Journal of Pharmaceutical and Biomedical Analysis 154 (2018) 332–338

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Nuclear magnetic resonance spectroscopy as a tool for the quantitative analysis of water and ions in pharmaceuticals: Example of heparin

Yulia B. Monakhova [a,b,c,*], Bernd W.K. Diehl [a]

[a] Spectral Service AG, Emil-Hoffmann-Straße 33, 50996 Köln, Germany
[b] Institute of Chemistry, Saratov State University, Astrakhanskaya Street 83, 410012 Saratov, Russia
[c] Institute of Chemistry, Saint Petersburg State University, 13 B Universitetskaya Emb., St Petersburg 199034, Russia

ARTICLE INFO

Article history:
Received 19 January 2018
Received in revised form 12 March 2018
Accepted 14 March 2018
Available online 15 March 2018

Keywords:
NMR spectroscopy
Heparin
Water content
Inorganic ions

ABSTRACT

Heparin is a linear, highly sulfated glycosaminoglycan (GAG), which consists of repeating disaccharide units of β-D-glucuronic acid or α-L-iduronic acid and α-D-glucosamine. While robust analytical approaches exist for the characterization of organic composition of heparin preparations, there is a lack of methods for the simultaneous quantification of inorganic compounds (water, anions, cations) in this matrix.

A nuclear magnetic resonance (NMR) spectrometric method for heparin characterization described in US Pharmacopeia was extended to simultaneous analysis of the inorganic ions (sodium, calcium, and chloride), acetate as well as water content. NMR control of these parameters is possible with only one sample preparation according to the US Pharmacopeia using just four sequential NMR experiments (^{1}H, ^{2}D, ^{23}Na, and ^{35}Cl) with a total measurement time less than 20 min. Validation results in terms of precision, reproducibility, limit of detection and recovery demonstrated that the developed method is fit-for-purpose for the authentic heparin samples.

The quantitative data for a representative set of more than hundred Na- and Ca- heparin and low-molecular weight heparin (LMWH) samples were discussed regarding animal origin and the type of anticoagulant. NMR spectrometry represents a unique analytical method suitable for the simultaneous quantitative control of organic and inorganic composition of heparin.

1. Introduction

Heparin is a polydisperse mixture of linear acidic polysaccharides, which has been used as an anticoagulant drug in medicinal practice for over 80 years [1]. To guarantee the overall quality and safety of this complex biological drug, several complementary analytical methods have been developed and introduced in US and European Pharmacopeias [2–4]. One of the most powerful and versatile of them, high resolution nuclear magnetic resonance (NMR) spectroscopy, has been widely applied to investigate the structures of heparin and related products [5–8]. During the latest heparin contamination crisis, 1D and 2D NMR techniques were crucial for the identification and quantitative determination of organic contaminants and impurities (such as oversulfated chondroitin sulfate (OSCS) and dermatan sulfate (DS)) [7–11]. NMR spectroscopy was also successfully applied to determine qualitative characteristics of heparin and low-molecular weight heparins (LMWHs) such as animal origin (porcine, bovine and ovine) and brand [6,12–14]. To complete the full characterization of heparin, average molecular weight, which is closely correlated with heparin pharmaceutical activity, was quantitatively determined by a fast 2D diffusion-ordered spectroscopy (DOSY) NMR measurement combined with multivariate regression analysis [15].

In addition to these characteristics, it would be advantageous to determine other important parameters such as water content and inorganic components (e.g., Na^{+}, Ca^{2+}, Cl^{-}, CH_3COO^{-}, F^{-}, PO_4^{3-}, etc.) in the same NMR run. Indeed, moisture content is a key control parameter for pharmaceutical products, which can be responsible for poor stability and low pharmaceutical activity. The determination of water content can be determined by thermogravimetric analysis and Karl Fisher (KF) titration, which requires additional equipment and sometimes fails [16–18].

* Corresponding author at: Spectral Service AG, Emil-Hoffmann-Straße 33, 50996 Köln, Germany.
E-mail address: monakhova@spectralservice.de (Y.B. Monakhova).

https://doi.org/10.1016/j.jpba.2018.03.028
0731-7085/

As for inorganic part, different salts and buffers are used in the commercial production of heparin [19]. As the result, free undesired anions including chloride and acetate are present in heparin and their presence needs to be monitored during manufacturing process as well as in finished pharmaceuticals. Other, positively charged ions, such as calcium and sodium, are essential constituents of any heparin material [20].

