Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 302–308

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

An assessment of polydispersed species in unfractionated and low molecular weight heparins by diffusion ordered nuclear magnetic resonance spectroscopy method

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ARTICLE INFO

Article history:
Received 5 February 2010
Received in revised form 25 March 2010
Accepted 26 March 2010
Available online 2 April 2010

Keywords:
Unfractionated (UFH) and low molecular weight heparins
Oversulfated chondroitin sulfate (OSCS)
Dermatan sulfate (DS) Diffusion ordered NMR spectroscopy

ABSTRACT

The primary goal of this project is to extend a ^{1}H NMR based method, which combines elements of separation on the basis of molecular size with the information specific to ^{1}H-1D NMR, to the assessment of the heparin contaminant oversulfated chondroitin sulfate (OSCS) and process related impurity dermatan sulfate (DS), and their polydisperse degradation products in samples of unfractionated heparins (UFHs) and low molecular weight heparins (LMWHs) used as the active pharmaceutical ingredients (APIs) in finished pharmaceutical products. The method has been briefly introduced by us in a recent contribution (*vide infra*). We propose a labelling of the N-acetyl peaks in the ^{1}H NMR spectra of the UFHs and LMWHs with the parameter D_i, the translational diffusion coefficient available from DOSY NMR. It is shown how DOSY can be applied for screening lots of unfractionated and depolymerised heparins for obtaining molecular size information for heparins and any impurities when using ^{1}H NMR. The evidence has been presented that title method can be applied as a routine means for assessment of the OSCS and DS contaminants and the polydisperse chemical entities present in the UFHs and LMWHs used as the APIs in heparin pharmaceuticals.

1. Introduction

The biopolymeric carbohydrate drug heparin is a member of the glycosaminoglycan (GAG) family, extracted from animal tissues. Unfractionated heparin (UFH) is a polydisperse biopolymer. A second GAG, dermatan sulfate (DS), is a process impurity in UFH, present in small amounts [1,2]. Heparin is the oldest widely used anticoagulant [3], and has been used clinically for over 75 years. A thorough account of the quality assessment of more than one hundred unfractionated heparin samples from international markets has appeared [4]. ^{1}H-1D NMR signal deconvolution was performed for quantification of two major contaminants, DS and the semi-synthetic sulfated GAG oversulfated chondroitin sulfate (OSCS). More recently, low molecular weight heparins (LMWHs), obtained by partial depolymerization of UFH in controlled chemical or enzymatic processes, have been introduced as anticoagulant and antithrombotic agents [5]. Capillary/gel electrophoresis, CE/GE, has been used to map the oligosaccharide composition [6,7], and NMR spectroscopy has aided successfully in the structure characterization and elucidation of heparin conformation in solution [8–10] and assessment of contaminants [11,12]. Physicochemical properties of heparins have been studied mainly via dynamic light scattering with respect to the role of selfassociation in the gelation of heparin-functionalized polymers [13,14]. It was found that heparin is mainly monomeric in solution at ca. 2.5 wt.% concentration and does not aggregate on prolonged standing.

Recently, some heparin lots, both unfractionated and LMWHs, have been contaminated with oversulfated chondroitin sulfate (OSCS) [15], the impurity being linked with adverse clinical events [16]. In a timely study, the chemical stability of OSCS in five depolymerization reactions, similar to those used in the preparation of LMWHs, has been investigated [17]. It was shown that base-catalysed β-elimination partially degrades OSCS, and hydrogen peroxide treatment results in its complete degradation, whereas nitrous acid, heparin lyase and periodate oxidation treatments leave the OSCS essentially intact. Therefore it was tentatively suggested that depolymerized OSCS could be a possible contaminant

Abbreviations: GAG, glycosaminoglycan; LMWHs, low molecular weight heparin(s); UFHs, unfractionated heparin(s); DOSY, diffusion ordered spectroscopy; OSCS, oversulfated chondroitin sulfate; CE, capillary electrophoresis; GE, gel electrophoresis; NMR, nuclear magnetic resonance; DS, dermatan sulfate; MW, molecular weight; API, active pharmaceutical ingredient.

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doi:10.1016/j.jpba.2010.03.037

of pharmaceuticals with enoxaparin or ardeparin as the APIs [18]. In the same way, dermatan sulfate (DS) may depolymerise and its polydisperse degradation products might be present as contaminants in LMWHs used as API. Data concerning the degradation of DS and heparin under enzymatic treatment by chondroitin lyases [19] and heparin lyases [20], respectively, have been published.

