The MS conditions previously optimised³⁸ and used for the
55 detection of procainamide-labelled disaccharides in the posi-
tive ESI mode were as follows; the capillary voltage was set at 3.1
kV and the probe was positioned 4.83 and 7 mm horizontally
and vertically respectively. The source and desolvation

temperature were set at 110 °C and 250 °C respectively. The cone and source gas flow were set at 1 and 800 L h⁻¹ respectively. A cone voltage of 50 V was used for the TIC and subsequently optimised for each procainamide-labelled disaccharide and used in the SIR chromatogram. The cone voltage was varied between 20–75 V to achieve this. The needle draw rate for the sample injector was set at 140 μL min⁻¹ and samples were maintained at 10 °C in the autosampler. The instrument was operated, and data collected and analysed using MassLynx® (v 4.1).

Quantitative analysis

A mixture of eight procainamide labelled CS disaccharides and internal standard was analysed using optimal ULPC-MS conditions over a concentration range of 0.005–20 μg mL⁻¹. This was used to determine the linearity (r^2) of the method. Five replicate injections were made, and data obtained was used to calculate the intra-assay coefficient of variation (CV) at three different concentrations (0.078, 1.25 and 10 μg mL⁻¹) and the method detection limit (MDL). The MDL was defined as the concentration with a signal distinguishable from the noise at a 99% confidence interval.

Ethics considerations

Informed consent was obtained from each volunteer and ethical approval was granted by the Barnet, Enfield & Haringey NHS and Hammersmith and Queen Charlotte's & Chelsea Hospitals NHS research ethics committee for the collection of placenta samples. Natural Science Ethics Committee at Middlesex University, London granted ethical approval for the current study (project number 1989).

Tissue preparation and clinical groups

Placenta tissues were collected and processed within 1 h. Samples were collected near the maternal surface adjacent to the maternal decidua. Tissues were washed in Hank's balanced salt solution to remove contaminants and red blood cells. The amnion and decidua tissue were removed, and the resulting villous trophoblast-enriched tissue was fixed in 80% ethanol until use. Trophoblast enrichment ensures that placenta tissue samples used in the current study are largely free of maternal tissue and amniotic membrane contamination.

Twenty-seven placenta samples were collected from gestational aged-matched pregnancies and used in this study. They were grouped as follows; term delivery from uncomplicated pregnancy prior to labour onset (term non-labour; TNL, $n = 10$), uncomplicated pregnancy at term after labour onset (Term labour; TL, $n = 7$), pre-term delivery upon spontaneous pre-term labour (pre-term labour; PTL, $n = 5$) and pre-term delivery from women with pre-eclampsia prior to labour onset (PE, $n = 5$). The onset of regular uterine contractions with cervical dilation before the 37th week of gestation was designated pre-term labour.⁴⁰

Women with pregnancies complicated by pre-eclampsia tend to deliver pre-term, and often *via* caesarean section. Therefore, in this study we have utilised other pregnancy groups

– preterm labour and uncomplicated pregnancy prior to labour onset (term non-labour, *i.e.*, term pregnancy delivered *via* a caesarean section prior to labour onset) to enable us understand whether chondroitin sulfation is also influenced by gestational age or labour.¹⁹

Sample preparation

Placenta tissue GAGs were extracted using a method adapted from ref. 29 and 41. Tissues were cut into tiny pieces and defatted using three sequential chloroform : methanol (v/v) solutions; 2 : 1, 1 : 1 and 1 : 2 each for 10 h. The solvent was removed, and the tissues were allowed to air dry for 20 min at room temperature. Tissues were then re-suspended in 750 μL of water and proteolysed for 18 h at 55 °C using 250 μL of actinase E (20 mg mL⁻¹ in 0.1 M Tris/0.1 M CaCl₂). The resulting solution was filtered using 0.22 μm syringe filter to remove particulates and peptides were removed using a 10 kDa molecular weight cut-off filter. The retentate was collected, lyophilised and re-suspended in 8 M urea and 2% CHAPS (w/v). This was then loaded onto an anion exchange column, washed with 0.2 M NaCl, and GAGs were eluted using 16% NaCl. The extracted GAGs were desalted using a 3 kDa molecular weight cut-off filter and stored at –20 °C prior to use.

Samples containing placenta-extracted GAGs were lyophilised and re-suspended in a buffer solution comprising 50 mM Tris and 60 mM sodium acetate, pH 8.0. CS was then enzymatically depolymerised by incubation with 10 mIU chondroitinase ABC for 24 h at 37 °C. CS and HA standard (20 μg) were also depolymerised similarly in triplicate for quantitative analysis. The reaction mixture was heated in a boiling water bath for 10 min to inactivate the enzymes and centrifuged at 12 000 g for 10 min. The supernatant containing CS disaccharides was collected and 0.5 μg of disaccharide internal standard (ΔUA,2S – GlcNCOEt,6S) was added. The sample was then lyophilised, disaccharides were labelled with procainamide and an aliquot (2 μL) was injected onto the UPLC column.

