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Qualitative and quantitative analysis of heparin and low molecular weight heparins using size exclusion chromatography with multiple angle laser scattering/refractive index and inductively coupled plasma/mass spectrometry detectors

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ABSTRACT

Heparin, a highly sulfated glycosaminoglycan, has been used as a clinical anticoagulant over 80 years. Low molecular weight heparins (LMWHs), heparins partially depolymerized using different processes, are widely used as clinical anticoagulants. Qualitative molecular weight (MW) and quantitative mass content analysis are two important factors that contribute to LMWH quality control. Size exclusion chromatography (SEC), relying on multiple angle laser scattering (MALS)/refractive index (RI) detectors, has been developed for accurate analysis of heparin MW in the absence of standards. However, the cations, which ion-pair with the anionic polysaccharide chains of heparin and LMWHs, had not been considered in previous reports. In this study, SEC with MALS/RI and inductively coupled plasma/mass spectrometry detectors were used in a comprehensive analytical approach taking both anionic polysaccharide and ion-paired cations heparin products. This approach was also applied to quantitative analysis of heparin and LMWHs. Full profiles of MWs and mass recoveries for three commercial heparin/LMWH products, heparin sodium, enoxaparin sodium and nadroparin calcium, were obtained and all showed higher MWs than previously reported. This important improvement more precisely characterized the MW properties of heparin/LMWHs and potentially many other anionic polysaccharides.

1. Introduction

Heparin, a highly sulfated glycosaminoglycan, has been widely used as a clinical anticoagulant since the 1930's [1]. It is one of the few polysaccharide drugs, and one of the most structurally complex drugs in use today [2]. Heparin is composed of the major repeating disaccharide unit, 2-*O*-sulfo-α-L-iduronic acid (IdoA2S) 1 → 4-linked to 6-*O*-sulfo, *N*-sulfo-α-D-glucosamine (GlcNS6S), as well as variable disaccharide units comprised of a β-D-glucuronic acid (GlcA), α-L-iduronic acid (IdoA) or IdoA2S residue 1 → 4-linked to 6-*O*-sulfo and/or 3-*O*-sulfo and/or *N*-sulfo (GlcNS) or *N*-acetylated (GlcNAc) α-D-glucosamine residues [3]. Rare pentasaccharide sequences comprise the antithrombin III (AT)-binding site, including GlcNAc/NS6S (1–4) GlcA (1–4) GlcNS3S, 6S (1–4) IdoA2S (1–4) GlcNS6S, and are important for heparin's anticoagulant activity [4]. Heparin has polydisperse molecular weight (MW) and complex structural composition [5]. Different MW of heparin chains can lead to different activities [6]. Relatively short heparin chains retain some of their anticoagulant activity, through their AT-binding site, but are often too short to retain their full anticoagulant activity associated with heparin's bleeding side effects by forming heparin-AT-thrombin ternary complexes [3]. This property promoted the development and wide spread clinical application of low molecular weight heparins (LMWHs) [7]. Different strategies have been applied to produce LMWHs from heparin, including enzymatic and chemical β-elimination and oxidative degradation using nitrous acid [8]. The MW properties of LMWHs corresponding these various processes differ [7]. Thus, accurate determination of MW properties is essential for controlling their quality, for their

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identification and structural characterization and for purity analysis [9].

Size-exclusion chromatography (SEC) using a refractive index (RI) detector has been widely used to determine the MW of heparins and LMWHs [10]. A series of standards of known MWs are required to establish the relationship between MW and retention time determined by GPC-RI [10,11]. A relative MW value can be obtained through calculating the proportion of sample with shorter or longer retention times compared to series of MW standards using extrapolation or interpolation. However, most common MW standards show different performance on different columns and the retention times of heparin or LMWHs vary [9,12]. Commonly used MW standards, such as globular proteins or neutral linear polysaccharides (dextran), have different conformations in solution and display different column interactions than heparin and LMWHs, leading to inaccurate results [13]. The preparation of verified MW markers using heparin fractions, having similar structural and chromatographic properties as heparin and LMWH, can be used to more accurately calibrate columns, but these are difficult to obtain and can be expensive [14].

