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Pharmaceutical grade chondroitin sulfate: Structural analysis and identification of contaminants in different commercial preparations

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ABSTRACT

The aim of the present study was to characterize 16 pharmaceutical grade chondroitin sulfate (CS) samples, concerning the structure and presence of contaminants, in comparison to USP and analytical grade CS. Agarose gel electrophoresis has shown that only 5 samples were >90% CS, while 11 contained less than 15% CS. FACE (fluorophore-assisted carbohydrate electrophoresis) revealed that maltodextrin was the main contaminant in nine of them, and lactose in two. Raman spectroscopy corroborated these results. Concerning the structure of the CS present in the five CS-rich samples, the ratios 4-sulfated:6-sulfated disaccharides varied from 0.9 to 1.7, and their modal molecular weight was 20–29 kDa. Also, they were all contaminated by small amounts of keratan sulfate (<1%). In conclusion, our findings indicate that the composition of CS preparations not always corresponds to the manufacturers' descriptions, and indicate that further characterization should be required for the registry and license of pharmaceutical grade CS.

1. Introduction

Osteoarthritis is a major public health problem for which there are few effective medical remedies. In the 1990s, chondroitin sulfate (CS) and glucosamine (GlcN) were introduced as "nutraceutical agents" for treatment of osteoarthritis (Reginster, Gillot, Bruyere, & Henrotin, 2000), and soon it was shown that these compounds also present anti-inflammatory and chondroprotective activities (Iovu, Dumais, & du Souich, 2008; Vallières & du Souich, 2010). Because they are extracted from natural sources, and due to their safety, treatment with CS and GlcN acquired substantial popularity. They are absorbed from the gastrointestinal tract (Ronca, Palmieri, Panicucci, & Ronca, 1998), and are excreted in the urine, part as intact polymers and part as partial degradation products (Baccarin, Machado, Lopes-Moraes, Vieira, & Michelacci, 2012; Michelacci, Boim, Bergamaschi, Rovigatti, & Schor, 1992), indicating their systemic distribution. Furthermore, they were tested in a number of clinical trials, which demonstrated their efficacy in osteoarthritis (review in McAlindon, LaValley, Gulin, & Felson, 2000).

CS and GlcN are currently recommended by The European League Against Rheumatism (EULAR) as a "SYSADOA" – symptomatic slow-acting drug for the treatment of osteoarthritis. It means that they provide pain relief and increased joint mobility only after a relatively long period of regular administration (1–2 months), despite the fact that their effects last long after the end of treatment (2–3 months).

It is well established that CSs are composed of alternating 1,3-*N*-acetyl-β-D-galactosamine and 1,4-β-D-glucuronic acid units, which bear 4-*O*- and/or 6-*O*-sulfations at the *N*-acetylgalactosamine units. Most CS polymers are hybrid structures, composed of two or more types of disaccharide units, disposed in specific patterns (Michelacci & Dietrich, 1976, 1986). Depending on the disaccharide unit that predominates, CS receive different names, and may have different biological activities. For instance, it was shown that the CS of human adult articular cartilage is predominantly 6-sulfated, while in human growth cartilage it is 50% 4-sulfated and 50% 6-sulfated (Michelacci, Mourão, Laredo, & Dietrich, 1979). The distribution of these units in the polymer is very specific and well defined.

In fact, some authors report that CS has little symptom-modifying activities (Distler & Anguelouch, 2006), while others include CS in the guidelines for osteoarthritis treatment (Cutolo, Berenbaum, Hochberg, Punzi, & Reginster, 2015). These different

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activities described by different authors could be due either to differences in the CS structures or to the presence of contaminants (or both).

CSs are isolated and purified from animal sources and, as already mentioned, have complex structures. Hence, it is very important to ensure the quality of the pharmaceutical grade CSs commercially available, which are currently used as raw materials by compounding pharmacies to prepare remedies to fit the unique need of each patient. Thus, the first aim of this study was to analyze the structure of the pharmaceutical grade CSs available in local compounding pharmacies (Juiz de Fora, MG, Brazil).

These compounds were submitted to agarose gel electrophoresis, which has shown that 11, out of 16 samples, contained much less CS than declared by their respective "Certificates of Analysis". Consequently, our second aim was to identify the main contaminants. Fluorophore-assisted carbohydrate electrophoresis (FACE), Raman spectroscopy, immunoblotting, and paper chromatography were used to identify the main contaminants.

FACE is a method introduced in 1990 for the analysis of the carbohydrate moiety of glycoconjugates (Jackson, 1990). This method is based on the separation, by polyacrylamide gel electrophoresis, of saccharides that have been labeled with a suitable fluorophore. In principle, carbohydrates that have a reducing aldehydic carbon are reacted with a fluorophore that has a primary amino group. The resulting Schiff base is stabilized by reductive amination with sodium cyanoborohydride forming the stable fluorescently labeled derivatives, which are separated by high resolution PAGE.

Raman spectroscopy is a vibrational technique based on scattered radiation, which has been successfully applied to investigate structure of different carbohydrates (Almeida et al., 2010; Mainreck et al., 2012). It has been applied to the analysis of both sulfated and non-sulfated polysaccharides, including heparin (Beni, Limtiaco, & Larive, 2011). It is a non-destructive technique that requires small amount of sample and is not time consuming. In the present study, Raman spectroscopy was used to confirm the identification of CS and contaminants.

Immunoblotting was used to identify keratan sulfate, and paper chromatography to identify and quantify inorganic sulfate.

2. Experimental

2.1. Materials

Sixteen samples of pharmaceutical grade CS raw material were obtained from seven pharmaceutical dealers in Juiz de Fora, MG, Brazil. These samples were numbered – samples #1–16 – and, according to the "Certificate of Analysis" of each product, all are extracted from bovine trachea. These samples came from the following countries: sample #6 from Brazil; samples #1, 2, 5, 7, 8, 9, 10, 11, and 14 from USA; samples #3, 4, 12 and 16 from China; and samples #13, and 15 from Germany.