Data analysis

The HPLC-peak areas were used to determine the concentrations of CS disaccharides from standard curve for each analyte and normalised with respect to the disaccharide internal standard (ΔUA,2S – GlcNCOEt-6S). Individual CS disaccharide concentrations were converted into a percentage of total CS disaccharides in a particular biological sample. ANOVA was used to determine significant differences between groups. A generalised linear model (GLM) analysis was used to determine the independent influence of pre-eclampsia (PE), labour (TL, TNL) and pre-term delivery (PTL, PE) on placental GAG disaccharide composition. All statistical analysis was done using Minitab® (v 17).

Results & discussion

LC separation of procainamide-labelled CS disaccharides

As previously described,³⁸ procainamide disaccharide labelling by reductive amination was utilised for chondroitin sulphate

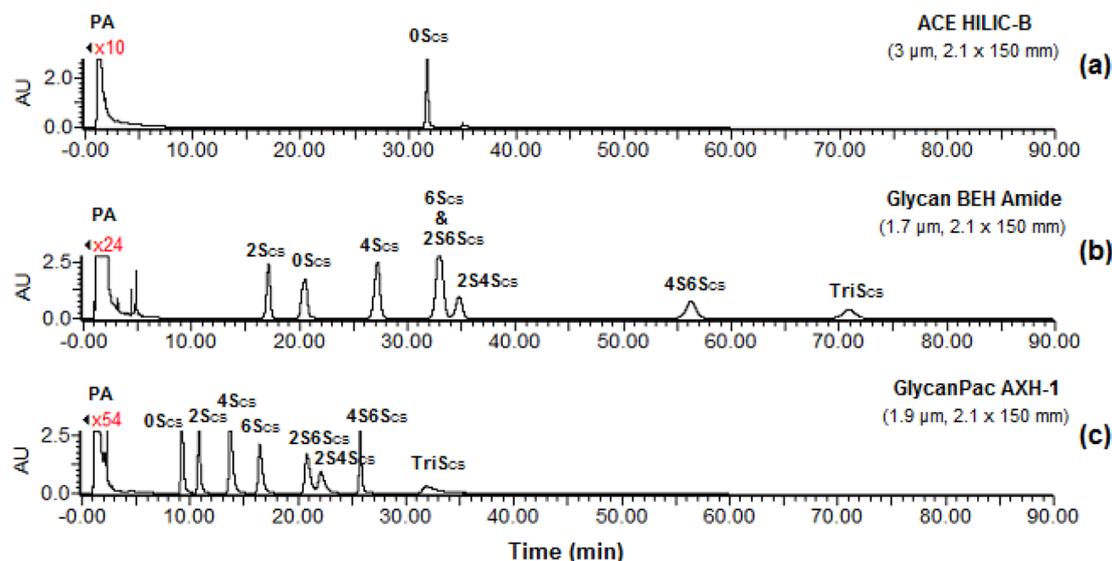


Fig. 1 LC separation of procainamide-labelled CS disaccharides using HILIC columns (a) ACE HILIC-B (b) glycan BEH amide and (c) GlycanPac AXH-1. Mobile phase A, B and C was ammonium formate (pH 4.4), acetonitrile and water respectively. Detection was diode array, and a wavelength of 299 nm was used for extraction of chromatograms. PA (procainamide peak).

disaccharides. Three different HILIC based column chemistries were then tested for the separation of these procainamide-labelled CS disaccharides. Due to the structural similarities of these disaccharides and thus resulting difficulty in LC separation, isocratic elution conditions were initially employed. Solvent A, B, and C were ammonium formate (150 mM, pH 4.4), ACN and UPLC water respectively. With all three columns, excess unreacted procainamide was poorly retained and eluted before the derivatised disaccharides (Fig. 1). This was expected as procainamide is less hydrophilic relative to procainamide-labelled CS disaccharides.

Procainamide-labelled CS disaccharides could not be separated using the ACE HILIC-B column and only the unsulphated disaccharide was eluted using a solvent composition of A : B : C of 20 : 80 : 0%, v/v (Fig. 1a). The other procainamide-labelled CS disaccharides could not be eluted even by increasing composition of the aqueous solvent in the mobile phase up to 50% A. Reducing the concentration of solvent A to 50 mM did not improve elution either. Strong adsorption of acidic analytes on HILIC columns is possible, due to their high affinity for aminopropyl-silica.⁴² The ACE HILIC-B column is a pH-responsive positively charged proprietary material, bonded to silica, that can be adjusted within a suggested operational pH span of 2.0 to 7.0. This may explain the seemingly irreversible retention of highly negatively charged sulphated CS disaccharides. The glycan BEH amide column was tested with different isocratic conditions ranging from 10–20% A (0% C). There was excellent resolution between procainamide-labelled CS disaccharides except for 6S_{CS} and 2S6S_{CS} which co-eluted (Fig. 1b). The use of 100 mM, 50 mM and 10 mM ammonium formate (pH 4.4) did not significantly improve resolution between these two CS disaccharides. Interestingly, the more hydrophilic mono-sulphated 2S_{CS} eluted before the unsulphated 0S_{CS}; thus, the order of elution did not strictly follow an increasing degree of

sulphation as expected. This phenomenon was also observed for procainamide-labelled heparan sulphate disaccharides on the glycan BEH Amide column but at a decreased buffer concentration.³⁸