SEC utilizing multiple angle laser scattering (MALS) and RI detectors has been successfully used to accurately analyze the weight average MW of heparin and other natural polysaccharides without the requirement of MW calibration standards [9,13–15]. In MALS increasing light-scattering intensity is proportional to the molecular weight when the analyte is in a diluted solution or is properly eluted from an SEC column [16]. The difference in light-scattering intensity between the chromatographic analyte and the background is the most important factor for accurately calculating the MW of heparin/LMWHs [15].

SEC-MALS-RI can also be used as a simple, rapid and accurate method, to quantitatively analyze polymers including natural polysaccharides extracted from plants and heparins [9,15–17]. The difference in refractive index between chromatographic analyte and background is the most important factor for accurately calculating the mass content of polysacchairdes [15,16].

The salt concentration of the mobile phase in SEC is isocratic and affords a uniform concentration of eluting cations. The concentration of cations surrounding the analyte, the anionic polysaccharide chains of heparin/LMWHs, is identical to the chromatographic background [13]. Thus, the contributions of the original cations, surrounding these anionic polysaccharides and increasing their light-scattering intensity, are neglected. Thus, only polysaccharide portion is considered when using current methods to analyze the MW and mass recovery of heparin and LMWH analytes making these values inaccurate.

In this study, the SEC performance of heparin/LMWH with its ion-paired cations was investigated in various mobile phases. A universal SEC method was developed to accurately analyze both the heparin/LMWH polysaccharide and ion-pairing cation component using MALS/RI and inductively coupled plasma coupled-mass spectrometry (ICP-MS) detectors. Three commercial heparin products, heparin sodium, the LMWH enoxaparin sodium (widely used in US), and the LMWH nadroparin calcium (widely used in Europe), were analyzed using SEC-MALS/RI and SEC-ICP/MS, to provide a full profile of both polysaccharide and ion-pairing cation. This approach improves the accuracy of the MW determination.

2. Materials and experiments

2.1. Materials

Two heparin sodium samples were obtained from Celsus Laboratories, Inc. (USP grade, Cincinnati, Ohio) and Sigma Chemical (from porcine intestine, St. Louis, MO). Enoxaparin sodium and nadroparin calcium standards were obtained from the USP (Rockville, MD) and EP (Strasbourg, France), respectively. Cation exchange resin was purchased from Sigma Chemical (St. Louis, MO), (Dowex R 50WX4 hydrogen form 50–100 mesh). Cation standards solution was purchased from o2si smart solutions (Charleston, SC, Matrix: H_2O; Ca^{2+}, 1000 ppm; Na^+, 200 ppm, NH_4^+, 400 ppm; Mg^{2+}, 200 ppm; K^+, 200 ppm; Li^+, 50 ppm). Other chemical reagents were all LC–MS grade.

2.2. Preparation of H^+ form heparin

Heparin sodium (Celsus, USP grade ~10 mg) was dissolved into ~1 mL water and passed through a 5 mL cartridge column packed with ~4 mL Dowex H^+ exchange resin. The eluate was collected and lyophilized. The dried sample (~5 mg, white powder) was weighed out accurately and dissolved into a proper volume of water to afford to a stack solution with concentration of 10.0 mg/mL just before analysis.

2.3. Preparation of analyte solutions

Heparin sodium, enoxaparin sodium, nadroparin calcium standards (three batches, respectively) and heparin sodium from Sigma were dried by vacuum drying oven to a constant weight. Each dried sample (~5 mg) was weighed out accurately and dissolved into a proper volume of water to afford to a stock solution with concentration of 10.0 mg/mL. The stock solutions were diluted to 0.02 mg/mL with water and ready for SEC-ICP-MS analysis. The stock solution of each sample was also diluted to 0.2, 0.4, 0.8, 1.2, 1.6 and 2.0 mg/mL with water, 50 mM ammonium acetate (NH_4OAc) and 50 mM sodium sulfate (Na_2SO_4) for detection of the individual dn/dc value.

Cation standard solution was diluted to 0.1 ppm, 0.5 ppm, 1.0 ppm, 5.0 ppm and 10.0 ppm (Ca and Na, respectively) for cation quantitative analysis with SEC-ICP-MS.