United States Pharmacopeia standard CS (USP-CS) was obtained from the United States Pharmacopeia (Rockville, MD; cat. 1133570, Lot H1K241). Analytical grade chondroitin 4-sulfate (Ch4S, from bovine trachea), chondroitin 6-sulfate (Ch6S, from shark cartilage), dermatan sulfate (DS, from porcine mucosa), maltodextrin, lactose, and sodium sulfate were purchased from Sigma–Aldrich Co (St. Louis, MO, USA). Heparan sulfate (HS, from bovine pancreas) and chondroitin sulfate lyases AC and B (from *Flavobacterium heparinum*) were prepared by methods previously described (Aguiar, Lima, Berto, & Michelacci, 2003; Dietrich & Nader, 1974). Agarose (standard, low Mr), nitrocellulose and Zeta-Probe membranes were purchased from Bio-Rad Laboratories (Richmond, CA, USA). MST1 anti-KS monoclonal antibody was obtained as previously described (Alves, Straus, Takahashi, & Michelacci, 1994). 2-Aminoacridone (AMAC), mercuric acetate, sodium cyanoborohydride, glycine, *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED), and Dowex AG50W-X8 (200–400 mesh) were purchased from Sigma–Aldrich Co. Tetramethylbenzidine was from KPL (Gaithersburg, MD, USA), and SuperSignal West Pico chemoluminescent substrate was from Thermo Scientific (Rockford, IL, USA). Standard unsaturated disaccharides ΔDi0S, ΔDi2S, ΔDi4S, and ΔDi6S were from Sigma–Aldrich Co., and standard ΔDi2,6S, ΔDi4,6S, ΔDi2,4,6S were from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). These disaccharides were a kind gift from Prof. Dr. Mauro Pavão, UFRJ, Rio de Janeiro, RJ, Brazil. Standard ΔDi0S, ΔDi4S, and ΔDi6S were also prepared by methods previously described (Michelacci & Dietrich, 1976).

2.2. Agarose gel electrophoresis of CS samples

All CS samples were submitted to agarose gel electrophoresis as previously described (Dietrich & Dietrich, 1976). Briefly, 1 mg/mL solutions of CS samples were prepared, and aliquots (5 μL) were applied to agarose gel slabs in 0.05 M 1,3-diaminopropane-acetate buffer, pH 9 (PDA). After fixation with cetyltrimethylammonium bromide (cetavlon) and Toluidine Blue staining, the glycosaminoglycans were quantified by densitometry of the gel slabs (Epson Expression 1680 Flatbed Scanner, with QuickScan Win 2000, Helena Laboratories, Beaumont, TX, USA). Analysis was performed in triplicates, using Sigma–Aldrich chondroitin sulfate as standard. These compounds were further characterized by enzymatic degradation with bacterial glycosaminoglycan lyases (chondroitinase AC and chondroitinase B from *Flavobacterium heparinum*), as already described (Petricevich & Michelacci, 1990).

2.3. Analytical data

Aminosugars were determined after acid hydrolysis (4 M HCl for 6 h at 100 °C) by a modified Elson–Morgan reaction (Rondle & Morgan, 1955). Uronic acid was determined by a modification of the carbazole reaction (Di Ferrante et al., 1971). Total sulfate was measured by paper chromatography after acid hydrolysis (8 M HCl for 6 h at 100 °C), as previously described (Nader & Dietrich, 1977), and soluble protein was measured by a modified Lowry procedure with bicinchoninic acid (BCA Protein Kit Assay, Pierce, IL, USA), using bovine serum albumin as standard (Smith et al., 1985). Reducing sugars were measured by the method of 3-5-dinitrosalicylic acid (DNS, Miller, 1959).

2.4. Identification of main contaminants of CS-poor samples by FACE (fluorophore-assisted carbohydrate electrophoresis) and Raman spectroscopy

2.4.1. Treatment of unsaturated disaccharides with mercuric acetate

Aliquots of standard unsaturated disaccharides (<50 nmol) were frozen on dry ice, and lyophilized on a vacuum concentrator until dry. These samples were resuspended in 100 μL of 17.5 mM mercuric acetate, 50 mM sodium acetate, and incubated at room temperature (30 min, Calabro, Benavides, Tammi, Hascall, & Midura, 2000). The mercuric ion was removed by addition of 30 μL of a 50% slurry of Dowex H^+ resin, which was removed by filtration through a glass wool plugged pipette tip. Both the reaction tube and the glass wool were washed with 100 μL of ultrapure water, and the trapped volume was recovered by centrifugation (2000 × g). The samples were again frozen and vacuum dried.

2.4.2. Fluorescent derivatization with 2-aminoacridone (AMAC)

The derivatization was performed as described by Calabro et al. (2000), with modifications. Dried samples (20–200 nmol of saccharides) were derivatized by addition of 5 μL of 50 mM AMAC (250 nmol) in 85% DMSO/15% acetic acid. After 15 min incubation at room temperature, 5 μL of a newly prepared 1 M sodium cyanoborohydride solution was added, incubated for 16 h at 37 °C, and then 30 μL of 30% glycerol were added. One aliquot (2 μL) of each derivatized sample was immediately analyzed, and the remaining was stored in the dark at −80 °C.

2.4.3. Fluorophore-assisted carbohydrate electrophoresis

Electrophoresis were carried out in Mini-Protean Tetra Cell (Bio-Rad Laboratories, Richmond, CA, USA) in two alternative buffer systems: nonborate buffer system (anionic oligosaccharides) and borate-containing buffer system (for neutral sugars).

