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Residual keratan sulfate in chondroitin sulfate formulations for oral administration

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ABSTRACT

Chondroitin sulfate is a biomedical glycosaminoglycan (GAG) mostly used as a dietary supplement. We undertook analysis on some formulations of chondroitin sulfates available for oral administration. The analysis was based on agarose-gel electrophoresis, strong anion-exchange chromatography, digestibility with specific GAG lyases, uronic acid content, NMR spectroscopy, and size-exclusion chromatography. Keratan sulfate was detected in batches from shark cartilage, averaging ~16% of the total GAG. Keratan sulfate is an inert material, and hazardous effects due to its presence in these formulations are unlikely to occur. However, its unexpected high percentage compromises the desired amounts of the real ingredient specified on the label claims, and forewarns the pharmacopeias to update their monographs. The techniques they recommended, especially cellulose acetate electrophoresis, are inefficient in detecting keratan sulfate in chondroitin sulfate formulations. In addition, this finding also alerts the manufacturers for improved isolation procedures as well as the supervisory agencies for better audits. Analysis based on strong anion-exchange chromatography is shown to be more reliable than the methods presently suggested by standard pharmacopeias.

1. Introduction

Glycosaminoglycans (GAGs) are widely used as therapeutic agents (Gesselbauer & Kungl, 2006). In particular, heparin has been largely exploited for treatments and preventions of thrombosis, and in procedures involving extracorporeal circulation (Blossom et al., 2008). More recently, chondroitin sulfate, eventually in combination with glucosamine (Clegg et al., 2006), has been employed as an alternative medicine in therapies for osteoarthritis, osteoarthrosis and possibly osteoporosis. Chondroitin sulfate formulations for oral administration are also used as a nutraceutical to prevent lesions of joint cartilage, for example, in cases of continuous physical impact on the knees (Clegg et al., 2006; Volpi, 2007). In contrast to heparin, which is used through intravascular or subcutaneous route; as a dietary supplement, chondroitin sulfate is taken orally.

Chondroitin sulfate formulations are derived from different cartilage sources such as bovine tracheal, shark and whale cartilage. However, the structure of chondroitin sulfate obtained from these tissues varies significantly, essentially due to variations of sulfation patterns of the *N*-acetylgalactosamine (GalNAc) residues, such as 4-sulfation (CS-A) and 6-sulfation (CS-C) (Sugahara et al., 2003). Other minor structural variations also occur, mainly as sulfation and epimerization extensions on the glucuronic acid (GlcA) residues (Sugahara et al., 2003). The molecular size of chondroitin sulfate chains may also vary markedly among cartilage types (Leta, Mourão, & Tovar, 2002). Another aggravating source of heterogeneity in preparations of chondroitin sulfate could be the undesirable presence of trailing other GAG types due to imperfections in purification processes since these formulations are derived from animal sources. In particular, keratan and heparan sulfates are other well-known GAG components from cartilaginous proteoglycans. The former GAG type has more structural similarities to chondroitin sulfates than the latter. These similarities comprise the presence of large extension in 6-*O*-sulfation, the lack of biosynthetic processing at the *N*-position of hexosamines, and perhaps, polydispersity. Hence, keratan sulfate is likely to present closer physicochemical properties to chondroitin sulfate, and this may leave some trailing

Abbreviations: Chase AC, chondroitin AC lyase; COSY, correlation spectroscopy; CS(s), chondroitin sulfate(s); Eur, European; GAG(s), glycosaminoglycan(s); Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; GARP, globally optimized alternating phase rectangular pulses; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single quantum coherence; KS, keratan sulfate; KSase, keratanase; LC, liquid chromatography; NMR, nuclear magnetic resonance; OSCS, oversulfated chondroitin sulfate; PAGE, polyacrylamide-gel electrophoresis; SAX, strong anion exchange; SEC, size-exclusion chromatography.

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amounts in large-scale production of chondroitin sulfates by raw purification procedures.

Herein we have analyzed several batches of chondroitin sulfate formulations readily available for oral administration, compared to standards from the USA and European pharmacopeias. We undertook analysis using agarose-gel electrophoresis, strong-anion exchange (SAX) and size-exclusion chromatography (SEC), both coupled to a high-pressure liquid chromatography (HPLC) system, digestibility with specific GAG lyases, estimation of uronic acid levels, and 1D + 2D nuclear magnetic resonance (NMR) spectroscopy. We found that keratan sulfate averages around 16% of the total GAG amount found in the formulations specifically originating from shark cartilage, including even the standard of the European pharmacopeia which is essentially based on this cartilage type. The keratan sulfate amount is far from a simple trace expectation, and strikingly indicates that more rigorous quality control tests on chondroitin sulfate formulations are urged in order to assure the proper efficacy, and correct amount of the bioactive ingredient in these formulations of chondroitin sulfate. Moreover, improved purification methods must be undertaken by manufacturers of this material as well as audits from regulatory agencies.

2. Materials and methods

2.1. Samples of chondroitin sulfate formulation

Seventeen batches of chondroitin sulfate formulations readily available for oral administration (fourteen from shark and three from bovine cartilage) were obtained from Brazilian pharmaceutical companies. All batches come from a single manufacturer. Its name is kept anonymous due to ethical principles. Pharmacopeial standards of chondroitin sulfate were obtained from the USA (Rockville, MD; cat. 1133570, Lot HOF184) and the European (Strasbourg, code Y0000593, ID. 002SJ4) pharmacopeias. Commercially available chondroitin sulfate from shark (CS-C, predominantly 6-sulfated) and whale (CS-A, mostly 4-sulfated) cartilage were from Sigma–Aldrich (St. Louis, MO, USA). Oversulfated chondroitin sulfate (OSCS) was prepared as described previously (Fonseca et al., 2010).

