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Ion-pairing reversed-phased chromatography/mass spectrometry of heparin

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Abstract—Heparin and heparin-derived components are widely applied anticoagulant drugs used for amongst other applications medical treatment of deep vein thrombosis and pulmonary embolism. Depolymerisation of native heparin results in complex mixtures of sulfated linear oligosaccharides that are usually not well characterised. In order to further characterise such mixtures, two on-line ion-pairing reverse-phased chromatography electrospray ionisation (ESI) mass spectrometry methods have been developed. One of the systems allows the determination of more than 200 components in a medium molecular weight heparin preparation, whereas the other system can be used to separate isomeric heparin oligosaccharides after previous separation according to size. This latter system allows semi-preparative isolation of isomeric heparin oligosaccharides. The experimental setup includes on-line cation exchange in order to prevent the ion-pairing reagent from entering the mass spectrometer.

Keywords: Heparin; Ion-pairing reversed-phase chromatography; Time-of-flight mass spectrometry; Electrospray ionisation; On-line cation exchange

1. Introduction

The glycosaminoglycan family of compounds includes linear sulfated polysaccharides such as heparin, heparan sulfate, dermatan sulfate, chondroitin sulfate and keratan sulfate. The various samples can be difficult to analyse structurally due to variations in chain lengths, sulfation pattern and stereochemistry. Heparin (found in mast cells) and low molecular weight heparin (LMWH) are widely applied anticoagulant drugs and consist of repeating units of (1→4)-linked glycosamine and hexuronic acid, either iduronic acid or glucuronic acid. These repeating units have varying degrees of sulfation and acetylation (a general structure is shown in Fig. 1). The most common disaccharide subunit contains three sulfate groups and no acetyl groups; making heparin the most highly charged known biomolecule (reviewed in Ref. 1).

The presence of the many sulfate groups in heparin oligosaccharides has made them difficult to analyse by mass spectrometry in the past. The sulfate groups are very labile and mild ionisation conditions are required to avoid fragmentations when analysing these molecules. The first successful mass spectrometric analyses of heparin oligosaccharides reported were performed by using fast atom bombardment ionisation or ^{252}Cf plasma desorption.[2–4] However, considerable sulfate loss was observed for both techniques, and a derivatisation based strategy was developed to circumvent this problem.[5,6] A matrix-assisted laser desorption/ionisation (MALDI) method was later developed that allowed

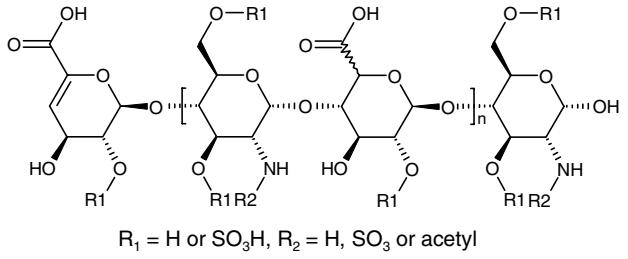

Figure 1. General structure of the most abundant components in preparations obtained by heparin lyase I digestion of heparin.

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analysis of heparin oligosaccharides with much less in-source fragmentation than previously observed.[7,8] This method was later used in conjunction with NMR spectroscopy to elucidate structures of purified heparin oligosaccharides with degrees of polymerisation (dp) up to 10.[9–11]

Electrospray ionisation (ESI) has been used to ionise heparin oligosaccharides and other glycosaminoglycans in the negative ion mode.[12–24] ESI mass spectrometry can be coupled on-line with different types of liquid chromatography. ESI MS combined with ion-pairing reversed-phase (IPRP) chromatography has previously been used to analyse heparin oligosaccharides.[13,14] Alkyl ammonium acetates were used as ion pairing reagents. However, the compatibility between the presence of alkyl ammonium ions in the eluate and achieving reasonable ionisation of the heparin oligosaccharides was critical. By keeping the concentration of the ion pairing reagent dibutyl ammonium acetate in the eluent as low as at 5 mM, Kuberan et al.[13] were able to separate six different heparan sulfate disaccharides. Thanawiroon et al.[14] were able to analyse a preparation of enzymatically polymerised heparin sample and obtained spectra of heparin oligosaccharides with a dp up to 14 by simultaneously spraying a second eluent solution into the ion source.

