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

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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].

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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.