2.2. Agarore gel electrophoresis

Aliquots of chondroitin sulfate (5 μg of each) were applied to a 5 mm-thick 0.5% agarose-gel, then run for 1 h at 110 V in 0.05 M 1,3-diaminopropane:sodium acetate (pH 9.0). The GAGs in the gel were fixed with 0.1% *N*-cetyl-*N,N,N*-trimethylammonium bromide solution. After 12 h, the gel was dried and stained with 0.1% toluidine blue in acetic acid:ethanol:water (0.1:5:5, v:v). This method is similar to one recommended by both USA (cellulose acetate electrophoretic version) and European pharmacopeias for analysis of chondroitin sulfate formulations (European Pharmacopeia, 2007; United States Pharmacopeia, 2008). It is hard to accurately predict the amounts of GAGs based solely on agarose gel electrophoresis, since this procedure involves multiple steps such as precipitation of the glycans in the gel with *N*-cetyl-*N,N,N*-trimethylammonium bromide, staining with toluidine blue, etc.

2.3. SAX and SEC

GAG samples (1 mg of each) were applied to a SAX Mono-Q column pre-equilibrated with 10 mM Tris:HCl containing 0.5 M NaCl, pH 7.4 and connected to HPLC system (Amersham Biosciences). The column was then washed with 10 mL of the same Tris buffer and eluted at a flow rate of 1.0 mL min^{-1} using a linear NaCl gradient of 0.5–3.0 M NaCl, total volume of 40 mL. The eluent was checked continuously by absorbance at 215 nm. Chondroitin sulfates from bovine cartilage (CS-A), from shark cartilage (CS-C), and keratan sulfate from shark cartilage, eluted from the SAX-HPLC (Mono-Q column) at 1.48, 1.66, and 2.2 M of NaCl, respectively.

For the preparation of large amounts of the individual GAG fractions, 10 mg of chondroitin sulfate formulation derived from shark cartilage was applied to the column, which was eluted as described in the previous paragraph. The fractions were individually collected, desalted by dialysis against distilled water and freeze-dried. The uronic acid content of these fractions was estimated using the carbazole reaction (Bitter & Muir, 1962), and glucuronolactone as standard.

For SEC, samples of chondroitin sulfates (20 μg of each) were analyzed with gel filtration columns (Tosoh TSK gel G4000 SW × 1 and G3000 SW × 1, both 7.5 mm i.d. × 300 mm) linked to an HPLC system. To widen the molecular-weight exclusion limits, a combination of one G4000 column followed by one G3000 was used. The columns were eluted with 0.1 M ammonium acetate, at room temperature (~20 °C) with a flow rate of 0.3 mL min^{-1}. The eluent was monitored by refractive index. The column was properly calibrated using GAG standards with known molecular size.

2.4. Digestions with specific GAG lyases

Fractions of GAGs obtained from shark cartilage (100 μg each) were separately incubated with 0.01 units of chondroitin AC lyase (Chase AC) (Sigma–Aldrich, St. Louis, MO) or 0.2 units keratan sulfate lyase I (KSase) (Seikagaku American Inc, East Falmouth, MA), in 100 μL 0.05 M Tris:HCl (pH 8.0), with 5 mM EDTA and 15 mM sodium acetate. The mixtures were kept at 37 °C for 12 h. The samples were then heated at dried-bath at 80 °C for 15 min to neutralize the reaction through enzyme denaturation. These samples were subsequently analyzed on polyacrylamide-gel electrophoresis (PAGE) as described previously (Pomin, Valente, Pereira, & Mourão, 2005). Essentially, aliquots containing 5 μg of the different fractions incubated in the absence or presence of the GAG lyases were applied to a 1-mm-thick 10% polyacrylamide-slab gel in 0.02 M Tris:Cl (pH 8.6). After electrophoresis (100 V for ~40 min), the GAGs were stained with 0.1% toluidine blue in 1% acetic acid and washed for about 1 h in 1% acetic acid.

2.5. NMR spectroscopy

^{1}H and ^{13}C, one-dimensional and two-dimensional spectra of the fractions obtained from shark cartilage were recorded using a Bruker DRX 800 MHz apparatus with a triple resonance probe as detailed previously (Pomin et al., 2005). About 5 mg of each sample was dissolved in 0.6 mL 99.9% deuterium oxide (Cambridge Isotope Laboratory, Cambridge, MA). All spectra were recorded at 35 °C with HOD suppression by presaturation. The 1D ^{1}H NMR spectra were recorded using 16 scans and inter-scan delay set to 1 s. The 2D $^{1}H/^{1}H$ COSY spectrum was recorded using states-time proportion phase incrementation (states-TPPI) for quadrature detection in the indirect dimension. The $^{1}H/^{13}C$ edited-HSQC spectrum was run with 1024 × 256 points and globally optimized alternating phase rectangular pulses (GARP) for decoupling. Chemical shifts are displayed relative to external trimethylsilylpropionic acid at 0 ppm for ^{1}H and relative to methanol for ^{13}C.

3. Results and discussion

Seventeen batches of chondroitin sulfate formulations readily available for oral administration were analyzed by agarose-gel electrophoresis, showing a single band with the same mobility as the standards from USA and European pharmacopeias (Fig. 1). No difference was observed between the electrophoretic migration of chondroitin sulfates from shark and bovine cartilage.