It is thus clear that the complex mixtures produced in any of the depolymerization processes of contaminated heparin, with OSCS and/or DS, would make analysis of the impurity profile of LMWHs difficult using either capillary electrophoresis or ^{1}H-1D NMR. The polydispersity of the degraded OSCS and DS prevents characterization of the contamination by the CE using known standards. More importantly, it is not clear whether the chemical shifts of the N-acetyl signals in degraded OSCS and DS coincide with the chemical shift of the OSCS and DS standards at 2.15 and 2.08 ppm, respectively.

In the past, neither the USP nor the Ph. Eur. monographs provided methods for identification and/or quantification of OSCS. Therefore, the regulatory authorities have suggested that LMWHs and UFH are examined by ^{1}H-1D NMR, utilizing the established differences in the N-acetyl chemical shifts of the major component of the finished pharmaceutical product, UFH (2.04 ppm), the process related impurity DS (2.08 ppm) and the contaminant OSCS (2.15 ppm). A general Ph. Eur. chapter on ^{1}H-1D NMR has been elaborated [21] and a protocol for assessment of OSCS in unfractionated heparin sodium has been published [22]. In addition, the USP monograph now contains a protocol for a separation method based on anion exchange chromatography for the assessment of heparin contamination with OSCS [23].

We have recently shown how DOSY can be used in the assessment of OSCS contaminant [18]. From the considerations discussed above it seemed likely that separately neither separations methods nor ^{1}H-1D NMR alone can guarantee reliable evaluation of contaminants for heparin pharmaceuticals. It is possible to devise a method which will label each signal at given chemical shift in the ^{1}H-1D NMR spectrum with an approximate particle size of the species it represents, by using the translational diffusion coefficient, D_i (m^2/s), which is readily available from DOSY NMR. DOSY combines information which is specific to ^{1}H-1D NMR chemical shifts and related to molecular weight distribution for a given species (see Section 3). It offers therefore in one experiment an orthogonal approach to the assessment of polydispersed contaminants in heparin pharmaceuticals [18] as compared to other strategies which utilise separately or combined, CE, anion exchange chromatography and 1D and/or 2D NMR [6,12,23,22]. Some questions in the application of the method still remain unanswered. While we have already addressed several problems which should be clarified before eventually implementing the method into routine pharmaceutical practice, i.e. defining the D_i values of reference materials in standard conditions or the effect on its D_i value of spiking pure heparin with OSCS or DS [16], additional answers are required. These concern the unambiguous identification of polydisperse contaminants such as OSCS, or natural DS both in UFH and in depolymerised LMWHs. These issues we address in the present contribution. Here we present a protocol which enables an assessment of all polydisperse chemical entities in heparin APIs by examining in ^{1}H NMR the diffusion coefficient labelled chemical shifts of OSCS, DS and heparin.

2. Materials and methods

2.1. Experimental details

Some batches of heparins used were collected from international markets by office of General Pharmaceutical Inspector. Heparin samples A–L were part of an investigative panel of samples supplied by the National Institute for Biological Standards and Control (NIBSC).

The ^{1}H NMR spectra were run on VARIAN NMR SYSTEM 500 MHz spectrometer using Nalorac ID probe equipped with the 60 Gauss/cm z gradient unit, at 25 °C. The chemical shifts were referenced *vs* internal TSPA standard dissolved in D_2O.

Free induction data of 32 K complex points were summations of 16, 64 or 256 acquisitions recorded with 90° pulse widths, 8000 sweep widths and 8 s relaxation delays. Data sets were archived to computer disk and apodized with an exponential window function using a 0.3 Hz line broadening factor before Fourier transformation into spectra and manual phase correction into pure absorption mode. Presaturation of residual HDO was not applied to avoid the influence on signal intensity.

2.2. Sample preparation and handling

All samples were prepared as solutions of ca. 16 ± 3 mg/ml of D_2O, or as otherwise indicated, with internal TSPA standard. Prior to measurements samples were allowed to reach temperature equilibrium in the NMR probe for at least 0.5 h until desired resolution on TSPA signal (ca. 1 Hz) was obtained. The equilibration step was particularly important when spiking experiments with OSCS and DS were performed and with UFHs.