We have previously demonstrated that on-line cation exchange enables efficient ionisation of larger heparin oligosaccharides with a 30 mM NH_4HCO_3 buffer used as eluent in an on-line size-exclusion chromatography system.[24] In the present paper, two different ion-pairing reversed-phase mass spectrometry systems are presented in this paper, system I and system II. Both take advantage of on-line cation exchange to remove alkyl ammonium ions from the eluate. System I is suitable for analysis of complex mixtures of larger heparin oligosaccharides and polysaccharides, which is demonstrated for a preparation of medium molecular weight heparin where over 200 components are identified. The largest of the identified components have a dp of 30 and a molecular weight exceeding 8000. Previously, the largest heparin oligosaccharides to have their mass determined by mass spectrometric analysis had a dp of 18 and molecular weights around 5000.[24] System II is applicable for separation of isomeric heparin oligosaccharides, which is demonstrated for three different preparations of heparin oligosaccharides, predominantly containing tetrasaccharides, hexasaccharides and octasaccharides, respectively.

2. Experimental

2.1. Chemicals and samples

Medium molecular weight heparin (MMWH) was produced by enzymatic depolymerisation of heparin from pig mucosa. The heparin tetrasaccharide fraction, the heparin hexasaccharide fraction and the heparin octa/decasaccharide fraction were prepared by enzymatic depolymerisation of porcine heparin followed by gel-filtration on Sephadex G-25. All heparin containing samples were provided by LEO Pharma.

Pure water was obtained using a Milli-Q system from Millipore (Billerica, MA, USA). HPLC grade methanol and acetic acid were purchased from Merck (Damstadt, Germany). Polyethylene 600 diacid used for MS calibration in the negative ion mode and butyl amine were purchased from Aldrich (Steinheim, Germany). Tripropyl amine was purchased from Fluka (Steinheim, Germany).

2.2. Chromatography

The chromatographic systems consist of a Waters (Milford, MA, USA) 2795 HPLC system with a Waters XTerra MS C-18 column 5 μm (2.1 mm (ID) * 250 mm). The flow rate was 0.2 mL/min.

For both system I and system II, an eluent A containing 100% H_2O and an eluent B containing H_2O–MeOH (1:9) were used. In the case of system I eluent A and eluent B both contain 25 mM tripropyl amine and 30 mM acetic acid. The gradient system that was developed for analysis of the MMWH goes from 40% to 50% B in 15 min followed by 50–65% B in 50 min. For system II eluent A and eluent B contain 40 mM butyl amine and 40 mM acetic acid. Three different gradient systems were developed for the three different samples. The system for the heparin tetrasaccharide sample was isocratic at 21% B for 60 min. The gradient system for the heparin hexasaccharide sample goes from 29% to 37% B in 60 min while the gradient system for the heparin octasaccharide system goes from 35% to 45% B in 60 min. The samples were dissolved in 200 mM aqueous solutions of tripropyl ammonium acetate or butyl ammonium acetate prior to the injection into system I or system II, respectively.

A photo-diode array detector (Waters 996) recording at 231–233 nm is placed after the reverse phase column. The eluate was subjected to on-line cation-exchange before reaching the mass spectrometer using a Dionex (Sunnyvale, CA, USA) 2 mm ASRS-Ultra II Column run in the chemical suppression mode according to the manufacture's manual.

2.3. Mass spectrometry

All ESI mass spectra were acquired in the negative ion mode on a Micromass (Manchester, UK) LCT time-of-flight mass spectrometer. The spray conditions were optimised to achieve minimal fragmentation in the spray interface resulting in the following conditions: cone voltage 10 V, extraction cone voltage 1 V, capillary voltage

3000 V and source temperature 110 °C. Nitrogen was used as drying gas with a temperature of 350 °C and a flow rate of 600 L/h. The probe tip position was found critical and should be as far back and as far left as possible in the ion source.

