Quality of different chondroitin sulfate preparations in relation to their therapeutic activity

Nicola Volpi

Department of Biologia Animale, Biological Chemistry Section, University of Modena and Reggio Emilia, Modena, Italy

Abstract

Objectives Chondroitin sulfate is currently recommended by the European League Against Rheumatism (EULAR) as a SYSADOA (symptomatic slow acting drug for osteoarthritis) in Europe in the treatment of knee and hand osteoarthritis based on research evidence and meta-analysis of numerous clinical studies. Furthermore, recent clinical trials demonstrated its possible structure-modifying effects. Chondroitin sulfate, alone or in combination with glucosamine or other ingredients, is also utilized as a nutraceutical in dietary supplements in Europe and the USA. However, it is derived from animal sources by extraction and purification processes. As a consequence, source material, manufacturing processes, the presence of contaminants and many other factors contribute to the overall biological and pharmacological actions of these agents. We aim to review the quality control of chondroitin sulfate in pharmaceutical-grade preparations and nutraceuticals.

Key findings Pharmaceutical-grade formulations of chondroitin sulfate are of high and standardized quality, purity and properties, due to the stricter regulations to which this drug is subjected by local national health institutes as regards production and characteristics. On the contrary, as several published studies available in literature indicate, the chondroitin sulfate quality of several nutraceuticals is poor. Additionally, there are no definite regulations governing the origin of the ingredients in these nutraceuticals and the origin of the ingredients in natural products is the most important factor ensuring quality, and thus safety and efficacy, in particular for chondroitin sulfate, due to its extraction from different sources.

Conclusions Due to the poor chondroitin sulfate quality of some nutraceuticals, we conclude that stricter regulations regarding their quality control should be introduced to guarantee the manufacture of high quality products for nutraceutical utilization and to protect customers from low-quality, ineffective and potentially dangerous products. There is a need for specific and accurate analytical procedures, which should be enforced to confirm purity and label claims both for raw materials and finished chondroitin sulfate products, and also to govern the origin of ingredients. Until these stricter regulations are in place, then it is strongly recommended that pharmaceutical-grade chondroitin sulfate is used rather than food supplements.

Keywords chondroitin sulfate; glycosaminoglycans; nutraceuticals; osteoarthritis

Structural heterogeneity of chondroitin sulfate

Chondroitin sulfate, belonging to the class of natural complex polysaccharides named glycosaminoglycans (GAGs), is composed of alternate disaccharide sequences of differently sulfated residues of d-glucuronic acid (GlcA) and of N-acetyl-d-galactosamine (GalNAc) linked by β(1→3) bonds. Depending on the disaccharide nature, chondroitin sulfates with different carbohydrate backbones are known. Chondroitin-4-sulfate (CSA), contains disaccharides sulfated in position 4 of GalNAc, while chondroitin-6-sulfate (CSC) is mainly composed of a disaccharide unit sulfated in position 6 of GalNAc. However, even if the known chondroitin samples are mainly composed of various percentages of these two kinds of disaccharide unit, disaccharides with a different number and position of sulfate groups can be located, in various percentages, within the polysaccharide chains. For example, the nonsulfated disaccharide is present, generally in low amounts, in the chondroitin sulfate backbone while the monosulfated disaccharide in position 2 of the GlcA is very uncommon in this natural polymer. On the contrary, disulfated disaccharides having two sulfate groups O-linked in various positions, such as
of GlcA and 6 of GalNAc (disaccharide D), or in position 4 and 6 of GalNAc (disaccharide E) or 2 of the GlcA and 4 of GalNAc (disaccharide B) may be present in the chondroitin sulfate backbone in various percentages in relation to specific animal sources. Finally, a fully trisulfated disaccharide may be located inside the chondroitin sulfate chains, generally in minimal quantities.

Besides the above-mentioned structures, a peculiar chondroitin sulfate polymer, known as CSB or dermatan sulfate, consists of a prevailing disaccharide unit formed of L-iduronic acid (IdoUA) and of GalNAc mainly sulfated in position 4 with minor concentrations of disulfated disaccharides, in particular sulfated in position 4 of GalNAc and 2 of the IdoUA unit (Figure 1). However, although the principles of the biosynthetic process have not yet been fully elucidated, it is well known that this process results in the generation, within the polymer chain, of highly modified oligosaccharide domains separated by regions of relatively low-degree structural modifications, introducing further high heterogeneity. Thus, the dermatan sulfate chain has a hybrid co-polymeric structure consisting of low-modified (chondroitin sulfate) and highly modified (dermatan sulfate) domains.

The sulfation heterogeneity is responsible for a great charge density variability as well as for the presence of low- or highly-sulfated sequences inside the carbohydrate backbone. Furthermore, the number of disaccharide units forming the chondroitin sulfate polymer is another key factor influencing biological and pharmacological activity. As a consequence, molecular mass parameters are of paramount importance for chondroitin sulfate properties.