For nonborate conditions, gels were prepared in Tris–HCl buffer and run in Tris–glycine buffer. Polyacrylamide separating gels (20%) were prepared as described by Oonuki, Yoshida, Uchiyama, and Asari (2005), with some modifications. Acrylamide-bisacrylamide stock solution (40% containing 5% bisacrylamide, 5 mL) plus 1.5 M Tris–HCl buffer, pH 8.9 (1.5 mL) were diluted with 3.5 mL of pure water. This solution was degased for a few minutes, and then mixed with 0.1 mL of 10% APS and 10 μL of TEMED. The gel solution was poured into sets of glass boards with butanol added to the top, and was left until completion of gelling. Then 0.5 mL of the acrylamide–bisacrylamide solution (40%) was mixed with 0.66 mL of 0.5 M Tris–HCl buffer (pH 6.7), and diluted with 3 mL of pure water. Also this "stacking gel solution" was degassed. After the butanol was taken away from the top of the separating gel, 5 μL of TEMED and 50 μL of 10% APS were added to the stacking gel solution, which was poured on top of the separation gel and a sample comb was set into the stacking gel. Stock electrode buffer, 10× was 1.92 M glycine, 0.25 M Tris base, pH 8.3. The run (100–220 V, 70 min) was followed with the aid of an UV-lamp (320–400 nm).

For borate-containing conditions, the stock resolving gel buffer 4× was 0.75 M Tris-0.5 M boric acid, adjusted to pH 7.0 with concentrated HCl; the stock stacking gel buffer, 4× was 0.5 M Tris-0.5 M boric acid, adjusted to pH 6.8 with concentrated HCl, and the stock electrode buffer, 5× was 0.5 M glycine, 0.6 M Tris base, and 0.5 M boric acid, final pH 8.3 (Gao & Lehrman, 2003).

After electrophoresis, gels were removed from the apparatus, illuminated with a UV transilluminator (365 nm), and photographed (GelDoc-It Imaging System, UVP). Quantitative results were obtained with TotalLab TL120 1D v2009 software, Nonlinear Dynamics Ltd.

2.4.4. Raman spectroscopy

Fourier transform Raman measurements were carried out using a Bruker RFS 100 instrument equipped with a Nd:YAG laser, a Germanium detector cooled with liquid nitrogen, operating at 1064 nm. The *in situ* analysis of each solid sample was obtained with 1000 scans collected at resolution of 4 cm^{-1}, with a power of 35 mW. These parameters were selected in order to obtain the best signal-to-noise ratio while the physical and chemical integrity of the samples was maintained; this was achieved by repeating each one of the spectra and observing the possible changes in position and intensity over all the spectrum bands. The FT-Raman spectra in the range of 3500–50 nm were obtained from the reference standard chondroitin 4-sulfate, maltodextrin, lactose, sodium sulfate, and sixteen pharmaceutical chondroitin sulfate samples. The peak assignment was based on Bansil, Yannas, and Stanley (1978) and on Ellis, Green, and Winlove (2009).

2.5. Structural analysis of the CS of the "CS-rich"

Aliquots of CS samples (100 μg, 10 μL of 10 mg/mL solutions) were incubated with 10 mU of chondroitin lyase AC (10 μL) in 0.05 M ethylenediamine-acetate (EDA) buffer, pH 8.0, at room temperature. After 18 h incubation, aliquots (2 μL) were submitted to agarose gel electrophoresis as described in Section 2.2. The remaining 18 μL were applied to Whatman #1 filter paper and submitted to descending chromatography in isobutyric acid: 1.25 M NH_4OH, 5:3, v/v. After 24 h, the reducing products were located by silver nitrate staining.

Alternatively, 20 μg of substrate were incubated with 2 mU of chondroitin lyase AC in EDA buffer, 50 μL final volume. After 18 h incubation at room temperature, the samples were vacuum dried, derivatized with AMAC as described in Section 2.4.2., and submitted to FACE as in Section 2.4.3.

The molecular weight of the chondroitin sulfates was determined by polyacrylamide gel electrophoresis as previously described (Dietrich & Nader, 1974; Hilborn & Anastassiadis, 1971).

2.6. Detection of keratan sulfate by immunoblotting

Keratan sulfate was detected in the samples by immunoblotting probed with MST1, a monoclonal antibody that recognizes keratan sulfate both as free chains and as proteoglycans (Alves et al., 1994; Baccarin et al., 2012; Pereira, Aguiar, Hagiwara, & Michelacci, 2004). After agarose gel electrophoresis, the glycosaminoglycans were transferred to nitrocellulose and Zeta-Probe nylon membranes. After blocking, membranes were probed with MST1. Then, the membranes were incubated with peroxidase-conjugated rabbit anti-mouse IgG secondary antibody, and either tetramethylbenzidine (TMB) or SuperSignal West Pico was used as substrates. Quantitative analysis were performed by ELISA (Alves et al., 1994), using keratan sulfate purified from bovine nucleus pulposus as standard.

3. Results and discussion

3.1. Agarose gel electrophoresis of CS samples

Sixteen pharmaceutical grade CS samples were analyzed by agarose gel electrophoresis in PDA buffer, in comparison to USP-CS and analytical grade Ch4S. USP-CS contained less CS (96.4%) then the analytical grade Ch4S. That is why the analytical grade Ch4S was used as standard in the following experiments. Fig. 1 shows representative agarose gel slabs (B), as well as a standard curve of Ch4S (A). The bands were quantified by densitometry, and the curve was linear (R^2 = 0.9998) between 1 and 8 μg of CS (European Pharmacopoeia, 2007; United States Pharmacopoeia, 2008). For all CS samples, a single band appeared, migrating as the standard Ch4S (Fig. 1B). However, only five samples – #3, 4, 6, 12 and 16 – were more than 90% CS (average 96.5%), while the remaining eleven were less than 15% CS (average 10%) (Table 1). Thus, these samples were classified as "CS-rich" (#3, 4, 6, 12 and 16) and "CS-poor" (#1, 2, 5, 7, 8, 9, 10, 11, 13, 14, 15) samples.