Using the GlycanPac AXH-1 column, the previously co-eluting disaccharide critical pair (6S_{CS} and 2S6S_{CS} on the glycan BEH amide column) was completely resolved using a mobile phase composition composed of A, B and C of 11 : 85 : 4%, v/v (Fig. 1c). Furthermore, all procainamide-labelled CS disaccharides except for the sulphational positional di-sulphated isomer 2S6S_{CS} and 2S4S_{CS} were completely separated to baseline. However, it was possible to integrate the HPLC-peak area for both these disaccharides (Fig. 1c). Consequently, this column was chosen for further analyses. A reduction in the concentration of Solvent A (ammonium formate, pH 4.4) to 50 mM did not further improve resolution but resulted in co-elution of 2S4S_{CS} and 2S6S_{CS} and an overall decrease in the retention of all procainamide-labelled CS disaccharides (ESI Fig. S-1†). Therefore, the final LC conditions used are as follows; Solvent A/B/C (11/85/4%, v/v from 0–23 min, transitioning to 11/60/29 from 23–26 min). The composition was maintained until 35 min after which the composition returned to the initial conditions at 38 min. Following each injection, the column was re-equilibrated for 22 min. The gradient step in the elution conditions was made after the separation of the critical pair, to shorten the method run time.

Of the three HILIC based columns tested for procainamide-labelled CS disaccharides, optimal separation was achieved using the GlycanPac AXH-1 column. Separation of procainamide-labelled disaccharides was achieved within 40 min on this column. Elution was in the order of increasing sulphation and hydrophilicity demonstrating the effectiveness of the HILIC and WAX separation mechanisms on the GlycanPac AXH-1 column; the unsulphated disaccharide (0S_{CS})

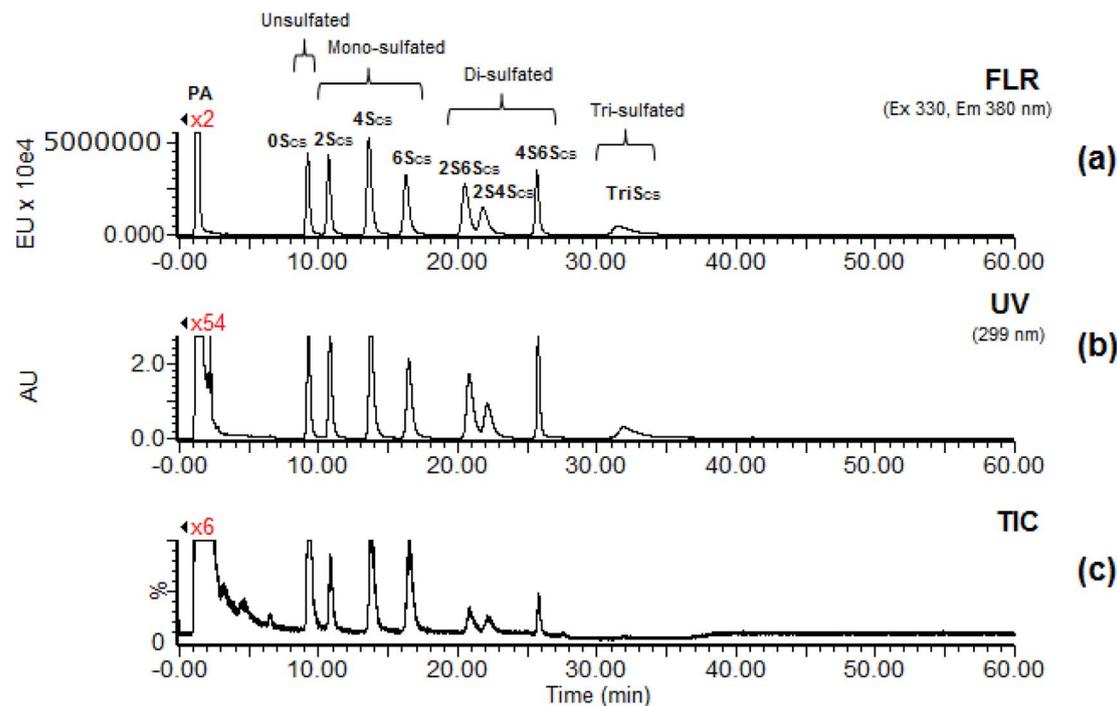


Fig. 2 Separation of procainamide-labelled CS disaccharides on GlycanPac AXH-1 (a) fluorescent (b) UV and (c) TIC. EU (emission units), PA (procainamide peak).

eluted first, followed by the mono- (2S_{CS}, 4S_{CS}, 6S_{CS}), di- (2S6S_{CS}, 2S4S_{CS}, 4S6S_{CS}), and tri-sulphated disaccharide (TriS_{CS}). These disaccharides were detected using fluorescent, UV and mass spectrometric detection (Fig. 2).

