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Quantitative capillary electrophoresis determination of oversulfated chondroitin sulfate as a contaminant in heparin preparations

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ABSTRACT

A simple, accurate, and robust quantitative capillary electrophoresis (CE) method for the determination of oversulfated chondroitin sulfate (OSCS) as a contaminant in heparin (Hep) preparations is described. After degradation of the polysaccharides by acidic hydrolysis, the hexosamines produced (i.e., GlcN from Hep and GalN from OSCS) were derivatized with anthranilic acid (AA) and separated by means of CE in approximately 10 min with high sensitivity detection at 214 nm (limit of detection [LOD] of ~200 pg). Furthermore, AA-derivatized GlcN and GalN showed quite similar molar absorptivity, allowing direct and simple quantification of OSCS in Hep samples. Moreover, a preliminary step of specific enzymatic treatment by using chondroitin ABC lyase may be applied for the specific elimination of interference in the analysis due to the possible presence in Hep samples of natural chondroitin sulfate and dermatan sulfate impurities, making this analytical approach highly specific for OSCS contamination given that chondroitin ABC lyase is unable to act on this semisynthetic polymer. The CE method was validated for specificity, linearity, accuracy, precision, LOD, and limit of quantification (LOQ). Due to the very high sensitivity of CE, as little as 1% OSCS contaminant in Hep sample could be detected and quantified. Finally, a contaminated raw Hep sample was found to contain 38.9% OSCS, whereas a formulated contaminated Hep was calculated to have 39.7% OSCS.

Heparin (Hep)[1] is a linear sulfated natural polysaccharide consisting of 1→4 linked pyranosyluronic acid (uronic acid, either α-L-iduronic or β-D-glucuronic acid with some *O*-sulfo substitution) and 2-amino-2-deoxyglucopyranose (α–D-glucosamine [GlcN] with either *N*-sulfo or *N*-acetyl substitution) repeating units [1]. It belongs to the family of glycosaminoglycans (GAGs) endowed with anticoagulant and antithrombotic properties [1–3] used clinically over the past half-century as an anticoagulant drug [1]. Unfortunately, Hep possesses several undesirable side effects that include dangerous haemorrhagic complications [4,5]. It was for this reason that low-molecular-weight (LMW) Heps (average molecular mass of 3000–8000) were introduced as Hep substitutes having reduced side effects, more predictable pharmacological action, sustained antithrombotic activity, and improved bioavailability [6,7].

Recently, patients presented, within several minutes after intravenous infusion of Hep, angioedema, hypotension, swelling of the larynx, and related symptoms that in some cases ended in death [8–10]. The contaminant was identified as an unusual oversulfated form of chondroitin sulfate (OSCS) present in high content in suspect lots of Hep [8]. Furthermore, dermatan sulfate (DS), a known impurity of Hep [11], was also found in selected samples with no other contaminant or impurities observed. Finally, initial reports have suggested that this OSCS macromolecule was also present in LMW Hep formulations [10] with a greater difficulty to be detected depending on the depolymerization process adopted [10].

The structure of the OSCS contaminant, present within specific lots of Hep, has been fully identified by using multiple orthogonal techniques, including multidimensional nuclear magnetic resonance (NMR), to overcome the challenges inherent in the analysis of complex polysaccharides, including Hep [8]. The structure of OSCS was definitively confirmed as formed of disaccharide repeat units of D-glucuronic acid linked β1 → 3 to a β-*N*-acetyl-D-galactosamine (GalN). The disaccharide unit was found to possess an unusual sulfation pattern, being sulfated at the 2-and 3-positions of the glucuronic acid as well as at the 4- and 6-positions of the GalN unit [8].

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[1] *Abbreviations used:* Hep, heparin; GAG, glycosaminoglycan; LMW Hep, low-molecular-weight heparin; GlcN, glucosamine (2-amino-2-deoxy-d-glucose); OSCS, oversulfated chondroitin sulfate; DS, dermatan sulfate; NMR, nuclear magnetic resonance; GalN, galactosamine (2-amino-2-deoxy-d-galactose); CE, capillary electrophoresis; AA, anthranilic acid (2-aminobenzoic acid); CS, chondroitin sulfate; Rib, ribose; CSA, chondroitin sulfate A; CSC, chondroitin sulfate C; API, active pharmaceutical ingredient; UV, ultraviolet; LOD, limit of detection; LOQ, limit of quantification; MT, migration time; PA, peak area; CV%, relative standard deviation percentage; REC%, recovery ratio percentage.

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Due to the nature of this contaminant, traditional screening tests and analytical approaches are unable to differentiate between contaminated and uncontaminated lots. Furthermore, the methodology used for the initial characterization of this contaminant (i.e., multiple orthogonal techniques, including multidimensional NMR [8]) is complex, expensive, and not useful for quality assurance in quality control laboratories because it is unable to process many samples in a short time and to give quantitative results with low coefficient of variation values. In contrast, capillary electrophoresis (CE) has been applied in the analysis of intact GAGs and GAG-derived oligosaccharides and disaccharides, such as monosaccharides, affording concentration and structural characterization data due to its high resolving power and sensitivity [12]. In this article, a validated CE method has been developed for the quantitative determination of OSCS in Hep preparations after degradation of the polysaccharides to produce hexosamine units (i.e., GlcN from Hep and GalN from OSCS), their derivatization with anthranilic acid (AA, 2-aminobenzoic acid), and separation by means of CE at 214 nm. Furthermore, a preliminary step of specific enzymatic treatment may be applied for the specific elimination of interference in the analysis due to the possible presence in Hep samples of natural chondroitin sulfate (CS)/DS [11]. Finally, this analytical approach has been performed on raw material such as Hep final formulations and is also potentially applicable to LMW Hep preparations.