Electrophoretic techniques are recommended by both the European Pharmacopeia (agarose gel electrophoresis in barium acetate buffer) and the United States Pharmacopeia (cellulose acetate electrophoresis in barium acetate buffer) for CS analysis. Not more than 2% of any individual impurity is acceptable. Furthermore, both pharmacopeias describe a photometric titration procedure with cetylpyridinium chloride (CPC), which forms insoluble complexes with CS and precipitate. Nevertheless, this procedure is not specific, since other glycosaminoglycans, nucleic acids, and even inorganic salts interfere with the CS quantification (Sim et al., 2005; Volpi,

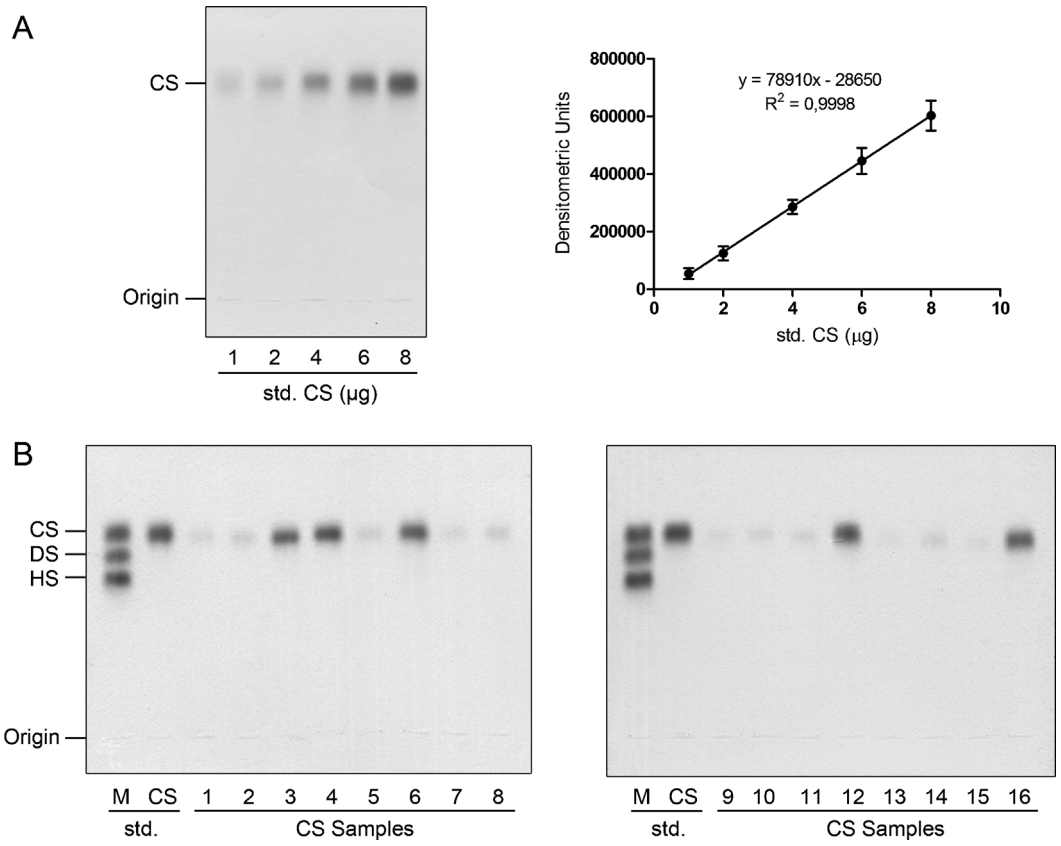

Fig. 1. Agarose gel electrophoresis of CS samples. (A) Calibration curve for CS. Standard chondroitin 4-sulfate (std CS) solutions were prepared: 0.2 mg/mL, 0.4 mg/mL, 0.8 mg/mL, 1.2 mg/mL, 1.6 mg/mL, and 5 µL aliquots of each were applied to 0.55% agarose gel in 0.05 M 1,3-diaminopropane-acetate buffer, pH 9.0 (PDA). After the run, the glycosaminoglycans were precipitated in the gel by 0.1% cetyltrimethylammonium bromide (cetavlon), the gel was dried, and stained by Toluidine Blue, as previously described (Dietrich & Dietrich, 1976). CS was quantified by densitometry, and mean ± standard deviation for three independent determinations are shown. The curve was linear from 1 to 8 µg of CS. A representative gel of the 16 CS samples here analyzed is shown in (B). Aqueous solutions (1 mg/mL) of each CS sample (1–16) were prepared, and 5 µL aliquots were submitted to agarose gel electrophoresis as above described. CS was quantified by densitometry in comparison to standard CS run in the same gel, and also to the CS standard curve. Quantitative results are mean ± standard deviation of three independent determinations. M, mixture of standard glycosaminoglycans containing chondroitin sulfate (CS), dermatan sulfate (DS), and heparan sulfate (HS) (5 µg each).

Table 1
Analytical data of sixteen samples of pharmaceutical grade CS.

	CS #	CS content (%)	Protein (%)	Reducing sugars (µg/100 µg of sample)	GlcUA (µg/100 µg of sample)	GalNAc (µg/100 µg of sample)	Sulfate (µg/100 µg of sample)
CS-rich	3	91.0	3.6	8.3	38.2	33.4	27.3[a]
	4	94.0	3.5	8.1	36.3	29.0	17.9[a]
	6	97.8	1.9	9.0	37.5	32.0	29.3[a]
	12	97.8	3.6	9.7	39.7	40.3	25.5[a]
	16	102.0	3.5	9.2	36.8	33.7	24.6[a]
	Mean ± SD	*96.5 ± 4.2*	*3.2 ± 0.7*	*8.9 ± 0.7*	*37.7 ± 1.3*	*33.7 ± 4.1*	*24.9 ± 4.3*
CS-poor	1	10.4	3.0	33.3	19.0	5.5	–
	2	13.1	3.2	34.8	19.6	6.5	–
	5	12.5	3.5	29.2	21.1	7.3	–
	7	9.6	3.5	32.3	20.9	8.9	–
	8	13.4	3.2	31.2	19.8	11.0	–
	9	7.3	3.4	32.7	20.7	10.4	–
	10	8.9	2.9	29.6	20.3	9.6	–
	11	11.0	3.1	33.3	24.8	6.9	–
	13	5.4	10.5	21.9	6.7	9.5	26.3[b]
	14	11.4	3.1	32.4	23.6	11.8	–
	15	6.8	11.1	19.8	7.9	8.0	26.1[b]
	Mean ± SD	*10.0 ± 2.7*	*4.6 ± 3.1*	*30.0 ± 4.9*	*18.6 ± 5.8*	*8.7 ± 2.0*	*26.2*
Standards	Ch4S	100.0	1.3	8.9	43.0	45.9	27.7[a]
	Ch6S	100.0	0.0	7.5	42.0	39.5	24.0[a]
	Maltodextrin	–	3.4	35.4	17.7	3.3	–
	Lactose	–	9.1	54.5	14.1	3.3	–