**Biological role of chondroitin sulfate**

Recent evidence from glycobiology studies suggests that proteoglycans, and their complex polysaccharidic macromolecules, are not only structural components, but they also participate in and regulate many cellular events and physiological processes. Growing recent evidence suggests that chondroitin sulfate (and dermatan sulfate) chains...
have intriguing functions in central nervous system development, wound repair, infection, growth factor signalling, morphogenesis and cell division, differentiation and migration, in addition to osteoarthritis (OA; see below) and their conventional structural roles (for more complete and specialized papers see references[12–18]).

**Chondroitin sulfate as a drug**

Chondroitin sulfate is currently recommended by the European League Against Rheumatism (EULAR) as a SYSADOA (symptomatic slow acting drug for OA) in Europe in the treatment of knee[19] and hand20 OA based on research evidence and meta-analysis of numerous clinical studies. Moreover, chondroitin sulfate, alone or in combination with glucosamine or other ingredients, is utilized as a nutraceutical in Europe and the USA.[21–23] In Europe, the publication of the EULAR recommendations for the treatment of knee OA in 2003 listed oral chondroitin sulfate as evidence 1A (A represents the highest level for a therapeutic strategy).[19] Furthermore, recent clinical trials demonstrated possible structure-modifying effects of chondroitin sulfate[24,25] as an additional very important factor to consider with respect to its well-documented symptom-modifying properties.

Evidence for the clinical efficacy of oral chondroitin sulfate as a drug able to significantly improve the functional symptoms of osteoarthritic disease has been discussed in a set of randomized clinical studies published in 2008.[26] Additionally, an exploratory post-hoc analysis of GAIT (Glucosamine/chondroitin Arthritis Intervention Trial) patients[27] suggested a statistically significant improvement in joint swelling in chondroitin sulfate-treated patients with moderate-to-severe pain compared with placebo-treated patients.[28] Taking these important points into account, we definitively have enough clinical data available to support the view that oral chondroitin sulfate is a valuable and safe symptomatic (and structure-modifying) treatment for OA.

However, chondroitin sulfate, like other natural polysaccharides, is derived from animal sources by extraction and purification processes.[1] As a consequence, besides its natural structural heterogeneity, source material, manufacturing processes, the presence of contaminants, purity and many other factors contribute to the overall pharmacological (and biological) action of this agent.

**Analytical tools for the evaluation of the properties and purity of chondroitin sulfate**

Chondroitin sulfate, like other natural macromolecules, has a complex structure that is known to change with the source tissue, organ and species.[1] As a consequence, the determination of the origin is of paramount importance. Commercial manufacture of chondroitin sulfate relies on bovine[29] porcine[29] chicken[30] or cartilaginous fish (such as shark[31] and skate32,33) by-products, in particular cartilage, as raw material. Chondroitin sulfate from different sources contains disaccharides possessing sulfate groups in different positions and in different percentages within the polysaccharide chains.[34] Furthermore, extraction and purification processes may introduce further modifications of the structural characteristics and properties. Finally, as a result of the biosynthetic processes related to specific tissues and species, chondroitin sulfates with different grades of polymerization may be biosynthesized, producing macromolecules having various molecular masses and polydispersity. Due to these structural variations, and also to the possible presence of specific oligosaccharide sequences, and to the purity (see below) of the preparations for therapeutic application or for nutraceuticals, chondroitin sulfate may have different properties and capacities.

The evaluation of the chondroitin sulfate content and purity of a tissue extract is a key point in the product preparation, not only for pharmaceutical application but also for nutraceuticals. Chondroitin sulfate, as previously illustrated, is generally produced by means of extraction and further purification from tissues, in particular cartilages. Depending on the purification protocols, the extracts may be more or less rich in chondroitin sulfate, having a variable grade of purity due to the presence of polluting side-products.[1] Furthermore, the chondroitin sulfate content and purity may change according to the manufacturing processes or tissue sources.

In addition to a specific and sensitive analytical technique, an accurate and reproducible quantitative assay requires a reference standard having high specificity, purity and well-known physico-chemical properties and structure.[34] This is not a simple task in the case of natural polysaccharides due to their high heterogeneity related to the variability of their properties and structures, which mainly depend on the source and purification protocols, as previously discussed. At the moment, one reference standard, manufactured by Bioiberica (www.bioiberica.com/) and approved in 2004 as a chemical reference substance (CRS) by the European Pharmacopoeia Commission, is available.[34] This chondroitin sulfate standard is produced from bovine cartilage and has a purity of > 98%, as declared by the company.