2.4. SEC-MALS-RI and SEC-ICP-MS

All SEC was performed on an Agilent 1260 system equipped with dual pumps (Agilent, Santa Clara, CA). The separation was achieved on a size exclusion column (Waters, Millford, MA, SEC BEH 200 Å, 1.7 μm, 4.6 × 150 mm) at 0.1 mL/min with an isocratic mobile phase. Different mobile phases were tried in this work, such as water, 50 mM NH_4OAc and 50 mM Na_2SO_4. The column temperature was set at 30 °C.

The SEC systems eluted with different mobile phases were coupled with a MALS (DAWN HELEOS II, Wyatt Technol., Goleta, CA) and a RI (Wyatt, Optilab T-rEX) detector to analyze MW and mass content of polysaccharide. The MALS instrument was equipped with a He-Ne laser (λ = 662 nm) and calibration constant was determined as 3.0047×10^{-5} RIU/pixel. The Wyatt ASTRA 6.1 software was utilized for data acquisition and analysis. The dn/dc value of each sample was measured using the RI detector at the same wavelength (658 nm). Six different concentrations were infused by syringe pump and the refractive index value measured at each concentration to achieve the dn/dc. The dn/dc values obtained with water, 50 mM NH_4OAc and 50 mM Na_2SO_4 were applied to the experiments performed with corresponding mobile phases. The injection volume is 20 μL of each sample (10 mg/mL). Each experiment was done in triplicate.

The SEC system eluted with 50 mM NH_4OAc was coupled with ICP-MS (Agilent 7900) to analyze cations in heparin and LHWHs qualitatively and quantitatively. The operating parameters of ICP-MS instrument were as follows: RF power, 1550 W; RF matching, 1.80 V; S/C temperature, 2 °C;Sample depth, 8.0 mm; Carrier gas flow rate, 0.95 L/min; Makeup gas, 0.2 L/min; Nebulizer pump flow

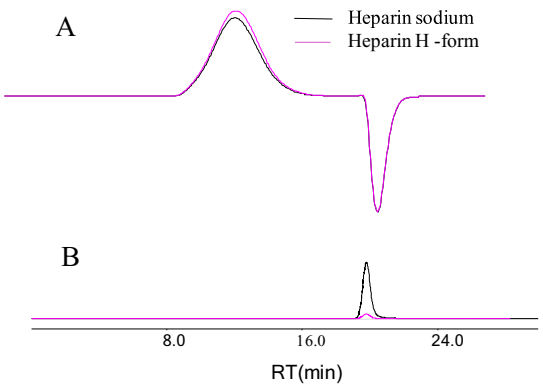

Fig. 1. Sized exclusion chromatograms of heparin sodium and heparin H with RI and ICP-MS (^{23}Na EIC) detection.
A, Sized exclusion chromatograms of heparin sodium and heparin H with RI detection; B, ^{23}Na extracted ion chromatograms (EIC) of heparin sodium and heparin H.

rate, 0.1 rps; Tuning solvent: 7 Li, 89 Y, 205 Tl. Analytes monitored were ^{23}Na, ^{44}Ca and integration time were 0.3 s, 0.6 s, respectively. The software, MassHunter workstation, was utilized for data acquisition and analysis. The injection volume is 20 μL of each sample (0.02 mg/mL). Each experiment was done in triplicate.

3. Results and discussion

3.1. Optimization of mobile phase in SEC method

Water cannot be used as mobile phase of SEC as analytes, such as heparin adsorb to the stationary phase through hydrogen bonding [18]. Non-volatile salt solutions, such as Na_2SO_4 solution, have typically been used as mobile phase in previous reports [17]. However, the contribution of original cations attached to heparin/LMWHs could be hidden by the salt in the element solution when they are analyzed with MALS/RI, as this measurement relies on the difference between signal intensity of analyte and background signal in each chromatogram. Using SEC-MALS/RI method with mobile phase of 50 mM Na_2SO_4, the mass recovery of heparin sodium was measured as ~90%, but that of heparin H^+ was measured as ~100%, confirming the missing ion-paired cation component of heparin sodium using this method (Table S1). Here, the mass recovery is defined as the ratio of the measured amount with this method to accurate mass in the solution. NH_4OAc has been used as salt in mobile phase of SEC recently [9,13–15]. Though the mass recovery of heparin sodium was also measured as ~90% (Table S1) when 50 mM NH_4OAc was used as mobile phase, the cation component, Na^+, could be measured with following ICP-MS separately, as NH_4OAc is compatible with MS analysis. Thus, the 50 mM NH_4OAc are selected as proper mobile phase of SEC, which could be coupled with MALS/RI and ICP-MS to measure polysaccharide and cation portions of heparin/LMWHs, respectively (Fig. S1 and Fig. 1)

Table 1
The reflex index increments (dn/dc) in 50 mM NH_4OAc.