LC separation of native CS disaccharides using WAX-HILIC has been previously reported.⁴³ However, only four disaccharides were separated and LC co-elution of sulphation positional disaccharide isomers (4S_{CS}, 6S_{CS}) in a single peak was observed which required a mass spectrometer to distinguish them. In comparison, the current study demonstrates the separation of all eight commercially available CS disaccharides. Although a longer run time was required, good resolution (1.3) is achieved between 4S_{CS} and 6S_{CS} which are typically the major disaccharides in biological samples. To the best of our knowledge, this is the first method demonstrating the procainamide labelling and separation of all eight unsaturated CS disaccharides using a mixed mode WAX HILIC. The ability for fluorescent detection is an advantage of pre-column procainamide labelling which allows CS disaccharide analysis in laboratories which have a fluorescent detector without the need for a relatively more expensive mass spectrometer.

Optimisation of MS conditions

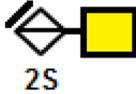
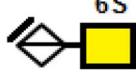
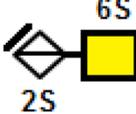
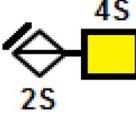
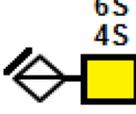
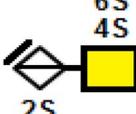
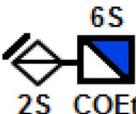
Labelling of CS disaccharides with procainamide resulted in a mass increase of 219 Da (Table 1), which is consistent with previous studies of procainamide-labelled HS disaccharides³⁸ and N-linked glycans.⁴⁴ Procainamide-labelled CS disaccharides standards and the internal disaccharide standard were then used to optimise MS conditions for their detection in the positive electrospray ionisation mode. Multiple ions were

observed in the MS spectrum for these disaccharides corresponding to $[M + H]^+$, $[M - SO_3 + H]^+$, $[M - 2SO_3 + H]^+$ and $[M - 3SO_3 + H]^+$ species (Table 1, ESI Fig. S-2†). The sulphonic acid group of GAG disaccharides are heat-labile, and their MS in-source fragmentation ($-80 m/z$) is well known.^{31,38} The $[M + H]^+$ ion was the most intense for the procainamide-labelled unsulphated CS disaccharide ($\Delta U A - GalNAc$), $[M - SO_3 + H]^+$ for the mono- and di-, while $[M - 2SO_3 + H]^+$ was the most intense ion for the tri-sulphated disaccharide (ESI Fig. S-2†). The MS signals of these ions were further optimised by varying their cone voltages. The optimum cone voltage which yielded the most intense MS signals for the chosen disaccharide ion (Table 1, ESI Fig. S-3†) was used to set up the selected ion recording method (SIR) (Fig. 3). In the SIR chromatogram, the procainamide-labelled internal disaccharide standard co-eluted with 2S4S_{CS}. However, this posed no issue since the MS ions utilised for detection had different m/z values. The optimised SIR method was used for quantitative analysis of disaccharides in biological samples.

Quantitative analysis

For the quantitative analysis of procainamide-labelled CS disaccharides and internal disaccharide standard, the following method validation parameters were determined using the HILIC-UPLC-MS method with SIR chromatogram and shown in Table 2. Excellent linearity (r^2) was obtained (0.998–1) over a concentration range of 0.005–20 $\mu\text{g mL}^{-1}$. Differential ionisation efficiency was observed for these disaccharides (ESI Fig. S-3†) reflecting the different method detection limits (MDL). The procainamide labelled disaccharides 0S_{CS} and 4S_{CS}

Table 1 Disaccharide code, symbol, theoretical molecular weight (MW), observed MS ions, assignment and optimum MS cone voltage for procainamide-labelled disaccharides

Disaccharide code	Symbol	Theoretical MW (native disaccharide)	Observed ions (procainamide labelled, m/z)	Assignment	Optimum MS cone voltage (V)
0S _{CS} (Δ UA – GalNAc)		379.0	599.4	$[M + H]^+$	60
2S _{CS} (Δ UA,2S – GalNAc)		459.0	679.4 599.4	$[M + H]^+$ $[M - SO_3 + H]^+$	70
4S _{CS} (Δ UA – GalNAc,4S)		459.0	679.4 599.4	$[M + H]^+$ $[M - SO_3 + H]^+$	70
6S _{CS} (Δ UA – GalNAc,6S)		459.0	679.4 599.4	$[M + H]^+$ $[M - SO_3 + H]^+$	75
2S6S _{CS} (Δ UA,2S – GalNAc,6S)		539.0	759.4 679.4 599.4	$[M + H]^+$ $[M - SO_3 + H]^+$ $[M - 2SO_3 + H]^+$	55
2S4S _{CS} (Δ UA,2S – GalNAc,4S)		539.0	759.4 679.4 599.4	$[M + H]^+$ $[M - SO_3 + H]^+$ $[M - 2SO_3 + H]^+$	55
4S6S _{CS} (Δ UA – GalNAc,4S,6S)		539.0	759.4 679.4 599.4	$[M + H]^+$ $[M - SO_3 + H]^+$ $[M - 2SO_3 + H]^+$	55
TriS _{CS} (Δ UA,2S – GalNAc,4S,6S)		619.0	839.6 759.4 679.4 599.5	$[M + H]^+$ $[M - SO_3 + H]^+$ $[M - 2SO_3 + H]^+$ $[M - 3SO_3 + H]^+$	55
Internal standard (Δ UA,2S – GlcNCOEt, 6S)		553.0	773.4 693.4 613.5	$[M + H]^+$ $[M - SO_3 + H]^+$ $[M - 2SO_3 + H]^+$	55