Recently, ion chromatography (IC) was used to analyze different anions (fluoride, acetate, chloride, bromide, nitrate, phosphate and sulfate) present in glycosaminoglycans including heparin, heparan sulfate, chondroitin sulfate and dermatan sulfate with a limit of quantification (LOQ) of 0.1 ppm [21]. An alternative method for the determination of free sulfate, chloride, fluoride, phosphate and acetate anions in heparin and LMWH based on capillary electrophoresis with indirect UV detection was also reported [22,23]. High performance anion exchange chromatography (HPAEC) has been developed to quantify free sulfate with high resolution and sensitivity [24]. However, chromatographic IC columns are expensive and can be easily contaminated by the sample matrix [22,23]. Moreover, the proposed methods are not straightforward and require complementary measurements to investigate ions of interest [12–23]. In this contribution we continue our publication series focused on the qualitative and quantitative control of heparin preparations [12,13,15]. In particularly, existing NMR methodology was extended to the simultaneous control of free ions including chloride, acetate, sodium, and calcium as well as water content in heparin products. To the best of our knowledge, there is no method for simultaneous analysis of organic and inorganic composition in pharmaceutical products (and in heparin in particularly) using single sample preparation and one instrumental technique. Moreover, there is the first application of ^{1}H NMR for the determination of water content in pharmaceuticals.

2. Materials and methods

2.1. Samples and chemicals

In total more than one hundred heparin (bovine, ovine, porcine and Ca-heparin) and LMWH samples were included in this representative study. Deuterated water of 99.8% purity containing 0.1% trimethylsilyl propanoic acid (TSP) as an internal standard was purchased from Euriso-top (Saarbrücken, Germany). Cs_2CO_3, NaCl and $CaCl_2$ were provided by Sigma Aldrich (Steinheim, Germany). Ethylenediaminetetraacetic acid (EDTA) and dimethylformamide were obtained from AppliChem (Munich, Germany). Acetic acid was purchased from VWR International (Leuven, Belgium).

2.2. Sample preparation and quantification

70 mg of a heparin (LMWH) sample was mixed with exactly 0.70 ml of D_2O. For the determination of Ca^{2+}, Cs-EDTA solution (pH 7.5) was added to heparin to form Ca-EDTA complexes. Stock Cs-EDTA solution was prepared as previously described [25,26].

According to the USP Monograph the recommended heparin concentration is 20 mg/ml. However, it is not prohibited to use higher concentrations (100 mg/ml in our case). The main requirement is that signal-to-noise level of at least 1000/1 of the *N*-acetyl heparin signal is achieved. This is granted with only 16 number of scans (NS) in our case (see Section 2.3), which leads to fast and high throughput measurements. Moreover, the addition of EDTA (maximum 12 μg/ml) is allowed, which was used for Ca^{2+} determination [2].

Na^{+} and Cl^{-} were quantified by ^{23}Na NMR (at δ −0.2 ppm) and ^{35}Cl NMR (at δ −3.6 ppm), respectively, using external calibration curves based on the NaCl stock solution. For calculations, ^{23}Na and ^{35}Cl integral values obtained from calibration solutions and samples were divided by the ^{2}D NMR integral (originated from D_2O solvent) for the normalization.

Ca^{2+} was quantified based on the signal of it complex with EDTA at δ 2.6 ppm using $CaCl_2$ – EDTA stock solution for constructing calibration curves [25,26]. CH_3COO^{-} was quantified based on the routine USP ^{1}H NMR experimental protocol and external calibration with acetic acid. In both cases Ca – EDTA and CH_3COO^{-} integral values were adjusted by the ^{2}D NMR integral and by the actual receiver gain values.

For the quantification of water content, the routine USP ^{1}H NMR experimental protocol was used. First, three specific ranges (δ 1.9–2.2 ppm, δ 2.9–4.6 ppm and δ 4.8–5.8 ppm) were integrated and the sum was normalized to 50. Than the water peak was integrated (δ 4.7–4.8 ppm) and the resulted relative value (ranged from 40.7 to 95.8 for authentic samples) was correlated to the water content in heparin samples found by the reference methods (Fig. 1).

The NMR protocols were validated in terms of linearity, quantification limits, recovery and repeatability. The limit of detection (LOD) was calculated based on the residual standard deviation of the response and the slope of the calibration curve near the expected detection limit. To assess repeatability, five separate standard sample preparations were performed for several types of heparin samples and measured within a short period of time. The recovery rates were ascertained by adding standard solutions at different concentrations to several heparin samples.

2.3. NMR measurements

NMR measurements were performed at 297 K on Bruker Avance III 600 MHz and 500 MHz spectrometers (Bruker Biospin, Rheinstetten, Germany) with BBO cryo probe and BBFO^{PLUS} Smart probe, respectively, equipped with Bruker Automatic Sample Changer (B-ACS 120). ^{1}H NMR spectra were recorded with standard pulse program (zg30 in Bruker language) using 16 scans (NS) and 2 prior dummy scans (DS). The data of 132 k points (TD) were acquired with a spectral width (SW) of 24.0155 ppm, an acquisition time (AQ) of 4.5438 s. Automated receiver gain (RG) adjustment was used.

The following parameters were selected for other NMR experiments: ^{35}Cl NMR: NS = 1024, DS = 4, TD = 4k, SW = 398.4 ppm, RG = 362; ^{23}Na NMR: NS = 128, DS = 4, TD = 32 k, SW = 198.8 ppm, RG = 1290; ^{2}D NMR: NS = 4, DS = 0, TD = 16k, SW = 20 ppm, RG = 1. The total measurement time (^{1}H + ^{35}Cl + ^{23}Na + ^{2}D) was 20 min per sample.

