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Theme Issue Article

Heparins: Process-related physico-chemical and compositional characteristics, fingerprints and impurities

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Summary

During the past 25 years, heparin extraction and purification processes have changed. The results of these changes are reflected by the continuous increase in potency of the International Standard for heparin. This increase is due not only to a higher purity, but also to a number of changes in the physico-chemical characteristics of heparin. For long time, all these changes have been disregarded as non-critical by regulatory authorities. Heparin marketing authorisation was reviewed only two years ago and Pharmacopoeia monographs were reviewed just for the addition of new tests, mainly aimed at tackling the oversulfated chondroitin sulfate (OSCS) crisis. Currently, heparin monographs are again under revision. Such changes, different for each manufacturer, have caused a further increase in the heterogeneity of individual batches of heparin. This review aims at showing that chemical, physical and biological characteristics of heparin (such as disaccharide composition, amount of low sulfated and high sulfated sequences, molecular weight profiles [MW], activities, structural artifacts, fingerprints and glycosaminoglycans impurities) are all process-dependent and may significantly vary when different processes are used to minimise the content of dermatan sulfate. The wide heterogeneity of the physico-chemical characteristics of currently marketed heparin and the lack of suitable and shareable reference standards for the identification/quantification of process-related impurities caused, and are still causing, heated debates among scientific institutions, companies and authorities.

Keywords

Heparin, dermatan sulfate, process-related impurities

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Introduction

A synthetic tetrasulfated chondroitin sulfate, not found in nature, was recently identified as a fraudulent contaminant in the recalled heparins originally sourced from China and distributed in Europe and USA, where several fatalities and other serious adverse events were reported after intravenous administration of the contaminated heparins to dialysed patients (1, 2). After disclosure of these findings, the Food and Drug Administration (FDA) indicated also dermatan sulfate (DS) and chondroitin sulfate (CS) as impurities of heparin, but, because of the natural occurrence of these glycosaminoglycans (GAGs) in all organs and tissues from which heparin is extracted, these GAGs should be better defined as "process-related impurities". All other Regulatory Agencies proposed to set a mandatory limit and specific tests for DS.

USP (United States Pharmacopoeia) suggested that this DS limit should be revisited in order to tighten it to not more than 1% (3), and is currently recommending the specification not more than 1% of galactosamine, assessed by the galactosamine method (4). The corresponding weight/weight specification for DS and/or chondroitin sulfate is even less than 1%. At the same time, the Committee for Medicinal Products for Human Use of the European Medicines Agency "was of the opinion that low-molecular-weight heparins (LMWHs) particularly enoxaparin, that contains low levels of oversulfated chondroitin sulfate (OSCS) (less than 5 %) can continue to be used, avoiding intravenous route, until they are replaced by OSCS-free batches" (5). These conflicting proposals prove the need of harmonisation among regulatory agencies.

In this paper we show that the compliance with tighter specifications for DS, designed for improving the quality of heparin preparations, can be obtained only with changes of manufacturing processes, and that such changes might lead to modifications of physico-chemical and compositional characteristics of some brands of currently marketed heparins. Such modifications will

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Table 1: Experiments H-3796, H-3854, H-3857. Molar fractions of major heparin disaccharides, of linkage region (l.r.), of major atypical disaccharides (Δ-GalA-GlcNS and Δ-GalA-GlcNS,6S), of the major tetrasaccharide containing 3-O-sulfated glucosamine and atypical disaccharide and tetrasaccharide containing the 1,6-anhydro rings. Molar fractions of Σ-6-OSO_3^-, Σ-IdoA2-SO_3^- and Σ-N-SO_3, Mw and dispersion (D) of different fractions of heparin.