Samples	dn/dc(mL/g)
Heparin sodium	0.1311 ± 0.0050
Enoxaparin sodium	0.1341 ± 0.0013
Nadroparin calcium	0.1382 ± 0.0034

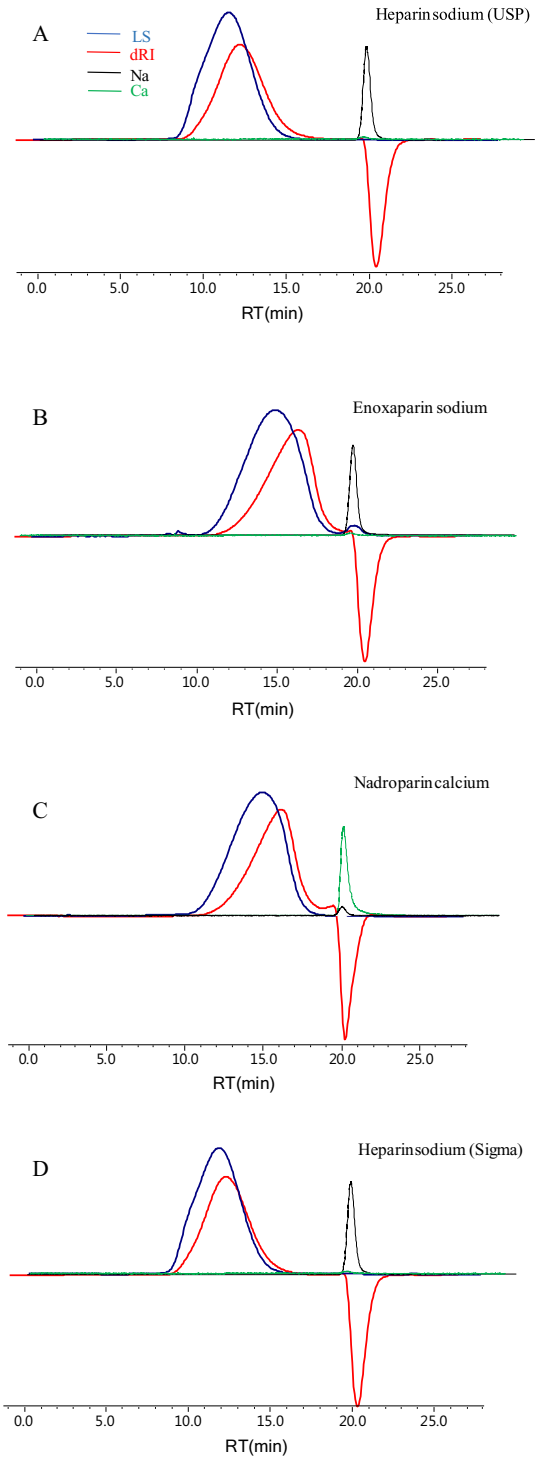

Fig. 2. Sized exclusion chromatograms of heparin sodium (USP), enoxaparin sodium nadroparin calcium and heparin sodium (Sigma) with RI, MALS and ICP-MS (^{23}Na and ^{44}Ca EIC) detection.

3.2. Selection of columns in SEC method

Different brands of SEC columns were also used to analyze heparin sodium (USP grade), such as Superdex, TSK (GE Healthcare, Pittsburgh, PA, Supporting information Fig. S1) and Waters BEH. Heparin has the best chromatographic performance on a Waters BEH column (Fig. 1). The black and red traces show the RI signals of heparin sodium and H^+ forms of heparin, respectively (Fig. 1A). The broad peaks observed at ~12 min in the chromatogram correspond to polysaccharide portion of heparin. The retention times, peak distributions and peak shapes of heparin sodium and heparin H^+ were identical. The peak height of heparin H^+ was slightly higher than that of heparin sodium, suggesting the concentration of this portion corresponding to the broad peak of heparin H was higher than that of heparin sodium. Using the same SEC conditions ICP-MS was next used for detection to analyze these two samples. The black and red lines show the Na^+ (M = 23) extracted ion chromatogram (EIC) of heparin sodium and heparin H^+, respectively (Fig. 1B). Relatively sharp peaks were observed at ~20 min, but no Na^+ signal was observed at ~12 min, where heparin polysaccharide portion eluted. The peak height of heparin H^+ in Fig. 1B was much lower than that of heparin sodium, confirming the most of the Na^+ had been removed using cation exchange. The separation polysaccharide and sodium with SEC suggests that the Na^+ originally ion-paired to heparin is exchanged by the NH_4^+ in mobile phase forming NaOAc which elutes later as a salt from the SEC column.

