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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.

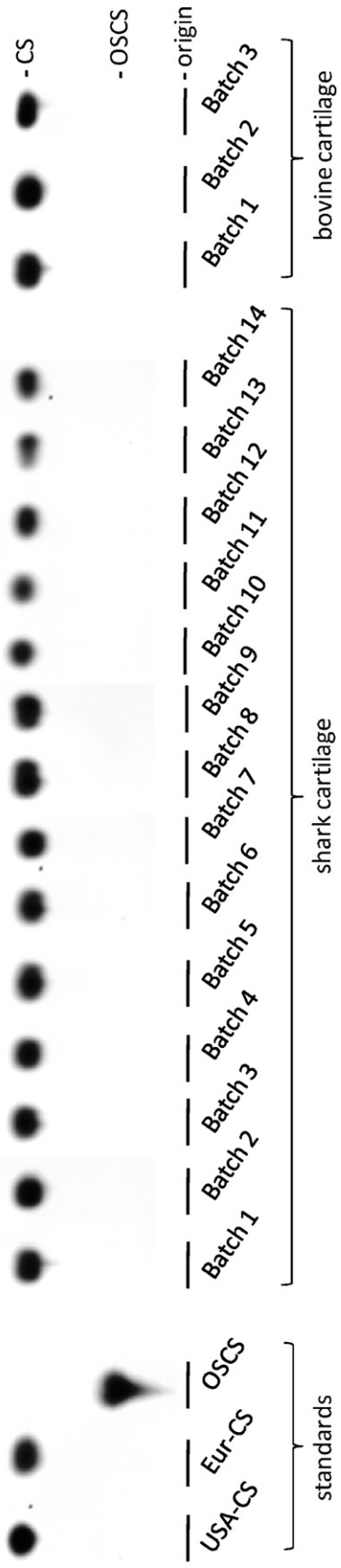

Fig. 1. Agarose-gel electrophoresis of chondroitin sulfate standards from reference pharmacopeias (American, USA-CS, and European, Eur-CS), oversulfated chondroitin sulfate, and of formulation batches derived from shark and bovine cartilage.

Table 1
Proportions of chondroitin sulfate (CS) and keratan sulfate (KS) in batches of chondroitin sulfate formulations for oral administration, obtained from shark or bovine cartilage, and pharmacopeial standards.[a]

Source	Number of batches	CS	KS
		% of total as mean ± SD	
Shark cartilage	14	84.2 ± 1.4	15.8 ± 1.4
Bovine cartilage	3	100	<1
Standard from USA pharmacopeia	1	100	<1
Standard from European pharmacopeia	1	90	10

[a] The proportions were obtained from the integrals of the SAX profiles of Fig. 2. Table S1 shows the amounts of each batch.

Clearly, OSCS was not detected in any of these batches using this method. Although USA and European pharmacopeias (European Pharmacopeia, 2007; United States Pharmacopeia, 2008) have established cellulose acetate for analysis of chondroitin sulfate formulations, agarose-gel electrophoresis is a similar methodological version of horizontal electrophoresis.

Subsequently, the elution profiles of chondroitin sulfate formulations obtained from shark and bovine cartilage were compared using a SAX-HPLC (Mono-Q column) (Fig. 2). Bovine chondroitin sulfate displayed a single peak (Fig. 2B) while batches obtained from shark cartilage unexpectedly showed two distinct components (Fig. 2A). The preponderant peak eluted as standard chondroitin sulfate, notated as CS. The minor component, designated KS, eluted at a higher NaCl concentration and accounts for approximately 16% of the total GAG amount found in the batches of chondroitin sulfate formulations obtained from shark cartilage analyzed herein (Table 1). The standard obtained from USA pharmacopeia, derived from bovine cartilage, showed just the single fraction CS, as expected. The standard from European pharmacopeia obtained from shark cartilage, conversely revealed both fractions as well (Fig. 2B, Table 1).