	H- 3788	H- 3796 non-retained	H-3796 / 0.15M NaCl	H-3796 / 0.67M NaCl	H-3796 / 1.5 M NaCl	H-3854 (1.0M)	H-3857 (1.5M)
	Molar %	Molar %	Molar %	Molar %	Molar %	Molar %	Molar %
Δ Glu-Gal-Gal-Xyl-O-Ser	0.9	1.2	1.1	0.8	0.7	0.9	1.1
Δ UA-GlcNAc	5.1	8.8	5.4	3.7	2.6	3.8	2.8
Δ Glu-Gal-Gal-Xyl-O-CH_2COOH	0.9	1.2	1.1	0.7	0.4	0.7	0.5
Δ GalA-GlcNS	0.1	0.4	0.2	0.1	0.0	0.1	0.0
Δ UA-GlcNS	3.4	6.8	3.6	2.3	1.3	2.5	0.9
Δ UA-GlcNAc, 6S	3.8	5.4	4.6	3.0	2.0	3.0	1.9
Δ UA, 2S-GlcNAc	1.4	2.2	1.6	1.2	0.7	1.2	0.5
Δ UA, 2S-GlcN, 6S	0.3	0.5	0.1	0.0	0.0	0.1	0.1
Δ GalA –GlcNS, 6S	1.3	1.9	1.2	0.9	0.9	0.7	0.6
Δ UA – GlcNS, 6S	12.0	12.5	13.2	11.9	10.5	10.5	10.2
Δ UA, 2S -GlcNS	7.2	12.1	8.3	5.9	3.5	5.9	3.1
Δ UA, 2S –GlcNS 1,6 anhydro	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Δ UA, 2S –GlcNAc, 6S	1.5	1.4	1.6	1.5	1.4	1.3	1.2
Δ UA, 2S –GlcNS, 6S	59.7	44.3	56.0	65.7	71.2	67.1	73.0
Δ UA –GlcNAc, 6S – GlcA – GlcNS, 3S, 6S	2.3	1.1	1.9	2.3	3.7	2.3	3.5
Δ UA, 2S –GlcNS, 6S –IdoA, 2S	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Δ UA, 2S –GlcNS, 6S –IdoA, 2S –ManNS 1,6 anhydro	0.0	0.0	0.0	0.1	0.0	0.1	0.5
SO_3^-/COO^-	2.36	2.07	2.30	2.48	2.58	2.48	2.59
Σ-2S (%)	68.4	59.9	66.5	72.8	75.1	74.0	75.9
Σ-6S (%)	81.3	67.5	79.0	85.6	91.1	85.2	90.9
Σ-NS (%)	84.2	78.3	82.9	87.1	88.0	87.0	88.8
Activity aXa (IU/mg)	214	135	209	258	282	206	253
Activity APTT (IU/mg)	182	95	150	231	279	196	254
Mw	15400	12400	11300	15000	21100	19200	25000
D	1.38	1.47	1.29	1.17	1.19	1.25	1.19

affect some molecular features, like the degree of sulfation, the distribution of molecular masses, the affinity to antithrombin (AT) and the related biological activities.

DS is easily co-purified with heparin and it has been a naturally occurring "unofficial" component of heparin preparations (6, 7) for years without any incident. It has been found in the 4th International Standard of heparin too (8). In the heparin not intended for parenteral administration (i.e., crude heparin), extracted from porcine mucosa, DS amounts could be as high as 15%.

DS binds to a number of proteins (9), and the binding to heparin cofactor II (HCII) contributes, at least in part, to its antithrombotic / thrombolytic properties.

DS is contained in the drug substance Sulodexide [57821–29–1], (Merck Index 14th, 8986) at about 20%, the remaining part being an heparin-like substance ("fast-moving heparin"). The combination of heparin and DS in such a drug substance provides a more effective antithrombotic mechanism of action than heparin or LMWH alone: heparin and LMWH catalyse the antiprotease actions of both antithrombin and heparin cofactor II (HCII), while DS catalyses thrombin inhibition only by HCII (10). DS is contained also in Danaparoid Sodium (Merck Index 14th, 2811) in the range 8 – 16 %, as quoted in the European Pharmacopoeia Monograph 01/2008:2090. Heparin and other iduronic acid-containing GAGs, such as DS, exert their anticoagulant properties primarily by accelerating the rate of inhibition of the natural protease inhibitors antithrombin, which inhibits both Xa and thrombin, and heparin cofactor II, which selectively inhibits thrombin (11).