Results are given as mean ± standard deviation.
[a] Sulfate detected only after acid hydrolysis (HCl 8 M, 6 h, 100 °C).
[b] Inorganic free sulfate.

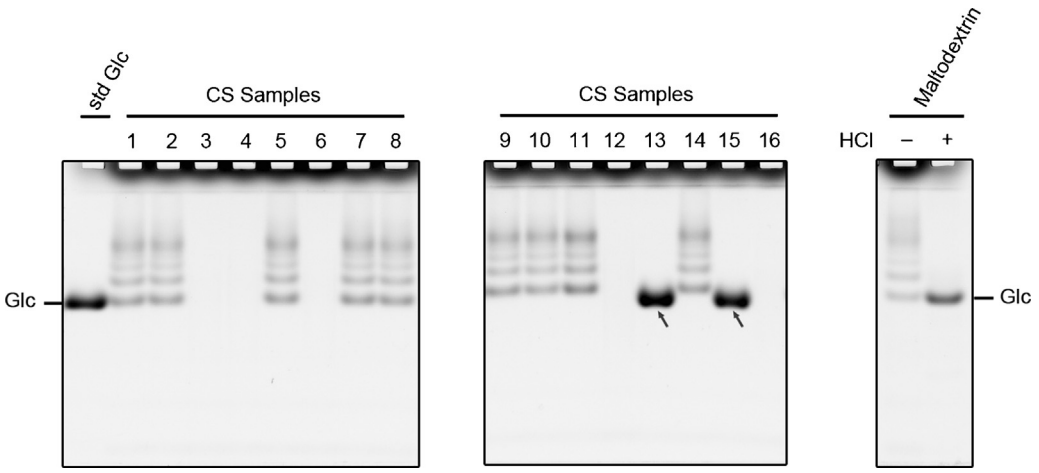

Fig. 2. Fluorophore-assisted carbohydrate electrophoresis (FACE) of CS samples in Tris-borate-glycine buffer. The CS samples were analyzed by FACE in 0.12 M Tris-borate-glycine buffer, pH 8.3, in order to identify the main contaminants. CS samples (50 μg) were derivatized with AMAC, and aliquots (2.5 μg) were submitted to electrophoresis as described in Section 2.4. Gel images were obtained under UV (λ = 365 nm) with a scientific grade CCD Camera (*GelDoc-It Imaging System, equipped with GelCam 310*). For comparison, maltodextrin was equally derivatized with AMAC and analyzed by FACE, under the same conditions. A band patter similar to that of maltodextrin was observed in CS samples 1, 2, 5, 7, 8, 9, 10, 11 and 14, but did not appear in the CS-rich samples (3, 4, 6, 12 and 16), as well as in samples 13 and 15, which presented only one band (arrow).

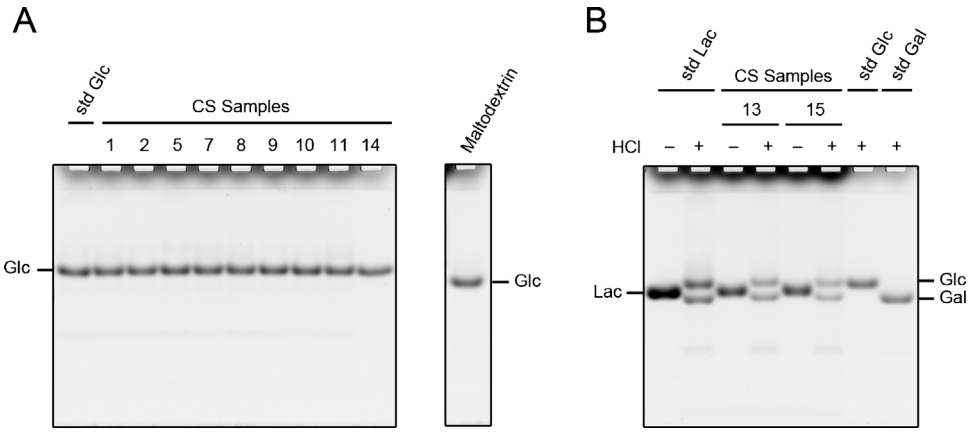

Fig. 3. Acid hydrolysis of CS-poor samples. To further characterize the main contaminants of CS-poor samples (15% CS or less), aliquots (20 μg) were submitted to acid hydrolysis (6 M HCl, 100 °C, 6 h), and then derivatized by AMAC and analyzed by FACE, under the same conditions described in Fig. 2. For comparison, maltodextrin was equally submitted to acid hydrolysis, treated with AMAC and analyzed by FACE, under the same conditions. Now, the electrofluorogram showed a single band for samples 1, 2, 5, 7, 8, 9, 10, 11, 14 and maltodextrin, migrating as glucose (Glc) (A). In contrast, for samples 13 and 15, which have shown only one band, migrating as lactose (Lac) before hydrolysis, two bands appeared after acid hydrolysis, migrating as glucose (Glc) and galactose (Gal) (B). Gel images were obtained as described in Fig. 2.

