Analytical Biochemistry 461 (2014) 46–48

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Notes & Tips

Heparin stability by determining unsubstituted amino groups using hydrophilic interaction chromatography mass spectrometry

Li Fu [a,b], Lingyun Li [a], Chao Cai [a], Guoyun Li [a,c], Fuming Zhang [b], Robert J. Linhardt [a,b,d,e,*]

[a] Department of Chemistry and Chemical, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA
[b] Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA
[c] College of Food Science and Technology, Ocean University of China, Qingdao, Shandong 266003, China
[d] Department of Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA
[e] Department of Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

ARTICLE INFO

Article history:
Received 29 March 2014
Received in revised form 23 May 2014
Accepted 29 May 2014
Available online 6 June 2014

Keywords:
Heparin
Sulfate
Stability assay
Amino group
Mass spectrometry

ABSTRACT

The thermal instability of the anticoagulant heparin is associated, in part, with the solvolytic loss of *N*-sulfo groups. This study describes a new method to assess the increased content of unsubstituted amino groups present in thermally stressed and autoclave-sterilized heparin formulations. N-Acetylation of heparin samples with acetic anhydride-d_6 is followed by exhaustive heparinase treatment and disaccharide analysis by hydrophilic interaction chromatography mass spectrometry (HILIC–MS). The introduction of a stable isotopic label provides a sensitive probe for the detection and localization of the lost *N*-sulfo groups, potentially providing valuable insights into the degradation mechanism and the reasons for anticoagulant potency loss.

Heparin is a sulfated polysaccharide that is widely used as an anticoagulant drug in extracorporeal therapy, surgical procedures, and treatment of clotting disorders [1]. The complex chemical structure of this heterogeneous (multiple disaccharide sequences), polydisperse (average molecular weight ~16,000 Da with $M_W/M_N \sim 1.6$) polysaccharide makes its analysis quite difficult [2]. This is exemplified by the recent introduction of a detailed compendium by the U.S. Pharmacopeia (USP)[1] to assess the identity and purity of heparin active pharmaceutical ingredient (API). Interest in heparin analysis peaked in 2008 during a contamination crisis in which heparin was adulterated, leading to many severe reactions, including patient deaths [3,4].

As part of a continued focus on developing improved methods for heparin analysis, our laboratory has been assessing stability-determining assays for heparin that are critical in ensuring drug potency and establishing appropriate shelf life for formulated heparin products. Surprisingly, there is very little published literature on stability-determining assays for heparin, and most commonly its anticoagulant potency is the sole method used to assess its stability on formulation and storage. Stability studies of thermally stressed and pH-stressed heparin formulations, first published by our group nearly 20 years ago [5], suggested several major mechanisms of non-oxidative decomposition of heparin, leading to loss of potency. The hydrolysis of glycosidic linkages and the β-elimination of glycosidic linkages to uronic acid residues result in decreased molecular weight, whereas the solvolytic loss of sulfate, particularly the more labile *N*-sulfo groups, can reduce heparin potency without significantly affecting its molecular weight (Fig. 1). The loss of potency in autoclaving-formulated heparin has been attributed to damage done to the heparin antithrombin III (AT)-binding site, resulting in decreased binding to AT, as determined by competitive binding studies using surface plasmon resonance (SPR), leading to loss in anticoagulant potency [6]. Heparin (and the structurally related polysaccharide heparan sulfate) contains a small number of unsubstituted amino groups [7], further complicating the analysis of such groups as they form when heparin decomposes. These unsubstituted amino groups can be determined using nuclear magnetic resonance (NMR) or through fluorescent labeling [7], and the inorganic sulfate formed can be determined using ion chromatography [8]. The current study explored the use of hydrophilic interaction chromatography mass

* Corresponding author at: Department of Chemistry and Chemical, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA. Fax: +1 518 276 3405.
E-mail address: linhar@rpi.edu (R.J. Linhardt).