Although AT and HCII are structural homologues, only heparin binds AT, whereas HCII has different binding sites for heparin and DS. The binding site of heparin for AT is a pentasacchar-

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Table 2: Comparison of unfractionated heparin manufactured by manufacturers A and B. Molar fractions of major heparin disaccharides, of linkage region (l.r.), of major atypical disaccharides (Δ-GalA-GlcNS and Δ-GalA-GlcNS,6S) of major tetrasaccharide containing 3-O-sulfated glucosamine and atypical disaccharide and tetrasaccharide containing the 1,6-anhydro rings. Molar fractions of 6-OSO_3^-, IdoA2-SO_3^- and NSO_3^- of unfractionated heparin (HFHs) manufactured by manufacturers A and B.

	Manufacturer A			Manufacturer B			
Batches	**17772**	**17937**	**17804**	**17933**	**17935**	**18045**	**18219**
	Molar %	**Molar %**	**Molar %**	**Molar %**	**Molar %**	**Molar %**	**Molar %**
Δ Glu-Gal-Gal-Xyl-O-Ser (l.r.)	2.1	1.9	1.5	0.5	0.5	0.5	0.8
Δ UA-GlcNAc	4.6	4.0	3.6	5.3	4.9	4.8	5.0
Δ Glu-Gal-Gal-Xyl-O-CH_2COOH (l.r.)	.0.2	0.2	0.2	1.3	1.3	1.3	0.7
Δ GalA-GlcNS	0.0	0.0	0.1	0.1	0.1	0.1	0.1
Δ UA-GlcNS	2.1	2.5	2.3	3.4	3.4	3.2	3.2
Δ UA-GlcNAc, 6S	3.0	2.8	2.8	3.7	3.6	3.8	3.8
Δ UA, 2S-GlcNAc	1.4	1.2	1.2	1.3	1.4	1.3	1.6
Δ UA, 2S-GlcN, 6S	0.0	0.1	0.0	0.0	0.1	0.1	0.0
Δ GalA –GlcNS, 6S	0.5	0.4	0.5	0.7	0.7	0.8	0.8
Δ UA – GlcNS, 6S	10.1	10.6	10.4	11.7	11.7	11.9	11.4
Δ UA, 2S -GlcNS	6.6	6.7	6.1	7.6	7.4	7.4	7.4
Δ UA, 2S –GlcNS 1,6 anhydro	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Δ UA, 2S –GlcNAc, 6S	1.3	1.3	1.5	1.5	1.5	1.5	1.7
Δ UA, 2S –GlcNS, 6S	65.4	65.9	66.8	60.5	60.8	60.7	60.6
Δ UA –GlcNAc, 6S – GlcA – GlcNS, 3S, 6S	2.5	2.4	2.5	2.4	2.3	2.3	2.7
Δ UA, 2S –GlcNS, 6S –IdoA, 2S	0.1	0.1	0.2	0.0	0.0	0.0	0.0
Δ UA, 2S –GlcNS, 6S –IdoA, 2S –ManNS 1,6 anhydro	0.4	0.0	0.3	0.3	0.2	0.2	0.2
$SO3^-/COO^-$	2.45	2.46	2.49	2.37	2.38	2.38	2.38
Σ-2S (%)	73.5	73.5	74.5	69.6	69.9	69.7	69.8
Σ-6S (%)	83.3	84.0	85.2	80.8	81.3	81.6	81.7
Σ-NS (%)	85.4	86.5	86.9	84.6	84.7	84.7	84.2
IU/mg	204	207	205	190	195	194	199

ide sequence, but only one third of the heparin chains contains this sequence; HCII binding sequences of heparin and DS are less specific and are contained in almost all of the GAG chains (11). Structures contributing to the HCII-mediated inhibition of thrombin are represented in DS by naturally oversulfated sequences (12). DS active site for HCII has been isolated from both beef mucosa and pig skin; it is represented by a nonasaccharide fragment containing the four-disaccharide sequence [α-L-iduronic acid 2-O-sulfate (1,3)-β-D-N-acetyl-galactosamine 4-sulfate] (13). In DS from pig mucosa, oversulfated structures are composed by both the sequences (IdoA2SO_3-GalNAc4SO_3)n and (IdoA-GalNAc-4,6SO_3)n with n = 2 or 3 (14). Protein binding and associated biological activities of heparin and DS are modulated by the "plasticity" of their iduronic acid residues, due to the availability of up to three equienergetic conformations among which the protein can select the one favoring the most stable complex (11).