2.3. Pulsed gradient spin echo (PGSE)

Experiments were performed according to the following conditions: 16 spectra were acquired using the convention compensated double stimulated echo pulse sequence [24] without suppression of residual HDO. The z-gradient coil constant was calibrated using known diffusion coefficients of water ($D_t = 19.02 \times 10^{-10}$ m^2/s) [25]. The gradient strengths were incremented as a square dependence in the range from 2 to 50 Gauss/cm in 16 steps. The diffusion time (Δ) and the duration of magnetic field gradients (δ) were 300 and 2.5 ms, respectively. Other parameters include the following: a sweep width of 8000 Hz, 32 K complex data points, 64 scans and an acquisition time 2 s and relaxation delay of 2 s and 16 dummy scans. In order to achieve resolution enhancement sufficient to discriminate between components of the composite signal at 2.05 ppm in the heparin spectra [26], a line broadening factor of −1.2 and Gaussian factor of 0.4 were used for apodization; exponential line broadening with a factor of 1.0 gave the appearance of a single broad line. The data were processed using either Varian implemented DOSY [27] for resolution enhancement presentation or DECRA procedure [28] (obtained from M. Nillson as a DOSYToolbox. v._0.54_15Mar08 package) in a purpose to obtain an averaged diffusion coefficient by fitting the data with one exponential function. D_i values obtained in this way are presented in Tables. It should be noted that, in general, the DECRA procedure is originally designed to perform multi exponential analysis for multicomponent mixtures. Most probably due to severe line overlap this procedure does not work in a present case. In Figs. 1–4 we have therefore presented DOSY results with resolution enhanced ^{1}H NMR of N-Ac signal in a top projection and related dispersion of D_i values for each line in a side projection.

It is essential that the data are processed in the same way if the spectra have to be compared.

3. Results and discussion

Here we show how the DOSY can be applied as a routine method for screening different lots of heparins for obtaining an impurity profile, and approximation of the population weighted, average molecular size of heparin products used as API.

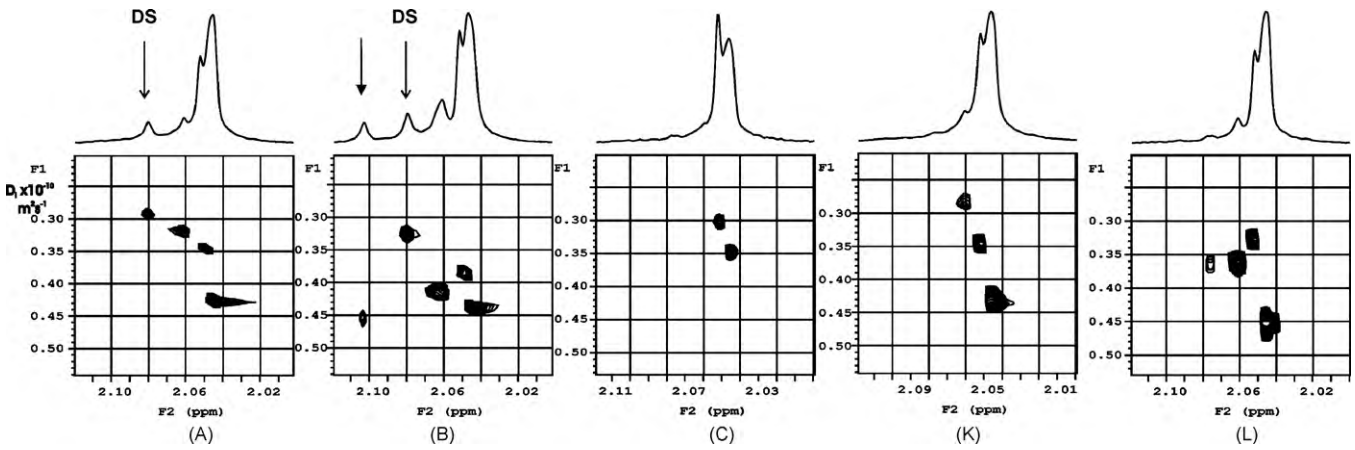

Fig. 1. Diffusion profiles for market unfractionated heparins (UFH), candidates for API standards (N-Ac signal shown). Concentrations were as follows: heparin A (16.2 mg/ml D_2O + 0.2% OSCS + 1.5% DS); heparin B (16.2 mg/ ml + 0.18% OSCS + 1.64% DS); heparin C (11.1 mg/ml); heparin K (15.6 mg/ml); heparin L (16.6 mg/ml). Open arrow shows spiked DS signal. Full arrows indicate the unidentified contaminant at 2.10 ppm originally present in heparin B.