showed the greatest ionisation efficiency and consequently the lowest MDL ($0.005 \mu\text{g mL}^{-1}$). The tri-sulphated disaccharide (TriS_{CS}) showed the least ionisation efficiency and thus the highest MDL ($0.108 \mu\text{g mL}^{-1}$). A similar pattern of decreasing ionisation efficiency with increasing sulphation was previously observed for procainamide labelled-HS disaccharides³⁸ and is expected considering that these disaccharides were detected using ESI operated in the positive ionisation mode. As an LC injection volume of only $2 \mu\text{L}$ was used in the current study, the on-column sensitivity is in the pico – nano-molar range. A previous study using the same WAX HILIC column chemistry reported a higher method sensitivity.⁴³ However, their column was repacked in-house to achieve a smaller internal diameter ($250 \mu\text{m}$) allowing for microscale chromatography. Furthermore, a more sensitive QTOF MS was used which is more

expensive than the single quadrupole MS used in the current study. Nevertheless, our method allows for the detection of CS disaccharides from as little as $2.5 \mu\text{g}$ of starting material. All calculated MDLs had an observed signal to noise ratio of at least 3 : 1. The use of procainamide-labelled internal disaccharide standard (Δ UA,2S – GlcNCOEt, 6S) allowed for correction of ion suppression/enhancement which is a significant advantage of the SIR WAX HILIC UPLC-MS method developed in the current study. The intra-assay precision determined by the coefficient of variation (CV) at three different disaccharide concentrations were as follows: 1.59–8.62% at $0.078 \mu\text{g mL}^{-1}$, 1.07–2.98% at $1.25 \mu\text{g mL}^{-1}$ and 0.88–2.79% at $10 \mu\text{g mL}^{-1}$. Overall, the CV ranged from 0.88–8.62% which is well within acceptable ranges of <15%.

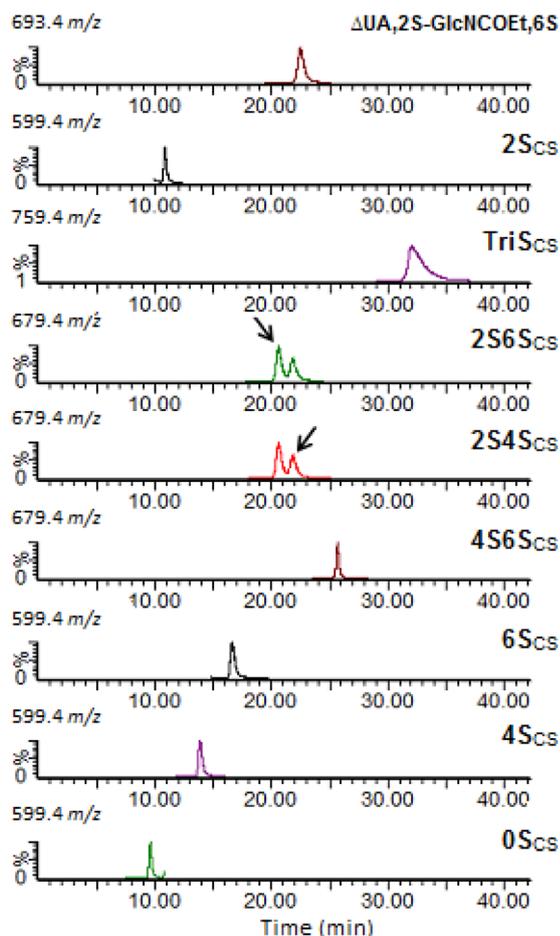


Fig. 3 SIR chromatogram of procainamide-labelled CS disaccharides and internal disaccharide standard ($20 \mu\text{g mL}^{-1}$ each) using optimum MS conditions. The monitored MS ion for each disaccharide is also shown.

CS disaccharide analysis of trophoblast-enriched placenta tissue

Prior to the disaccharide compositional analysis of CS extracted from trophoblast-enriched placenta tissue, the suitability of the developed and optimised HILIC-UPLC-MS method was first tested on commercially sourced bovine cartilage CS. This revealed a total of six CS constituent disaccharides: 0S_{CS} , 4S_{CS} , 6S_{CS} , $4\text{S}6\text{S}_{\text{CS}}$, $2\text{S}4\text{S}_{\text{CS}}$ and $2\text{S}6\text{S}_{\text{CS}}$ (Fig. 4).