2007). For instance, it was recently shown that propylene glycol alginate sulfate sodium, also known as alginic sodium diester (ASD), and Zero One (Z1), a water-soluble agent recently identified as sodium hexametaphosphate, are also titratable anions and form ionic pairs with CPC, therefore interfering with the CPC titration (Weiguo et al., 2014). The authors suggest that CPC titration and electrophoresis to be used in combination to detect CS adulterants ASD and Z1.

The agarose gel electrophoresis here used, developed by Jaques, Ballieux, Dietrich, and Kavanagh (1968) and modified by Dietrich and Dietrich (1976), using a special buffer system – PDA – to permit the separation and identification of different glycosaminoglycans, is much more precise and specific. After electrophoresis, the glycosaminoglycans are precipitated in the gel by a cationic detergent (cetavlon), the gel is dried under a sheet of paper and hot air flow to remove excess cetavlon and buffer, and stained by Toluidine Blue. Only metachromatic bands are glycosaminoglycans. These bands are quantified by densitometry.

3.2. Analytical data

Table 1 shows the analytical data of our CS samples, and standards Ch4S, Ch6S, maltodextrin and lactose. Only two of the CS-poor samples – #13 and 15 – contained significant amounts of soluble proteins (~10%). All other samples contained ~3% protein. In contrast, all the "CS-poor" samples contained much more reducing sugars than the "CS-rich" samples (about 3 times), while the amounts of glucuronic acid (GlcUA) and galactosamine (GalNAc) were lower (a half for GlcUA, and one third for GalNAc). These results are in agreement with the findings shown in Fig. 1. It is also important to notice that the GlcUA measurement may be overestimated in some "CS-poor" samples due to the possible presence of neutral sugars (see Table 1 and Section 3.3). Free inorganic sulfate was also detected only in samples #13 and 15 (before acid hydrolysis). In the "CS-rich" samples, as well as in standard Ch4S and Ch6S, sulfate was measured after acid hydrolysis of the polymers (HCl 8 M, 6 h, 100 °C).

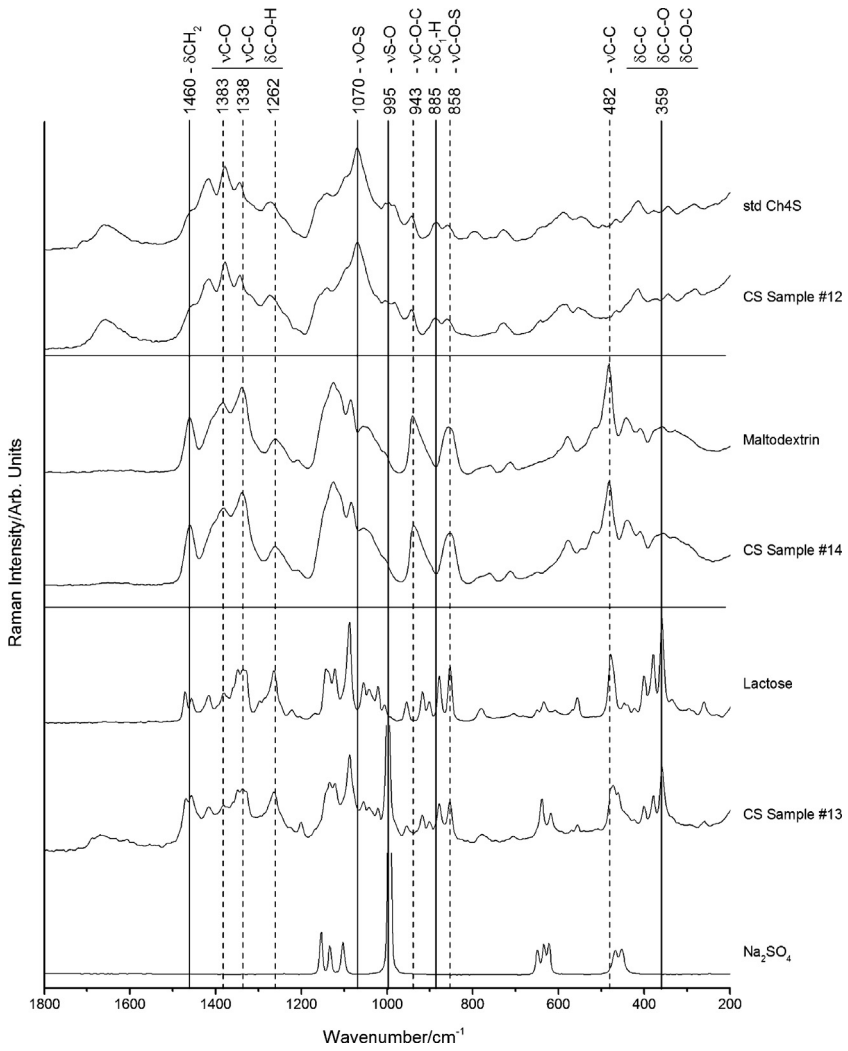

Fig. 4. Raman spectra of CS pharmaceutical samples and standards. *In situ* analysis of reference standards and representative samples by macro-Raman spectroscopy operating at 1064 nm. The spectra were obtained with 1000 scans of powder samples. Fingerprint bands are shown in the region of 1800–200 cm^{-1}.

3.3. Identification of main contaminants of CS-poor samples by FACE and Raman spectroscopy

As CS was only a minor component of 11 (out of 16) CS samples, corresponding to only 5–13% of total weight, we decided to investigate the main contaminants. So, the "CS-poor" samples were submitted to FACE in Tris-borate-glycine buffer system (Fig. 2). In 9, out of these 11 "CS-poor" samples – #1, 2, 5, 7–11, 14 – several bands appeared. These bands migrated as those obtained from maltodextrin (Fig. 2). The remaining two "CS-poor" samples – #13 and 15 – presented a single intense band (arrow, Fig. 2). In the "CS-rich" samples, no bands appeared.