This same separation of polysaccharide and sodium was observed with other brands of SEC columns (Supporting information Fig. S1).

3.3. Analysis of polysaccharide portions of heparin and LMWHs with SEC-MALS-RI

The MW of heparin and LMWHs were analyzed with SEC-MALS-RI. Polysaccharides of different structures, sizes and shapes exhibit different RI increment (dn/dc) values. These values are important for the MALS detector to accurately analyze each specific polysaccharide. The dn/dc values of heparin sodium, enoxaparin sodium and nadroparin calcium were obtained with the correlation between the series of concentration of each sample and their intensities of signals from RI detector (Table 1). The dn/dc values of two heparin sodium from different venders are same.

The chromatograms in Fig. 2 show blue, red, black and green traces corresponding to laser, RI signals, ^{23} Na and ^{44} Ca EIC, respectively. The retention times observed with laser signal are always shorter than that with RI signal, and the retention times observed from RI signal are more reliable [15]. Based on their retention times, heparin sodium has highest MW and the two LMWHs have lower, but similar MWs. The MWs of heparin sodium (USP grade), heparin H^+, enoxaparin sodium, and nadroparin calcium detected using their individual dn/dc values of 15.9, 15.6, 4.3, and 4.5 kDa, respectively (Table 2), and are consistent with those provided in the pharmacopeia [9,19–21]. The MW of heparin sodium from Sigma was detected as 16.0 kDa, consistent with that of USP grade heparin sodium. The MWs of heparin Na and heparin H^+ measured with the mobile phase of NH_4OAc or Na_2SO_4 are the same (Table S1 and Table 2), suggesting the only polysaccharide component is being measured and that the Na^+ associated heparin is not considered when the MW of heparin is directly analyzed with SEC-MALS-RI.

Heparin and LMWH concentrations can be calculated based on the refractive index difference based on the equation [15] $C_i = \alpha(V_i - V_{i,baseline})/(dn/dc)$, where C_i is the detected concentration of heparins or LMWHs, α is the RI calibration constant, and V_i and $V_{i,baseline}$ are the RI voltages of sample and baseline, respectively. Here, a unique dn/dc value is important to determine accurate concentrations of particular sample. Mass recovery can be obtained from C_i/C_w, where C_w is the weighed concentration. The mass recoveries of heparin sodium (USP grade), heparin H^+, enoxaparin sodium, nadroparin calcium and heparin sodium (Sigma) were 90.2, 98.5, 91.5, 91.3% and 89.8%, respectively (Table 2). The low cation content results in the high mass recovery for heparin H^+.

3.4. Quantitative analysis of cations in heparin and LMWHs with SEC-ICP-MS

ICP-MS has been widely used for qualitative and quantitative metal analysis [22,23]. Cations in four samples, heparin sodium (USP grade), enoxaparin sodium, nadroparin calcium and heparin sodium (Sigma), were identified and quantified using ICP-MS detection of the SEC separation. A series of concentrations (0.1 ppm, 0.5 ppm, 1.0 ppm, 5.0 ppm and 10.0 ppm) of Na^+ and Ca^{2+} standard solutions were eluted with the same SEC conditions. The ^{23}Na and ^{44}Ca signals were recorded through corresponding channels in ICP-MS and their EICs are shown in Fig. 3. The peak areas of standard Na^+ and Ca^{2+} in their EICs are plotted as functions of their concentrations. Both show good linearity over a wide range of concentrations (0.1–10 ppm, inserts of Fig. 3) and their equations and correlation coefficients are provided in Fig. 3. The black and green traces in the chromatograms of heparin sodium (USP grade), enoxaparin sodium, nadroparin calcium and heparin sodium (Sigma) (Fig. 2) are MS signals of ^{23}Na and ^{44}Ca, respectively. The calculated contents of Na^+ and Ca^{2+} in these five samples including heparin H^+ are provided in Table 2. Na^+ was observed in heparin sodium, heparin H^+, enoxaparin sodium, nadroparin calcium and heparin sodium (Sigma) at 10.69, 0.87, 10.56, 0.66% and 11.24%, respectively. Ca^{2+} was only observed in nadroparin calcium at 10.01%. The sodium contents of heparin sodium and enoxaparin sodium was suggested as 9.5 ~ 12.5% and 11.3 ~ 12.5% with absorption spectrophotometry (AAS) in USP and EP, [19,20] which is similar to the results