The data were recorded automatically under the control of ICON-NMR (Bruker Biospin, Rheinstetten, Germany). All NMR spectra were manually phased and baseline-corrected using Topspin 3.2 (Bruker Biospin, Rheinstetten, Germany).

2.4. Reference methods

Water content was separately measured for 26 products including 2 ovine, 3 bovine and 12 porcine Na-heparin as well as 4 Ca-heparin and 5 LMWH (enoxaparin sodium) samples.

For the reference water determination coulometric Karl Fischer titration was applied (Karl Fischer Titrator TitroLine KF (Schott Instruments GmbH, Mainz, Germany)). Dimethylformamide was used as a solvent due to poor heparin solubility in methanol. The measurements were performed in triplicate. Accuracy was successfully verified according to European Pharmacopoeia 6.0 (chapter 2.5.32) [27]. For three heparin samples, water content was determined by “loss on drying” at 80 °C in oven.

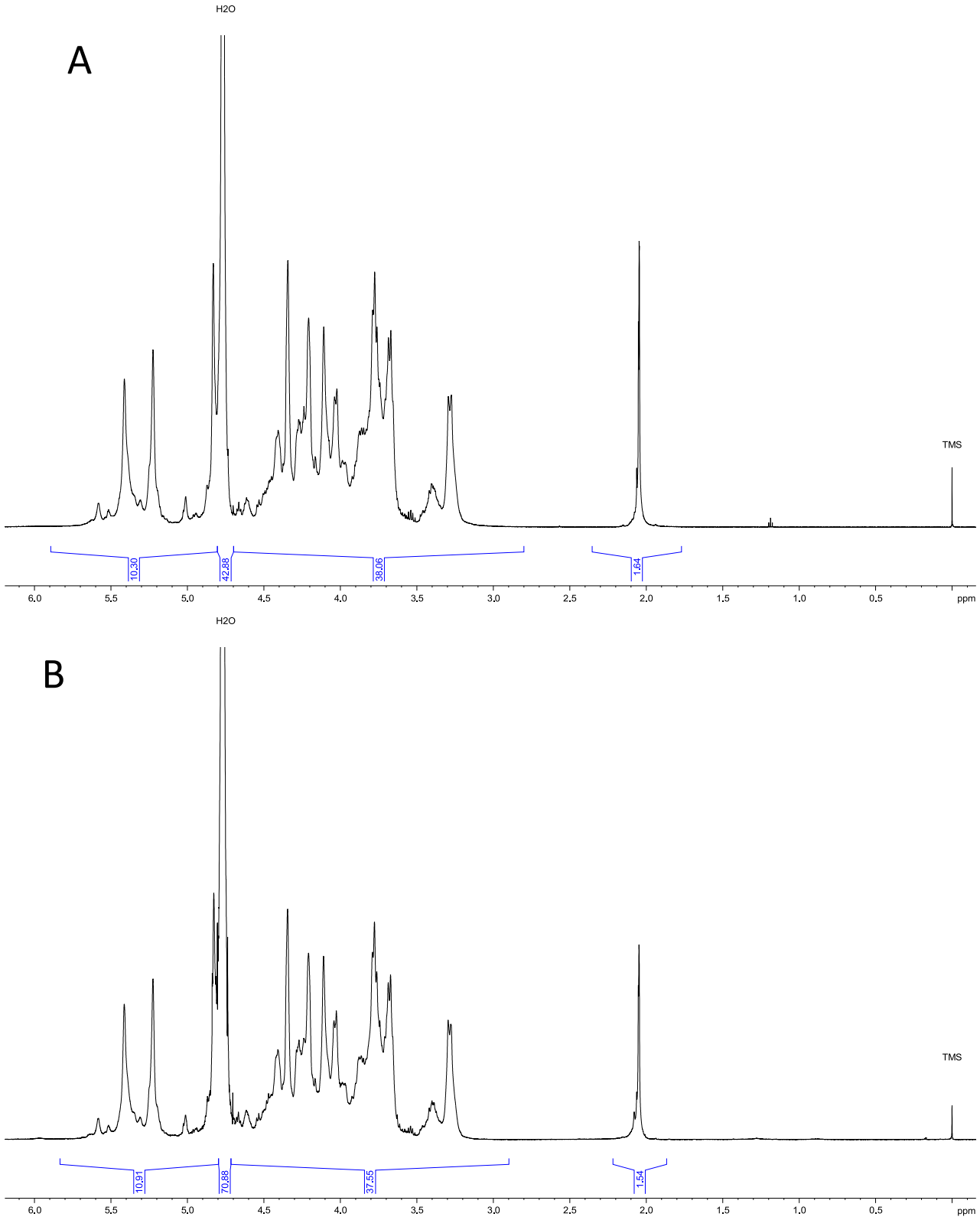

Fig. 1. ^{1}H NMR spectra of porcine heparin (A) and Ca-heparin (B) with water content of 2.6 w/w% and 11.9 w/w%, respectively. Intensity axis [A.U.] is not shown for simplicity.