Large amounts of the chondroitin and keratan sulfates fractions were prepared from representative batches of shark cartilage (Fig. 3A), and used afterwards for further uronic acid estimation, susceptibility to specific GAG lyases, and NMR structural analysis. Fractions designated CS and KS were ultimately characterized as chondroitin and keratan sulfate, respectively, based on the following data. Firstly, CS fraction contains uronic acid, which it is absent in KS fraction (Fig. 3B). Secondly, CS and KS fractions were susceptible to digestions with chondroitin AC lyase (Chase AC) and keratanase (KSase), respectively, and properly resistant in treatments using the unrelated lyases (Fig. 3C). More surprisingly was that on agarose-gel electrophoresis, these two fractions were seen undistinguishable since they co-migrate through this method (Fig. 3D). Thirdly, these two fractions were properly characterized through high-field NMR spectroscopy (Figs. 4, 5 and S2). The ^{1}H-signal distribution in the spectrum of fraction CS is also similar to that of CS-C obtained from Sigma–Aldrich (compare Figs. 4B *vs* S1A). The GalNAc units in these samples are preponderantly 6-sulfated (Pomin et al., 2012). ^{1}H NMR spectra of CS-A obtained from whale or bovine tracheal cartilage showed an intense signal at 5.4 ppm assigned as H4 of 4-sulfated GalNAc units (Fig. S1B). Overall, these observations clearly indicate that fraction denoted CS is a chondroitin sulfate mostly 6-sulfated.

However, more significantly, an ^{1}H NMR spectrum of fraction KS showed a distinct peak distribution of ^{1}H-signals compared with those from fraction CS (Fig. 4B *vs* C). The ^{1}H/^{1}H COSY spectrum (Fig. S2), and especially the ^{1}H/^{13}C-HSQC spectrum (Fig. 5) of fraction KS enabled proper assignment of many typical signals of keratan sulfate-like molecule. Through the edited ^{1}H/^{13}C-HSQC spectrum, in which the phased CH signals (orange signals in Fig. 5)

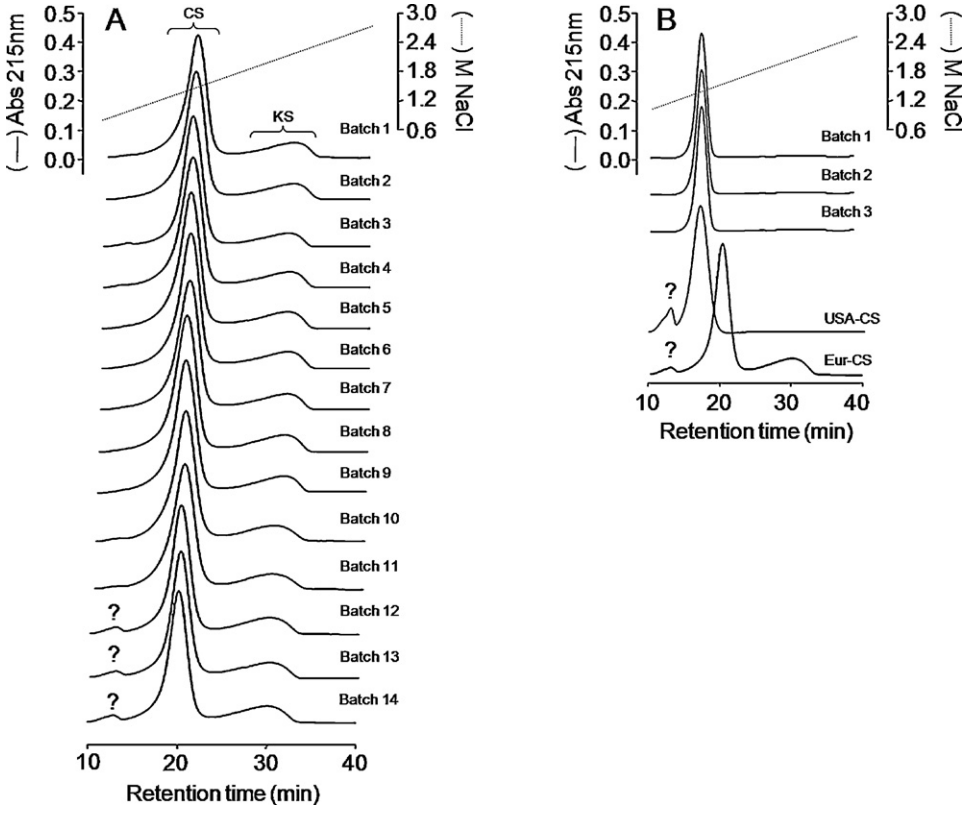

Fig. 2. SAX-HPLC (Mono-Q column) profiles of chondroitin sulfate formulations readily available for oral administration obtained from shark (A), or bovine (B) cartilage. Standards from pharmacopeias (USA-CS of bovine cartilage, and Eur-CS of shark cartilage origin) are shown in the bottom of panel (B). Peaks labeled with a question mark are uncharacterized materials.