In order to further characterize the contaminant carbohydrates, the "CS-poor" samples were again submitted to acid hydrolysis (HCl 6 M, 6 h, 100 °C), and analyzed by FACE in Tris-borate-glycine buffer system (Fig. 3). A single band migrating as glucose was observed in all samples that presented the "maltodextrin-type" band pattern – #1, 2, 5, 7-11, 14, Fig. 3A. In contrast, the "CS-poor" samples that presented only one intense band in Fig. 2, exhibited, after acid hydrolysis, two bands of the same intensity, migrating as glucose and galactose (Fig. 3B). Actually, the single band observed in the non-hydrolyzed material had the same migration of lactose.

These results were confirmed by Raman spectroscopy. Fig. 4 shows representative spectra of standard CS, maltodextrin, lactose, and sodium sulfate, as well as selected CS samples (#12, 13, and 14). The main component of CS #12 presents a Raman profile similar to that of standard CS, while the spectrum of sample #14 is similar to that of maltodextrin, and the sample #13 spectrum resembles that of lactose and sodium sulfate.

So, our data show that the main contaminant of samples #1, 2, 5, 7–11, and 14 was maltodextrin, while samples #13 and 15 contain predominantly lactose (51% and 46%, respectively), sulfate (~26%), and protein (~10%), with only 5–6% CS. This finding is alarming since medicines prepared from these raw materials will not only contain much less of the desired active principle (CS), but also could have serious side effects from the contaminants.

Unconformities regarding CS content in nutraceuticals, as well as the presence of other contaminant sugars and glycosaminoglycans have also been reported by others (Adebowale, Cox, Liang, & Eddington, 2000; Sim et al., 2005; Volpi, 2009).

To our knowledge, this is the first study to describe pharmaceutical preparations having less than 15% CS, and in which the main contaminants of eleven preparations were totally characterized. It is still important to note that these contaminants could not be impurities from the extraction and purification

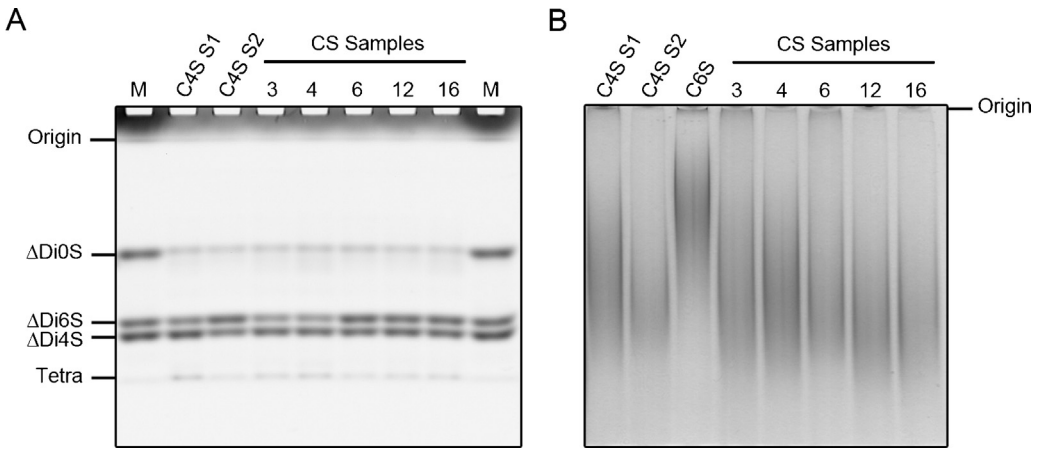

Fig. 5. FACE (Tris-glycine buffer), and PAGE of the five "CS-rich" samples. To access the CS sulfation pattern and modal molecular weight, the "CS-rich" samples were further analyzed by FACE after enzymatic degradation, and by PAGE. Aliquots (20 μg) of each sample were incubated with *Flavobacterium heparinum* chondroitin lyase AC (2 mU). The products formed were derivatized with AMAC, and analyzed by FACE in 0.025 M Tris-glycine buffer, pH 8.3. Gel images were obtained as described in Fig. 2. A representative gel is shown in (A). The bands were quantified by densitometry using TotalLab TL120 software. Two independent derivatizations were made for each sample, and three gels were run for each derivatization. For the determination of CS modal molecular weight, aliquots (10 μg) of CS samples were applied to a 7.5% polyacrylamide gel in 20 mM Tris-HCl buffer, pH 7.4, as previously described (Hilborn & Anastassiadis, 1971; Dietrich & Nader, 1974). After the run (100 V, 50 min) the gels were stained with 0.1% Toluidine Blue in 1% acetic acid (B), and analyzed by densitometry. ΔDi0S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; Tetra, tetrasaccharides; M, mixture of standard unsaturated disaccharides; Ch4S, standard CS extracted from bovine trachea (Sigma–Aldrich); Ch6S, standard CS from shark cartilage (Sigma–Aldrich); 3, 4, 6, 12 and 16, CS pharmaceutical samples.

procedures, since they are not found in the same animal sources of CS.

3.4. Structural analysis of the CS of the "CS-rich"

Standard disaccharides were characterized by FACE, and also by treatment with mercuric acetate, followed by FACE (nonborate system). The products formed from the "CS-rich" samples were compared to these standards.

Aliquots (20 μg) of the "CS-rich" samples – #3, 4, 6, 12, 16 – were incubated with *F. heparinum* chondroitin lyase AC as described in Section 2.5, and derivatized with AMAC (40 μL final volume). Aliquots (2 μL, containing ~1 μg of CS products) were submitted to FACE, as described in Section 2.4.3. Representative results are shown in Fig. 5, and quantitative data are given in Table 2. All the

Table 2
Disaccharide composition and molecular weight analysis of 5 pharmaceutical CS samples with CS content above 90%.