The mono-sulphated 4S_{CS} ($\sim 50\%$), was the most abundant disaccharide followed by 6S_{CS} , ($\sim 40\%$) and then the non-sulphated disaccharide (0S_{CS} , $\sim 8.4\%$). The three di-sulphated disaccharides ($4\text{S}6\text{S}_{\text{CS}}$, $2\text{S}4\text{S}_{\text{CS}}$ and $2\text{S}6\text{S}_{\text{CS}}$) were each less than 1% (Fig. 4). The mono-sulphated 2S_{CS} and the tri-sulphated TriS_{CS} were not detected in commercially available bovine cartilage CS. These results are in broad agreement with previous studies including one which analysed a reference standard approved by the European Pharmacopoeia Commission. Review of these studies of enzymatically depolymerised bovine cartilage CS show that 2S_{CS} and TriS_{CS} were also not reported while the non-sulphated 0S_{CS} ranged from about 3–8%, the di-sulphated disaccharides from non-detection to 2%, and a $4\text{S}_{\text{CS}}/6\text{S}_{\text{CS}}$ ratio of 1.5–2.0.^{45–47} The $4\text{S}_{\text{CS}}/6\text{S}_{\text{CS}}$ ratio is about 1.3 in the current study. The similarities between the results obtained in the current study and those in published literature demonstrate that the developed HILIC-UPLC-MS method can be reliably used for CS disaccharide compositional analysis.

Interestingly, an additional LC-peak eluting close the non-sulphated CS disaccharide was observed in the chromatogram of bovine cartilage CS which was suspected to be the non-sulphated disaccharide of hyaluronic acid (0S_{HA}) (ESI Fig. S-4[†]). This was subsequently confirmed using chondroitinase ABC-treated HA standard. As chondroitinase ABC or AC II can also depolymerise HA,^{48,49} the ability of the developed HILIC-UPLC-MS method to differentiate between 0S_{CS} and 0S_{HA} is an important advantage of the method as HA is often present in biological samples. In addition, HA is a known contaminant of commercially available CS standard and CS-based pharmaceutical formulations.^{48,50}

To investigate whether CS sulphation is altered in pre-eclampsia, disaccharide compositional analysis of CS extracted from the placenta of women following term delivery from uncomplicated pregnancy prior to labour onset (Term non-labour; TNL), uncomplicated pregnancy at term after labour onset (Term labour; TL), pre-term delivery upon spontaneous pre-term labour (Pre-term labour; PTL) and pre-term delivery from women with pre-eclampsia (PE) were analysed. This revealed a total of six CS disaccharides (0S_{CS} , 4S_{CS} , 6S_{CS} , $4\text{S}6\text{S}_{\text{CS}}$, $2\text{S}4\text{S}_{\text{CS}}$ and $2\text{S}6\text{S}_{\text{CS}}$) in the placenta of all four clinical groups (Fig. 5a). The mono-sulphated 2S_{CS} and the tri-sulphated TriS_{CS} were also not detected in the placenta. Only one other study²² has characterised the disaccharide composition of placental CS

Table 2 Method parameters; linearity (r^2), method detection limit (MDL) and precision measured as coefficient of variation (% CV)

Disaccharide	r^2	Concentration range ($\mu\text{g mL}^{-1}$)	MDL ($\mu\text{g mL}^{-1}$) [nM]	%CV (10 $\mu\text{g mL}^{-1}$)	%CV (1.25 $\mu\text{g mL}^{-1}$)	%CV (0.078 $\mu\text{g mL}^{-1}$)
0S_{CS}	0.998	0.005–20	0.005 [13.2]	1.62	1.60	1.59
2S_{CS}	0.999	0.005–20	0.006 [13.1]	2.79	2.45	1.92
4S_{CS}	0.999	0.005–20	0.005 [10.9]	0.88	1.27	1.83
6S_{CS}	0.999	0.005–20	0.007 [15.3]	1.11	1.38	2.49
$2\text{S}6\text{S}_{\text{CS}}$	1	0.078–20	0.023 [42.7]	1.40	1.07	7.88
$2\text{S}4\text{S}_{\text{CS}}$	0.999	0.019–20	0.022 [40.8]	1.56	1.76	7.55
$4\text{S}6\text{S}_{\text{CS}}$	1	0.078–20	0.025 [46.4]	1.86	2.98	8.62
TriS_{CS}	1	0.156–20	0.108 [17.4]	1.49	2.30	—
$\Delta\text{UA},2\text{S} - \text{GlcNCOEt},6\text{S}$	1	0.019–20	0.007 [12.7]	1.30	2.57	2.46