Table 2
Measured and calculated MWs and mass recoveries (n = 3).

Samples	MW_p (kDa)	Mass recovery%$_p$	Content%$_c$ (Na/Ca)	MW_r (kDa)	Mass recovery%$_g$	Reported MW (kDa)
Heparin sodium (USP)	15.9 ± 5.0%	90.2 ± 3.0%	10.60 ± 1.1%/n.d.	17.8 ± 4.9%	100.8 ± 2.8%	15.9 [9]
Enoxaparin sodium	4.3 ± 2.3%	90.5 ± 1.9%	10.56 ± 1.5%/n.d.	4.8 ± 2.1%	101.1 ± 1.8%	4.5 [19,20]
Nadroparin calcium	4.5 ± 3.2%	90.3 ±4.6%	0.66 ± 0.8%/10.01 ± 0.6%	5.0 ± 3.2%	101.0 ± 4.2%	4.3 [21]
Heparin H	15.6 ± 2.1%	98.5 ± 3.1%	0.87 ± 0.5%/n.d.	15.7 ± 1.7%	99.4 ± 3.1%	–
Heparin sodium (Sigma)	16.0 ± 2.7%	89.8 ± 4.1%	11.24 ± 0.7%/n.d.	18.0 ± 2.6%	101.0 ± 3.7%	–

MW_p, Weight average molecular weight of polysaccharides portion measured with SEC-MALS-RI.
MW_r,Real weight average molecular weight considered both polysaccharides and cation portions.
Mass recovery%$_p$, Mass recovery of polysaccharides portion measured with SEC-MALS-RI.
Mass recovery%$_g$, Gross mass recovery considered both polysaccharides and cation portions.
Content%$_c$, Cation content measured with SEC-ICP-MS.
$MW_r = MW_p/(1 - \text{content}\%_c)$.
Mass recovery%$_g$ = Mass recovery%$_p$ + content%$_c$.