3. Results and discussion

3.1. Determination of residual water in heparin preparations

The idea behind water determination by ^{1}H NMR is that the structure of heparin is highly uniform and consists of a repeating disaccharide unit (2-*O*-sulfated iduronic acid and 6-*O*-sulfated, *N*-sulfated glucosamine), which has defined molecular weight and the number of corresponding protons [1,5,8,28]. The heparin polysaccharide signals were grouped in three NMR spectral ranges corresponding to the anomeric (5.0–5.7 ppm), the sugar ring (2.9–4.5 ppm) and the acetamidomethyl protons (1.9–2.2 ppm)

[28] (Fig. 1). The remaining peak at δ 4.7 ppm in the NMR spectrum represented the sum of exchangeable OH-groups of heparin itself (e.g., —OH and —COOH), the residual H_2O in D_2O solvent (0.2 w/w%) and water originated from heparin. It should be mentioned that the OH-signal at δ 4.7 ppm is also slightly overlapped by the small heparin matrix resonances. However, the sum of the impurities signals within OH integral is also constant relative to the area of the other heparin spectral regions.

Given that exactly the same amount of solvent is added to heparin and its constant structure, the sum of the first two components is constant. Therefore, a linear correlation should exist between the water content and the relative OH-integral. Fig. 1 showed ^{1}H NMR spectra of two Na-heparin samples with different water content of 2.6 w/w% and 11.9 w/w%, for which the relative OH-integral values were 42.9 and 70.9, respectively. Therefore, relative OH-integral values increased simultaneously with the actual water content in heparin.

The normalized OH-peak areas (ranged from 40.7 to 95.8) were plotted against reference water content values for 26 heparin samples. Initially, using KF reference data set for water content, three outliers were identified (marked with "X" in Fig. 1 in Supplementary information). These samples had the high relative OH integral values (about 19) but, according to the KF analysis, contained only about 12 w/w% of water. However, plausible values between 18 w/w% and 19 w/w% were obtained for these samples using "loss on drying" as an alternative method, which is not affected by chemical cross reactions or solubility in organic solvents (outlined with a circle, Fig. 1 in Supplementary information). Therefore, water content above 12 w/w% obtained by KF titration was strongly underestimated, while NMR measurements were free of these interferences.

The final calibration equation was determined as $Y = (33.4 \pm 0.9) + (3.2 \pm 0.1) * X$ with a linear correlation coefficient of 0.99 over a reference range of water content between 2.4 w/w% and 18.9 w/w% (Fig. 1 in Supplementary information). The accuracy of the NMR determination cannot be more precise than those of Karl Fischer/loss-on-drying methods (about 3%). These moisture levels corresponded to the water content found in previous studies (mean range 0.7–18.4 w/w%) [17,18]. This linear equation can be used to predict water content in heparin.

Validation of the method was first conducted by repeated sample preparation of different types of heparin samples (Table 1). The intraday repeatability of OH peak area values (5 separate sample preparation) varied between 0.3% for Ca-heparin to 0.70% for porcine Na-heparin. Accuracy was assessed by the recovery experiments using untreated and dried heparin material. Five different amounts of distilled water were spiked to three heparin samples. The recovery values for heparin samples with 2.4 w/w%, 7.3 w/w% and 12.4 w/w% water content were in the range of 95–105%, demonstrating that the developed method is accurate enough for samples with various water contents.

Generally, KF is the most commonly applied approach for the moisture content determination as the equipment is fairly inexpensive and the method is easily to perform [16–18]. Volumetric titration was successfully used for heparin in the study of Sommers et al. [17]. Columetric KF titration is another widely used technique to determine residual water content in biologicals including heparin [16,18]. However, problems were encountered using this method for biopharmaceuticals as methanol can react with ketons and aldehydes [16]. Underestimation of KF water content was also observed in our study. Therefore, modifications are needed in case of heparin analysis, for example, change of solvent, cleaning cell or preliminary separation of water by evaporation [16,18].

Residual moisture determination is a key quality control test for biopharmaceutical manufactures and we recommend NMR analysis for this purpose. Recently, relatively high water content (up to 31 w/w%) was found in OSCS-contaminated heparin batches [18]. Water content of heparin can be simply predicted based on our linear equation and the relative OH integral values using the routine USP ^{1}H NMR experimental protocol. The important feature of the developed method is its universality because the same linear equation is applicable for Na-heparin of different origin and Ca-heparin as well as for LMWHs.

3.2. Anion profiling in heparin (Cl^- and CH_3COO^-)

Apart from water, several other undesirable constituents can be present in heparin. For example, sodium chloride is abundantly applied in heparin production processes, therefore, chloride is a major free anion observed in heparin [21]. The presence of chloride has to be constantly monitored, because chloride content can reflect the inefficient desalting.

Inorganic chloride cannot be determined by the routine ^{1}H NMR run, however, an additional ^{35}Cl NMR experiment can be used for its detection and quantification. Some examples of Cl^- peaks from authentic heparin samples with different chloride content were present in Fig. 2.

