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Determination of galactosamine impurities in heparin sodium using fluorescent labeling and conventional high-performance liquid chromatography

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ABSTRACT

Heparin is a sulfated glycosaminoglycan (GAG), which contains *N*-acetylated or *N*-sulfated glucosamine (GlcN). Heparin, which is generally obtained from the healthy porcine intestines, is widely used as an anticoagulant during dialysis and treatments of thrombosis such as disseminated intravascular coagulation. Dermatan sulfate (DS) and chondroitin sulfate (CS), which are galactosamine (GalN)-containing GAGs, are major process-related impurities of heparin products. The varying DS and CS contents between heparin products can be responsible for the different anticoagulant activities of heparin. Therefore, a test to determine the concentrations of GalN-containing GAG is essential to ensure the quality and safety of heparin products. In this study, we developed a method for determination of relative content of GalN from GalN-containing GAG in heparin active pharmaceutical ingredients (APIs). The method validation and collaborative study with heparin manufacturers and suppliers showed that our method has enough specificity, sensitivity, linearity, repeatability, reproducibility, and recovery as the limiting test for GalN from GalN-containing GAGs. We believe that our method will be useful for ensuring quality, efficacy, and safety of pharmaceutical heparins. On July 30, 2010, the GalN limiting test based on our method was adopted in the heparin sodium monograph in the Japanese Pharmacopoeia.

1. Introduction

Heparin is a glycosaminoglycan (GAG), which consists of repeating disaccharide units composed of uronic acid (D-glucuronic acid [GlcA] or L-iduronic acid [IdoA]) and D-glucosamine (GlcN) with 0–4 (mean value, 2.6) sulfates [1–3]. Heparin, which is generally obtained from healthy porcine intestines, is widely used as an anticoagulant during dialysis and treatments of thrombosis such as disseminated intravascular coagulation. Heparin sodium active pharmaceutical ingredients (APIs) contain dermatan sulfate (DS), which consists of repeating disaccharide units of IdoA and galactosamine (GalN), as one of the major process-related impurities. In addition, APIs contain small amounts of chondroitin sulfate (CS), which consists of repeating disaccharide units of GlcA and GalN. The levels of GalN-containing GAGs vary between batches or

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lots of heparin APIs [4] and these levels can affect anticoagulant activities [5–8]. Therefore, a test to determine the concentration of GalN-containing GAGs is essential to ensure the quality and safety of heparin products.

Currently, several methods have been used for the determination of GalN-containing GAGs in heparin samples, such as proton nuclear magnetic resonance (^{1}H NMR) [9,10], capillary electrophoresis [11,12], anion-exchange high-performance liquid chromatography (HPLC) [13], and nitrite degradation combined with anion-exchange HPLC [14]. The GalN limiting test described in the heparin sodium monograph in the 2009 United States Pharmacopeia (USP) included a combination of acidic hydrolysis and strong anion exchange HPLC with pulsed amperometric detection (SAX-HPLC/PAD) [15]. This method is well established and sensitive and allows detection of 0.04% GalN in total hexosamine without using reference standards for GAG. However, the only suitable column for this method is the Dionex CarboPac PA20 column. On the other hand, the limiting test for GalN-containing GAGs in the heparin sodium and heparin calcium monographs in the 2010 European Pharmacopoeia (EP) includes a combination of nitrite degradation and SAX-HPLC with ultraviolet (UV) detection [16]. Although this method is easy and sensitive, the reference standard for GalN-containing GAGs is required for assignment of peaks.

In this study, we developed an alternative method for determination of GalN using combination of simple fluorescent (FL) labeling for monosaccharides and conventional HPLC system using a C18 column. The monosaccharide compositional analysis typically involves labeling the monosaccharides with a phosphorogen or chromogen [17] such as 2-aminopyridine [18,19], 2-aminobenzoic acid [20], 1-phenyl-3-methyl-5-pyrazolone [21,22], or ethyl 4-aminobenzoate (ABEE) [23] and visualization using UV or FL detections. Because of ease of labeling, we chose to label hexosamines with ABEE. We performed a study in collaboration with heparin manufacturers and suppliers and method validation and evaluated the utility of ABEE-labeling followed by reversed-phase HPLC (ABEE/RP-HPLC) with FL and UV detection as the limiting test for determining relative GalN content in GalN-containing GAGs in heparin sodium.

