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Quality assessment of unfractionated heparin using ^{1}H nuclear magnetic resonance spectroscopy

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ABSTRACT

Due to problems, especially anaphylactoid reactions, raised by impure unfractionated heparin the quality assessment of heparin has to be reconsidered. Neither the USP nor the European Pharmacopoeia are able to guarantee the purity of heparin, i.e., the limitation of oversulfated chondroitin sulfate (OSCS) which was found to be the reason for the allergic adverse effects. In the first run the regulatory authorities ask for ^{1}H NMR spectroscopic and capillary electrophoretic measurements in order to characterize the impurity profile of heparin. Using an optimized ^{1}H NMR method the limit of detection for OSCS was found to be 0.1%. In addition, it is possible to reliably quantify both OSCS and dermatan sulfate (DS), the latter being an indicator of poor purification of the unfractionated heparin. Screening of more than 100 heparin samples collected from international markets revealed a high number of samples containing substantial amounts of DS and a number of samples containing OSCS in an amount higher than 0.1%.

1. Introduction

The unfractionated heparin calcium and sodium are preparations containing the calcium or sodium salt of a sulfated glucosaminoglycan present in mammalian tissues. It is prepared mostly from the intestinal mucosae of oxen, pigs or sheep [1]. The 10–15 glucosaminoglycans, each containing 200–300 monosaccharide units, result in a molecular mass of 750–1000 kDa. The glucosaminoglycan can undergo a series of modifications including *N*-deacetylation and *N*-sulfation of the glucosamine, epimerization of D-glucuronic acid to L-iduronic acid, *O*-sulfation of iduronic and glucuronic acid residues at position C2, and *O*-sulfation of the glucosamine at C3 and C6 position (see Fig. 1). Additionally, the final product may contain acetic acid and sulfuric acid.

Unfractionated heparin is produced via a series of protein denaturation procedures by means of NaOH/ammonium sulfate and trypsin, filtration and precipitation as barium and eventually as sodium or calcium salts. Due to the structural similarity heparin always contains varying amounts of dermatan sulfate (DS). Moreover, the content of DS is an indicator of the quality of the purification.

Heparin is widely used in dialysis settings and in initial treatment of venous thrombosis, pulmonary embolism and acute coronary syndrome. Beside the potential bleeding risk the most serious side effect is the heparin-induced thrombocytopenia [2]. Recently, hundreds of cases of adverse effects of anaphylactoid character in addition to several deaths were reported to the Food and Drug Administration in USA and to the competent European authorities in Germany, France, Italy and UK (see Ad hoc heparin meeting [3]). These serious adverse effects [4] could be assigned by means of NMR spectroscopy to the contaminant oversulfated chondroitin sulfate (OSCS) [5] using sophisticated two-dimensional NMR techniques [6]. Since OSCS is a synthetic glucosaminoglycan product it must have been added to the heparin deliberately. Preliminary screening of contaminated heparin batches recently collected from different sources by means of ^{1}H NMR spectroscopy and capillary electrophoresis [7] revealed four different groups, i.e., pure heparin with almost no DS, heparin-containing DS and chondroitin sulfate A and C in varying amounts, heparin with OSCS, and heparin with OSCS and varying amounts of DS and chondroitin sulfate A and C. Most interestingly, in 1998 Holzgrabe et al. published ^{1}H NMR spectra of heparin which already contained the contaminant OSCS [8].

Since the methods currently described in the European Pharmacopoeia (EP6 [1]) are not able to prove the quality of heparin it is necessary to look for new methods which are able to limit the contaminant OSCS and to evaluate the content of DS. Here we report

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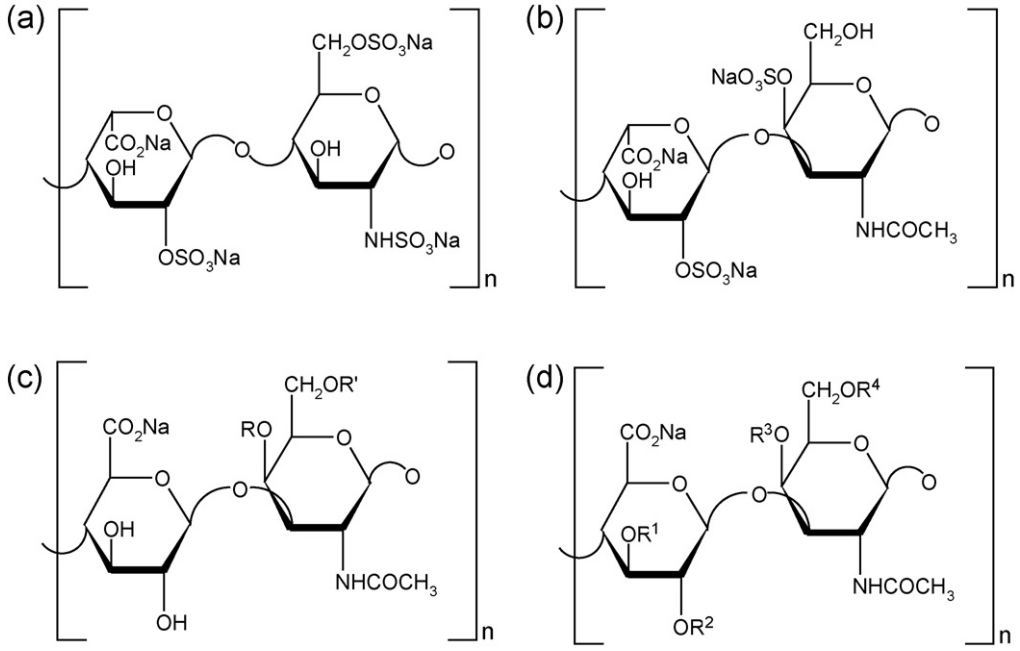