3. Results and discussion

3.1. On-line cation exchange

The alkyl ammonium ions were removed from the eluate by on-line cation exchange using a Dionex ASRS Ultra II column. The ASRS Ultra II column contains two regenerant compartments and one eluent compartment separated by cation exchange membranes. Sulfuric acid (75 mM) is passed through the two regenerant chambers at a flow rate of 6–7 mL/min, while the eluate containing alkyl ammonium ions is passed through the column in the opposite direction. Hydronium ions from the regenerant chambers continuously migrate into the eluent chamber through the membrane. Simultaneously, alkyl ammonium ions from the eluent chamber migrate into the regenerant chambers. The net result is the exchange of alkyl ammonium with protons in the eluent, and as a consequence the eluate contains acetic acid instead of alkyl ammonium acetate buffer when it enters the ion source of the mass spectrometer.

The column can also be operated in 'Auto-Suppression' mode where water is passed through the regenerant chambers and the hydronium ions are produced by electrolysis. This mode of operating the ASRS column would usually be preferable for use in combination with MS since the use of sulfuric acid is avoided. It is, however, not applicable for systems such as the ones presented here where the content of organic solvent in the eluent exceeds 40%.

It should be noted here that a small amount of sulfuric acid gets transferred into the eluent and will enter the ion source as observed by the presence of ions such as $[2SO_4+3H]^-$ (m/z 195) and $[3SO_4+5H]^-$ (m/z 293) in the mass spectra. The small amount of sulfuric acid in the eluate causes black or dark brown coating in some areas in the ion source over time. This coating is, however, relatively easy to remove by washing with water.

3.2. Selecting the ion-pairing reagents

Four different ion-pairing reagents were tested: tributyl ammonium acetate, dibutyl ammonium acetate, butyl ammonium acetate and tripropyl ammonium acetate. Peaks corresponding to alkyl ammonium adducts were observed in the mass spectra when tributyl ammonium acetate or dibutyl ammonium acetate where used as ion-pairing reagents. The formation of this type of ions is undesirable, since they complicate interpretation of spectra of complex mixtures. Conversely, negligible amounts or no alkyl ammonium adducts were observed with the use of butyl ammonium acetate or tripropyl ammonium acetate and were hence used in all the following experiments.

When tripropyl ammonium acetate was used as the ion pairing reagent, the separation of the heparin oligosaccharides was found to be dominated by the number of sulfate groups. However, the retention time was also influenced by the number of *N*-acetyl groups, dp and saturation or nonsaturation of the hexuronic acid at the nonreducing end. In systems containing butyl ammonium acetate separation of isomers with same dp and degree of substitution was observed. Consequently, tripropyl ammonium acetate is preferred for analysis of complex mixtures of larger heparin chains where the isomer separation is not the goal (system I) whereas butyl ammonium acetate is advantageous when the goal is to separate different isomers in fractions of heparin oligosaccharides with limited dp range (system II).

3.3. On-line analysis of a complex MMWH mixture by IPRP LC/MS

The total ion current (TIC) chromatogram resulting from IPRP LC/MS of the MMWH preparation using system I is shown in Figure 2. Extracted ion traces for five components in the MMWH mixture disregarding the presence of isomers are shown in Figure 3a–e. The components are assigned by (X, Y, Z) for heparin chains containing unsaturated hexuronic acid at their nonreducing end, X being the number of saccharide units, Y the number of sulfate groups and Z the number of *N*-acetyl groups. Heparin chains that contain the original saturated hexuronic acid at their nonreducing end are assigned by HO-(X, Y, Z).