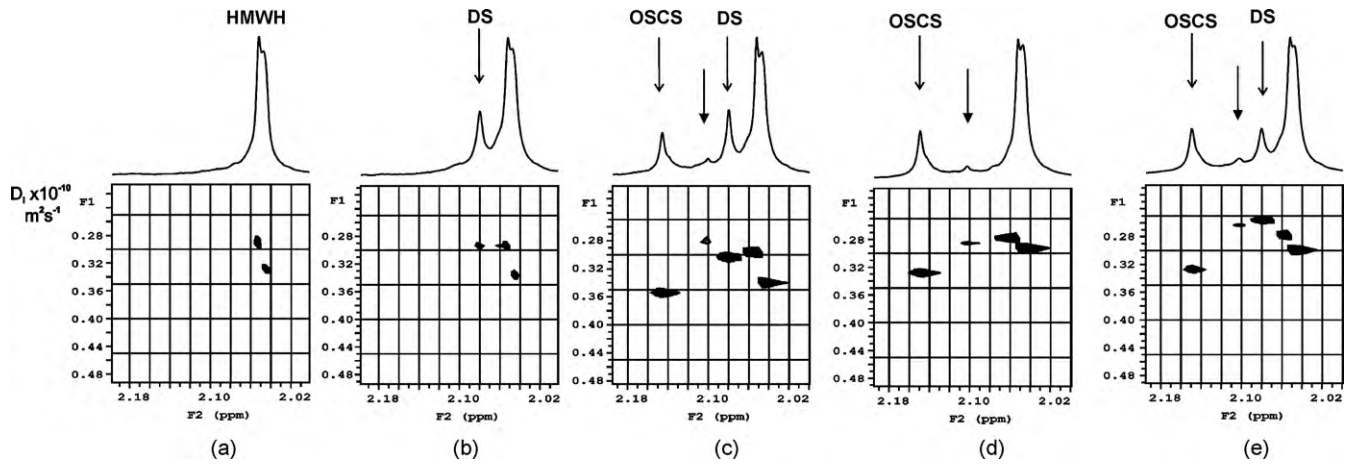

Fig. 2. Spiking experiments of heparin C at different concentrations with the similar concentrations of OSCS and increasing concentrations of DS. Concentrations were as follows: (a) 11.05 mg heparin C; (b) 11.05 mg heparin C + 4.4% DS; (c) 11.05 mg heparin C + 4.4% DS + 4.7% OSCS; (d) 20.74 mg heparin C + 5.2% OSCS; (e) 20.74 mg heparin C + 5.2% OSCS + 7.6% DS.

In a first instance we have checked the concentration dependence of UHF heparin A and B. A small, linear decrease of D_i with increasing concentration, is expected because of friction, as a result of crowding of a solute species [29]. Such a situation is observed in Table 1. This concentration dependence was also established for the OSCS and dermatan, DS, shown in Table 1, as these are the species which are of interest as major contaminants of API in the heparin pharmaceuticals. Furthermore, different concentrations of UFH with respect to the OSCS or DS in the contaminated samples have to be taken into account if quantitative comparisons of a diffu-