Fig. 1. Structural formulae of heparin (a), dermatan sulfate (b), chondroitin sulfate A and C (c), and oversulfated chondroitin sulfate (d). For chondroitin sulfate A, R marks the sulfated moiety, as for chondroitin sulfate C the residual group R′ is sulfated. For OSCS, R^1–R^4 label possibly sulfated moieties.

on the optimization and partially validation of the ^{1}H NMR spectroscopic methods which are able to limit the contaminant OSCS to 0.1% and to assess the amount of DS. Additionally, a statistical evaluation of more than 100 batches is presented.

2. Experimental

2.1. Instrumentation

^{1}H NMR spectra were recorded on a Bruker Avance 400 MHz and Bruker Avance 300 MHz spectrometer operating at 400.13 and 300.133 MHz, respectively, using 5 mm probes. All data were processed using Bruker's XWIN-NMR software (version 3.5, Bruker Analytik, Rheinstetten, Germany).

2.2. Quantitative NMR spectroscopy

For the 400 MHz ^{1}H NMR spectra of all heparin batches analyzed, 32 scans were collected into 64 K data points over a spectral width of 4789 Hz (12 ppm) with the transmitter offset at 5.00 ppm, yielding a digital resolution of 0.15 Hz per point. The acquisition time was 6.84 s, followed by a relaxation delay of 1 s, resulting in a total pulse recycle time of 7.84 s to ensure full T_1 relaxation of the *N*-acetyl signal of heparin and its impurities (T_1 relaxation values were determined to be about 1.44 s for all components). All spectra were recorded at 315 K (for better signal shape and shifting the HOD signal out of the fingerprint region) using a flip angle of 30° and the chemical shifts were reported to trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) as external standard at 0.00 ppm. An exponential line broadening window function of 0.3 Hz was used in the data processing before Fourier transformation. Baseline corrections were mostly done automatically, only in a very few cases it was additionally manually operated. Phasing was always performed manually.

For the determination of the limit of detection of OSCS the spectra were acquired using 90° pulses with 128 scans, all other parameters were set as mentioned above.

^{1}H NMR high-temperature spectra at 353 K were measured at 300.13 MHz using a QMP probe. Thirty two scans were collected into 64 K data points over a spectral width of 6172.84 Hz (20.56 ppm). A flip angle of 30° was used. The acquisition time was 5.31 s, followed by a relaxation delay of 1 s. An exponential line broadening window function of 0.3 Hz was used in the data processing.

2.3. Chemicals

The batches of unfractionated heparin were collected from international markets by the Federal Institute of Drugs and Medical Devices (BfArM, Bonn, Germany). Batches of oversulfated chondroitin sulfate were provided by Sanofi-Aventis and US Food and Drug Administration. Deuterium oxide (99.9% D) was purchased from Euriso-top (Saarbrücken, Germany). Dermatan sulfate was purchased from Sigma–Aldrich (Steinheim, Germany).

2.4. Solutions

For characterization of the heparin batches an amount of 25 mg of the corresponding heparin batch was dissolved in 700 μl D_2O. Thereof a standardized sample volume of 650 μl was transferred to a NMR tube and measured at 400 MHz.

In order to determine the LOD of OSCS different predefined amounts of the contaminant were spiked to pure samples of heparin with a concentration of 25 mg/700 μl. Again, a standardized sample volume of 650 μl was transferred to a NMR tube and measured at 400 MHz.

For the quantification of DS 30 mg of heparin sodium were dissolved in 1 ml D_2O and constantly shaken for several minutes. The samples were transferred into a 5-mm NMR tube. For quantification of DS 30 mg of heparin sodium was spiked with 2, 4, 6, and 10% DS reference compound. Therefore, a stock solution was prepared by dissolving 30 mg DS in 2970 mg D_2O. Reproducibility was tested by analyzing a heparin test item six times using individual initial weights.

