Journal of Pharmaceutical and Biomedical Analysis 149 (2018) 128–132

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Novel method for the determination of average molecular weight of natural polymers based on 2D DOSY NMR and chemometrics: Example of heparin

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ARTICLE INFO

Article history:
Received 1 September 2017
Received in revised form
26 September 2017
Accepted 1 November 2017

Keywords:
NMR spectroscopy
DOSY
Molecular weight
Heparin
Partial least squares regression

ABSTRACT

Apart from the characterization of impurities, the full characterization of heparin and low molecular weight heparin (LMWH) also requires the determination of average molecular weight, which is closely related to the pharmaceutical properties of anticoagulant drugs.

To determine average molecular weight of these animal-derived polymer products, partial least squares regression (PLS) was utilized for modelling of diffused-ordered spectroscopy NMR data (DOSY) of a representative set of heparin (n = 32) and LMWH (n = 30) samples. The same sets of samples were measured by gel permeation chromatography (GPC) to obtain reference data. The application of PLS to the data led to calibration models with root mean square error of prediction of 498 Da and 179 Da for heparin and LMWH, respectively. The average coefficients of variation (CVs) did not exceed 2.1% excluding sample preparation (by successive measuring one solution, n = 5) and 2.5% including sample preparation (by preparing and analyzing separate samples, n = 5). An advantage of the method is that the sample after standard 1D NMR characterization can be used for the molecular weight determination without further manipulation. The accuracy of multivariate models is better than the previous results for other matrices employing internal standards. Therefore, DOSY experiment is recommended to be employed for the calculation of molecular weight of heparin products as a complementary measurement to standard 1D NMR quality control. The method can be easily transferred to other matrices as well.

1. Introduction

Heparin is a polysaccharide polymer drug isolated from glycosaminoglycans released from animal tissues [1]. This medicinal product consists of alternating highly sulfated glucosamine and uronic acid monosaccharide fragments, most of which have molecular weight between 5 and 30 kDa [1,2].

Like all other natural polysaccharides, heparin is a polydisperse mixture containing a large number of chains with varying molecular weights [2]. The variations in fractionating procedure among manufacturers result in differences in the MW distribution of the finished heparin products. On the other side, the chain length is one of the parameters highly affecting biological activity of heparin and low molecular weight heparin (LMWH) as well as therapeutic and pharmacological properties [2–4]. Therefore, an accurate determination of MW is particularly important for the heparin characterization.

The evaluation of average MW represents one of controversial aspects concerning characterization of polymer materials. In this regard, heparin is a challenging matrix due to its sequence heterogeneity, high degree of polydispersity and its polysaccharide nature with long length chains [3]. Among other techniques, liquid chromatography with mass spectrometry (LC–MS) has been used to profile heparin preparations [5,6]. However, for large polymers overlapping MW patterns prevent accurate interpretation of experimental data [5,6]. For LMWH an alternative method based on UV/refractive index ratio of a sample prepared by beta-elimination is also available [6–8].

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https://doi.org/10.1016/j.jpba.2017.11.016
0731-7085/

The most common method for the determination of MW profiles of heparin and LMWH is gel permeation chromatography (GPC) with refractive index or light scattering detection [2,9,10]. Recently a GPC method for the evaluation of polymer samples was developed, which combines the performance of light-scattering detector, refractometer and viscometer [3]. However, the main disadvantage of GPC based methods is their dependency on a set of reference standards with well-defined average MW and narrow MW distribution (except recently proposed triple detection [3]), which are expensive and are not currently produced on a large scale [2,9]. An alternative universal calibration, which relates the retention times of a polymer to its hydrodynamic volume, has not been applied to heparin so far [11]. Moreover, GPC experiments are often time-consuming and require large amounts of organic solvents.

It is well known that nuclear magnetic resonance (NMR) spectroscopy is a recognized instrumental method for heparin surveillance regarding quantitative assessment of contaminant levels and qualitative features such as animal origin or brand [12–14].

Recently the NMR method was implemented as a mandatory identity test in European Pharmacopoeia (EP) and US Pharmacopoeia (USP) [15,16]. Several attempts were also made regarding using NMR to determine MW of heparin and LMWH in a standardless manner [17,18]. For example, the ^{13}C NMR signal intensities of the reducing end and internal anomeric carbons were used to calculate MW of heparin and LMWH [17,18]. However, measurement times required for such analysis with sufficient accuracy even using modern NMR equipment are unacceptable for routine quality control of this medicinal drug [17,18].

