Impact of Autoclave Sterilization on the Activity and Structure of Formulated Heparin

JULIE M. BEAUDET,[1] AMANDA WEYERS,[1] KEMAL SOLAKYILDIRIM,[1] BO YANG,[1] MAJDE TAKIEDDIN,[2] SHAKER MOUSA,[2] FUMING ZHANG,[3] ROBERT J. LINHARDT[1,3,4,5]

[1]Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, Troy, New York 12180

[2]The Pharmaceutical Research Institute, Albany College of Pharmacy and Health Sciences, Albany, New York 12208

[3]Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, New York 12180

[4]Department of Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180

[5]Department of Biology, Rensselaer Polytechnic Institute, Troy, New York 12180

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ABSTRACT: The stability of a formulated heparin was examined during its sterilization by autoclaving. A new method to follow loss in heparin binding to the serine protease inhibitor, antithrombin III, and the serine protease, thrombin, was developed using a surface plasmon resonance competitive binding assay. This loss in binding affinity correlated well with loss in antifactor IIa (thrombin) activity as well as antifactor Xa activity as measured using conventional amidolytic assays. Autoclaving also resulted in a modest breakdown of the heparin backbone as confirmed by a slight reduction in number-averaged and weight-averaged molecular weight and an increase in polydispersity. Although no clear changes were observed by nuclear magnetic resonance spectroscopy, disaccharide composition analysis using high-performance liquid chromatography–electrospray ionization–mass spectrometry suggested that loss of selected sulfo groups had taken place. It is this sulfo group loss that probably accounts for a decrease in the binding of autoclaved heparin to antithrombin III and thrombin as well as the observed decrease in its amidolytic activity. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:3396–3404, 2011

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INTRODUCTION

Heparin is a highly sulfated, linear glycosaminoglycan that is abundantly found in mucosal tissues such as the lungs and intestines. The structure of this approximately 10–20 kDa polysaccharide is predominantly made up of a major repeating disaccharide unit, α-l-IdoA2S (1→4)-α-d-GlcNS6S (where IdoA is idopyranosyluronic acid, S is sulfo, and GlcN is 2-deoxy, 2-amino glucopyranose).[1] The structure and sulfation pattern of the molecule are integral to its therapeutic value. In particular, a unique pentasaccharide sequence present in heparin having the structure, →GlcNAc6S→GlcA →GlcNS3S6S→IdoA2S→GlcNS6S→, where GlcA is d-glucopyranosyluronic acid and Ac is acetyl, is responsible for its specific binding to the serine protease inhibitor antithrombin III (ATIII), resulting in its conformational activation and leading to the inhibition of major coagulation cascade proteases, including thrombin [factor (F) IIa] and FXa.[1] Thrombin binds, with less specificity, to an adjacent decasaccharide domain comprised of heparin's abundant trisulfated disaccharide repeating unit, →IdoA2S→GlcNS6S→, resulting in a ternary thrombin–heparin–ATIII complex.[1] Unlike thrombin, FXa does not form a ternary complex as it does not directly interact with heparin but, rather, is inhibited

Correspondence to: Robert J. Linhardt (Telephone: +518-276-3404; Fax: +518-276-3405; E-mail: linhar@rpi.edu); Fuming Zhang (Telephone: +518-276-6839; Fax: +518-276-3405; E-mail: zhangf2@rpi.edu)

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directly by conformationally activated ATIII. Thus, the inactivation of thrombin by ATIII requires a longer heparin chain (having >15 saccharide units) than the small pentasaccharide capable of promoting the ATIII inactivation. Thus, although heparin exhibits equal anti-FXa/anti-FIIa activity, low-molecular-weight heparins (LMWHs) show greater anti-FXa activity than anti-IIa activity.[1]