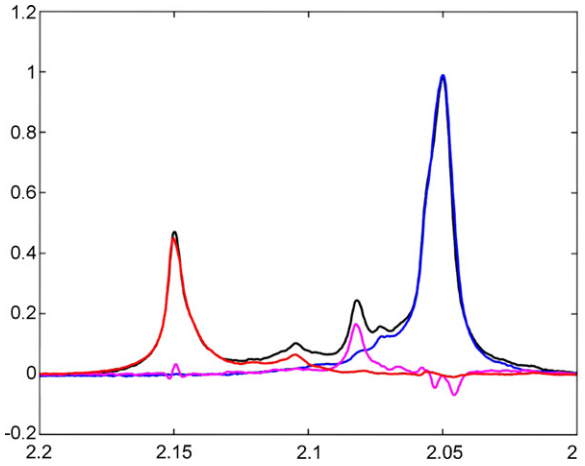

Fig. 2. Decomposition of spectrum 89 (black line) into the heparin *N*-acetyl (blue), the DS signal (magenta), and OSCS signal (red). Relative concentrations are 0.09 and 0.44 for DS and OSCS, respectively.

2.5. Numerical data analysis

Data analysis has been done using MatLab 7.4 (The MathWorks Inc., USA). Prior to multivariate statistics and quantification, spectra were aligned and normalized to the maximum of the NMR signal of the *N*-acetyl groups resonating between $\delta = 2.02$ and 2.08 ppm. Bucketing of ^{1}H NMR spectra was performed by segmentation of the spectra into $N = 60$ consecutive integrated spectral regions (buckets) of fixed bucket width (0.005 ppm) covering the range from 2.0 to 2.3 ppm. Principal component analysis (PCA) on the bucket table (Table 1) was performed using the standard MatLab function PRINCOMP.

Spectral deconvolution and quantification was done using in-house developed MatLab routines. Both deconvolution and quantification was done locally within the region from 2.0 to 2.2 ppm. Two reference spectra were defined, i.e., spectrum 25 and 19 representing the situation in pure heparin and heparin strongly polluted with OSCS but without DS. In order to separate the signals from heparin *N*-acetyl, OSCS and DS, spectrum 25 was scaled to the respective intensities of the *N*-acetyl signals of the different samples via least squares fit and subtracted from all other spectra. As a result, within the 2.0–2.2 ppm only pure OSCS and DS signals remained, while residual contributions of *N*-acetyl signals were small and negligible. In a second step, the same procedure was repeated using the residue of spectrum 19 for fitting and subtraction. In this way, signal intensities of OSCS and DS could be separated and quantified with reference to the *N*-acetyl signal (see Fig. 2).

3. Results and discussion

Due to the purification procedure many heparin batches contain the solvents methanol (signal at $\delta = \sim 3.4$ ppm, singlet) and ethanol (signal at $\delta = \sim 1.2$ ppm, triplet) as well as acetate (signal at $\delta = \sim 1.9$ ppm, singlet) in varying amounts. Fig. 3 displays a heparin sample containing OSCS, DS and the solvents.

The spectra of heparin, OSCS and the various dermatan sulfates can be inspected using the signals of the *N*-acetyl groups resonating between $\delta = 2.0$ and 2.3 ppm (see Fig. 4) or/and the signals in the fingerprint region between $\delta = 4.4$ and 5.6 ppm (see Fig. 5). Especially for the latter evaluation it is advantageous to perform the measurements at higher temperature, e.g., at 315 or even better at 353 K, in order to shift the HDO signal out of the region to $\delta = 4.2$ ppm (at room temperature 4.8 ppm).