DS, heparin, heparan sulfate (HS) and other GAGs are key biological response modifiers, acting as stabilisers, cofactors and/or co-receptors for growth factors, cytokines and chemokines (15). They are regulators of enzyme activities and signaling molecules in response to cellular damage, such as wounding (16), infections and tumorigenesis (9, 17).

The most important steps of the standard manufacturing process of heparin are: enzymatic hydrolysis of proteoglycans, purification of heparin from related impurities (which can be recovered as by-products) by strong or weak anion exchange resins, fractionated precipitation by solvents, selective treatments at low and high pH, selective precipitations as salts of different counter-cations, concentration by tangential flow filtration through membranes with different cut-off, and some further treatments, mainly oxidative, for virus removal and bleaching.

Of course, different types of anion exchange resins, different concentrations of buffers used for the elution of GAGs (as in the following examples), different ratios of solvent used in the fractional precipitation, as well as the strength of chemical treatments, can originate different characteristics in heparin and in other GAG components. It is usual that a few per cent of chains of naturally occurring oversulfated dermatan sulfate fractions remain in heparin as an impurity and, vice versa, traces of "under-

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sulfated" heparin fractions remain in preparations of DS. Furthermore, different strengths of treatments, aimed at the viral removal, bleaching and purification from proteins, can affect, selectively, different heparin structures, to a different extent, and generate peculiar fingerprints. The same considerations hold for "heparinoid" drug substances, represented by mixtures of GAGs (e.g. Sulodexide and Danaparoid), which are the results of the selective fractionations from by-products of heparin manufacturing.

Current therapeutic heparin is characterised by its own heterogeneity (18), which is process-dependent, and DS is just a heterogeneous process-related impurity of heparin. The changes in the manufacturing processes of heparin for minimising the content of DS will change some characteristics of heparin, as well as those of its process-related impurities.

Materials and methods

Fractionations

Injectable-grade heparin samples were supplied by two Chinese manufacturers. Heparinoid IC290 was from SPL (Waunakee, WI, USA). Heparinoids like IC290 are by-products of manufacturing of heparin and starting material for Sulodexide.

The widely used DEAE-Sepharose resin was from GE Healthcare (Milan, Italy). A Pilot scale column (ID 15 cm, bed eight 25 cm) was operated at a linear flow rate of 150 cm/h. Stepwise elutions with sodium chloride at different concentrations in 10 mM acetate buffer, pH 5.0, were monitored with a refractive index detector. Eluted fractions were desalted and concentrated by tangential flow filtration on Pellicon2 cassettes, regenerated cellulose, cut-off 3 Kda (Millipore; Milan, Italy). The samples were recovered by precipitation and freeze-drying.

An experiment (H-3796) of fractionation of a porcine, injectable-grade, heparin by weak anion exchange chromatography with DEAE-Sepharose, using a ratio weight of heparin/volume of resin 6:100 and a step elution with four buffers of increasing concentration of sodium chloride, was carried out, obtaining four fractions: not-retained (0.00 M), 0.15 M, 0.67 M and 1.50 M. The disaccharide composition of these fractions is reported in Table 1, in comparison with the parent heparin H-3788.

In examples H-3854 and H-3857, the same heparin was loaded on the same column in a 0.15 M NaCl buffer solution: the elution with 1.0 M NaCl solution originated the sample H-3854. In a second run, the same batch of heparin was fractionated with an elution with a 1.5 M NaCl buffer, originating the sample H-3857. These two samples were characterised; results are reported in Table 2.

In all these three examples, the not-retained fraction contained fast-moving heparin and about 3% of DS (data not shown).

A different injectable-grade heparin preparation containing, as impurities, heparan sulfate and about 1% of DS, has been purified by fractionation on DEAE-Sepharose by NaCl buffers. The first fraction D-3860/0.15M contained DS characterised by a disaccharide composition similar to the one of DS directly extracted from porcine mucosa (14) and a second heparan sulfate-like compound (HS-like), always present in the by-products of manufacturing of heparin already described (19, 20). Data related to these fractions are reported in Table 3.