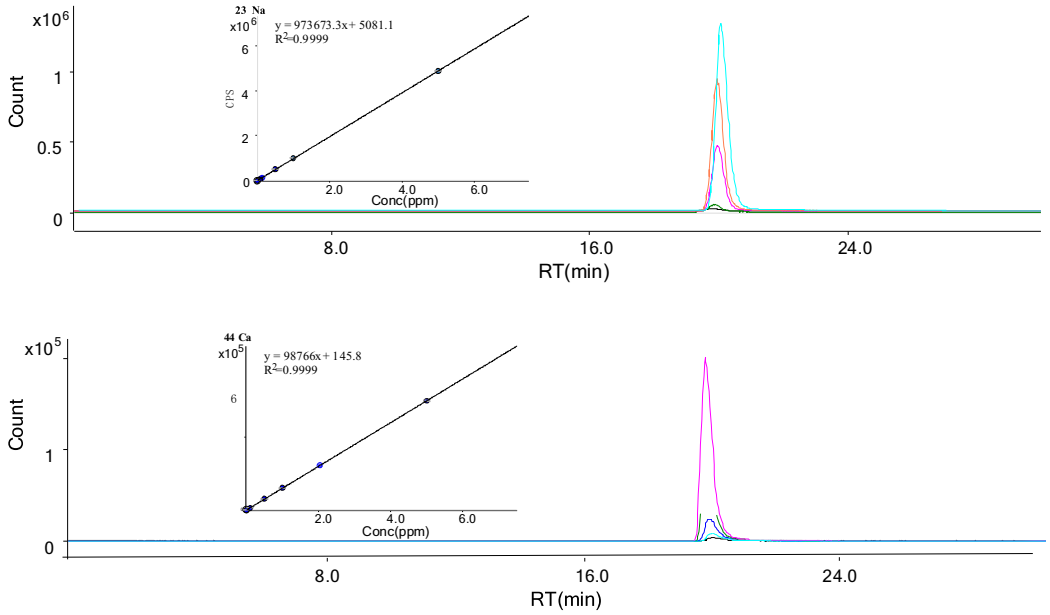

Fig. 3. Quantitation of cations with SEC-ICP-MS.
A, ^{23}Na EICs with Na^+ standard solutions with concentrations 0.1 ppm, 0.5 ppm, 1.0 ppm, 5.0 ppm and 10.0 ppm, and standard curve; B, ^{44}Ca EICs with Ca^{2+} standard solutions with concentrations 0.1 ppm, 0.5 ppm, 1.0 ppm, 5.0 ppm and 10.0 ppm, and standard curve.

listed in our work with SEC-ICP-MS. The small amount of sodium and the major cation, calcium, in nadroprin was also quantitated with this method. Based on the average number of sulfo groups per disaccharide units of heparin sodium, enoxaparin sodium and nadroparin calcium reported previously [19–21,24], their theoretical contents of cations were calculated as ~12%, ~11% and ~10%, which are matched by our results.

3.5. Analysis of accurate weight average MW and mass contents of heparin and LMWHs

The accurate weight average MW of heparin and LMWHs should consider both anionic polysaccharide and cation components. The final MWs of samples considering both of these components were calculated using the equation $MW_r = MW_p/(1 - \text{content}\%_c)$, where MW_r is 'real' molecular weight consisting of both polysaccharide and cation components, MW_p is the MW of polysaccharide measured using SEC-MALS-RI, and content%$_c$ is the content of cation measured using SEC-ICP-MS. The calculated MW of heparin sodium (USP grade), heparin H^+, enoxaparin sodium, nadroparin calcium and heparin sodium (Sigma) were 17.8, 15.7, 4.8, 5.0 and 18.0 kDa, respectively (Table 2). These MW_r values are all higher than the previously reported MW_p values [9,19–21]. The calculated MW of heparin H^+ did not significantly change from the measured data as little sodium remained in the sample after the cation exchange step used in its preparation.

Mass recovery also needs to consider both the polysaccharide and cation components of a heparin. The gross mass recovery was calculated using the equation Mass recovery%$_g$ = Mass recovery%$_p$ + content%$_c$, where Mass recovery%$_g$ is gross mass recovery, and Mass recovery%$_p$ is the mass recovery of polysaccharide portion measured with SEC-MALS-RI. The gross mass recovery of heparin sodium, heparin H^+, enoxaparin sodium, nadroparin calcium and heparin sodium (Sigma) were 100.8, 99.4, 1, 101.0% and 101.0%, respectively (Table 2). Thus, the mass recovery of anionic polysaccharides can be accurately measured with this strategy considering both polysaccharide and cation components.

4. Conclusion

Heparin and LMWHs are highly negative-charged anionic polysaccharides. The sulfo groups and carboxyl groups in the polysaccharide chains are ion-paired to cations. The level of occupancy depends on the pH of the sample and at neutral pH values the sulfo groups are completely occupied with cations. The cation present in most heparin products is Na^+ but Ca^{2+} or other metal can often be found in some heparin products [24]. SEC-MALS-RI methods have been widely used to measure the MW and mass content of polysaccharides such as heparin [9,15,25]. However, the current study demonstrates that this method only considers polysaccharide component of a heparin product and ignores the cation component. A SEC method using volatile salt 50 mM NH_4OAc was applied in this work. It is suitable for MALS-RI detection, which analyzes only polysaccharide component and it is also compatible with ICP-MS detection, which qualitatively and quantitatively analyzes the cation component. Both anionic polysaccharide and cation components need be considered to accurately determine the MW and mass recovery of heparin and LMWHs using SEC. The use of SEC-MALS-RI together with SEC-ICP-MS provides a comprehensive strategy for the accurate determination of MW and mass recovery of heparin and LMWH products and should be an important improvement or supplement to previously reported SEC related methods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2017.09.040.

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