Table 1
Results of relative quantification

Sample	Scaling	Heparin (2.05 ppm)	DS	OSCS	Rel. DS	Rel. OSC
1	1.70E+06	82.51	0.91	0	0.01	0
2	1.51E+06	82.51	0.52	0	0.01	0
3	1.42E+06	82.51	1.46	1.34	0.02	0.02
4	1.33E+06	82.51	2.14	47.85	0.03	0.58
5	1.02E+06	82.51	2.66	41.99	0.03	0.51
6	8.88E+05	82.51	0.6	0	0.01	0
7	1.01E+06	82.51	9.45	0	0.11	0
8	1.15E+06	82.51	9.39	0.33	0.11	0
9	1.11E+06	82.51	16.51	2.78	0.2	0.03
10	1.76E+06	82.51	7.33	0.22	0.09	0
11	1.86E+06	82.51	6.87	0.4	0.08	0
12	1.91E+06	82.51	7.14	0.71	0.09	0.01
13	1.58E+06	82.51	8.71	39.56	0.11	0.48
14	1.81E+06	82.51	13.54	0.22	0.16	0
15	1.15E+06	82.51	13.2	0.25	0.16	0
16	1.12E+06	82.51	0.53	0	0.01	0
17	1.53E+06	82.51	0	0.65	0	0.01
18	1.45E+06	82.51	0	41.15	0	0.5
19	1.26E+06	82.51	0	99.95	0	1.21
20	1.31E+06	82.51	0	0	0	0
21	1.74E+06	82.51	0.3	0.92	0	0.01
22	1.47E+06	82.51	0.77	0	0.01	0
23	1.26E+06	82.51	1.95	44.24	0.02	0.54
24	1.35E+06	82.51	0.8	23.61	0.01	0.29
25	9.42E+05	82.51	0	0	0	0
26	1.14E+06	82.51	0	0	0	0
27	7.05E+05	82.51	0	80.91	0	0.98
28	1.16E+06	82.51	5.34	0	0.06	0
29	1.45E+06	82.51	2.92	0	0.04	0
30	1.29E+06	82.51	7.41	1.25	0.09	0.02
31	1.46E+06	82.51	9.31	0	0.11	0
32	1.15E+06	82.51	0.6	0	0.01	0
33	1.32E+06	82.51	0.26	0	0	0
34	1.49E+06	82.51	1.16	0.5	0.01	0.01
35	1.29E+06	82.51	5.76	26.95	0.07	0.33
36	1.58E+06	82.51	6.77	0.21	0.08	0
37	1.02E+06	82.51	2.51	1.29	0.03	0.02
38	1.45E+06	82.51	1.68	32.04	0.02	0.39
39	1.47E+06	82.51	1.53	30.46	0.02	0.37
40	1.52E+06	82.51	0.54	25.48	0.01	0.31
41	1.81E+06	82.51	2.63	0	0.03	0
42	1.40E+06	82.51	0.97	14.62	0.01	0.18
43	1.12E+06	82.51	1.56	0.84	0.02	0.01
44	1.20E+06	82.51	1.91	0.39	0.02	0
45	7.58E+05	82.51	0.15	0.37	0	0
46	1.28E+06	82.51	0	0	0	0
47	1.56E+06	82.51	0	0.16	0	0
48	1.58E+06	82.51	3.06	0.46	0.04	0.01
49	1.10E+06	82.51	0.5	0	0.01	0
50	1.12E+06	82.51	1.45	0.14	0.02	0
51	1.07E+06	82.51	11.35	1.82	0.14	0.02
52	1.43E+06	82.51	0	0.16	0	0
53	1.81E+06	82.51	10.11	1.35	0.12	0.02
54	1.66E+06	82.51	7.46	0.32	0.09	0
55	1.21E+06	82.51	2.6	0.23	0.03	0
56	1.44E+06	82.51	2.75	0.49	0.03	0.01
57	1.46E+06	82.51	1.52	0.52	0.02	0.01
58	1.36E+06	82.51	2.32	0.62	0.03	0.01
59	1.91E+06	82.51	7.59	0.03	0.09	0
60	8.53E+05	82.51	0	0.35	0	0
61	1.60E+06	82.51	6.31	0	0.08	0
62	1.73E+06	82.51	6.84	0.32	0.08	0
63	7.76E+05	82.51	0	40.93	0	0.5
64	9.83E+05	82.51	0.4	77.92	0	0.94
65	1.22E+06	82.51	1.76	0.2	0.02	0
66	8.64E+05	82.51	0	0	0	0
67	1.17E+06	82.51	0	13.27	0	0.16
68	9.98E+05	82.51	0	0	0	0
69	7.82E+05	82.51	13.51	31.57	0.16	0.38
70	1.26E+06	82.51	5.58	0.59	0.07	0.01
71	8.11E+05	82.51	0	0	0	0
72	1.18E+06	82.51	0	0	0	0
73	1.32E+06	82.51	9.07	0.4	0.11	0

Table 1 (*Continued*)