An extraction of electrophoretically pure DS from the heparinoid IC290 has been carried out. The sodium salt of the heparinoid was treated with a calcium chloride buffer, precipitated and the obtained product was fractionated on the DEAE-Sepharose column. The first eluted fractions were concentrated by tangential flow filtration (TFF) and purified by nitrous acid cleavage (21, 22) for removing all heparin-like substances, i.e. all substances containing N-sulfated glucosamine. The DS fraction was further purified by TFF and freeze-dried. The sample DS D3541 was contained in the first eluted fractions (NaCl < 0.75 M); other fractions obtained by this purification procedure were used to prepare different compounds. Four families of components were recovered: DS, heparan sulfate, fast-moving and slow-moving heparins.

Composition analysis with complete enzymatic digestion

Composition analysis of heparin and heparin-like samples has been carried out by enzymatic cleavage with heparinases I, II, III (Grampian Enzymes, Orkney, UK) and by separation of fragments by SAX- HPLC, using a Spherisorb S5 SAX, 4.6 x 250 mm, chromatographic column (Waters; Milan, Italy), with UV detection at 234 nm. The elution condition was a linear gradient of sodium perchlorate, pH 3.0 (from 30 to 800 mM in 60 min.), at 1.4 mL/min. (23).

Disaccharide analysis of DS samples has been carried out by enzymatic cleavage with chondroitinase ABC (Sigma-Aldrich; St. Louis, MO) and separation by SAX- HPLC (strong anion exchange), using the same chromatographic column and UV detection. The elution condition was 5 min. at 0.2 M of sodium chloride, pH 3.5 and a linear gradient, from 0.2 to 1.0 M in 40 min., at 1.4 mL/min. (24).

The relative standard deviation (RSD%) of the intermediate precision of these methods ranges between 2.3 and 0.2 %, according to area of each peak.

Results and discussion

Large differences between the parent heparin H-3788 and its fractions H-3796 were found for a number of parameters. The most relevant differences are shown in Table 1 and are: the percentages of disaccharide ΔUA,2S-GlcNS,6S, of the undigested tetrasaccharide ΔUA-GlcNAc,6S-GlcA-GlcNS,3S,6S (a marker of the pre-existing pentasaccharides present in the binding site for AT) (25, 26), of molar fractions Σ-$2SO_3$, Σ-$6SO_3$, and Σ-NSO_3. All these parameters show an increase from the first to the last fraction (from the lowest to the highest concentration of sodium chloride), as well as molecular masses and the relevant biological activities, aXa and APTT. Fractions H-3796/0.15M, 3796/0.67M, 3796/1.5M, 3854/1.0M, 3857/1.5M are different, but all equally pure, that is without DS and HS-like impurities.

Data in Table 1 show that it is possible to manage the chromatography process in order to obtain different fractions, with different potencies and, maybe, different *in vivo* activities.

The current European Pharmacopoeia monograph states: "the potency [of heparin] is not less than 150 IU/mg". Fractions H-3796 0.15M, 0.67M and 1.50M are in compliance with that definition, but they are not similar and not all of them could be

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Table 3: Comparison of byproducts from experiments 3860 and IC290. A) Molar fractions of disaccharides and molecular mass of two dermatan sulfates prepared from heparin (D3860) and heparinoid (D3541) samples. B) Molar fractions of major disaccharides and of 6-OSO3-, IdoA2-SO3– and NAc of two heparan sulfate-like samples prepared from heparin (HS3860) and heparinoid (HS3535) samples.

A

	D3860 Molar %	D3541 Molar %
Δ-Di-0S	3.6	2.3
Δ-Di-6S	5.3	2.2
Δ-Di-4S	80.9	86.3
Δ-Di-UA2S	0.0	0.0
Δ-Di-6S,UA2S	0.3	0.2
Δ-Di-4,6diS	4.2	4.2
Δ-Di-4S,UA2S	5.6	4.8
Δ-Di-4,6diS,UA2S	0.2	0.0
Mw	30100	25800
D	1.26	1.11