In this study we report on the development of a fast and reliable method for the determination of average MW of heparin and LMWH based on diffusion ordered spectroscopy (DOSY) NMR experiments. DOSY represents a method for the discrimination of species with unequal molecular size in their mixture through the measurement of diffusion coefficients (logD) [19]. Access to a series of calibrant compounds with defined molecular weight allowed determination of MW within ±10% deviation through diffusion coefficient – MW analysis [20]. This approach was further improved by using normalized diffusion coefficients and taking also the shape of the molecules into account [21]. For example, plot of the log of the determined diffusion coefficients versus the log of the MW was linear in case of series of N-acetyl-chitooligosaccharide complexes, pullulan fractions, a set of oligo-/polysaccharides and kinetic samples from controlled polymerization [21–23]. DOSY was also previously employed as an approach to determine the stoichiometry of intermolecular oligosaccharide and organometallic complexes [20,21].

In contrast to these previous studies, in this report multivariate calibration, namely partial least squares regression (PLS), was used to correlate the 2D NMR data with the data of reference GPC analysis. The models were constructed and validated using representative datasets of heparin and LMWH samples derived from different animal tissues (porcine, ovine, bovine). The method is suitable for routine quality control of commercial heparin and LMWH products according to international USP and EP guidelines, because no additional sample preparation is necessary. Software package for the analysis of DOSY NMR data is available to automate the process.

2. Materials and methods

2.1. Samples and sample preparation

A total of thirty-two heparin (12 bovine, 9 ovine, and 11 porcine) and thirty LMWH (8 ovine and 22 porcine) samples were investigated. Deuterated water of 99.8% purity containing 0.1% trimethylsilyl propanoic acid (TSP) as internal standard was purchased from Euriso-top (Saarbrücken, Germany). For sample preparation, 70 mg of a heparin (LMWH) sample was mixed with 0.7 mL of D_2O.

2.2. NMR measurements

NMR measurements were performed on Bruker Avance III 600 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) with BBO cryo probe equipped with Bruker Automatic Sample Changer (B-ACS 120) at 297 K. NMR spectra were recorded with standard pulse program (zg30 in Bruker language) using 16 scans and 2 prior dummy scans. The data of 132 k points were acquired with a spectral width of 24.0155 ppm, a receiver gain of 72, an acquisition time of 4.5438 s.

2D DOSY (diffusion ordered spectroscopy) experiments were performed using standard DOSY pulse sequence with longitudinal eddy current delay (LED) with bipolar gradient pulse pair and 2 spoil gradients. The length of the gradient pulse (δ) was set to 1400 μs and diffusion time (Δ) was set to 0.05 s 2 scans provided enough sensitivity for heparin measurements (this parameter was varied between 2 and 16). The measurement took only 5 min for one sample.

For the processing of DOSY spectra the following diffusion fit function was used:

$f(x) = I_0 + e^{\left(-y^2 g^2 \delta^2 \left(\Delta - \frac{\delta}{3}\right) D\right)}$, where D is the diffusion coefficient, g is the gradient strength and y is the gyromagnetic ratio. I_0 and I represent the maximum and observed signal intensity. The 2D plots show diffusion coefficient values D in [m^2/s].

The DOSY spectra were baseline corrected and were normalized to TSP signal at −9.3 m^2/s and δ 0.0 ppm. The data points within the range of δ 6.0-1.8 ppm for heparin and δ 6.2-1.8 ppm for LMWH were pre-processed by bucketing with 0.01 ppm width. The buckets were scaled to total intensity using in-house developed Matlab script. The water peak other solvent signals were excluded from the consideration. Each resultant matrix was unfolded to an array of 1 × 10500 (heparin) or 1 × 13200 (LMWH). Finally, the data were normalized to total intensity before multivariate modelling.

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).

2.3. Chemometric modelling and validation

Matlab 2015a (The Math Works, Natick, MA, USA) and SAISIR package for MATLAB [24] were used for statistical calculations. Principal component analysis (PCA) was first applied to the datasets for outlier detection.

NMR spectra were correlated with the results of the GPC analysis by PLS regression. No weighing was performed for the models. Validation of the models was first performed using leave-one-out cross validation (LOOCV) to select the number of latent variables (LVs). The simplest models regarding the number of LVs with the minimum value of root-mean-square error of validation (RMSEV) were chosen.

Afterwards, PLS models were validated using independent test set (eight samples for each data set). The splitting into calibration and validation sets was performed ten times. The samples from different animal origin were always included in both subsets. Average values of root mean square of prediction (RMSEP) were used as a quality criterion for model performance.

To assess interday and intraday precision, the analysis of selected samples was performed using separate sample preparations (n = 5) or while staying the autosampler (n = 5).