[1] *Abbreviations used:* USP, U.S. Pharmacopeia; API, active pharmaceutical ingredient; AT, antithrombin III; SPR, surface plasmon resonance; NMR, nuclear magnetic resonance; HILIC–MS, hydrophilic interaction chromatography mass spectrometry; CNDS, chemically N-desulfonated heparin; MWCO, molecular weight cutoff; 1D, one-dimensional; 2D, two-dimensional; HSQC, heteronuclear single-quantum coherence.

http://dx.doi.org/10.1016/j.ab.2014.05.028
0003-2697/

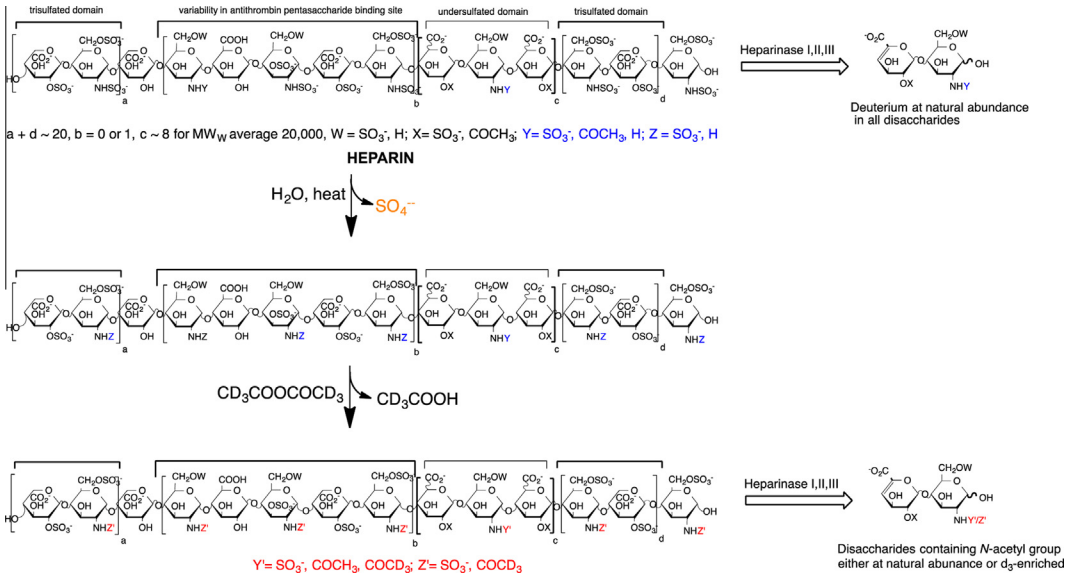

Fig.1. Schematic of solvolytic loss of heparin *N*-sulfo groups, leading to decreased AT affinity and loss of potency in formulated heparin samples, and method for determining *N*-sulfo group loss.

Table 1
Structure of disaccharides detected by HILIC–MS analysis.

Disaccharide unit	*m/z*	Deuterated disaccharide	*m/z*
ΔUA-GlcNAc (0S)	$[378.10]^{1-}$	ΔUA-GlcNAc-d_3 (0S)	$[381.12]^{1-}$
ΔUA2S-GlcNAc (2S)	$[458.06]^{1-}$	ΔUA2S-GlcNAc-d_3 (2S)	$[461.07]^{1-}$
ΔUA-GlcNAc6S (6S)	$[458.06]^{1-}$	ΔUA-GlcNAc-d_3 6S (6S)	$[461.07]^{1-}$
ΔUA2S-GlcNAc6S (2S6S)	$[268.50]^{2-}$, $[538.01]^{1-}$	ΔUA2S-GlcNAc-d_3 6S (2S6S)	$[270.01]^{2-}$, $[541.03]^{1-}$
ΔUA-GlcNS (NS)	$[416.04]^{1-}$		
ΔUA2S-GlcNS (NS2S)	$[247.50]^{2-}$, $[496.01]^{1-}$		
ΔUA-GlcNS6S (NS6S)	$[247.50]^{2-}$, $[496.01]^{1-}$		
ΔUA2S-GlcNS6S (Tri S)	$[287.48]^{2-}$, $[575.96]^{1-}$		

spectrometry (HILIC–MS) to measure unsubstituted amino groups formed in thermally stressed heparin formulations.