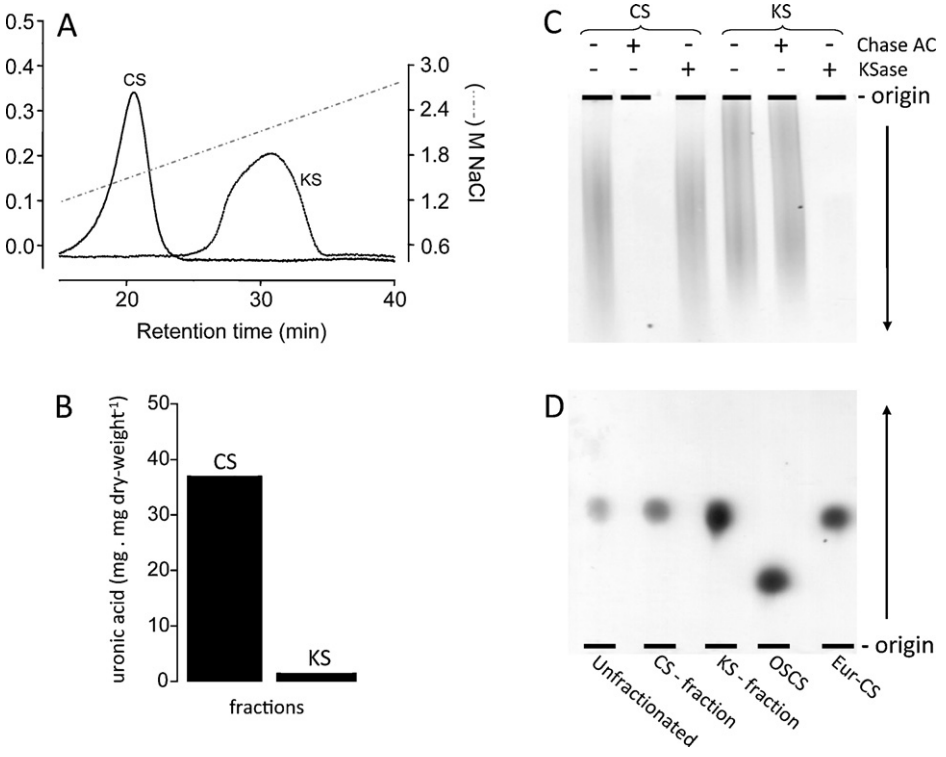

Fig. 3. Analysis of purified CS and KS fractions through SAX-HPLC (Mono-Q column) (A), estimation of uronic acid content (B), PAGE before and after chondroitin AC lyase (Chase AC), or keratanase (KSase) treatment (C) and agarose-gel electrophoresis of a untreated representative chondroitin sulfate batch, purified fractions CS and KS, OSCS and standard from European pharmacopeia (Eur-CS).