The calibration curve for chloride anion based on the NaCl stock solution was linear in the range from 0.05 w/w% and 30 w/w% of Cl^- anion (Table 1). For the standard solutions and authentic samples, the repeatability values expressed as relative standard deviations (RSD) were always below 2%. The recovery rates ascertained at five different concentrations were between 94% and 106%. These short validation results showed that ^{35}Cl NMR method is appropriate to detect and quantify the presence of free chloride ion in heparin. The method can be simply transferred to other pharmaceuticals as well.

The developed methodology was used for analysis of 90 authentic heparin samples including Na- and Ca- heparin of different animal origin. The results were presented in the form of the box plot inserted in Fig. 2. From the analyzed 90 samples, 78 (87%) contained chloride above the detection limit of 0.05 w/w%. The chloride content in the majority of samples was lower than 0.5 w/w%. The average and median concentrations of free chloride anion were 0.33 and 0.20 w/w%, respectively. The relatively high chloride content (2.4. w/w%) was observed in one heparin sample (Fig. 2). According to our knowledge, this is the first study, where Cl^- content was measured for a big dataset of authentic pharmaceutical samples by ^{35}Cl NMR.

Retrospective determination of free acetyl anion in heparin samples was another goal of this study. The acetate ion could result in heparin from the instability of the *N*-acetylated polysaccharides and/or could be introduced in the production process. The methyl group of free acetate ions was observed at δ 1.9 ppm in ^{1}H NMR spectra and the signal is free from interference from the *N*-acetyl signal originated from the polysaccharide [7]. Acetate anion in small quantities has been previously reported in heparin products [7,21]. Sodium acetate at the concentration up to 0.5% was found in OSCS-contaminated heparin batches in 2008 [18]. The validation parameters were listed in Table 1. According to this method, no free acetate above the detection limit of 0.002 w/w% was observed in 131 investigated heparin samples.

Our method for the determination of Cl^- and CH_3COO^- does not require additional sample preparation and analytical equipment. Only about five minutes are needed to perform additional ^{35}Cl and ^{2}D NMR runs. It should be mentioned that other anions such as phosphate and fluoride can be detected on demand by the additional ^{31}P and ^{19}F NMR experiments, respectively. These anions are normally not detected in heparin preparations [21]. Unfortunately, only sulfate anion cannot be quantified by ^{33}S NMR due to low sen-

Table 1
Validation results for water and ions in heparin by NMR.

Parameter	Water	Na^+	Cl^-	CH_3COO^-	Ca^{2+}
Required measurements	USP protocol	USP protocol ^{23}Na NMR ^{2}D NMR	USP protocol ^{35}Cl NMR ^{2}D NMR	USP protocol ^{2}D NMR	USP protocol ^{2}D NMR
Number of investigated samples	26	88	90	131	10
LOD [w/w%]	–[a]	0.05	0.2	0.002	0.5
Linear range [w/w%]	2.4–18.9	0.05–30	0.2–30	0.002–15	0.5–20
Repeatability [RSD, %][b]	0.7	<3	<2	<3	<2
Recovery [%]	95–105	92–104	94–106	96–102	97–107

[a] Was not estimated.
[b] RSD – relative standard deviation.

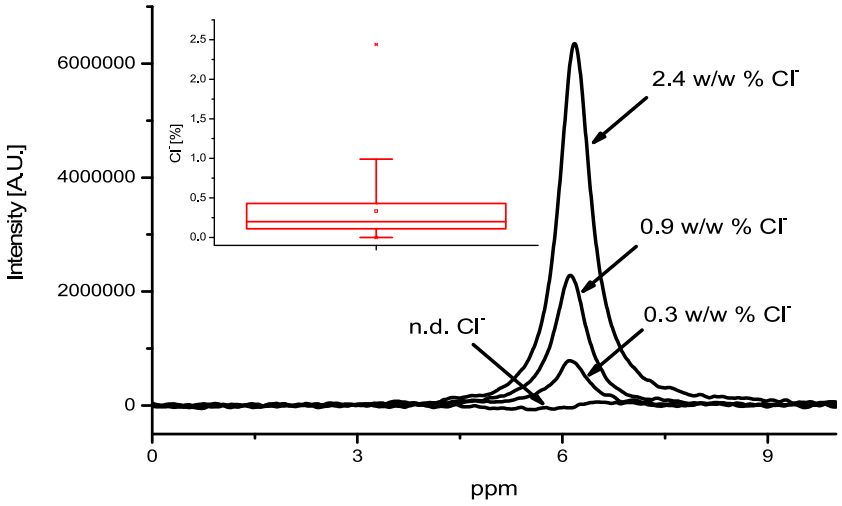

Fig. 2. ^{35}Cl NMR spectra of heparin samples with different Cl^- content. Inserted is box plot of Cl^- content for 90 heparin samples.

sitivity, which means unacceptable measurement time for routine analysis.

3.3. Determination of counterions (Na^+ and Ca^{2+}) in heparin

Positively charged ions such as calcium and sodium play important role in pharmacological action of anticoagulant drugs [20]. However, the content of Na^+ and Ca^{2+} in heparin has not been previously monitored in a systematic way.