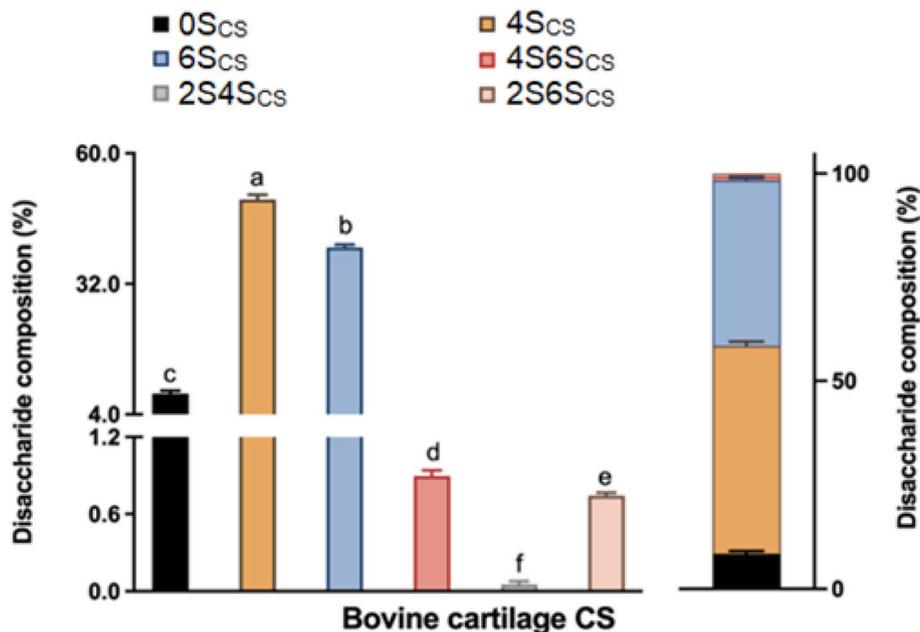


Fig. 4 Percentage disaccharide composition of bovine cartilage CS. Results are expressed as mean \pm standard deviation. Means that do not share a letter are significantly different (p values < 0.001 , ANOVA).

in pre-eclampsia. However, they detected only five and three CS disaccharides in the control and pre-eclampsia group respectively. The very small sample size ($n = 6$; three controls, three pre-eclampsia) and analytical method used in their study may have contributed to this. The di-sulphated CS disaccharides; 2S4SCS and 2S6SCS which could not be resolved from each other using their method and 4S6SCS were not detected in their study. Their lack of detection of these di-sulphated disaccharides may be due to method sensitivity as these disaccharides were each less than 1.5% of total CS disaccharides in the current study.

Overall, 6SCS was the most abundant disaccharide in the placenta in all clinical groups, followed by 0SCS and then by 4SCS (Fig. 5a). CSPGs account for more than 74% of total PGs in the placenta; 2 and 72% of cell-associated and fibrous tissue PGs respectively and their chains contain less than 10% and about 60% non-sulphated disaccharide respectively.⁷ In the current study, the non-sulphated 0SCS was about 35% of total CS disaccharides which likely reflects CS present both in the fibrous tissue and on cells of the placenta. This is similar to a previous study where placental cotyledon-CS comprised of about 30% 0SCS.²⁶ Compared to term labour, sulphated CS disaccharides; 4SCS, 2S4SCS and 2S6SCS but not 6SCS were decreased in pre-eclampsia but did not achieve independent statistical significance. Decreased mRNA expression of the enzymes responsible for the 4-O, 6-O and 2-O sulphation of CS; C4ST-1, C6ST and UA2ST respectively, has been previously reported in trophoblast-enriched pre-eclamptic placental tissues.²² However, extrapolation of this finding should be treated with caution as various isoforms of these enzymes are known except for UA2ST.

The use of three different clinical control groups (term non-labour, term labour and pre-term labour; PTL) allowed us to demonstrate the levels of CS disaccharides in pre-eclampsia independent of the effects of gestational age and labour which is a significant strength of the current study. Using a GLM analysis, decreased levels of 4S6SCS was independently associated with pre-eclampsia ($p = 0.003$), pre-term delivery ($p < 0.001$) and with labour ($p < 0.001$) (Fig. 5a). In addition, increased levels of other 4-O sulphated disaccharides were independently associated with labour; 4SCS ($p < 0.001$) and 2S4SCS ($p = 0.001$). Furthermore, decreased levels of the di-sulphated 2S6SCS were independently associated with pre-term delivery ($p = 0.018$) while decreased levels of the non-sulphated 0SCS was independently associated with labour ($p = 0.001$) (Fig. 5a). It is difficult to discuss the clinical significance of some of these results. However, highly sulphated CS comprising majorly 4S6SCS has been shown to bind growth factors such as HB-EGF, FGF-10 and FGF-2 (ref. 51) therefore, can mediate growth factor signalling. This disaccharide is decreased in pre-eclampsia in the current study but may play a limited role in placental growth factor binding as it comprises only about 1% of placental CS. When the disaccharides were grouped based on sulphation, increased levels of the mono- ($p = 0.002$), di- ($p = 0.001$) and total sulphated CS disaccharides ($p = 0.001$) were independently associated with labour (Fig. 5b). The consequent decrease in the levels of the non-sulphated CS disaccharide was also independently associated with labour ($p = 0.001$). This is an interesting finding which suggests that placental CS sulphation may be involved in preparing the placenta for labour. However, further studies are required to confirm this.