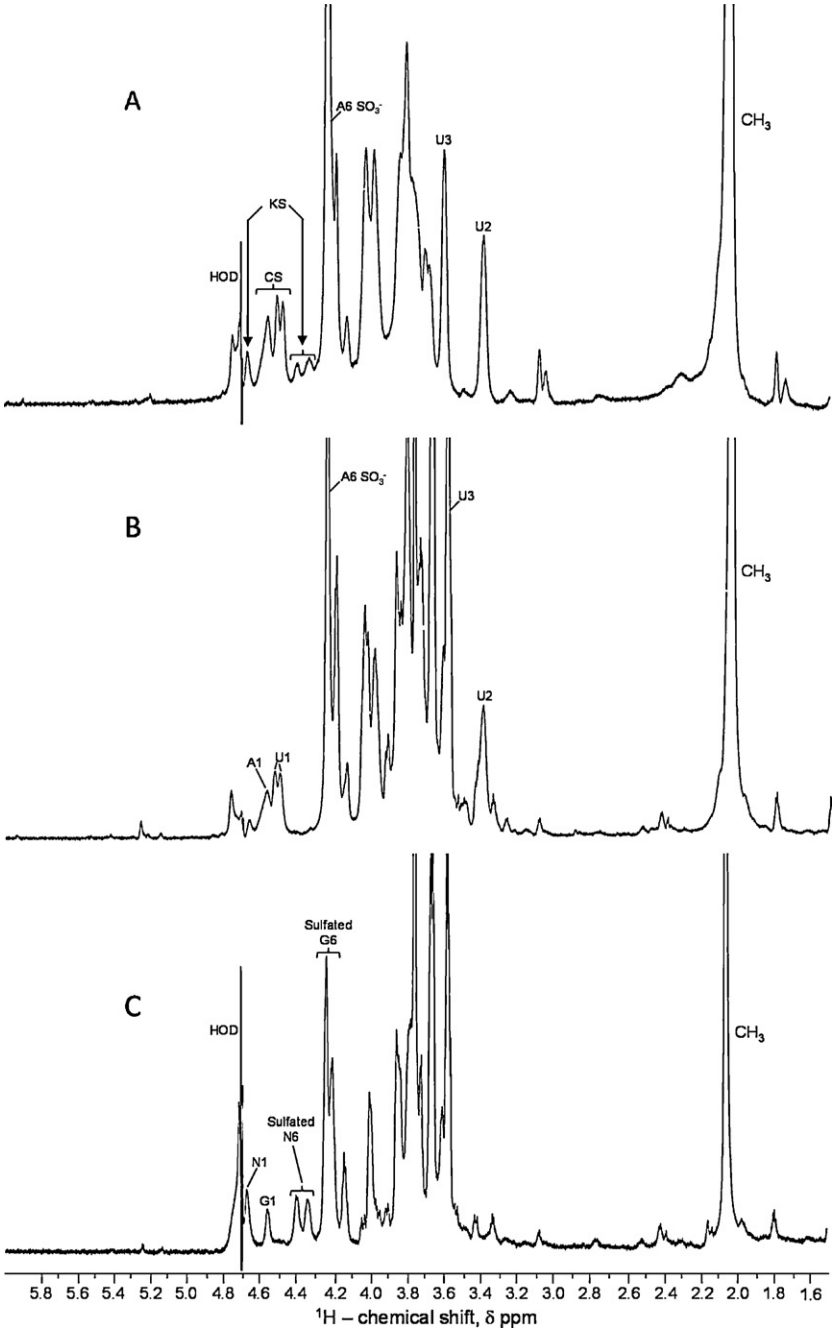

Fig. 4. 1D ^{1}H NMR spectra of a representative chondroitin sulfate preparation from shark cartilage (A), and its purified CS (B), and KS (C) fractions. U, A, N, and G stand for glucuronic acid, N-acetylgalactosamine, N-acetylglucosamine, and galactose, respectively. The numbers after these letters indicate positions of hydrogen atoms.

can be easily distinguished from anti-phased CH_2 signals (in green), several structural information were generated as the following.

Firstly, two characteristic β-anomeric ^{1}H/^{13}C-signals at 4.68/102.85 and 4.49/102.93 ppm were identified, and respectively attributed to residues of *N*-acetyl-β-glucosamine (GlcNAc) (denoted N1) and β-galactose (Gal) (denoted G1) (Fig. 5 and Table 2). These signals are in approximately equimolar proportions, conceived with the disaccharide repeating unit of a keratan sulfate-like molecule composed of alternating 4-linked GlcNAc and 3-linked Gal residues. Secondly, two clear signals involved in glycosydic bonds are at 3.70/78.91 and 3.69/82.28 ppm (denoted N4 and G3 respectively in Fig. 5 and Table 2), typically of ^{13}C-signals at downfield region, as expected in the case of glycosylation sites. These two cross-peaks belong to ^{1}H/^{13}C-signals of 4-linked GlcNAc and 3-linked Gal residues consistent with the glycosidic linkage type in keratan sulfate molecules (Table 2). Gal units had a ^{1}H-chemical shift of their H2 at ~3.55 ppm, as opposed to the upfield ~3.4 ppm for H2 of glucuronic acids (signal U2 of spectrum of Fig. 4B), and to the downfield ~3.8 ppm for H2 of GalNAc (signal N2 of spectrum at Fig. 5). Thirdly, the H2 assigned at ~3.55 ppm through ^{1}H–^{1}H COSY spectrum of the fraction KS (Fig. 2S) unequivocally confirms the presence of a Gal unit (Table 2), and keratan sulfate is the only GAG type that bears this neutral sugar instead of an uronic acid unit.