Sample	Scaling	Heparin (2.05 ppm)	DS	OSCS	Rel. DS	Rel. OSC
74	1.40E+06	82.51	2.03	0.9	0.02	0.01
75	1.17E+06	82.51	0.31	0.24	0	0
76	1.42E+06	82.51	11.44	1.07	0.14	0.01
77	1.36E+06	82.51	6.18	0.17	0.07	0
78	1.17E+06	82.51	28.62	12.83	0.35	0.16
79	1.32E+06	82.51	1.76	1.01	0.02	0.01
80	1.11E+06	82.51	0	12.61	0	0.15
81	1.26E+06	82.51	1.45	0.2	0.02	0
82	1.26E+06	82.51	2.18	0.22	0.03	0
83	1.05E+06	82.51	5.35	22.27	0.06	0.27
84	1.19E+06	82.51	2.17	0.4	0.03	0
85	8.30E+05	82.51	0.89	22.87	0.01	0.28
86	1.25E+06	82.51	2.54	0.84	0.03	0.01
87	1.14E+06	82.51	0.06	0	0	0
88	1.29E+06	82.51	1.31	0	0.02	0
89	1.55E+06	82.51	7.22	36.15	0.09	0.44
90	1.21E+06	82.51	3.13	1.03	0.04	0.01
91	9.22E+05	82.51	18.29	0	0.22	0
92	7.53E+05	82.51	0	0	0	0
93	1.15E+06	82.51	1	1.06	0.01	0.01
94	6.70E+05	82.51	1.67	0	0.02	0
95	1.25E+06	82.51	0.08	0	0	0
96	1.02E+06	82.51	0	1.24	0	0.02
97	7.24E+05	82.51	0.55	9.12	0.01	0.11
98	7.26E+05	82.51	0	14.24	0	0.17
99	7.13E+05	82.51	3.77	21.01	0.05	0.25
100	1.69E+06	82.51	4.19	0	0.05	0
101	1.86E+06	82.51	2.04	0.3	0.02	0
102	2.20E+06	82.51	2.65	0.3	0.03	0
103	1.24E+06	82.51	8.2	1.78	0.1	0.02
104	1.45E+06	82.51	9.69	2.39	0.12	0.03
105	1.48E+06	82.51	5.81	0.02	0.07	0
106	1.14E+06	82.51	9.79	1.71	0.12	0.02
107	1.37E+06	82.51	2.67	0.79	0.03	0.01
108	9.04E+05	82.51	3.09	0.24	0.04	0

3.1. Assignment

By means of measuring the individual components, the signals can be easily assigned: at $\delta = 2.04$ ppm the acetyl groups of chondroitin sulfate A and C appear, at $\delta = 2.05$ ppm the heparin acetyl group resonates, at $\delta = 2.08$ ppm DS can be detected and at $\delta = 2.15$ ppm OSCS can be found (see Fig. 4). In a mixture of components the signals might be slightly shifted.

Using the same procedure of individual measurement of all components, the signals in the fingerprint region can be assigned (see Fig. 5). Thus, the evaluation in the acetyl region is further supported by the contaminant signals in the fingerprint of the mixture in the region between 4.4 and 5.1 ppm (see Fig. 5).

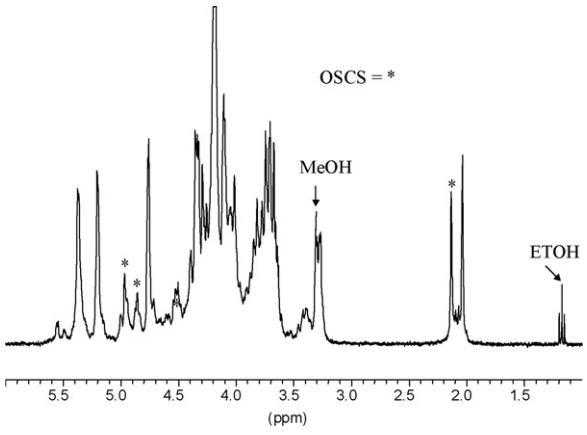

Fig. 3. ^{1}H NMR spectrum of a contaminated sample of heparin containing the solvents and the impurities DS and OSCS, 300 MHz. Signals of OSCS are assigned only.

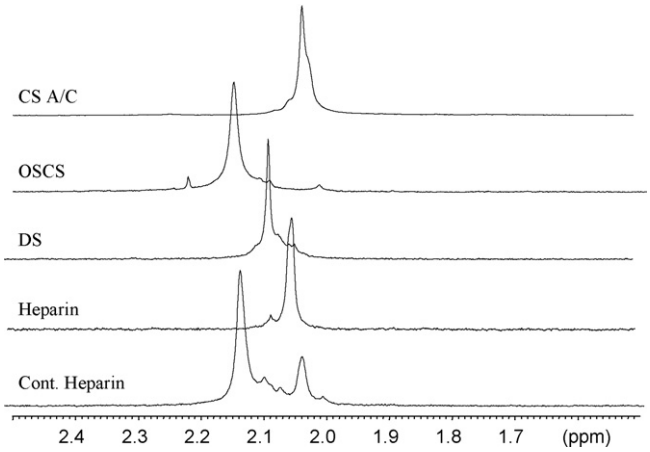

Fig. 4. ^{1}H NMR spectra (300 MHz) of the acetyl region of the chondroitin sulfate A and C (CS A/C), OCSC, DS, heparin and a contaminated heparin sample.

3.2. Limit of detection and quantification of OSCS and DS in heparin

Neither heparin nor DS or OSCS are defined molecules. Therefore a conventional quantification is impossible. Alternatively one can use the standard addition method which has the only restriction that at least one signal of heparin and the target analyte do not interfere. In case of OSCS and DS the acetyl signals are useful.