	Disaccharide composition					Modal molecular weight (kDa)
	Di0S	Di6S	Di4S	4S/6S	SO_3/UA	
Ch4S (standard)	10%	49%	41%	0.8	0.90	25.4
CS sample #3	12%	33%	55%	1.7	0.88	27.6
CS sample #4	13%	34%	53%	1.6	0.87	29.0
CS sample #6	11%	47%	42%	0.9	0.89	24.0
CS sample #12	9%	44%	47%	1.1	0.91	19.6
CS sample #16	10%	44%	46%	1.0	0.90	20.4

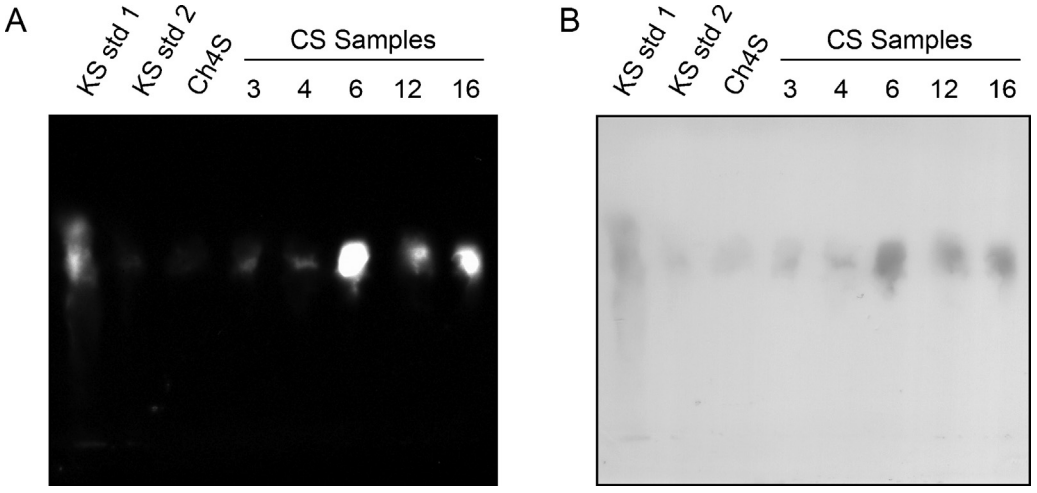

Fig. 6. Immunoblotting of keratan sulfate. Aliquots (5 μg) of the five CS-rich samples (3, 4, 6, 12 and 16) were submitted to agarose gel electrophoresis as described in Fig. 1, except that after the run the glycosaminoglycans were transferred to nitrocellulose membrane, and revealed by an anti-keratan sulfate monoclonal antibody – MST1 (Alves et al., 1994). The blotting was processed as previously described (Pereira et al., 2004). This antibody recognizes keratan sulfate both as free chains and as proteoglycans. Secondary antibody conjugated with horseradish peroxidase (HRP) was used, and two substrates were used in sequence: Super Signal West Pico chemiluminescent substrate (A), and tetramethylbenzidine (TMB) (B). KS std 1, standard keratan sulfate proteoglycan; KS std 2, standard keratan sulfate; Ch4S, standard chondroitin 4-sulfate (Sigma–Aldrich).

CS samples gave three bands migrating as ΔDi0S, ΔDi4S, ΔDi6S, and trace amounts of tetrasaccharide (Tetra). Nevertheless, the relative proportions of disaccharides varied. All of them contained ~10% Di0S, but samples #3 and 4 contained predominantly Di4S (Di4S:Di6S ratios 1.6–1.7), while samples #6, 12, and 16 contained similar amounts of Di4S and Di6S (Di4S:Di6S ratios 0.9–1.1). Fig. 5 also shows a polyacrylamide gel electrophoresis of the same samples, performed to determine the molecular weight of the polymers. All of them were polydisperse polymers, with modal molecular weight ~20 kDa.

Differences in the composition and biological activities of chondroitin sulfates of different origins were also reported by others (Martel-Pelletier, Farran, Montell, Vergés, & Pelletier, 2015; Michelacci & Dietrich, 1976; Michelacci et al., 1979).

3.5. Detection of keratan sulfate by immunoblotting

Although only one band migrating as CS was observed for all "CS-rich" samples in agarose gel electrophoresis (Fig. 1), trace amounts of keratan sulfate appeared on immunoblotting probed with monoclonal antibody (MST1) (Fig. 6). The identification of keratan sulfate was confirmed by incubation with *Pseudomonas* sp. keratanase. Quantitative analysis performed by ELISA using the same primary antibody has shown that keratan sulfate corresponds to less than 1% of total glycosaminoglycan of these samples. It is important to notice that even the standard Ch4S (Sigma–Aldrich) also contained trace amounts of keratan sulfate. This keratan sulfate probably comes from the same biological source – cartilage – and due to its polyanionic nature was not separated from chondroitin sulfate during the purification procedures.

The presence of keratan sulfate in CS preparations was also recently reported by others (Nakano & Ozimek, 2014).

4. Conclusions

In conclusion, our data show that, out of 16 CS samples prepared in different countries all over the world, only 5 contained more than 90% CS and were in conformity to their labels. The remaining 11 CS-poor samples contained maltodextrin or lactose as the main contaminants (both of which are commonly used as pharmaceutical excipients). It is important to reinforce that these "CS" preparations came from diverse locations, and all of them are clearly not in conformity to their label specifications.

This finding could explain, at least in part, the variations reported by different authors in the biological responses of osteoarthritis to CS treatment. In addition, some contaminants could lead to serious side effects, especially in sensible patients (for instance, lactose in intolerant patients).

We strongly suggest that more efficient and strict criteria should be applied to both licensing and quality control of raw materials, as well as to the final pharmacological preparations obtained from them.

The use of agarose gel electrophoresis, as well as FACE and Raman spectroscopy, to determine both the CS contents and the eventual presence/identification of contaminants, is recommended.

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