B

	HS3860 Molar %	HS3535 Molar %
Δ-UA-GlcNAc	21.7	46.1
Δ-UA-GlcNS	14.0	22.3
Δ-UA-GlcNAc,6S	6.8	11.3
Δ-UA-2S-GlcNAc	1.8	0.9
Δ-UA-2S-GlcN,6S	0.2	0.0
Δ-UA-GlcNS,6S	8.9	5.1
Δ-UA-2S-GlcNS	17.0	7.1
Δ-UA-2S-GlcNAc,6S	0.7	0.2
Δ-UA-2S-GlcNS,6S	28.7	7.1
SO_3^-/COO^-	1.62	0.81
Σ 6S	45.3	23.7
Σ 2S	48.4	15.3
Σ NAc	31.1	58.5

suitable as a "reference" standard in the analysis by capillary electrophoresis (CE) or nuclear magnetic resonance (NMR) recommended by FDA, if they are intended for providing quantitative results. The not-retained fraction 3796 contained fast-moving heparin and DS.

The results of experiments H-3854 and H-3857, reported in Table 1, prove that different strategies of fractionation on the resin originated heparin batches having different potencies and MW profiling, both for weight-average molecular-weight (Mw) and dispersion (D). Also in these examples the not-retained fraction contained fast-moving heparin and a small percentage of DS.

Tables 1 and 2 report also percentages of the most representative and important tetrasaccharide containing 3-O-sulfated glucosamine, of the building blocks containing unsaturated residue in galacturonic form, of the building block ΔGlu-Gal-Gal-Xyl-O-Ser and its derivative ΔGlu-Gal-Gal-Xyl-O-CH_2COOH, as reported by Mourier and Viskov (27), both belonging to the linkage region (l.r.).

The comparison of the characteristics of the heparin fractions (Table 1) with those of the injectable-grade heparins provided by two different Chinese manufacturers, A and B (Table 2), allows the following considerations about sulfation patterns and fingerprints in these batches:

i) the variability of biological activities could depend on the pattern of sulfation and on the different content of high affinity (HA) fractions which contain AT binding sites, characterised by the tetrasaccharide containing the 3-O-sulfated glucosamine. The different contents of the tri-sulfated disaccharide ΔUA2S-GlcNS,6S are always directly related to biological potencies.

ii) the processes of the two manufacturers A and B involve, maybe, different strengths in the oxidative steps, which could explain the higher amount of serine derivative in heparin produced by manufacturer A in comparison with B. Data show that the sum of the two building blocks is about the same for the two manufacturers, with a higher content of the serine derivative in heparin manufactured with a process using weaker conditions for the oxidation steps. The building blocks belonging to the linkage region (l.r.) can be identified also by ^{13}C-NMR spectra (data not shown), by signals at 84.6 and 85.0 ppm and at 106.1 and 106.4 ppm; they are arising from C-3 and C-1, respectively, of the two Gal residues (28).

iii) the content of disaccharides ΔGalA-GlcNS and ΔGalA-GlcNS,6S is a little bit higher in heparin of manufacturer B. Epimerisation of glucuronic acid into galacturonic is usually caused by alkaline treatments (29). In this case, the higher content of galacturonic acid as a fingerprint, could be due to a remote treatment of crude heparin in warm alkaline solution, most likely a stronger alkaline proteolytic digestion carried out by manufacturer B.

iv) the disaccharide ΔUA,2S,GlcNS-1,6-anhydro and the tetrasaccharide ΔUA,2S,GlcNS,6S-IdoA,2S-ManNS-1,6-anhydro are present in traces and only in a few heparins of Table 2 and not in purified fractions of Table 1. They result from the formation of bicyclic acetals (called 1,6 anhydro rings) at the reducing end terminals and, in the case of the tetrasaccharide, from epimerisation of the glucosamine at the reducing end terminal.

v)the trisaccharide Δ-UA,2S-GlcNS,6S-IdoA,2S is found in some heparins of Table 2, as a consequence of a peeling reaction.

These last three artifacts, or process-related fingerprints, are modified heparin structures that occur when alkaline treatments are used to extract or purify heparin (30).