The selectivity of the system can be exemplified by comparing the elution of the component $(12, 17, 0)$ (Fig. 3a) with that of similar components (Fig. 3b–d). The retention time is reduced by 1 min if one of the sulfate groups is replaced with a *N*-acetyl group, whereas the addition of one sulfate group leads to an increase in retention time by 1.2 min. A saturated hexuronic acid unit on the non-reducing end instead of an unsaturated iduronic acid results in 0.5 min earlier elution. This

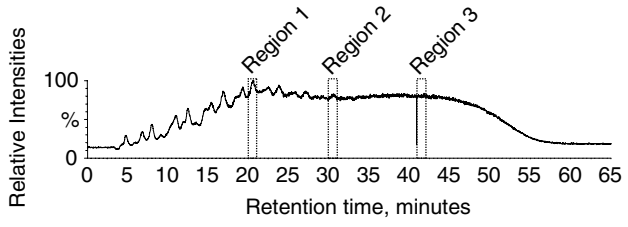

Figure 2. TIC chromatogram of a medium molecular weight heparin preparation separated using IPRP method I.

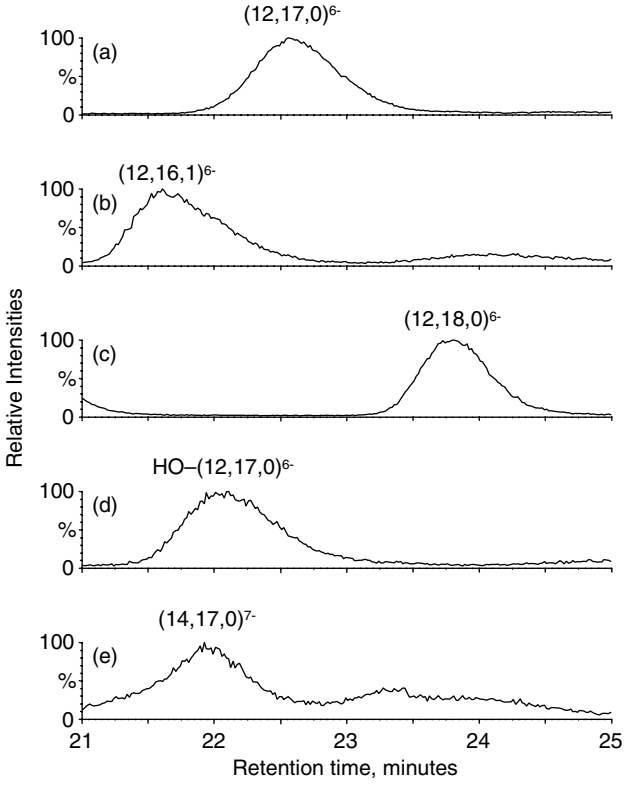

Figure 3. Extracted ion traces corresponding to different components in the MMWH mixture (same chromatographic run as shown in Fig. 2).