USP heparin sodium (200 U/mg, Celsus Laboratories, Cincinnati, OH, USA) was prepared at 1000 U/ml in 0.9% aqueous sodium chloride at pH 7.0. A heparin sample (60 ml) was divided into six equal portions. One portion was retained (control), and two portions were treated under 65 and 85 °C for 2 h, respectively (thermally stressed). The three remaining heparin samples were sterilized in an autoclave at 121 °C for 0.5, 1, and 2 h, respectively (sterilized heparin) [6]. For each sample, 1 ml (containing 5 mg of heparin) was lyophilized.

Samples (each consisting of 5 mg polysaccharide), including control heparin, thermally stressed heparins, sterilized heparins, and chemically N-desulfonated heparin (CNDS) prepared as described previously [9], were dissolved in 1 ml of 50 mM aqueous sodium carbonate containing 10% (v/v) methanol. Aliquots (8 μl) of acetic anhydride-d_6 (or acetic anhydride in the case of one of the CNDS samples [negative control]) were added into the heparin solution every 20 min over a total reaction time of 2 h. After the chemical N-acetylation, all samples were desalted using 3-kDa molecular weight cutoff (MWCO) membranes and lyophilized. One-dimensional (1D) ^{1}H NMR (600 MHz, Bruker Bio Spin, Billerica, MA, USA) of CNDS confirmed the presence of unsubstituted amino groups, and their conversion to *N*-acetyl groups following treatment with acetic anhydride (see Fig. S1 in online supplementary material) and deuterium labeling of CNDS was confirmed using 1D ^{2}H NMR (Fig. S2). Two-dimensional (2D) heteronuclear single-quantum coherence (HSQC) NMR allowed for spectral assignments and confirmed that all of the amino groups had been N-acetylated (Fig. S3) [2,10].

Treatment of each heparin sample (10 μl, 10 μg/μl) with recombinant *Flavobacterial* heparin lyases I, II and III [11] (10 mU each) in 90 μl of 40 mM ammonium acetate and 2 mM calcium acetate aqueous (pH 7.0) at 33 °C for 10 h completely degraded the heparin samples to their constituent disaccharides. Some residues with unsubstituted amino groups are resistant to heparin lyase degradation [7]. Moreover, most of the unsubstituted disaccharides are isobaric with other disaccharides. Thus, we decided to N-acetylate heparin samples containing such residues with acetic anhydride-d_6 to afford disaccharide products with unique masses with retention times matched to readily available disaccharide standards. After enzymatic treatment, samples were heated in a 100 °C water bath for 10 min to thermally inactivate the enzymes and were centrifuged (5000*g*, 10 min) to remove precipitated protein [10], and the supernatants were collected for liquid chromatography (LC)–MS analysis using a Luna HILIC column (2.0 × 50 mm, 200 Å, Phenomenex, Torrance, CA, USA) connected online to the standard electrospray ionization (ESI) source of an LTQ Orbitrap XL FT-MS instrument (Thermo Fisher Scientific, San Jose, CA, USA) [2]. The short column selected provides sufficient resolution for rapid analysis (base peak width of ~0.2–0.5 min) and removes salts and proteins from the analyte. Longer (2.0 × 150 mm) columns markedly improve resolution but result in lower sample throughput [12]. Gradient elution used 5 to 70% 5 mM aqueous ammonium acetate with 95 to 30% 5 mM aqueous ammonium acetate in 98% acetonitrile over 7 min at 250 μl/min. Unsaturated heparin disaccharide standards were analyzed at different concentrations to prepare a standard curve [2]. All of the disaccharides and deuterated disaccharides detected (see Fig. S4 in supplementary material) and their masses are provided in Table 1. The mass percentages of these

Table 2
Disaccharide compositional analysis of heparin samples following N-acetylation.