Chondroitin sulfate produced for pharmaceutical applications is strictly evaluated for quality, content, structural characterization and parameters (such as charge density and molecular mass) by means of sensitive, specific, validated and published analytical approaches (see Volpi[34] for a discussion of the analytical procedures applied to pharmaceutical grade chondroitin sulfate). This feature is of paramount importance to ensure therapeutic reproducibility and safe use, and to protect patients from ineffective or unsafe drugs. Is it possible to affirm the same for nutraceutical chondroitin sulfate preparations?

**Are the chondroitin sulfates used in nutraceuticals of the same grade as that of pharmaceutical chondroitin sulfate?**

A multianalytical approach has been developed, validated and published[22,23] to enable a complete evaluation of the quality of chondroitin sulfate in nutraceuticals, specifically in terms of amount present and characteristics, both of which are of paramount importance to assure biological and pharmacological effects. The amount of chondroitin sulfate was evaluated, in the presence of other additives and compounds, by two different approaches: by agarose-gel electrophoresis
which enables the quantitative detection of the intact macromolecule; and quantitative evaluation of chondroitin sulfate by measuring its constituent disaccharides by means of HPLC (Figure 3a–e). Furthermore, agarose-gel electrophoresis is able to detect, with very high sensitivity, the presence of other glycosaminoglycans, such as heparin, heparan sulfate, dermatan sulfate and hyaluronic acid, which could potentially pollute the formulations.\cite{34} Additionally, by means of the evaluation of chondroitin sulfate constituent disaccharides, it is possible to determine the origin of the chondroitin sulfate contained in food supplement formulations by virtue of the different disaccharide patterns in relation to the sources (Figure 3a–d). In fact, the disaccharide pattern evaluation gives us the means to determine the charge density values (as sulfate group number per disaccharide unit) and the 4-sulfated/6-sulfated ratio (4s/6s ratio – the ratio between the sulfated groups located in position 4 and 6 on GalNAc).

The chondroitin sulfate samples from shark (and skate) have peculiar charge density values, greater than approximately 1.0 due to the presence of disulfated disaccharides, and a 4s/6s
Figure 3  Strong-anion exchange HPLC separation of the disaccharides from the chondroitin sulfate samples of various origins (a–d) and quantitative evaluation (e). (a–d) Strong-anion exchange (SAX)-HPLC separation of the disaccharides from the chondroitin sulfate samples of bovine (a), porcine (b), chicken (c) and shark (d) origin. The HPLC equipment was a Jasco pump model PU-1580, UV detector model UV-1570, Rheodyne injector equipped with a 10-μl loop and software Jasco-Borwin rel. 1.5. The unsaturated disaccharides generated from chondroitin sulfate samples treated with chondroitin ABC lyase were separated by SAX-HPLC using an isocratic separation with 50 mM NaCl pH 4 for 5 min and a gradient separation from 50 mM NaCl pH 4 at 5 min to 1.2 M NaCl pH 4 at 60 min at a flow rate of 1.2 ml/min. UV detection was set at 232 nm. Quantitative evaluation is also illustrated (e) by SAX-HPLC separation of disaccharides from increasing amounts (1–20 μg) of chondroitin sulfate. Modified from Volpi and Maccari.23 ΔDi-0s, ΔHexA-GalNAc; ΔDi-6s, ΔHexA-GalNAc (6-OSO₃); ΔDi-4s, ΔHexA-GalNAc (4-OSO₃); ΔDi-2,6dis, ΔHexA (2-OSO₃)-GalNAc (6-OSO₃); ΔDi-2,4dis, ΔHexA (2-OSO₃)-GalNAc (4-OSO₃); ΔDi-4,6dis, ΔHexA-GalNAc (4-OSO₃, 6-OSO₃).
ratio lower than 0.70 (in the case of shark chondroitin sulfate) due to the presence of a higher percentage of 4-sulfated groups than 6-sulfated ones (Figure 3d). On the contrary, chondroitin sulfate samples from bovine, porcine and chicken trachea show the same charge density values, of 0.90–0.96, due to the absence of disulfated (and trisulfated) disaccharides and to the presence of 6.0–8.0% of the nonsulfated disaccharide. However, these chondroitin sulfate samples can be distinguished by the different 4s/6s ratios, as the 4-sulfated disaccharide content in bovine chondroitin sulfate is almost double that of the monosulfated disaccharide in position 6, producing a 4s/6s ratio in the range of 1.50–2.00. The percentage of the 4-sulfated disaccharide increases in comparison with the 6-sulfated one in chicken chondroitin sulfate (Figure 3c) producing a 4s/6s ratio in the range of 3.00–4.00, and porcine chondroitin sulfate (Figure 3b) has the lowest amount of the 6-sulfated disaccharide with a high 4s/6s ratio in the range of 4.50–7.00. Finally, the evaluation of the molecular mass parameters of chondroitin sulfate (Figure 4a–d) is a key factor related to pharmacological activity as degraded products are unable to produce comparable biological effects.[34]