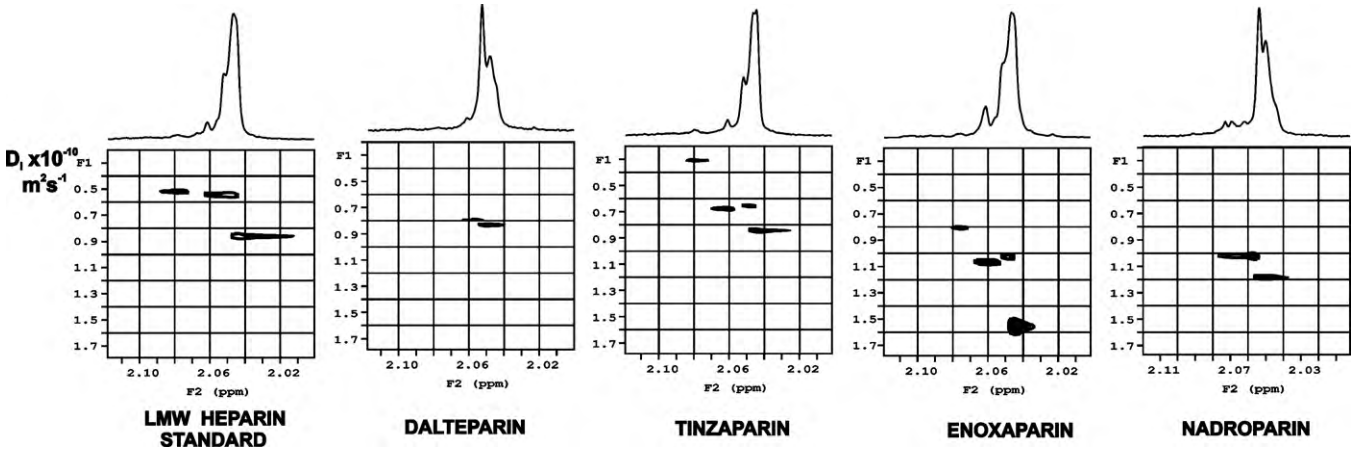

Fig. 3. Line shape of LMWH *vs* their diffusion coefficients, $D_i \times 10^{-10}$ m^2/s. Concentrations were as follows: LMW, 16.42 mg/ml; dalteparin, 17.69 mg/ml; tinzaparin, 16.64 mg/ml; enoxoparin, 27.73 mg/ml; nadroparin, 15.48 mg/ml.

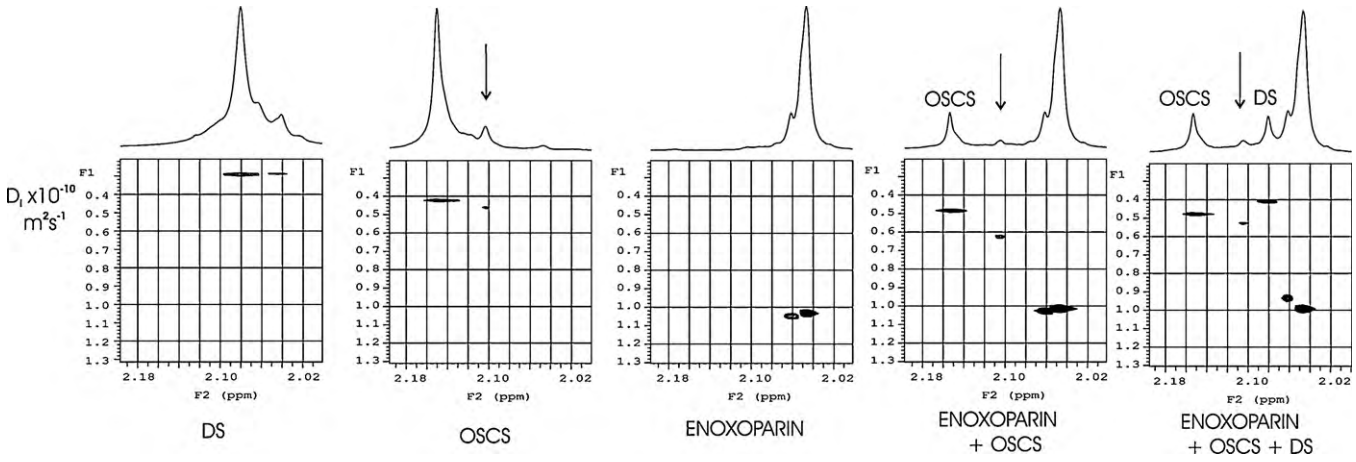

Fig. 4. Translation diffusion coefficient D_i, from left to right, for DS (16.6 mg/ml), OSCS (16.2 mg/ml), enoxaparin (27.7 mg/ml), enoxaparin (27.7 mg/ml) + 5% OSCS and enoxaparin (27.7 mg/ml) + 5% OSCS + 2.6% DS. Full arrow marks unidentified contaminant present in OSCS.

Table 1
Concentration (C) dependence of measured diffusion coefficient D_i for UFH A, UFH B, OSCS and DS.

UFH A		UFH B		OSCS		DS	
C (mg/ml)	$D_i \times 10^{-10}$ (m^2/s)	C (mg/ml)	$D_i \times 10^{-10}$ (m^2/s)	C (mg/ml)	$D_i \times 10^{-10}$ (m^2/s)	C (mg/ml)	$D_i \times 10^{-10}$ (m^2/s)
57.06	0.280	26.32	0.408	–	–	–	–
35.21	0.347	23.49	0.422	–	–	–	–
16.16	0.390	16.23	0.449	16.24	0.420	16.63	0.284
6.19	0.401	6.26	0.484	3.13	0.438	–	–
0.87	0.469	1.04	0.467	0.24	0.457	0.26	0.368

sion coefficient are made. During this study the concentration used in all samples was of the order of 14 ± 3 mg/ml, or, as otherwise indicated.

In a following step the N-acetyl signals of the OSCS and DS were labelled with diffusion coefficients in UFH and LMWHs in D_2O. Both parameters were obtained by spiking standards of both heparins with a stock solution of the DS and OSCS.