It is known that ^{23}Na is a medium sensitivity nucleus and yields slightly broad lines. Therefore, by analogy to Cl^- anion, free Na^+ cation can be determined by an additional ^{23}Na NMR experiment using the peak at δ −0.2 ppm. The validation parameters were comparable to those previously described for ^{35}Cl NMR, therefore, the method is sufficient for the screening of Na^+ content in heparin (Table 1). Furthermore, NMR results for 16 Na-heparin samples were compared with values obtained by the conventional atomic absorption spectroscopy (AAS) method. Statistical analysis using *T*-test between AAS and NMR data showed that at the 0.05 level, the population means were not significantly different.

The NMR results for Na^+ in 88 investigated samples (44 bovine, 22 ovine and 22 porcine) were summarized in Fig. 3 as the box plots for Na-heparins originating from different animal material. Generally the data showed a small variation in Na^+ with the average values of 11.5 w/w%, 11.3 w/w% and 11.7 w/w% for bovine, ovine and porcine heparin, respectively. The 25th and 75th percentiles for all Na-heparin samples were found to be 10.9 w/w% and 12.2 w/w%. Thus, Na^+ cannot be used to differentiate between heparin of different animal origin [12,13].

As for the Ca^{2+} cation, ^{1}H NMR methodology was already proposed for its determination in mineral water and *Aloe vera*

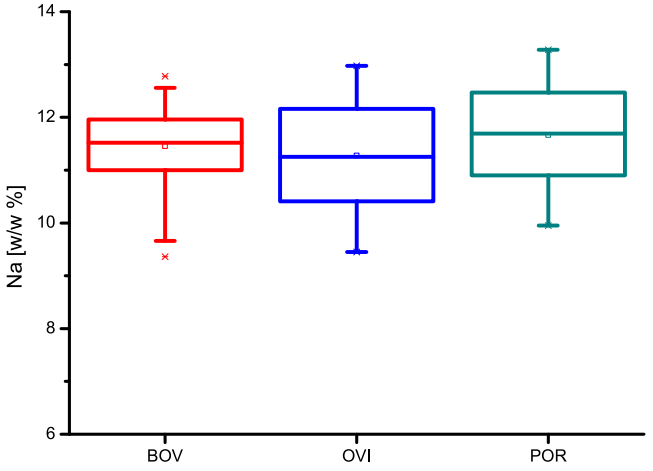

Fig. 3. Box plot of Na^+ content for bovine (n = 36), porcine (n = 35) and ovine (n = 17) heparin samples.

preparations [25,26]. For heparin, addition of the EDTA standard solution to Ca-heparin samples generated new signals in ^{1}H NMR spectrum (Fig. 4). The two peaks at δ 3.68 ppm and at δ 3.31 ppm indicated protons from free EDTA [25,26]. Ca-EDTA signals are centered at δ 3.13 ppm and at δ 2.56 ppm. The later signal is not overlapped with heparin matrix signals and was used for direct determination of Ca^{2+} in Ca-heparin preparations (Fig. 4).

Our validation results suggested that the coefficients of variation (CVs) were below 2% intraday (n = 5) and interday (n = 5) meaning good precision for the NMR method for the Ca^{2+} determination (Table 1). Linear correlation between the peak area and

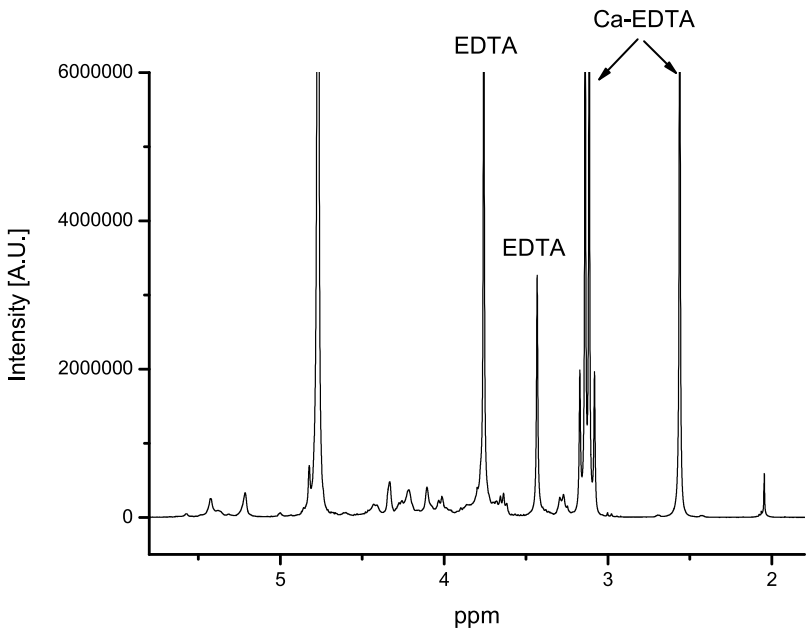

Fig. 4. ^{1}H NMR spectrum of Ca-heparin sample with the addition of Cs-EDTA buffer solution.