Finally, information about 6-sulfation of GlcNAc and Gal residues was easily deduced by analysis of the anti-phased peaks

Table 2
Major ^{1}H and ^{13}C-chemical shifts (ppm) from signals identified in the 2D NMR spectra of KS fraction compared with literature data.

Signal	Values from the present study		Literature data[a]	
	4-β-GlcNAc-1 (N)	3-β-Gal-1 (G)	4-β-GlcNAc-1	3-β-Gal-1
H1	4.69	4.49	4.76	4.54
H2	3.78	3.55	3.83	ND
H3	ND	*3.70*	ND	*3.79*
H4	*3.71*	ND	~3.80	ND
Sulfated H6	**4.37–4.23**	**4.20–4.08**	**4.32–4.39**	**4.22**
C1	102.87	102.93	105	105
C2	55.13	69.97	57	ND
C3	ND	*82.22*	ND	*85*
C4	*78.95*	ND	*81*	ND
Sulfated C6	**66.65**	**67.72**	**69**	**70**

The sulfation and glycosilation sites are highlighted in bold and italics, respectively. ND stands for not determined.
[a] Cockin, Huckerby, and Nieduszynski (1986).

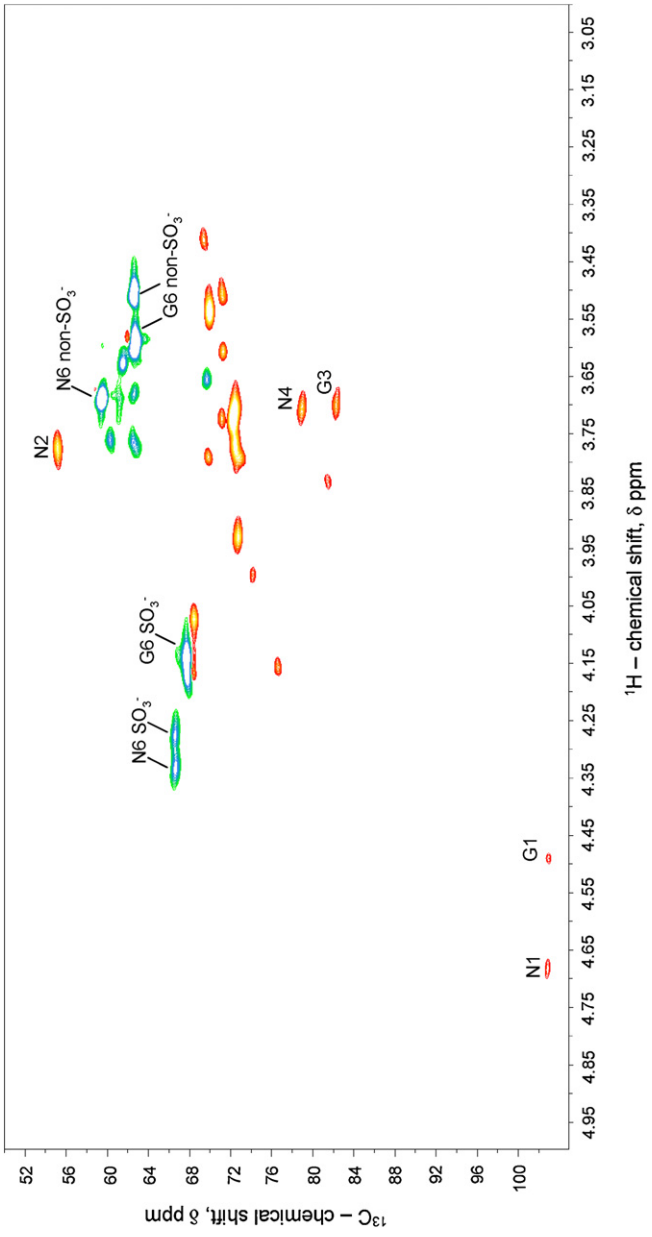

Fig. 5. $^{1}H/^{13}C$ edited HSQC spectrum of the fraction KS obtained. The letters N and G stand for N-acetylgalactosamine and galactose, respectively. The numbers following letters indicated position of $^{1}H/^{13}C$ cross-peaks. SO_3^- and non-SO_3^- stand for sulfated and non-sulfated sites. Green and orange peaks denote anti-phased (negative) CH_3 signals, and phased (positive) CH/CH_2 peaks, respectively.

shown in orange in Fig. 5. We can identify two signals at 4.37–4.23/66.58 and 4.20–4.08/67.67 ppm, ascribed to 6-sulfated GlcNAc (N6 SO_3^-) and Gal (G6 SO_3^-) units, respectively (Table 2). These signals are ~0.7 ppm shifted downfield in the ^{1}H-scale and ~8 ppm in the ^{13}C-scale from a more heterogeneous group of signals assigned to non-sulfated residues. Overall, these results indicated clearly that KS fraction is indeed a keratan sulfate-like molecule occurring with 6-sulfation at both residues, although 6-sulfation occurring more at GlcNAc units than usual (see peak-intensities of sulfated Gal *vs* GlcNAc in Fig. 4C).