The DOSY spectra of standard UFHs are shown in Fig. 1. The N-Ac signals processed under conditions of resolution enhancement show several closely lying peaks with D_i value within $0.39–0.45 \times 10^{-10}$ m^2/s. This spectrum can be considered as a fingerprint of each UHF heparin. In general, in all heparins the main N-Ac signal consists of a low frequency broad component centered at 2.046 ppm, and high frequency sharper one, centered at 2.052 ppm, characterized by smaller D_i value. It is important to note that, despite their average strong overlapping in the ^{1}H NMR frequency scale, at 2.05 ppm, all heparins differ in D_i coefficient which most likely reflects a dispersion of components differing in molecular weight. Labeling the N-Ac signal with D_i is therefore the ID label, at a given spectrometer frequency, of the source of each heparin. The processing of spectra shown in Fig. 1 was done under conditions of resolution enhancement (see Section 2.1, also Fig. 1S). It is therefore possible to partly resolve closely lying components. For routine heparin identification one can use line broadening which gives essentially one broader line characterized by an 'average' D_i coefficient. These values for each heparin are cited in Table 2. It is seen from Table 2 that UFHs are characterized by D_i coefficient $0.40 \pm 0.05 \times 10^{-10}$ m^2/s. There is one noticeable exception of heparin C which has the D_i value 0.30×10^{-10} m^2/s. The explanation can be deduced from Fig. 1 which shows that heparin C is the most homogenous with respect to a number of components and high frequency component, having smaller D_i value, has the highest population among of all heparins.

Table 2
Averaged diffusion coefficients D_i (av) of commercial UFHs and LMWHs[a,b].

HEPARIN	Concentration (mg/ml)	D_i (av)
A	16.16	0.39
B	16.23	0.45
C	11.05	0.31
D (1% OSCS)	13.42	0.39
E (0.5% OSCS)	13.06	0.40
F (2% OSCS)	14.34	0.41
G (2.5% OSCS)	15.16	0.41
H (4% OSCS)	13.39	0.41
L	16.61	0.40
K	15.55	0.40
LMWH mass calibration	16.42	0.88
Dalteparin sodium	17.69 (5600–6400)	0.82
Tinzaparin sodium	16.64 (6500 average)	0.81
Enoxoparin sodium	27.73 (3800–5000)	1.03
Nadroparin calcium	15.48 (3600–5000)	1.15

[a] Heparins D–H contained originally indicated amount of OSCS (see Fig. 1S for the ^{1}H NMR of N-Ac signal).
[b] The ranges of molecular weight for LMWHs are taken from relevant monographs in *European Pharmacopoeia, 6th Edition, 2008*.

We have spiked heparins A and B with the OSCS at LD level (0.2 weight %) and with DS at the level of 1.5%. The DS signal appears at expected position in both heparins A and B.

The heparin B contains an unidentified signal at 2.10 ppm (full arrow) with D_i 0.45×10^{-10} m^2/s. Both values are the same as found for the small component appearing in OSCS (see Fig. 4). For further studies heparin C was chosen because it is most homogenous of all examined and therefore the positions of spiked OSCS and DS should not be influenced by minor contaminants present in other heparins.

Fig. 2 shows the results of spiking UFH C with DS and OSCS in an attempt to identify these contaminants simultaneously in the presence of API heparin and verify the influence of concentration on contaminant position on the diffusion scale.

Spiking UFH C, shown in Fig. 2, allows important observations which could be useful in routine application of this method to the assessment of the contaminants in heparin pharmaceuticals. It appears that the diffusion parameter depends on several factors, making the method sensitive to different phenomenas. The value of this method therefore depends on relevant precautions which must be observed in its application.

Firstly, the position on a diffusion scale of the main signal at 2.05 ppm depends on concentration, as discussed above. As expected, in the spectra 2d and 2e heparin C (Fig. 2) has smaller D_i value, than in 2 a–c, due to the friction effect at higher concentration. The dispersion pattern of the main signal is reproducible at the same concentration but differs at different concentrations. It seems therefore essential to use the same concentrations if the identity of a heparin has to be assessed *vs* a given reference standard, especially at the high concentrations (>40 mg/ml) recommended for OSCS identification and quantification by NMR [20].

Secondly, at given concentration of a heparin the position of the contaminants on a diffusion scale is constant, irrespective of the presence of other contaminants. This is obvious from comparison of the spectra 2b and 2c for the signal of the DS and the spectra 2d and 2e for the OSCS signal.