Table 2
Determination of Ca^{2+} in Ca-heparin samples.

Sample	Ca^{2+} [w/w%]
S1	10.1
S2	10.0
S3	10.6
S4	10.1
S5	10.0
S6	9.9
S7	9.9
S8	10.2
S9	10.5
S10	10.2
Average [w/w%]	10.1
Relative standard deviation [%]	0.2

anion concentration from 1 to 20 w/w% was observed. The results are comparable with those obtained for mineral water and *Aloe vera* matrix [25,26].

Similarly to Na^{+} concentrations, the Ca^{2+} content in ten investigated samples differed only slightly. The average concentration was found to be 10.1 ± 0.2 w/w% (Table 2). The data demonstrated that Na-heparin and Ca-heparin samples showed constant structure regarding its counterion composition.

4. Conclusions

The major goal of our on-going research on heparin is to develop a robust and fast NMR methodology, which is applicable in quality control laboratories for heparin screening. Our data suggested that apart from previously developed techniques to determine heparin animal origin, type of manufacturing process, organic composition, and molecular weight, other relevant parameters (Cl^{-}, $CH3COO^{-}$, Ca^{2+}, Na^{+}, water content) can be additionally assessed using the simple sample preparation according to the USP [2,3,12,13,15,29]. Notably, for the quantitative analysis no internal standard has to be added to the matrix, and the exact amount of deuterated D_2O solvent was used for this purpose.

Different types of heparin were analyzed for the presence of free ions. Free chloride ion was observed in the majority of heparin samples examined. Presumably, chloride remained from the extraction and purification processes used to make these products. Acetate ion was not detected in the study but, potentially, it could have been released from *N*-acetyl groups of heparin or/and during blenching with peracetic acid, was not detected. The content of counterions (Ca^{2+} and Na^{+}) found in a representative set of heparin samples was quite uniform suggesting constant structure of natural polymer material.

In this study, a universal NMR method for water determination in aqueous solutions was developed for pharmaceuticals. Contrary to the conventional KF method, our methodology has no limitation regarding the range of moisture levels in heparin (confirmed between 2 w/w% and 19 w/w%). The method can be used in routine quality control of Na- and Ca- heparin and LMWH of different origin and can be transferred to other pharmaceuticals with the constant composition on demand.

Acknowledgements

We warmly thank K. Seitz and T. Lüke for their excellent technical assistance. We thank Professor Fareed and Dr. Yao for providing samples of ovine origin. Y. Monakhova acknowledges support of the Russian Ministry of Science and Education (project 4.1063.2017/4.6).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jpba.2018.03.028.

References

[1] R.J. Linhardt, Heparin: an important drug enters its seventh decade, Chem. Ind. 2 (1991) 45–50.
[2] US Pharmacopoeia, United States Pharmacopeia Heparin Sodium Stage 3 Monograph, US Pharmacopoeia, Rockville, 2014.
[3] European Pharmacopoeia, European Pharmacopeia Heparin Sodium Monograph PA/PH/Exp. 6/T(0) 42 PUB Monograph Number 333, EDQM, Strasbourg, 2010.
[4] A.Y. Szajek, E. Chess, K. Johansen, G. Gratzl, E. Gray, D. Keire, R.J. Linhardt, J. Liu, T. Morris, B. Mulloy, M. Nasr, Z. Shriver, P. Torralba, C. Viskov, R. Williams, J. Woodcock, W. Workman, A. Al-Hakim, The US regulatory and pharmacopeia response to the global heparin contamination crisis, Nat. Biotechnol. 34 (2016) 625–630.