In order to prove the adequacy of SAX compared to the other LC-based methods in detection of keratan sulfate in chondroitin sulfate formulations readily available for oral administration, we also undertook a systematic analysis using the SEC method. It is well known that the molecular size range of chondroitin sulfate (and probably keratan sulfate) varies significantly depending on the type of cartilage that is chosen for preparation (Leta et al., 2002). Hence, this would consequently lead to some variation in the SEC profiles for diagnostic purposes. Although chondroitin sulfate from shark and bovine tracheal (or whale) cartilage have very disperse molecular size, ranging from ~10 to ~60 kDa (Pomin et al., 2012), the major portions of each material are diagnostically different, and this difference can be used in assignments through SEC as shown at Fig. 6. SEC-HPLC is clearly able to differentiate the chondroitin sulfate types: shark cartilage CS-C *vs* bovine tracheal CS-A or whale cartilage CS-A (Fig. 6), but not to distinguish keratan sulfate from chondroitin sulfate, since the polydispersity of keratan sulfate overlaps the peaks of any chondroitin sulfate type (Fig. 6A). This reinforces that SAX chromatography coupled to automatic fast LC systems is the most suitable LC-based method for detecting keratan sulfate in formulations of chondroitin sulfates.

Chondroitin sulfates from bovine (CS-A), and from shark cartilage (CS-C) showed distinct SEC-HPLC profiles as seen by the pharmacopeial standards from USA and Europe, respectively (Fig. 6B). Samples mostly composed of C-type condroitin sulfate elute earlier than A-types on SEC. This rule can also be seen comparing the readily available chondroitin sulfate standards (Fig. 6C). In addition to the distinct SEC-HPLC profiles (Fig. 6C), these samples can also be distinguished and recognized by SAX-HPLC (Fig. S3). Besides the marked differences in their molecular weights (Fig. 6C) (Leta et al., 2002), these GAGs are well-known to differ in their 4-/6-sulfation ratios (Pomin et al., 2012; Pomin, Sharp, Li, Wang, & Prestegard, 2010), and this is the reason sustains SAX chromatography useful for diagnostics of these chondroitin sulfate types. Even though SEC reveals primarily a better resolution to distinguish between chondroitin sulfates (Figs. S3 *vs* 6C), this method has proven incapable of detecting keratan sulfate in chondroitin sulfate sample of any origin (Fig. 6).