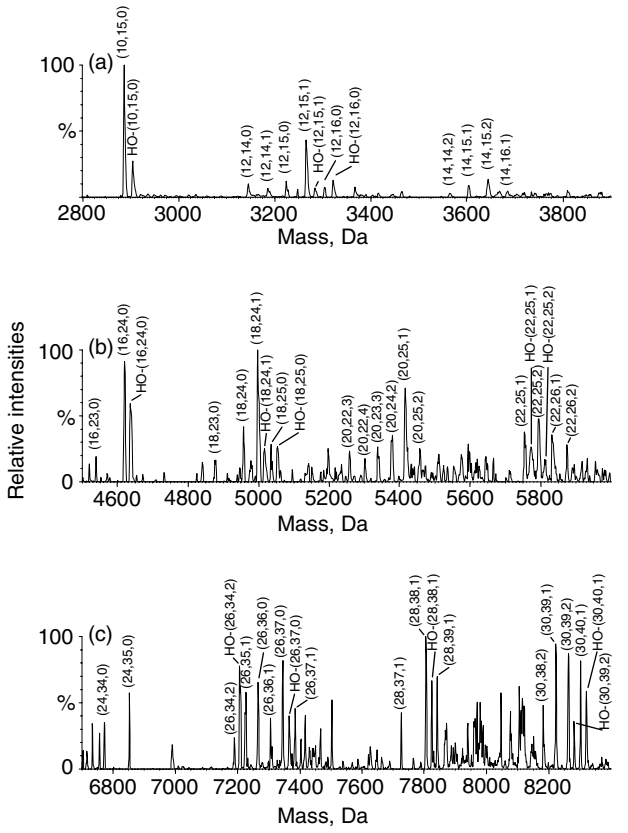

Figure 4. Deconvoluted spectra from the marked regions in the TIC chromatogram of MMWH shown in Figure 2.

might be explained by increased hydrophilicity due to the additional hydroxyl group. However, differences in conformation of the ring system might also influence the retention time. Increasing the dp by 2 decreases the retention time by 0.7 min.

The TIC chromatogram (Fig. 2) was divided in to regions and the spectra in each region summed and deconvoluted using the MaxEnt1 function of MassLynx. It was possible to identify more than 200 components from the peaks in the deconvoluted mass spectra, the largest with a dp of 30 and a molecular weight exceeding 8000. Three of the regions are shown in Figure 2 and the corresponding deconvoluted mass spectra in Figure 4a–c. An increase in dp without changing the substitution leads to a decrease in retention time. However, components with higher dp generally elute later because they carry more substituents. It was possible for each dp to identify components with a large range in a number of substituents. The least substituted identified component with dp 16 carries 17 substituents (~2 substituents pr. disaccharide unit) whereas the most substituted identified component with dp 16 carries 24 substituents (3 substituents pr. disaccharide unit). The two-dimensional approach combining IPRP LC and MS allows for the identification of components with similar molecular mass but different dp because they have different retention times. As an example the (16, 16, 2) and HO-(14, 21, 0) have similar molecular weights of 4063 and 4060, respectively, but elute at 21.4 and 26.8 min, respectively.

3.4. Separation of isomeric heparin oligosaccharides by IPRP guided by MS

High buffer concentration (40 mM butyl amine, 40 mM acetic acid) was chosen to make the system allow for a wide analyte concentration range. Maximal acceptable loads were 20, 60 and 100 μg for the samples containing tetrasaccharides, hexasaccharides and octasaccharides, respectively.

Figure 5a–c shows the separation using system II of a mixture of the heparin tetrasaccharide fraction from a heparinase lyase I digest. The UV chromatogram is shown in Figure 5a and the ion trace of m/z 357.9 corresponding to the (4, 5, 0) isomers and the ion trace of m/z 383.7 corresponding to the (4, 6, 0) isomers, in Figure 5b and c, respectively. The (4, 5, 0) component seems to be composed of two dominant isomers. The (4, 6, 0) component appears to be composed by one dominant isomer followed by a second appearing as a shoulder at higher retention time. Based on the knowledge of heparins,[1] it is very likely that the two isomers are

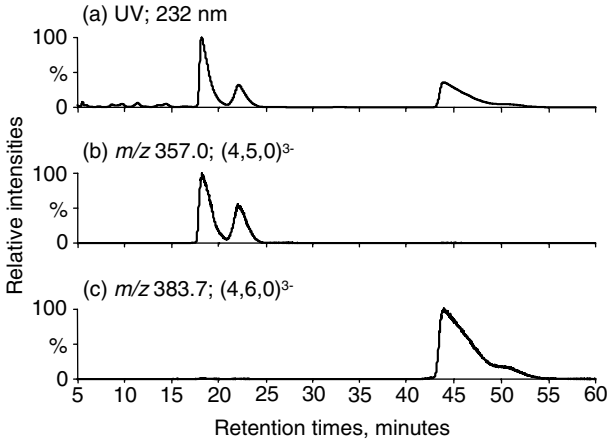

Figure 5. Separation of heparin tetrasaccharides using the IPRP LC/MS method II. (a) PDA chromatogram recorded at 231–233 nm. (b)–(c) Extracted ion traces showing the separation of isomeric tetrasaccharides.

epimers with the most abundant being dHexA(2S)-GlcN(6S,NS)-IduA(2S)-GlcN(6S,NS) whereas the later eluting (4,6,0) isomer is likely to be the epimer dHexA(2S)-GlcN(6S,NS)-GlcA(2S)-GlcN(6S,NS).

