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Fine structural characterization of sulodexide

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ABSTRACT

Sulodexide is a heparinoid which combines the properties of its components heparin and dermatan sulfate and is used not only for the prophylaxis and treatment of thromboembolic diseases but also for the treatment of diabetic nephropathy. Despite many clinical studies have been conducted to investigate its activity and safety, no data are available on the fine chemical characterization of its components. In this work, the in-depth investigation on the structural features of both the whole mixture and the isolated components was accomplished, involving the analysis of molecular weight distribution and of their mono, di and oligosaccharide composition by HP-SEC/TDA, 2D-NMR and HPLC–MS techniques. Moreover, also the separation of fractions endowed of graded affinity to antithrombin was achieved followed again by detailed structural analysis. The combination of different techniques permits to profile in depth the structural features of such a drug and offers a useful tool for possible analysis of batch production.

1. Introduction

Sulodexide is a heparinoid obtained by a patented process (U.S. 3,936,351) from porcine mucosa. It is described as a highly-purified mixture of 80% fast-moving heparin (Fm-Hep) fraction, defined on its electrophoretic mobility in the barium-propanediamine system, and 20% dermatan sulfate [1,2]. Fm-Hep component of sulodexide is described as a 7 kDa fraction with lower anticoagulant activity and a lower degree of sulfation than unfractionated heparin (UFH) [3]. Such as UFH, it is composed by 1,4-linked repeating disaccharide units containing a uronic acid, mainly α-L-iduronic (IdoA) or β-D-glucuronic (GlcA) acids, and a α-*N*-acetyl-glucosamine (GlcNAc) or more frequently a α-*N*-sulfated-glucosamine (GlcNS). O-sulfation can occur at different degrees in position 2 of IdoA and in position 6 and/or rarely in position 3 of glucosamine.

Dermatan sulfate (DeS) is constituted by →3)-β-D-galactosamine-*N*-acetylate-4-sulfate (1→4)-α-L-iduronic acid (GalNAc4S-IdoA) as the most representative unit. Galactosamine can be alternatively or additionally sulfated in position 6 and, in minor sequences, also IdoA can be sulfated in position 2 [4]. A mean molecular weight of 25 kDa is reported for DeS component of sulodexide [3].

Due to the concomitant presence of Fm-Hep with affinity for antithrombin III (AT) and of DeS with affinity for heparin cofactor II (HCII), sulodexide is reported to possess various biological activities: lipasemic (≥10 LRU/mg), anticoagulant (<100 IU/mg), anti-Xa (70–100 IU/mg), HCII (~180 U/mg), APTT (<50 U/mg) determined against the IV International Standard of Heparin and antithrombotic activity [3]. Thrombin inhibition by the simultaneous activation of AT and HCII would confer to sulodexide greater efficacy than heparin in preventing thrombus formation, such that a lower dosage of sulodexide can achieve an equivalent antithrombotic effect [5]. The pharmacological activities are mainly characterized by a prolonged half-life and reduced effect on global coagulation and bleeding parameters compared to heparin, in addition to the possibility of oral, subcutaneous and intravenous administration. Many clinical studies have demonstrated the safety and efficacy of sulodexide [3] not only for the prophylaxis and treatment of thromboembolic diseases but also for the treatment of diabetic nephropathy. Despite sulodexide has raised a notable interest as antithrombotic drug only a few structural and biochemical data are available in literature. In 1986, Radhakrishnamurthy and co-workers reported on the chemical composition only of a fraction of sulodexide (f-Sulodexide) which turned out to consist in a mixture of chondroitin sulfates and heparin, this latter with lower sulfate content than unfractionated standard heparin and with lower molecular weight [6]. In the present work, the fine structural characterization of sulodexide as a whole and of its isolated Fm-Hep and DeS components were achieved. Three samples

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were analyzed by HP-SEC/TDA to evaluate their molecular weight parameters, and by two-dimensional NMR (2D-NMR) to assess their mono/disaccharide composition. On one of the three samples an in-depth study was accomplished, by characterization of the two isolated glycosaminoglycan (GAG) components and subsequent enzymatic digestion with specific lyases (Heparinases I, II, and III and Chondroitinase ABC) and HPLC–MS analysis to profile their di-oligosaccharide composition. Also, the interaction of sulodexide with antithrombin (AT) was investigated through affinity chromatography on AT-Sepharose and both fractions devoid or endowed of affinity to AT were analyzed by NMR and HPLC–MS as above. The applied techniques proved to be a powerful combination for studying drugs based on GAG mixtures.

2. Material and methods

2.1. Chemicals and reagents

Sulodexide samples (Sldx-A, Sldx-B and Sldx-C) were from LDO, Italy. Heparin lyases I (EC4.2.2.7), II and III (EC4.2.2.8) were purchased from Grampian Enzymes, Aberdeen, UK. Bovine Serum Albumin (BSA), Chondroitinase ABC from *Proteus vulgaris*, Deuterium oxide (D_2O ≥99.9%), dibutylamine (DBA, ≥99.5%), methanol (LC–MS grade), acetonitrile (LC–MS grade), acetic acid (glacial 100%), Amberlite IR 120Na+, $NaNO_3$ (≥99%), NaN_3 (≥99%), NH_4 OAc (≥99%), formic acid (≥95%), HCl (37%), carbazole (≥98%), sulfuric acid (95–97%), NH_4Cl (≥99.5%), were purchased by Sigma Aldrich. Sodium acetate (≥99%) was purchased from Merck, Italy. Calcium acetate (>97%) and NaOH (0.1 M) were from BDH, Italy and Fluka, Italy, respectively. Antithrombin (Kybernin P1000) was obtained from CSL Behring, Italy. Unsaturated uronate (ΔU) disaccharide standards ΔU2S-GlcNS,6S, ΔU-GlcNS,6S, ΔU2S-GlcNS, ΔU-GlcNS, ΔU-GlcNAc,6S ΔU2S-GlcNAc, ΔU-GlcNAc, ΔU2S-GlcNAc,6S, ΔU-GalNAc, ΔU-GalNAc,4S, ΔU-GalNAc,6S, ΔU2S-GalNAc,4S, ΔU2S-GalNAc,6S, ΔU2S-GalNAc,4S,6S, ΔU2S-GalNAc were from Iduron, Manchester, UK. Heparin-sepharose 6-fast flow and cyanogen bromide activated sepharose (CNBr activated sepharose) were obtained from GE Healthcare, Italy. Deionized water was prepared with a reverse osmosis system.