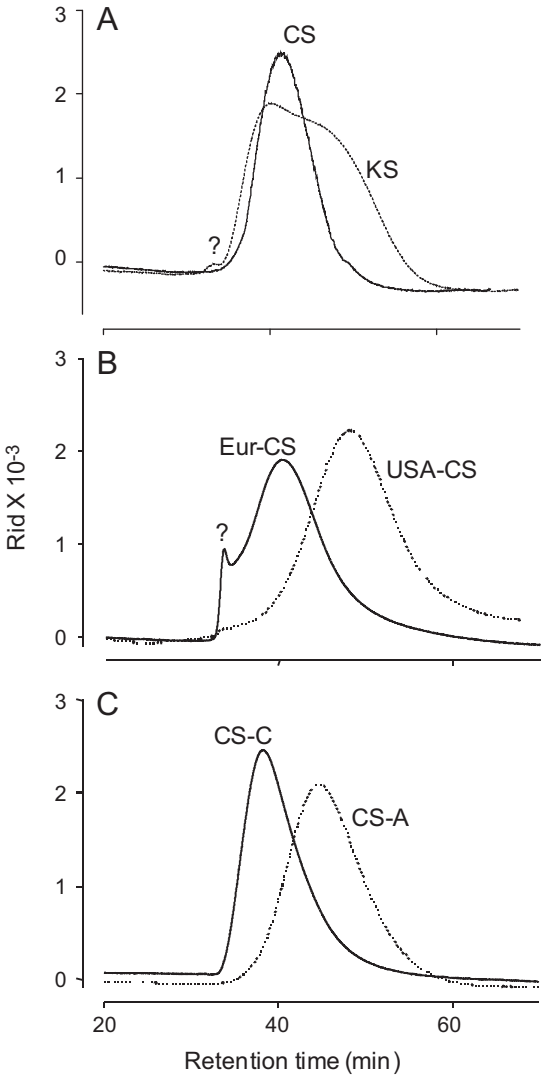

Fig. 6. SEC-HPLC (TSK G3000 + G4000 columns) profiles of chondroitin sulfates and keratan sulfate fractions. (A) Fractions CS (chondroitin sulfate) and KS (keratan sulfate), both obtained from SAX-HPLC of representative pharmaceutical batches of chondroitin sulfates from shark cartilage. (B) Chondroitin sulfates from European and American pharmacopeias (Eur-CS, and USA-CS, respectively). They are essentially composed of shark cartilage CS-C, and bovine cartilage CS-A, respectively. The European pharmacopeial CS (B) as well as both CS and KS fractions obtained from representative batches (A) revealed an extra unknown component assigned with question mark. (C) Readily available standards from Sigma–Aldrich: shark cartilage CS-C and whale cartilage CS-A. Peaks labeled with a question mark at panels (A) and (B) are uncharacterized materials.

In conclusion, formulations of chondroitin sulfate readily available for oral administration obtained from cartilage of shark and bovine origin differ in their content of residual keratan sulfate, in their molecular size and sulfate of their GalNAc units. Several methods may be employed to distinguish these two types of preparations, but SAX chromatography coupled to automatic LC systems appears to be more reliable and robust implementation not only for quality control of these GAG-based drug types but mainly to allow industrial preparation of chondroitin sulfate samples distained of large amounts of the residual keratan sulfate. Results from this suggestive method can be qualitatively enhanced by a combination with ^{1}H-based NMR analysis. This spectroscopic technique can ultimately ensure the proper structural integrity of the bioactive agent in biomedical formulations, together with a final fingerprint of the batches prior to their release into the market. Presence of extra minor material can be straightforwardly checked using NMR spectroscopy.