Based on the ion trace shown in Figure 6b, there seems to be two major isomers of (6,6,1) and in addition to these, at least seven minor isomers. There seems to be four major (6,7,1) isomers (see Fig. 6c). In addition to these four major components there seems to be at least one minor component eluting at 20 min. The dominating (6,8,0) isomer (Fig. 6d) elutes at 38.6 min. Based on the ion trace of (6,8,0) there seems to be at least six other isomers.

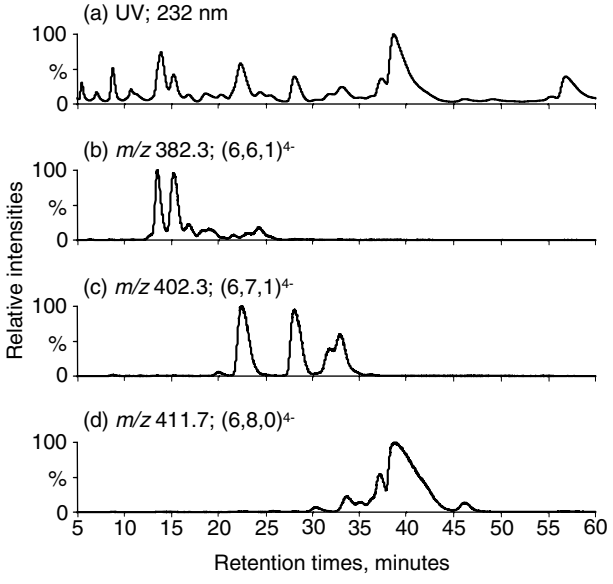

Figure 6. Separation of the heparin hexasaccharides using the IPRP LC/MS method II. (a) PDA chromatogram recorded at 231–233 nm. (b)–(d) Extracted ion traces showing the separation of isomeric hexaasaccharides.

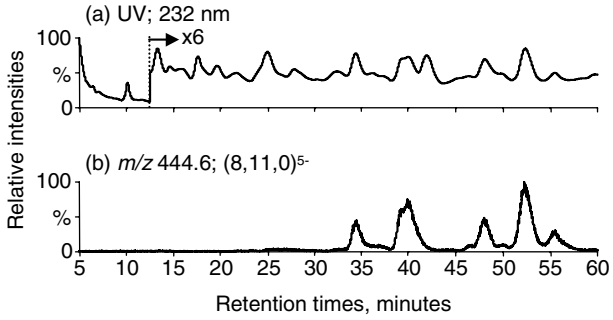

Figure 7. Separation of the heparin octasaccharides using the IPRP LC/MS method II. (a) PDA chromatogram recorded at 231–233 nm. (b) Extracted ion trace showing the separation of the (8,11,0) isomers.

The heparin octasaccharide mixture contains many different components (Fig. 7a). In the ion trace shown in Figure 7b corresponding to the (8,11,0) isomers, the complexity is reduced and there appears to be five major components eluting at 34.4, 39.2, 39.9, 48.0 and 52.2 min, respectively. In addition, there seems to be at least five minor (8,11,0) isomers.

The quality of the various fractions from the above experiments was examined by reinjecting them into the chromatographic system I. It was observed that the fractions decompose or isomerise to unknown products when lyophilised in the presence of butyl ammonium salts. Therefore, the content of butyl ammonium salts was removed from the samples by cation exchange prior to lyophilisation. The different fractions were also attempted and characterised by tandem mass spectrometry, but unfortunately conclusive distinction between different isomers with respect to the position of the sulfate groups was not successful.

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