Materials and methods

Materials

D-(+)-GlcN hydrochloride, D-(+)-GalN hydrochloride, D-ribose (Rib), AA, sodium cyanoborohydride, chondroitinase ABC, chondroitin ABC lyase from *Proteus vulgaris* (EC 4.2.2.4), 0.5 to 2.0 units/mg, were obtained from Sigma–Aldrich. Chondroitin sulfate A (CSA) from bovine trachea, chondroitin sulfate C (CSC) from shark cartilage, DS, and Hep from porcine intestinal mucosa were obtained from Sigma. Microcon YM-3 filters having a molecular mass cutoff of 3000 Da were obtained from Amicon. All other reagents were of analytical grade. OSCS was prepared from CS according to previously published procedures [8,10,13]. A contaminated Hep active pharmaceutical ingredient (API) sample, a contaminated raw Hep sample, and a contaminated formulated Hep sample were obtained from Hep manufacturers [10].

NMR analysis

The ^{13}C-NMR spectra of OSCS were recorded by a Bruker AMX400 WB spectrometer operating at 100.61 MHz. The sample was previously lyophilized three times with D_2O and finally prepared by dissolving 200 mg in 2.0 ml of D_2O at a high level of deuteration (99.997%). The spectra were recorded at a temperature of 33 °C and pH 6.5 unless otherwise specified.

Sample preparation

Stock solutions of GlcN and GalN standard were prepared by dissolving an accurately weighed amount of 50 mg in 5 ml (10 mg/ml) of doubly distilled water. A series of standard solutions were obtained by dilution of the stock solution in a standard volume of water (200 µl) and lyophilized.

Samples were prepared by dissolving an accurately weighed amount (10 mg) of sample in 10 ml (1 mg/ml) of doubly distilled water. Sample solutions (200 µl) were lyophilized and reconstituted with 200 µl of accurately prepared 2 M HCl. After 60 min at 110 °C (see below for the time course of chemical treatment), the samples were reconstituted with 2 ml of doubly distilled water and lyophilized.

In the case of pretreatment with chondroitinase ABC to degrade possible CS/DS impurity, Hep samples (200 µl of the above-prepared solutions) were incubated with enzyme (40 µl containing 100 milliunits) and 260 µl of 50 mM ammonium acetate at pH 8.0 for 12 h at 37 °C. After boiling for 5 min, the samples were filtered on the YM-3 centrifugal filters at 10,000*g* for 60 min. The undigested polysaccharides (i.e., Hep and OSCS) were recovered from the retentate, lyophilized, and submitted to chemical hydrolysis with 2 M HCl (as described above).

Derivatization of hexosamine with AA

Lyophilized GlcN and GalN standard solutions or treated samples, in the presence of internal standard Rib, were dissolved in 50 µl of 1% fresh sodium acetate and 50 µl of AA (30 mg) and sodium cyanoborohydride (20 mg) dissolved in 1 ml of methanol–acetate–borate solution (120 mg of sodium acetate and 100 mg of boric acid in 5 ml of methanol) [14]. Tubes were heated at 80 °C for 60 min. After cooling to room temperature, the samples were made up to 150 µl with doubly distilled water and analyzed by CE.

Capillary electrophoresis

CE was performed on a Beckman HPCE instrument (P/ACE system 5000) equipped with an ultraviolet (UV) detector set at 214 nm. Separation and analysis were performed on an uncoated fused-silica capillary tube (50 µm i.d., 85 cm total length, and 65 cm from the injection point to the detector) at 25 °C. The operating buffer was composed of 150 mM boric acid and 50 mM NaH_2PO_4 buffered at pH 7.0 with NaOH solution. The buffer was degassed by vacuum filtration through a 0.2-µm membrane filter, followed by agitation in an ultrasonic bath. Before each run, the capillary tube was washed with 0.1 M NaOH for 1 min, washed with doubly distilled water for 5 min, and then conditioned with the operating buffer for 5 min. The samples to be analyzed were injected automatically using the pressure injection mode in which the sample is pressurized for 10 s. The injection volume can be calculated with Poiseuille's equation as proposed by the manufacturer, giving an estimated volume of 6 nl per second of injection time. Electrophoresis was performed at 15 kV (~35 µA) using normal polarity. Peak areas were recorded and calculated using the Beckman software system Gold V810.

Validation of analytical method

The quantitative CE/UV method validations were established according to the Guidance for Industry: Bioanalytical Method Validation (U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine) published in May 2001 [15], including specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, recovery, and robustness tests. The detection limits were estimated as the quantity of GlcN and GalN producing signal/noise ratios of 3:1 for LOD and 10:1 for LOQ. The specificity of the CE/UV technique was determined with migration time (MT) and peak area (PA) of the two hexosamine peaks through the precision analysis assay. The calibration curves were constructed from PA versus concentrations of GlcN/GalN standard. Linear regression analysis was used to calculate the slope, intercept, and correlation coefficient (r^2) of the calibration curve. The precision of the method was assessed by determination of hexosamines with five replicates ($n = 5$) of five different concentrations (from 240 to 2400 pg) of standard solutions. Intra- and interday precision and accuracy of the method were estimated by relative standard deviation percentage