4. Conclusions

Herein, we have reported a systematic analysis on 17 batches of chondroitin sulfate formulations readily available for oral administration. Agarose-gel electrophoresis was proven unable to reveal residual keratan sulfate in these biomedical preparations since these two GAG type co-migrate (Fig. 3D). This electrophoretic method is similar to the cellulose acetate version recommended by USA and European pharmacopeias. In contrast, SAX-HPLC (Fig. 2), but not SEC-HPLC (Fig. 6), has now proven to be the most appropriate method for detection of the eventual presence of keratan sulfate, although SEC allows clear differentiation of chondroitin sulfates of different sources (bovine, whale and shark origin) (Fig. 6). Therefore, anion exchange-based methods should be implemented by manufacturers during large-scale production of chondroitin sulfates samples destined to the market. It is also origin worth mentioning that 1D ^{1}H NMR is very diagnostic for detection of the keratan sulfate presence in formulations of chondroitin sulfate as straightforwardly indicated by the well-resolved and isolated groups of signals at 4.68 and 4.37–4.30 ppm in contaminated samples (compare Fig. 4A *vs* C). Clearly, this spectroscopic method cannot be left aside in quality control tests of this drug type, and should be used in conjunction with the SAX method prior to release the final chondroitin sulfate preparation into the market. Although USA pharmacopeia recommends infrared spectroscopy and determination of the specific optical rotation, these methods have been supplanted by NMR spectroscopy for analysis of carbohydrates, especially in GAG-based biomedical preparations (Limtiaco, Jones, & Larive, 2012; Pomin et al., 2010; Rudd et al., 2011; Zhang et al., 2009).

More trustworthy and robust procedures should be implemented not only for control and regulation of chondroitin sulfate formulations for oral administration, but also during industrial preparation. And anion exchange-based methods have shown herein to be the most feasible one to be implemented during manufacturing. The occurrence of ~16%-keratan sulfate indicates that the biomedical formulations do not reflect the correct chondroitin sulfate amounts specified with their label claims, and it is far out from a simple expectation of trace amounts. A minimum of up-to 5% presence of this inert GAG would be tolerated however, the averaged amount evaluated herein extrapolate 3-times more the maximum value of this reasonable range, and thus points to considerable changes on the preparation and control procedures in chondroitin sulfate formulations. Besides chondroitin and keratan sulfate, a minor other substance was observed by LC, as marked with question marks at Figs. 2A, B and 6B, C. Since it represents much less than 5% of the tolerate percentage of impurities, this material was not characterized. Cautions must be adopted in purification procedures of these chondroitin sulfate-based products by the manufacturers, especially in products related with healthcare. Although, purification steps using anion exchange-based method would reduce drastically the amounts of products per rounds of preparation, the product generated would be much more reliable than that based heavily on a crude isolation method procedure. Curiously, contamination happened only on chondroitin sulfates from shark origin. This source may enhance the presence of contamination, and manufacturers are also free to change to other sources (whale or bovine cartilage rather than shark cartilage) if the general properties of the isolates are close to those of the real drug.

It is noteworthy to emphasize that only behind insulin the second mostly used natural drug is heparin (Pomin & Mourão, 2008).

Chondroitin sulfate is undoubtedly the second mostly exploited GAG type in therapies. Its market has been growing rapidly worldwide, especially in US as a dietary supplement, and therefore chondroitin sulfate may be assumed as the second carbohydrate-based bioagent utilized in the global market. It is very important to highlight that the biomedical use of chondroitin sulfate is not related just only as a nutraceutical substance, but also involved in many pharmaceutical applications such as in treatments of inflammation, virosis, and cancer, wound healing, neuroprotection, wound repair keratinocytes, wound healing in maxillary sinus mucosa, repair of the Central Nervous System, liver regeneration, and as potential antimalarial vaccine (Schiraldi, Cimini, & De Rosa, 2010).

The previous alarming cases of OSCS-contaminated heparin preparations (Blossom et al., 2008; Guerrini et al., 2008) strongly reinforce prompt and adequate reformulations of the standard procedures adopted by international pharmacopeias in quality controls of GAG-based therapeutic agents. It is imperative that GAG-based preparations destined to either clinical use or as a nutraceutical must be distained at the maximum of impurities, even though of those with apparently harmless nature like another physiological GAG such as keratan sulfate. This should be a standard procedure to be followed to any pharmaceutical or a nutraceutical product by any supplier committed with healthcare-related products. The amount of the real ingredient in these formulations must be correct.

Ultimately, it seems yet unclear the molecular basis for the beneficial effects of chondroitin sufates on osteoarthritis and osteoarthrosis. We cannot predict if minor structural components of this GAG type are in fact responsible for its beneficial effects. But we can anticipate that biomedical formulations of chondroitin sulfates covering a least uniform composition should be provided in order to maintain comparable clinical effects.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.06.009.

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