[5] M. Guerrini, A. Bisio, G. Torri, Combined quantitative ^{1}H and ^{13}C uclear magnetic resonance spectroscopy for characterization of heparin preparations, Semin. Thromb. Hemost. 27 (2001) 473–482.
[6] M. Guerrini, T.R. Rudd, L. Mauri, E. Macchi, J. Fareed, E.A. Yates, A. Naggi, G. Torri, Differentiation of generic enoxaparins marketed in the united states by employing NMR and multivariate analysis, Anal. Chem. 87 (2015) 8275–8283.
[7] Z. Zhang, B. Li, J. Suwan, F. Zhang, Z. Wang, H. Liu, B. Mulloy, R.J. Linhardt, Analysis of pharmaceutical heparins and potential contaminants using (1)H-NMR and PAGE, J. Pharm. Sci. 98 (2009) 4017–4026.
[8] T. Beyer, B. Diehl, G. Randel, E. Humpfer, H. Schäfer, M. Spraul, C. Schollmayer, U. Holzgrabe, Quality assessment of unfractionated heparin using 1H nuclear magnetic resonance spectroscopy, J. Pharm. Biomed. Anal. 48 (2008) 13–19.
[9] M. Guerrini, S. Guglieri, A. Naggi, R. Sasisekharan, G. Torri, Low molecular weight heparins: structural differentiation by bidimensional nuclear magnetic resonance spectroscopy, Semin. Thromb. Hemost. 33 (2007) 478–487.
[10] D.A. Keire, L.F. Buhse, A. al-Hakim, Characterization of currently marketed heparin products: composition analysis by 2D-NMR, Anal. Methods 5 (2013) 2984–2994.
[11] S. Alban, S. Lühn, S. Schiemann, T. Beyer, J. Norwig, C. Schilling, O. Rädler, B. Wolf, M. Matz, K. Baumann, U. Holzgrabe, Comparison of established and novel purity tests for the quality control of heparin by means of a set of 177 heparin samples, Anal. Bioanal. Chem. 399 (2011) 605–620.
[12] Y.B. Monakhova, B.W.K. Diehl, J. Fareed, Authentication of animal origin of heparin and low molecular weight heparin including ovine, porcine and bovine species using 1D NMR spectroscopy and chemometric tools, J. Pharm. Biomed. Anal. 149 (2018) 114–119.
[13] Y.B. Monakhova, B.W. Diehl, Combining 1H NMR spectroscopy and multivariate regression techniques to quantitatively determine falsification of porcine heparin with bovine species, J. Pharm. Biomed. Anal. 115 (2015) 543–551.
[14] P.A.J. Mourier, F. Herman, P. Sizun, C. Viskov, Analytical comparison of a US generic enoxaparin with the originatorproduct: the focus on comparative assessment of antithrombin-binding components, J. Pharm. Biomed. Anal. 129 (2016) 542–550.
[15] Y.B. Monakhova, B.W.K. Diehl, T.X. Do, M. Schulze, S. Witzleben, Novel method for the determination of average molecular weight of natural polymers based on 2D DOSY NMR and chemometrics: example of heparin, J. Pharm. Biomed. Anal. 149 (2018) 128–132.
[16] P. Matejtschuk, C. Duru, K. Malik, E. Ezeajughi, E. Gray, S. Raut, F. Mawas, Use of thermogravimetric analysis for moisture determination in difficult lyophilized biological samples, Am. J. Anal. Chem. 7 (2016) 260–265.
[17] C.D. Sommers, H. Ye, R.E. Kolinski, M. Nasr, L.F. Buhse, A. Al-Hakim, D.A. Keire, Characterization of currently marketed heparin products: analysis of molecular weight and heparinase-I digest patterns, Anal. Bioanal. Chem. 401 (2011) 2445–2454.
[18] T. Beyer, M. Matz, D. Brinz, O. Rädler, B. Wolf, J. Norwig, K. Baumann, S. Alban, U. Holzgrabe, Composition of OSCS-contaminated heparin occurring in 2008 in batches on the German market, Eur. J. Pharm. Sci. 40 (2010) 297–304.
[19] J.Y. van der Meer, E. Kellenbach, L.J. van den Bos, From farm to pharma: an overview of industrial heparin manufacturing methods, Molecules 22 (2017) 1025, http://dx.doi.org/10.3390/molecules22061025.
[20] G.P. Diakun, H.E. Edwards, D.J. Wedlock, J.C. Allen, G.O. Phillips, The relationship between counterion activity coefficients and the anticoagulant activity of heparin, Macromolecules 11 (1978) 1110–1114.
[21] L. Liu, R.J. Linhardt, Z. Zhang, Quantitative analysis of anions in glycosaminoglycans and application in heparin stability studies, Carbohydr. Polym. 106 (2014) 343–350.
[22] M. Zhang, D. Qiu, J. Kang, Determination of sulfate anions in heparin by capillary electrophoresis with improved indirect UV detection, Chromatographia 78 (2015) 833–837.
[23] M. Zhang, D. Qiu, J. Kang, Determination of anions in low molecular weight heparin by capillary electrophoresis with phthalate as the background electrolyte, Chin. J. Chromatogr. 35 (2017) 59–64.
[25] Y.B. Monakhova, G. Randel, B.W. Diehl, Automated control of the organic and inorganic composition of *Aloe vera* extracts using ^{1}H NMR Spectroscopy, J. AOAC Int. 99 (2016) 1213–1218.
[26] Y.B. Monakhova, T. Kuballa, C. Tschiersch, B.W.K. Diehl, Rapid NMR determination of inorganic cations in food matrices: application to mineral water, Food Chem. 221 (2017) 1828–1833.
[27] European Pharmacopoeia Commission, European Pharmacopeia, Microdetermination of Water, Council of Europe, Strasbourg, 2008, pp. 184–185.
[28] L. Fu, G. Li, B. Yang, A. Onishi, L. Li, P. Sun, F. Zhang, R.J. Linhardt, Structural characterization of pharmaceutical heparins prepared from different animal tissues, J. Pharm. Sci. 102 (2013) 1447–1457.
[29] Y.B. Monakhova, J. Fareed, Y. Yao, B.W.K. Diehl, Improving reliability of chemometric models for authentication of species origin of heparin by switching from 1D to 2D NMR experiments, J. Pharm. Biomed. Anal. 153 (2018) 168–174, http://dx.doi.org/10.1016/j.jpba.2018.02.041.