Not all the sequences of heparin can be completely recovered as disaccharides by enzymatic cleavage (31). Many oligosaccharides containing the N,3-disulfated glucosamine have been identified in heparins and characterised also on the basis of their affinity for AT. But not all the fragments containing 3-O-sulfated glucosamine are typical of the HA species, as demonstrated by bi-dimensional NMR experiments (32). At least five tetrasaccharides containing the 3-O-sulfated glucosamine have been identified by sequence analysis of CTA-SAX fractions from a LMWH (31):

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- ΔU-GlcNAc,6S-GlcA-GlcNS,3S,6S (ΔIIa-IIs_{glu})
- ΔU-GlcNS,6S-GlcA-GlcNS,3S,6S (ΔIIs-IIs_{glu})
- ΔU2S-GlcNS,6S-GlcA-GlcNS,3S,6S (ΔIs-IIs_{glu})
- ΔU2S-GlcNAc,6S-GlcA-GlcNS,3S,6S (ΔIa-IIs_{glu})
- ΔU-GlcNAc,6S-GlcA-GlcNS,3S (ΔIIa-IVs_{glu}).

The specificity and potency of different antithrombin-binding octasaccharides, containing the pentasaccharide characterised by the tetrasaccharide ΔIIa-IIs_{glu}, have been recently studied (33).

ΔIIa-IIs_{glu} is the most represented (up to 70%) of the GlcNS,3S-containing tetrasaccharides. It averages out about 2.3 % in injectable-grade heparins, but it progressively increases to 3.5% and 3.7% in fractions experimentally enriched in the sulfate content, as shown in Tables 1 and 2. The higher degree of sulfation, and consequently the increase in concentration of HA sequences and in potencies, in the examples, depend on the type and performance of the resin and on concentration of buffers used in the process. They are process-related characteristics (Table 3).

The HS-like sample, obtained from experiment H-3860, is comparable to the heparan sulfates described by Vongchan et al. (34). This compound, with a content of disaccharide ΔUA-GlcNAc as high as 21.7%, is another non-heparin species isolated from heparin preparations. Heparan sulfates, although devoid of anticoagulant activity, are indeed potent antithrombotic compounds (19).

In the experiment of fractionation of heparinoid IC290, four electrophoretically pure, different, fractions were obtained: DS D3541, HS-like HS3535, fast-moving and slow-moving heparin (35). The physico-chemical characteristics (MW, electrophoretic pattern, disaccharide building blocks etc.) and activities of these three last fractions were very different: in particular, the N-acetylglucosamine content is peculiar. Furthermore, also DS D3541 is different from the DS recovered as impurity of injectable-grade heparin for a number of physico-chemical characteristics (i.e. MW, distribution, disaccharide building blocks etc. Data shown in Table 3).

All these data about different fingerprints and different amounts of the same fingerprint can be used to understand differences in the process of heparin manufacturing.

The reported data about different fractionation processes of heparin show that it is possible to obtain heparin samples that have a very low content of process-related impurities, or no impurities at all, but different processes could lead to different kinds of heparin preparations.

Another problem could be the choice of suitable methods for the detection of such low levels of impurities.

At the time of heparin crisis, FDA recommended at least two tests for the assessment of OSCS: the ^{1}H-NMR and the CE.

These methods had been considered suitable to identify and evaluate both types of impurities in heparin: the not naturally occurring OSCS and the process-related impurities of DS and CS.

Other regulatory agencies in collaboration with pharmaceutical companies, started studying and implementing new methods and alternative techniques.

Additional requirements will be shortly recommended by USP (5) to cover overall resonance distribution of ^{1}H-NMR identification test for OSCS and DS. But the quantitative evaluation of DS remains a problem. The ^{1}H-NMR method relies on the determination of the ratio between the signal quantitatively variable of N-acetylglucosamine in heparin and the corresponding N-acetylgalactosamine in DS. Such a method should require an accurate and independent assessment of N-acetylglucosamine/ N-SO_3-glucosamine ratio in the heparin to be analysed for its content of DS.

The CE identification test could be replaced with a strong ion-exchange HPLC method. In fact the quantitative evaluation of DS through the CE technique might be possible only by comparison with a suitable reference of DS, after degradation of interfering HS and after selective degradation of CS by chondroitinase AC.

USP is expected to issue a limit test for impurities of DS in heparin shortly. It is based on galactosamine/glucosamine ratio, assessed after the hydrolysis of heparin by hydrochloric acid (5), but the drastic condition of hydrolysis of this method is expected to generate additional peaks from amino-sugars (maybe anhydro structures), complicating the necessary validation.