Sample	Unlabeled disaccharides (%)						Deuterium-labeled disaccharides (%)			Total deuterium-labeled disaccharides (%)
	0S	2S/6S	2S6S	NS	NS2S/NS6S	Tri S	0S	2S/6S	2S6S	
CNDS	9.91	13.9	5.90	68.50	0.36	1.38	0.04	<0.01	0.02	0.06
Ac-CNDS	3.85	14.0	81.7	0.18	0.01	0.23	<0.01	<0.01	0.02	0.02
Ac-d_3-CNDS	2.00	5.14	2.30	0.17	<0.01	0.25	1.69	10.3	78.2	90.1
Heparin ($n = 3$)	3.98 ± 0.42	4.97 ± 0.78	4.44 ± 0.43	7.40 ± 1.15	9.20 ± 0.98	69.7 ± 1.28	0.07 ± 0.01	0.04 ± 0.01	0.16 ± 0.01	0.27 ± 0.02
65 °C – 48 h	4.65	6.23	4.79	11.19	0.28	72.56	0.08	0.04	0.17	0.30
85 °C – 48 h	5.12	6.39	5.19	10.39	0.21	72.05	0.10	0.07	0.47	0.64
121 °C – 0.5 h	4.18	4.61	4.18	8.36	8.08	70.09	0.08	0.06	0.35	0.49
121 °C – 1 h	4.22	4.99	4.51	7.05	9.61	68.79	0.08	0.08	0.67	0.83
121 °C – 2 h	4.01	4.40	4.71	9.55	8.80	64.94	0.15	0.25	3.19	3.58

Note. The IC_{50} values determined in competitive AT binding measured by SPR (see Fig. S5 in supplementary material) are as follows: heparin, 1.38 U/ml; 65 °C – 48 h, 1.52 U/ml; 85 °C – 48 h, 1.76 U/ml; 121 °C – 0.5 h, 1.84 U/ml; 121 °C – 1 h, 1.96 U/ml; 121 °C – 2 h, 3.81 U/ml.

disaccharides are presented in Table 2. Little, if any, deuterated disaccharide (natural abundance of ~0.02%) was detected in the negative controls corresponding to CNDS (0.06%) and N-acetylated CNDS (0.02%) samples (Table 2). In contrast, the positive control, N-acetylated-d_3 CNDS, showed the expected high percentage (~90%) of deuterated disaccharide (see Fig. S6 in supplementary material). These data are consistent with the NMR results showing complete N-acetylation of unsubstituted amine groups and suggesting the low background and high sensitivity of this assay method. Heparin API, examined in triplicate, showed a relatively low level of unsubstituted amino groups (0.27 ± 0.02%), establishing the excellent repeatability and stability of the current method. The heparin sample treated for 48 h at 85 °C generated twice as many unsubstituted amino groups than the sample treated at 65 °C, and the autoclaved samples showed the greatest loss of *N*-sulfo groups. *N*-Sulfo groups appeared to be lost in equal amounts from monosulfated, disulfated, and trisulfated sequences, suggesting that this solvolysis reaction is not selective (Table 2). Prior studies have demonstrated that autoclaving heparin reduces its anticoagulant potency [2].

Solution competition studies between surface-immobilized heparin and different concentrations (0–10 μg/ml) of soluble heparin sample and soluble AT (250 nM) to measure IC_{50} were performed using SPR on a BIAcore 3000 instrument (GE Healthcare) as described previously [2]. AT-binding affinity of heparin is dependent on the presence of an *N*-sulfo group-containing AT-binding site within a heparin chain [1], and this measured IC_{50} correlates to in vitro potency assays [2]. As expected, increased temperature and longer heat exposure times result in higher *N*-sulfo group loss (Table 1) and higher IC_{50} values (Table 2).