Thirdly, comparison of the contaminant position on a diffusion scale can be misleading if the concentration of heparin differs substantially. It is clear from a comparison of the OSCS position in the spectra 2c and 2e, in which the OSCS concentration is comparable, but heparin C concentration increased twice going from the spectra 2c to 2e. This effect is most likely due to overlap of a Lorentzian line shape of closely lying signals, i.e. the OSCS at 2.15 ppm with heparin C at 2.05 ppm, and is enhanced with a higher concentration of the heparin C. This effect is even more pronounced for the DS signal which lies closer to the heparin signal than the OSCS signal.

Spiking the heparins with OSCS introduces a new signal in the analysed region, at 2.10 ppm, marked with full arrow (see to Fig. 4).

The origin of the species to which this signal belongs is not yet clear. It has the same D_i coefficient as OSCS, 0.45×10^{-10} m^2/s (Fig. 4), and an impurity at 2.10 ppm in the uncontaminated heparin B (Fig. 1) suggesting a common origin of both. In Fig. 2 this contaminant appears at ca. 0.28×10^{-10} m^2/s, this value tends to be smaller when going from the spectra 2c and 2d to Fig. 2e, most likely due to overlap from the DS and heparin signals and the higher heparin concentration in Fig. 2e.

In Fig. 3 the shapes of the LMWH's N-Ac signal under resolution enhancement processing are shown which allow the observation of molecular weight dispersion in each heparin. Unlike the case of UFH, where a signal profile was similar for all heparins, in the case of LMW heparins the signal profile is characteristic for the method of depolymerization. Thus, dalteparin and nadroparin, both processed under conditions of deaminative cleavage with nitrous acid, have much the same chemical shift profile. The same is seen in the case of tinzaparin and enoxaparin, both being processed in β-eliminative cleavage condition, although the former by heparinase digestion and the latter in alkaline conditions. More importantly, the presented method can be used not only to distinguish between degradation processes but also for the method of fractionation of resultant product. Thus nadroparin, with D_i value 1.15×10^{-10} m^2/s consists of species with lower molecular weight than dalteparin having D_i 0.82×10^{-10} m^2/s. This is in agreement with the European Pharmacopoeia data cited in Table 2 according to which the average molecular weight for dalteparin is higher than for nadroparin. Even more pronounced is this effect in a case of tinzaparin and enoxaparin. The latter one is the least homogeneous with respect to a molecular weight dispersity having components characterized with very high D_i coefficient, 1.6×10^{-10} m^2/s.

In Fig. 4 the assessment of contaminants in LMWHs is presented. Here the impurity profile is very sensitive to concentrations and chemical shift differences as we merge the components of very different molecular weights. As we have already presented in our recent account [18] spiking the enoxaparin with OSCS results in minor change of its diffusion coefficient, most likely because the signal is sufficiently distant from an enoxaparin main signal. Because of this it is a straightforward task to distinguish the original OSCS from its degradation product which can be a potential contaminant in LMWHs if UFH, contaminated with OSCS, was subjected to β-eliminative degradation in alkaline conditions. The unidentified impurity at 2.10 ppm, introduced with OSCS, appears more severely affected as it is much closer and an intense overlap with a tail of the enoxaparin Lorentzian signal occurs. A similar situation is observed with a signal of spiked DS which overlaps severely with the enoxaparin and its D_i coefficient acquires larger value. The impurity at 2.10 ppm is affected in diverse directions by both the DS and enoxaparin. It should be mentioned that these interactions do not affect the proper assessment of impurities as the origin of a line can be verified by spiking the contaminant. Another solution to the problem is an application of a higher NMR frequency giving higher chemical shift dispersion.

DOSY NMR, that measures the translational diffusion coefficient D_i, is a powerful method for analyzing mixtures of chemical species in solution [27,30]. Larger molecules diffuse typically more slowly than smaller ones. Each spectral line in the ^{1}H-1D NMR spectrum of a given chemical entity should be characterized by the same D_i, providing the line is separated from spectral lines of other species in solution, which are characterized by their own D_i. The N-acetyl signal for which diffusion coefficients are monitored in this work consists of closely lying overlapping singlets (easily exposed under resolution enhancement procedures) reflecting polydispersity of a given heparin in a narrow range of molecular weights. It should therefore be noted that the measured diffusion coefficient D_i for heparin is an average for the ensemble of polydispersed species in the narrow molecular weight range which it represents.