Collaborative studies are in progress in laboratories of the industry, aiming to validate methods based on HSQC-NMR (2D-heteronuclear single quantum coherence) experiments and SAX-HPLC assessment of DS and ChS, with and without depolymerisation of heparin structures by nitrous acid.

The European Directorate for the Quality of Medicines (EDQM) has not disclosed yet the new monograph of heparin and its relevant specifications.

We evaluated all these techniques aimed at the assessment of DS content in heparin and in heparinoid substances such as Sulodexide®. In all cases we had to face problems: different response factor for the same method, due to different needed reference standards, different reproducibility and sensitivity for different techniques.

During the past 25 years many new heparin-manufacturing sites have sprung worldwide (18), and a number of changes were introduced in heparin extraction and purification processes. As a consequence, the International Standard of heparin progressively changed from about 130 IU/mg in 1958 to about 170 IU/mg in 1973, about 190 IU/mg in 1983 and to values exceeding 200 IU/mg in 2000 (8). The increase in potency of International Standard demonstrates that heparin preparations with increasing average MW and affinity to AT have been manufactured and marketed. In spite of these large differences in physico-chemical properties and biological activities, which result from a tuning of extraction, fractionation and purification processes leading to therapeutic heparin, only two years ago the heparin marketing authorisation (as a generic) has been reconsidered in the light of the new criteria on biological substances.

In case a new International Standard of heparin, endowed with a potency higher than 229.5 IU/mg (the heparin V International Standard), will be available and in the case of a change of composition and molecular distribution of the currently marketed heparin, (e.g. as a consequence of the prescribed tightening of limits for CS and DS impurities) would a new marketing authorisation for this "new", or different, heparin be recommended?

New specifications for process-related impurities (DS, CS, HS) are now under evaluation by European regulatory auth-

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orities, but such tighter specifications, up to less than 1% according to the recent recommendation of USP, could involve a modification of some essential characteristics of heparin and of the process-related impurities too.

Due to the wide, both natural and process-related heterogeneity of these GAGs, it is quite evident that the compounds suggested as reference standards should be defined both with reference to the process (8) and to their characteristics. The characterisation methods have significantly improved since the '80s, and now many analytical techniques are suitable to determine these characteristics, provided that the correct reference is used. For these reasons, a full harmonisation should be advisable. To avoid an increase in mismatches of results among different methods and laboratories, such harmonisation should take into careful account specifications, methods and the reference standards for the process-related impurities and the heparin itself.

Manufacturers of heparin, which are not in compliance with the upcoming specifications, will have to make further changes to their purification processes. These adjustments are expected to change again the physico-chemical characteristics of some brands of currently marketed heparin.

Safety of heparin preparations should not be involved, unless preparations with potencies as high as that, e.g. of sample H-3857 (aXa 253 IU/mg), are marketed: in such a case, a revision of clinical protocols should be advisable. The same considerations should apply to LMWHs, with difficulties for the manufacturers to comply with specifications only in case of parent heparins with very high potencies.

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Abbreviations

APTT, activated partial thromboplastin time; AT, antithrombin; CE, capillary electrophoresis; CS, chondroitin sulfate; CTA; cetyltrimethylammonium; DS, dermatan sulfate; GAG, glycosaminoglycan; GalA, galacturonic acid; ΔGalA, 4,5-unsaturated GalA; GalN, galactosamine; GlcA, glucuronic acid; GlcN, glucosamine; Gal, galactose; Glu, glucose; HA, high affinity; HCII, heparin cofactor II; HPLC, high-performance liquid-chromatography; HS, heparan sulfate; HSQC, 2D heteronuclear single quantum coherence; IdoA, iduronic acid; LMWH, low-molecular-weight heparin; Man, mannose; ManN, mannosamine; MW, molecular weight; NMR, nuclear magnetic resonance; OSCS, oversulfated chondroitin sulfate; SAX, strong anion exchange; SEC, size exclusion chromatography; Ser, serine; TFF, tangential flow filtration; ΔUA, 4,5-unsaturated uronic acid; Xyl, xylose.

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