2.2. Digestion of sulodexide with chondroitinase ABC

100 mg of sample were dissolved in 50 ml of 50 mM Na phosphate/50 mM Na acetate buffer, pH 8 and incubated with 0.7 IU of Chondroitinase ABC (Ch.ase) for 48 h at 37 °C, added in three times. The digestion was inactivated by boiling for 5 min followed by filtration with 0.2 μm cut-off.

2.3. Digestion of sulodexide with heparinase III

250 mg of sample were dissolved in 2.5 ml of 2 mM Ca/50 mM Na acetate buffer, pH 7.4 and digested with 4U of Heparinase III for 48 h at 37 °C, then the digestion was inactivated by boiling for 5 min followed by filtration with 0.2 μm cut-off.

2.4. Depolimerization by reductive deamination

About 250 mg of sample were dissolved in 50 ml of water and kept in a cold bath and 57 mg of $NaNO_2$ were added. The pH of the solution was corrected to 2 with HCl and after 30 min was readjusted to neutrality with NaOH. Then, 78 mg of $NaBH_4$ were added and the pH was corrected to 3 with HCl for ten minutes in a cold bath, then readjusted again to 7.

2.5. Gel permeation chromatography

Desalting of samples was performed by GPC on a 5 × 85 cm column packed with TSK HW40 and eluting with 10% EtOH in water at 4.8/5.0 ml/min to separate salts and small oligosaccharides obtained by deamination or enzymatic digestion. Fractions collected by an automatic fraction collector were detected at 210 nm.

2.6. Size exclusion chromatography

Fractionation of samples was performed by GPC on a two-column system of 3 × 48 and 2 × 83 cm packed with Sephadex S30 connected to a peristaltic pump and an online UV detector and eluting with 0.25 M NH_4Cl at 5 ml/min.

2.7. Affinity fractionation of sulodexide

To separate no-affinity (NA) and high-affinity (HA) fractions of sulodexide, a semi-preparative chromatography onto a 180 ml AT-Sepharose column was performed. The column was prepared by coupling the high affinity fraction of antithrombin purified as previously described by Naggi et al. [7], to a CNBr-activated Sepharose according to Pixley and Danishefsky [8]. The column was equilibrated at 4 °C with equilibrium buffer (50 mM Tris-HCl/50 mM NaCl, pH 7.4). About 50 mg of Sldx-C were dissolved in 5 ml of the same buffer and loaded onto the column. A NaCl gradient system was used for elution at 2.5 ml/min using the equilibrium buffer and the elution buffer (50 mM Tris-HCl/3 M NaCl, pH 7.4) following the schedule (100% of equilibrium buffer for 216 min; a linear increment of elution buffer for 208 min; 100% elution buffer for additional 72 min). In the isocratic mode, fractions were collected every 12 mins while in the linear increment every 8 min. The uronic acid content was determined by the method of Bitter and Muir [9]. Fractions were collected according to the chromatographic profile, desalted by gel permeation onto a TSK column, followed by an ion exchange chromatography onto a 5 ml Amberlite IR 120 Na^+ column to exchange Tris-HCl with NaCl. Samples were then recovered by freeze-drying.

2.8. HP-SEC/TDA

Evaluation of the molecular weight distribution of sulodexide samples and of the isolated Fm-Hep and DeS components was performed by Size Exclusion Chromatography coupled with a multi-detector system (refractive index, right angle light scattering, viscometer, Viscotek mod. 305 Triple Detector Array) [10]. Chromatographic conditions were set up using TSKG2500 PWXL + TSKG3000 PWXL columns (Tosoh Bioscience, 7.8 mm ID × 30 cm) with an aqueous solution of 0.1 M $NaNO_3$ added with 0.05% NaN_3 pre-filtered onto 0.22 μm filter (Millipore), used as mobile phase at a flow rate of 0.6 ml/min. 100 μl of each sample were injected at about 5 mg/ml and chromatogram elaboration was performed using OmniSEC software version 4.6.2. For one sulodexide sample (Sldx-C), also silica columns were used for column performance comparison (TSKG4000 SWXL + TSKG3000 SWXL columns, Tosoh Bioscience, 7.8 mm ID × 30 cm), with an aqueous solution of 0.1 M NH_4 OAc added with 0.02% NaN_3 pre-filtered on 0.22 μm filter (Millipore) as mobile phase and by maintaining the same operating conditions.

2.9. NMR characterization

Heteronuclear Correlation NMR spectroscopy (HSQC) spectra of starting sulodexide (35 mg) were recorded in 0.6 ml of 0.15 M deuterated phosphate buffer, 0.003 mM EDTA, 0.002% TSP, pH 7.1 while spectra of fractions were recorder in deuterated water, pH 7