2. Materials and methods

2.1. Materials

D-Glucosamine hydrochloride, D-galactosamine hydrochloride, and D-mannosamine hydrochloride were purchased from Sigma–Aldrich (MO, USA), Seikagaku Biobusiness (Tokyo, Japan), and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Borane–pyridine complexes and trifluoroacetic acid (TFA) were purchased from Tokyo Chemical Industry Co., Ltd. and Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ABEE was obtained from Wako Pure Chemical Industries, Ltd. Commercially available heparin sodium and DS were purchased from Calbiochem (CA, USA), Seikagaku Biobusiness, and Sigma–Aldrich. Hydrochloric acid (HCl; content of hydrogen chloride, 35%) was purchased from Nacalai Tesque (Kyoto, Japan) and Kishida Chemical Co. Ltd. Acetonitrile (ACN) was purchased from ThermoFisher Scientific (MA, USA) and Sigma–Aldrich.

2.2. Hydrolysis conditions

Heparin sodium (2.4 mg) was dissolved in 1.0 mL of 5 M HCl, and 500 μL of the solution was incubated in a glass tube fitted with a screw cap at 100 °C for 6 h. When the solution cooled to room temperature, 100 μL of the solution was dried under vacuum.

2.3. High-performance anion exchange chromatography with PAD

A 5-μL aliquot of the sample solution was injected into a high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD) system (Dionex Co., CA, USA) equipped with a CarboPac PA1 column (250 × 4.0 mm; Dionex Co.) equilibrated with 8 mM NaOH solution. Hexosamines were eluted isocratically at a flow rate of 1 mL/min at 30 °C. In addition, incomplete degradation products from heparin sodium were eluted with 8 mM NaOH solution in 1 min and followed by linear gradient from 0% to 40% of 1 M sodium acetate in 15 min at a flow rate of 1 mL/min at 30 °C. The hexosamines and incomplete degradation products were detected by PAD by using the following pulse potentials: $E1 = 0.1$ V ($t1 = 0.0$–0.4 ms), $E2 = -2.0$ V ($t2 = 0.41$–0.42 ms), $E3 = 0.60$ V ($t3 = 0.43$ ms), and $E4 = -0.10$ V ($t4 = 0.44$–0.50 ms).

2.4. Labeling of hexosamines with ABEE

Labeling of hexosamines with ABEE was performed according to a previously established method [23]. Briefly, ABEE (280 mg) was dissolved in 600 μL of methanol at 50 °C; further, we added 170 μL of acetic acid and 145 μL of a borane–pyridine complex to the solution. After drying the hydrolyzed sample, 50 μL of methanol was added, mixed, and dried again under vacuum. The residue was dissolved in 10 μL of water, and 40 μL of ABEE solution was added to the solution. The mixture was incubated at 80 °C for 1 h and then dried under vacuum. Water (200 μL) and ethyl acetate (200 μL) were added to the residues. The solution was mixed vigorously and then centrifuged. The upper layer was removed, and 200 μL of ethyl acetate was added to the lower layer again. The solution was mixed and centrifuged. The lower layer (5 μL) was used as a sample solution.

2.5. HPLC

Buffer A was a solution of purified water and TFA (1000:1) and buffer B was a solution of buffer A and ACN (1:1). A 5-μL aliquot of the sample solution was injected into the HPLC system equipped with Capcellpack C18 MGII S3 column (150 × 4.6 mm; particle size, 3 μm; Shiseido Co., Ltd., Tokyo, Japan). The ABEE-labeled hexosamines were eluted isocratically using an eluent containing 14% buffer B. The eluted ABEE-labeled hexosamines were monitored using FL detection (excitation wavelength, 305 nm; emission wavelength, 360 nm) or UV detection (305 nm). The flow rate and column temperature were set at 1.0 mL/min and 45 °C, respectively. The HPLC apparatuses used in this study were as follows: Alliance 2695 (Waters Co., Milford, MA, USA) in laboratories A, C, E, and I; CLASS-VP (Shimadzu Co., Kyoto, Japan) in laboratory B; Agilent 1200 (Agilent Technologies, Inc., California, USA) in laboratories D and G; Prominence20A (Shimadzu Co., Kyoto, Japan) in laboratory F; and LC-2010CHT (Shimadzu Co., Kyoto, Japan) in laboratory H.

2.6. Method validation

The validation of ABEE/RP-HPLC method was performed at laboratory D. Linearity between DS concentration (%) and peak area ratio of ABEE-labeled GalN/GlcN was evaluated by using commercially available heparin sodium (2.4 mg/mL) spiked with 0.025, 0.05, 0.1, 0.5, 1.0, 2.0, and 5.0% DS. Coefficient of determination (R^2) was determined by using a least-squares linear regression model. The detection limit (DL) and quantification limit (QL) were evaluated on the basis of regression equations (DL = 3.3 × σ/slope and QL = 10 × σ/slope; σ = standard error). Repeatability was evaluated by calculating the relative standard deviation (RSD)% of peak area