Separation of the spectral lines is better achieved with higher magnetic fields. The larger the difference in MW of the two species, the larger the difference in D_i between them. This method can therefore be applied to differentiating between unfractionated heparins, the OSCS impurity of ca. 18 kDa MW [17] and the LMWHs of ca. 4–8 kDa. Because the D_i values may be measured at different fields, which affects the degree of overlap of closely lying components of a signal at 2.04 ppm, one should also be aware of the fact that the appearance of the 2D DOSY signal, as presented here in Figs. 1–4, can be field dependent whereas D_i values are not [22]. The interpretation of the data from DOSY requires therefore taking into consideration not only the MW of the species in solution but also the concentration dependence of D_i value and possible overlap of closely lying signals. A nonbonding interaction of the species in solution can also influence the D_i value, especially if one of these is present in large excess with respect to the other.

Table 1 shows the dependence of D_i values on sample concentration. The decrease of D_i value with increasing concentration is mainly assigned to viscosity, but also solute interaction, as mentioned above. There is an approach published recently [31] allowing obtaining values of D_i at infinite dilution, applied to the identification of homogeneous molecular species. It is based on comparison of D_i values of neat solvent and its value in a presence of a solute. In the present case this attractive approach is not easy to use, for a number of reasons. Firstly, the water signal is overlapped with heparin signals and involved in chemical exchange with heparin hydroxyls. Secondly, the difference in size and relaxation behaviour between the polysaccharide solutes and the solvent, or other small molecule in the solution such as TSPA, is considerable. Long recycle times between transients, and reduced concentration of solute to reduce viscosity, would make the procedure impractically lengthy for use in the routine screening of commercial heparin samples.

For this reason we suggest to use samples of heparins of the same concentration, at carefully controlled temperature as in Table 3. Our results provide an empirical demonstration of the utility of this simple approach.

Our observations confirm that comparisons between LMWH prepared by similar processes, and so giving rise to ^{1}H NMR spectra similar in appearance, can be made using the DOSY technique. For example, a comparison of the ^{1}H spectra of dalteparin and nadroparin suggests they are more similar than products produced by different processes [32,33], but differences in D_i due to their very different molecular weight distributions give rise to distinct DOSY spectra. Similarly, comparison of the DOSY spectra of enoxaparin and tinzaparin, both beta-elimination products, shows the considerable differences between their molecular weight profiles. Though the depolymerisation processes have an effect on the therapeutic properties of LMWHs, their anticoagulant activity profiles are more strongly dependent on their molecular weight distributions, therefore one can link the different biological properties inherent in each LMWH to their diffusion coefficients. This reasoning seems to be naturally confirmed by an established consensus that biological properties of LMWH are to a large extent dependent on the specific polydispersity of each product. As might also be expected, products prepared by distinctly different processes are dissimilar in physical, chemical, and biological properties [34].

4. Conclusion

In this account we have presented a new NMR method, based on the DOSY technique (diffusion ordered spectroscopy), for tracing the profile of molecular weight dispersion in unfractionated and depolymerised heparins and the assessment of contaminants in both types of these pharmaceuticals. Labelling the N-Ac signal in heparins with diffusion coefficient, D_i, allows the distinction between species which are indistinguishable by ^{1}H NMR chemical shift criteria but differ in their molecular weight. By doing so we have assigned to the N-Ac signals in a region 2.0–2.20 ppm in NMR spectrum of heparin a double label identifying the origin of the species to which the signal belongs, characterized by a given chemical shift, and a link to a molecular weight information by means of its diffusion coefficient, D_i. Thus the method combines ^{1}H NMR and size-based separations techniques.

These results demonstrate that ^{1}H NMR can be used for screening market samples for contamination with OSCS, DS or other yet not identified contaminants. It should be noted that the interpretation of the D_i values, likewise the interpretation of ^{1}H NMR chemical shifts, requires consideration of the overlap of closely lying spectral lines which can change their true D_i value, and more importantly, one needs to be aware that ^{1}H chemical shift can be identical for two species differing markedly in molecular weight. Finally, non-covalent binding of the two species in solution can influence the true D_i and chemical shift values. It appears then that the diffusion parameter depends on several factors, making the method sensitive to different phenomenas. This method may be of considerable utility, providing the relevant precautions are observed with its application. A full investigation is ongoing.

Acknowledgements

L.K. gratefully acknowledges the EDQM (European Directorate for Medicines Quality and Health Care, European Council, Strasbourg) authorities, in particular Jean-Marc Spieser and Richard Wanko, for their decision to include National Medicines Institute's NMR laboratory in a comparative study on heparins during which this contribution was created and the authors profited from discussions therein.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2010.03.037.

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