3.2.1. Limit of detection of OSCS and content evaluation

In order to determine the LOD of OSCS a stock solution of pure heparin was spiked with decreasing amounts of the contaminant, starting with 1.0%. Using this procedure we reached a final concentration of 0.1% OSCS where the contaminant could just be detected. For this concentration the intensity of the *N*-acetyl signal of OSCS at $\delta = 2.15$ ppm approximately equals the intensity of the ^{13}C satellites of heparin (see Fig. 6). Thus, with a higher number of scans and hence an improved signal-to-noise ratio, it is possible to verify for the presence of small amounts of OSCS even at frequencies of about 400 MHz.

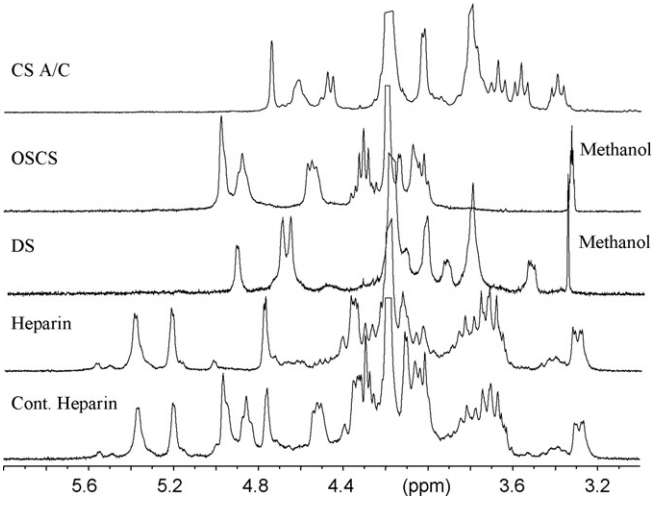

Fig. 5. ^{1}H NMR spectra (300 MHz) of the fingerprint region of the chondroitin sulfate A and C (CS A/C), OCSC, DS, heparin and a contaminated heparin sample.

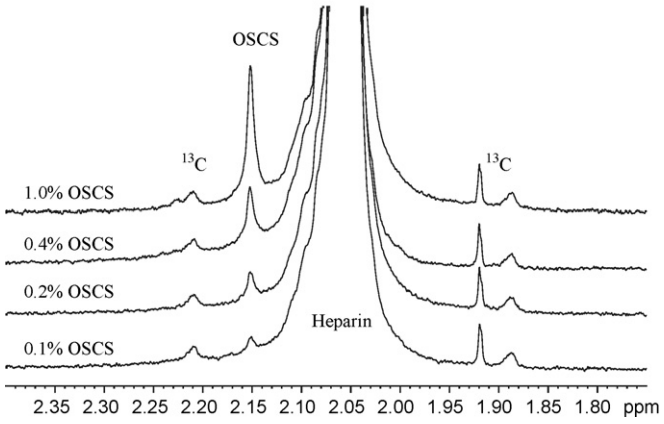

Fig. 6. *N*-Acetyl region for different amounts of OSCS added to a pure heparin sample, 400 MHz. At a concentration of about 0.1% of the contaminant the OSCS signal and the ^{13}C satellites of heparin are of same order of magnitude.

Note that at low concentrations the contaminant can be identified only in the *N*-acetyl signal; in the fingerprint region, the OSCS signals are hidden due to the contribution of heparin to the overall signal in the respective region.

To enable quantification without reference material an alternative way is practicable. A sample of contaminated heparin has to be spiked with a pure heparin sample in steps between 50 and 250% (w/w). Using the acetyl signal at $\delta = 2.15$ ppm a linear regression enables the calculation of the initial heparin amount ($y = 0.4627x + 38.409$; $r^2 = 0.9975$). The amount of OSCS *vice versa* can be calculated via the difference to 100%.