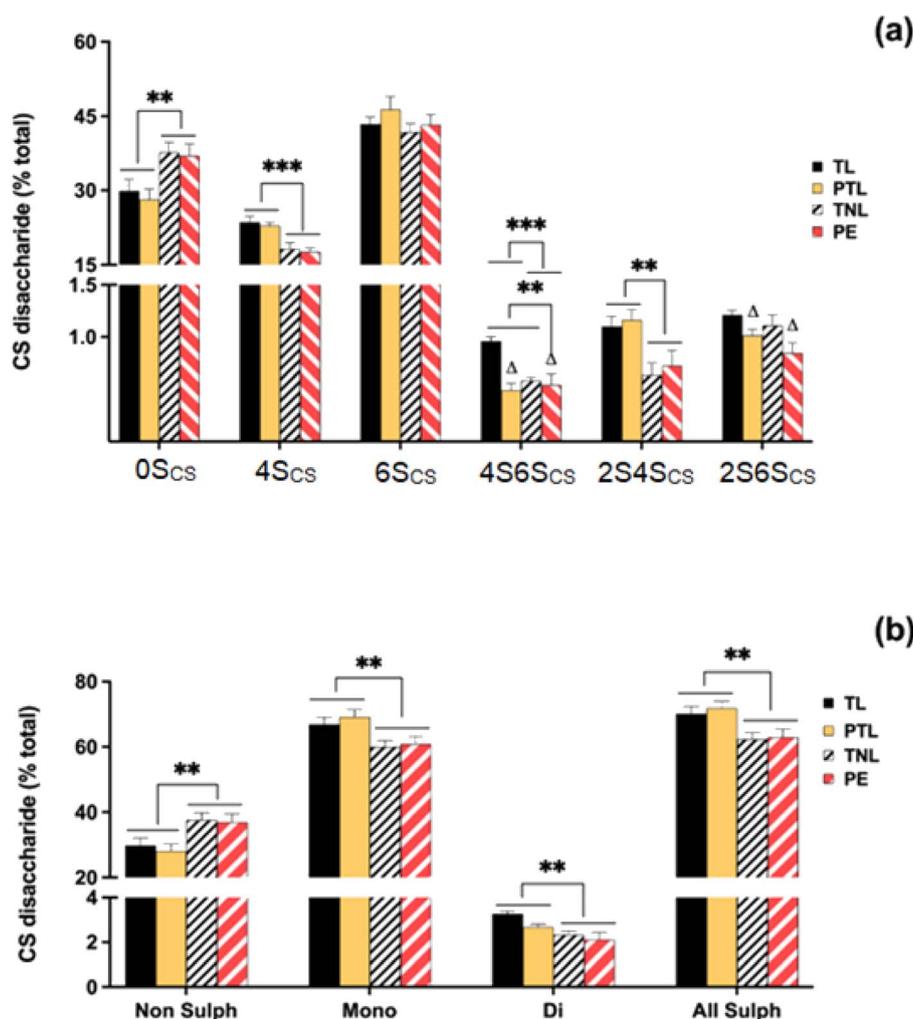


Fig. 5 The disaccharide composition of placenta tissue extracted-CS from different clinical groups (TL, PTL, TNL and PE) (a): the individual disaccharides, (b): disaccharides grouped based on sulphation. Results are reported as mean \pm SEM. ** = $p \leq 0.01$ and *** = $p \leq 0.001$ (GLM). Δ indicates disaccharides significantly decreased in association with pre-term delivery ($p < 0.05$, GLM).

Conclusion

In this study, we have developed a WAX HILIC-UPLC-MS method with procainamide labelling which allows for the separation and detection of all eight commercially available CS disaccharides. The method also allows hyaluronic acid non-sulphated disaccharide to be resolved from its isomer in CS thus enabling the detection of hyaluronic acid which is a common contaminant of CS samples. Furthermore, procainamide labelling enables fluorescent detection, therefore a mass spectrometry is not necessary for the implementation of this method. Using the developed method, disaccharide compositional analysis of bovine cartilage CS was performed with results similar to previous studies, confirming the reliability of the method. The method was then used to determine the disaccharide composition of placenta-extracted CS in pre-eclampsia with results showing that the disaccharide composition of CS in the placenta in pre-eclampsia and other pregnancy complications are different. The results also suggests that CS sulphation may be important in maternal labour.

Author contributions

Imeobong U Antia: investigation, formal analysis, conceptualisation, methodology, visualisation, writing – original draft and writing – review & editing. Frank A Hills: conceptualisation, sample collection, methodology, supervision, writing – review & editing. Ajit J Shah: conceptualisation, methodology, supervision, writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

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