Heparin is commonly used as an injectable anticoagulant during medical procedures. Despite heparin's widespread use for over 80 years, there are very few publications detailing its stability.[2,3] Previous work in our laboratory described one of the few stability studies on heparin active pharmaceutical ingredient (API) under highly acidic/basic conditions over extended periods of time.[2] This study demonstrated that under highly basic conditions, heparin chain length was reduced through β-elimination, whereas under acidic conditions, heparin chain length was reduced through hydrolysis. Loss of sulfation was also observed under acidic conditions but not under basic conditions. A previous study on the stability of enoxaparin, a form of LMWH, heated the compound for several days.[3] These researchers observed decrease in anti-FXa, a loss of sulfo groups, as well as an increase in unsubstituted amine groups and reducing capacity over the duration of the experiment. Both exhibited steep changes over the 24 h and then showed remarkably less change in the remaining more than 500 h of the experiment. The current study examines the stability of a heparin formulation following its sterilization by autoclaving. Autoclave sterilization is currently widely used in the pharmaceutical industry for preparing sterile heparin formulations for injection and parenteral use. The current study correlates changes in heparin binding determined by surface plasmon resonance (SPR) with changes in heparin's *in vitro* activity. Activity was measured using anti-FIIa (thrombin) and anti-FXa bioassays. Changes in heparin chain length were determined using polyacrylamide gel electrophoresis (PAGE), and changes in its fine structure were examined by nuclear magnetic resonance (NMR) spectroscopy and by disaccharide analysis using high-performance liquid chromatography (HPLC)–mass spectrometry (MS). A measure of heparin stability on autoclaving is provided.

Experimental

Materials

The United States Pharmacopeia (USP) heparin sodium (>180 U/mg) active pharmaceutical ingredient (API) was purchased from multiple commercial suppliers. Recombinant *Flavobacterial* heparin lyase I, II, and III were expressed in our laboratory using *Escherichia coli* strains, provided by Professor Jian Liu (College of Pharmacy, University of North Carolina, Chapel Hill, North Carolina). Polyacrylamide, alcian blue dye, 2-cyanoacetamide, tetra-*n*-butylamonium hydrogen sulfate, and all other reagents used in this study were from Sigma (St. Louis, Missouri, USA). Unsaturated heparin/HS disaccharides standards (Di-0S, ΔUA-GlcNAc; Di-NS, ΔUA-GlcNS; Di-6S, ΔUA-GlcNAc6S; Di-UA2S, ΔUA2S-GlcNAc; Di-UA2SNS, ΔUA2S-GlcNS; Di-NS6S, ΔUA-GlcNS6S; Di-UA2S6S, ΔUA2S-GlcNAc6S; and Di-triS, ΔUA2S-GlcNS6S) were obtained from Seikagaku Corporation (Tokyo, Japan). ATIII and human thrombin were acquired from Hyphen Biomed (Neuville-sur-Oise, France).

Formulation and Sterilization of Heparin

Heparin sodium (40 U/mL) in 5% dextrose (pH 5.8) was prepared and 1 L of this sample was divided into four equal portions. One portion was retained (unsterilized heparin solution). The three remaining heparin samples were sterilized at 121°C for 30, 60, and 120 min, respectively.

SPR Analysis

Preparation of Heparin Biochip

SPR was performed on a BIAcore 3000 (GE Healthcare, Uppsala, Sweden). Buffers were filtered (0.22 μM) and degassed. The biotinylated heparin was prepared by reaction of sulfo-*N*-hydroxysuccinimide long-chain biotin (Piece, Rockford, Illinois, USA) with free amino groups of unsubstituted glucosamine residues in the polysaccharide chain following a published procedure.[4] Biotinylated heparin was immobilized to streptavidin (SA) chip based on the manufacturer's protocol. In brief, 20 μL solution of the heparin–biotin conjugate (0.1 mg/mL) in HBS-EP [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM sodium chloride, 3 nM ethylenediaminetetraacetic acid (EDTA), 0.005% polysorbate surfactant P20 (pH 7.4) buffer] running buffer was injected over flow cell 2 of the SA chip (GE Healthcare, Uppsala, Sweden). at a flow rate of 10 μL/min. The successful immobilization of heparin was confirmed by the observation of a 100-resonance unit (RU) increase in the sensor chip. The control flow cell was prepared by 1 min injection with saturated biotin.

Measurement of Interaction Between Heparin, ATIII, and Thrombin Using SPR

The protein (ATIII or thrombin) samples were diluted in HBS-EP buffer (GE Healthcare, Uppsala, Sweden). Different dilutions of protein samples were injected at a flow rate of 30 μL/min. At the end of the sample injection, the same buffer was flowed over the sensor surface to facilitate dissociation. After a 3 min dissociation time, the sensor surface was fully