In conclusion, a facile assay for the presence and formation of unsubstituted amino groups within heparin has been described. This assay shows that loss of *N*-sulfo groups in formulated heparin is proportional to the temperature and length of exposure in thermally stressed samples. Moreover, this loss of *N*-sulfo groups, although nonselective, results in decreased heparin affinity for AT, corresponding to reduced potency.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2014.05.028.

References

[1] R.J. Linhardt, Heparin: structure and activity, J. Med. Chem. 46 (2003) 2521–2564.
[2] L. Fu, G. Li, B. Yang, A. Onishi, L. Li, P. Sun, F. Zhang, R.J. Linhardt, Structural characterization of pharmaceutical heparins prepared from different animal tissues, J. Pharm. Sci. 102 (2013) 1447–1457.
[3] M. Guerrini, D. Beccati, Z. Shriver, A.M. Naggi, A. Bisio, I. Capila, J. Lansing, S. Guglieri, B. Fraser, A. Al-Hakim, S. Gunay, K. Viswanathan, Z. Zhang, L. Robinson, G. Venkataraman, L. Buhse, M. Nasr, J. Woodcock, R. Langer, R.J. Linhardt, B. Casu, G. Torri, R. Sasisekharan, Oversulfated chondroitin sulfate is a major contaminant in heparin associated with adverse clinical events, Nat. Biotechnol. 26 (2008) 669–775.
[4] H. Liu, Z. Zhang, R.J. Linhardt, Lessons learned from the contamination of heparin, Nat. Prod. Rep. 26 (2009) 313–321.
[5] K.A. Jandik, D. Kruep, M. Cartier, R.J. Linhardt, Accelerated stability studies of heparin, J. Pharm. Sci. 85 (1996) 45–51.
[6] J.M. Beaudet, A. Weyers, K. Solakyildirim, B. Yang, M. Takieddin, S. Mousa, F. Zhang, R.J. Linhardt, Impact of autoclave sterilization on the activity and structure of formulated heparin, J. Pharm. Sci. 100 (2011) 3396–3404.
[7] T. Toida, H. Yoshida, H. Toyoda, I. Koshiishi, T. Imanari, R.E. Hileman, J.R. Fromm, R.J. Linhardt, Structural differences and the presence of unsubstituted amino groups in heparan sulphates from different tissues and species, Biochem. J. 322 (1997) 499–506.
[8] L. Liu, R.J. Linhardt, Z. Zhang, Quantitative analysis of free and covalent anions in glycosaminoglycans and application in heparin stability studies, Carbohydr. Polym. 106 (2014) 343–350.
[9] R.J. Linhardt, Chemical and enzymatic methods for the depolymerization and modification of heparin, in: H. Ogura, A. Hasegawa, T. Suami (Eds.), Carbohydrates: Synthetic Methods and Applications in Medicinal Chemistry, Kodansha/VCH, Tokyo, 1992, pp. 385–401.
[10] L. Fu, F. Zhang, G. Li, A. Onishi, U. Bhaskar, P. Sun, R.J. Linhardt, Structure and activity of a new low-molecular-weight heparin produced by enzymatic ultrafiltration, J. Pharm. Sci. 103 (2014) 1375–1383.
[11] J. Chen, C.L. Jones, J. Liu, Using an enzymatic combinatorial approach to identify anticoagulant heparan sulfate structures, Chem. Biol. 14 (2007) 986–993.
[12] L. Li, F. Zhang, J. Zaia, R.J. Linhardt, Top-down approach for the direct characterization of low molecular weight heparins using LC–FT–MS, Anal. Chem. 84 (2012) 8822–8829.