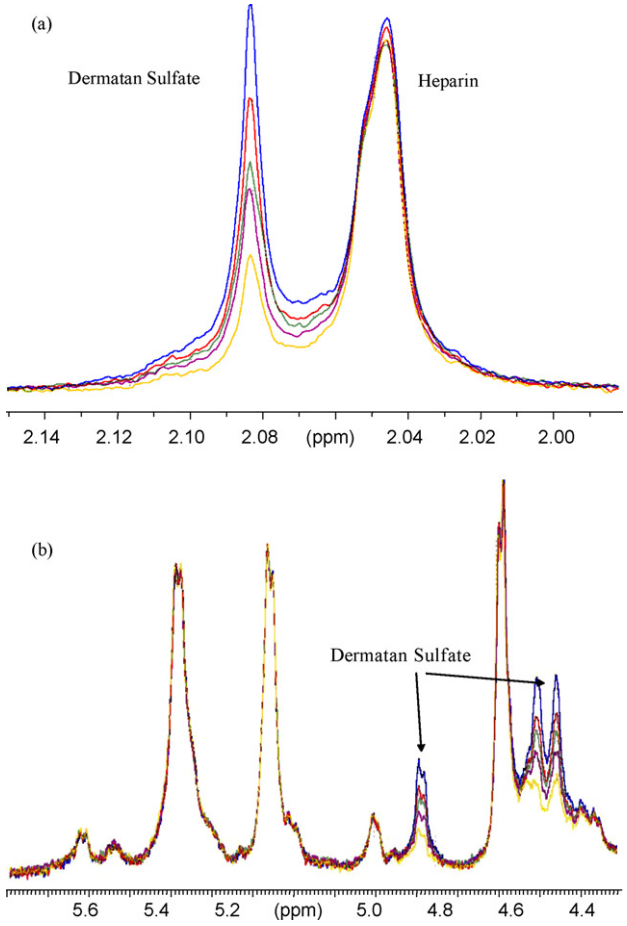

Fig. 7. (a) Increasing acetyl signals by adding DS to heparin, 353 K, 300 MHz; (b) increasing finger print signals by adding DS to heparin, 353 K, 300 MHz.

3.2.2. Quantification of DS in heparin

To a heparin sample containing an initial amount of DS a corresponding reference standard was added in steps of approximately 2, 4, 6, and 10% (w/w). Using the increasing acetyl signal at $\delta = 2.08$ ppm (see Fig. 7a), a linear regression enables the calculation of the initial DS amount ($y = 1.0832\ x + 4.4586$; $r^2 = 0.9956$) in relation to the heparin acetate signal. Thereby, the partial signal interference in the broad bases of heparin and DS acetyl signals have to be considered. Fig. 7b shows the alternative region to quantify DS at $\delta = 4.8$ ppm. Reproducibility of DS quantification in one heparin sample analyzed six times was proved with a mean value of 4.54% (w/w) ±0.08.

3.3. Principal component analysis

More than 100 samples of heparin collected from international markets were subjected to a principle component analysis.

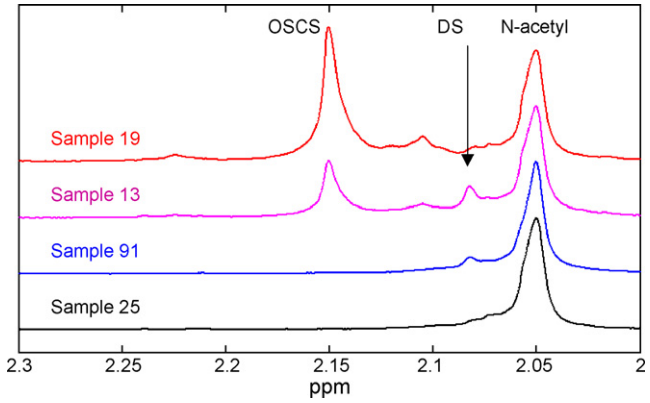

Fig. 8. Overlay of the acetyl region of four representative ^{1}H NMR spectra from the overall data set.

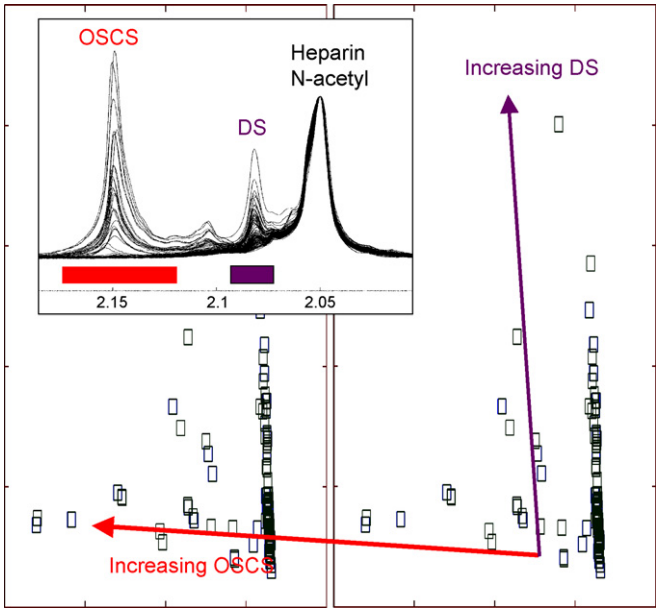

Fig. 9. Scores plot of the PCA analysis of the spectral data set. Prior to bucketing spectra were aligned and scaled to the *N*-acetyl signal of heparin at 2.05 ppm. For details see text.

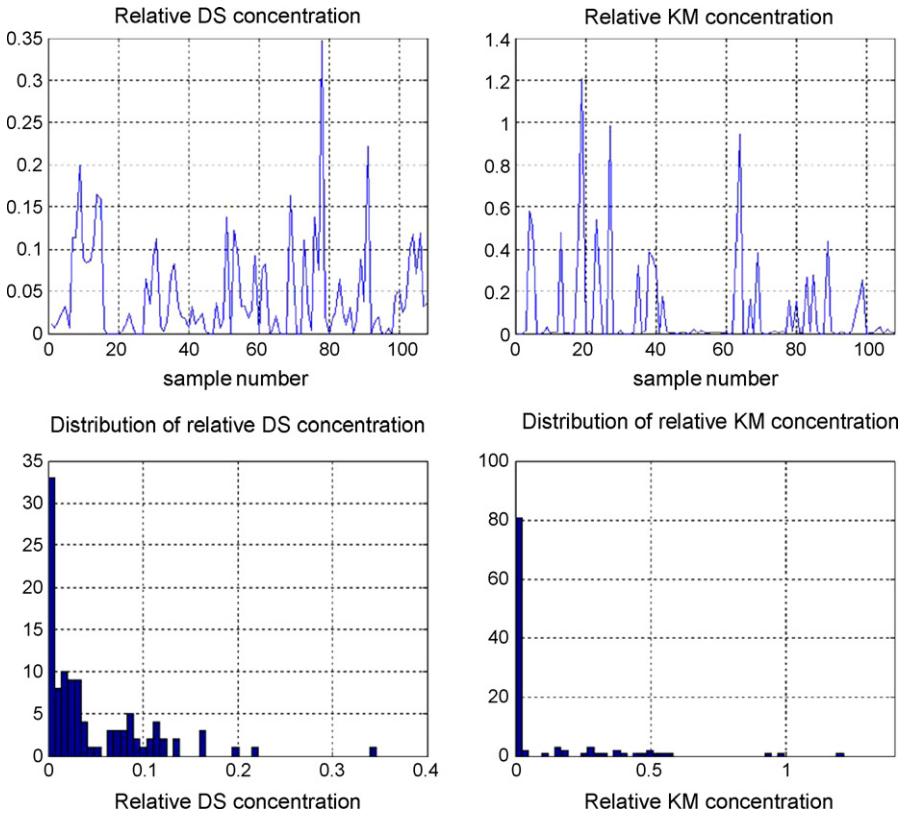

Fig. 10. Results of relative quantification of DS and OSCS (=KM) signals with respect to the *N*-acetyl signal of heparin (at 2.05 ppm). Upper plots give signal intensities as function of sample identity whereas lower plots provide relative concentration distributions.

An overlay of all spectra of the acetyl signal region (see Fig. 8) unraveled the different groups of batches: (1) pure heparin with a very small content of DS (sample 25); (2) relatively pure heparin with a higher content of DS (sample 91) in addition to free acetate (not shown); (3) contaminated heparin consisting of a small amount of OSCS, even higher amount of DS (sample 13) and free acetate and (4) a very impure heparin with high amounts of OSCS and less DS (sample 19) and free acetate. In the latter samples small additional acetyl signals of OSCS resonating upfield can be found which are likely to be caused by less sulfation. A comparison of ^{1}H NMR spectra of synthesized sulfated DS and oversulfated DS (=OSCS) supports this assignment (data not shown).

Bucketing has been applied to the spectra within the range of δ = 2.0–2.2 ppm, resulting into 60 buckets with 0.005 ppm bucket width. The principle components PC1 and PC2 scores represent 83.6 and 12.6% of the total variance in the bucket table, respectively. The PC1 scores are dominated by the effect of OSCS contamination whereas PC2 variation results from DS concentration variation (see Fig. 9). Both effects are rather independent, because the PC are orthogonal. Spectra containing either DS or OSCS are represented by points aligned along PC1 or PC2, respectively. Their scores values scale with relative concentration. Spectra containing both DS and OSCS are represented by linear combinations of PC1 and PC2, again with their scores values scaling with relative concentrations.

As can be seen in Fig. 10 and Table 1, many samples contain substantial amounts of DS indicating an insufficient purification of the crude unfractionated heparin. In addition some samples were found to consist of OSCS mostly in small amounts.

4. Conclusion

In this study we were able to show that the NMR spectroscopy at 300 or 400 MHz is sufficient to quantify and limit the impurities in heparin, especially OCSC and DS, by means of the *N*-acetyl signals. Thus, the quality of heparin can be guaranteed by routine NMR measurements when applying a sufficient high number of scans. However, higher field strengths and cryo probes can enhance these analytical possibilities.

Additionally, the heparin case unraveled the poor purification of the unfractionated heparin containing up to 30% DS.

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