In a recent study,[23] several European nutraceuticals, in particular from the Czech Republic, have been analysed by the proposed methodology and the chondroitin sulfate content and overall quality accurately evaluated. On the basis of the analytical results obtained (Figure 5), the chondroitin sulfate content in these food supplement products was found to conform to the label specifications in only four of the ten samples analysed. Four of the food supplement preparations

Figure 4  High-performance size-exclusion chromatography analysis of the chondroitin sulfate samples from bovine (a), porcine (b), chicken (c) or shark (d) origin. Each purified polysaccharide (100 μg) was injected into the column (HPLC model LC-1500 from Jasco: pump model PU-1580, UV detector model UV-1570, refractive index detector model RI-2031, software Jasco-Borwin rel. 1.5). The mobile phase was composed of 125 mM Na2SO4 and 2 mM NaH2PO4 adjusted to pH 6.0 with 0.1 N NaOH. The flow rate was 0.9 ml/min with a back pressure of 40 kg/cm². Standards were solubilized in the mobile phase at a concentration of 5 mg/ml. Ten microlitres (100 μg) was injected into the HPLC. Columns were Protein Pak 125 (Waters, code 84601, 7.8 mm × 30 cm) and Protein Pak 300 (Waters, code 77271L, 7.5 mm × 30 cm) assembled in series. For the molecular mass parameter determination (average molecular weight number (Mn), average molecular weight (Mw) and polydispersity index (Mw/Mn)), the retention times were plotted against the logarithm of molecular mass for standard chondroitin sulfate having different molecular mass values. The curve that fits the experimental data is a third-grade polynomial with the formula y(fx) = -ax³ + bx² -cx + d performed by the Jasco Borwin GPC software ver 4.1. Modified from Volpi and Maccari.[23]
were found to contain approximately 0–1% chondroitin sulfate in comparison with 47, 17, 12 and 6% (samples A, B, D and L, respectively) declared on the label. Two products (samples E and F) were found to have about 30–45% of the declared chondroitin sulfate content, and one preparation (sample L) was found to contain approximately 2% hyaluronic acid (Figure 6a–c). Samples C, G, H and I were found to be conform to the label specifications (even if the chondroitin sulfate was present in very low amounts in C and I).

HPLC separation of unsaturated disaccharides for nutraceutical chondroitin sulfate was also used to evaluate its quality and possible origin (Figure 7). The chondroitin sulfate contained in eight food supplements proved to be of bovine or porcine origin, one was from cartilaginous fishes and in one case it was not possible to determine the origin due to the very low chondroitin sulfate content. In fact, as evident from chromatograms in Figure 7 reported as examples, sample E was clearly produced from shark cartilage due to the peculiar presence of disulfated disaccharides and to a charge density value greater than 1.0, and sample G was found to be of porcine origin, with a 4s/6s ratio in the range of 4.50–7.00 (see also Volpi and Maccari[23]).

These results are comparable with those of another published study showing the poor quality of chondroitin sulfate and the serious lack of compliance with the label claim in some nutraceuticals sold in the USA.[35] Furthermore, the quality of the raw material and finished products was found to be poor on the Korean market,[36] and a great variability in the chondroitin sulfate origin was found in twelve products from Japan, which generally did not correspond to the declared content claimed on the label.[37]

Is the chondroitin sulfate used in nutraceuticals able to produce comparable biological and pharmacological effects to those of the chondroitin sulfate in the pharmaceutical-grade drug?

Clinical studies on chondroitin sulfate efficacy have been evaluated using a very pure product having specific properties and physico-chemical characteristics, as approved by the various national health institutes[19,20] (mentioned above). For example, to assess the long-term combined symptom- and structure-modifying effects of chondroitin sulfate, a highly purified preparation with a standardized structure, not less than 95%, produced from bovine cartilage was used.[25]

It is well known that specific activity depends on the chondroitin sulfate structure and properties.[2–18] Additionally, chondroitin is administered orally during therapy and bioavailability and pharmacokinetic parameters have been reported to change depending on its structural characteristics and origin.[38–40] As a consequence, chondroitin sulfate with a low quality of content and properties, generally present in nutraceuticals, would be unable to exert comparable pharmacological effects to those of the pharmaceutical-grade chondroitin sulfate, and the same clinical effects would not be produced unless various chondroitin sulfate preparations have a similar structure. Finally, clinical trials are required and should be performed to demonstrate efficacy of nutraceuticals.

Conclusions

From published studies available in the literature,[22,23,35–37] the chondroitin sulfate quality (content and standardization of its properties) of several nutraceuticals has been found to be poor. As a consequence, stricter regulations regarding quality control should be introduced to guarantee the manufacture of high quality products for nutraceutical utilization and to protect customers from low-quality, ineffective and potentially dangerous products. Furthermore, specific and accurate analytical procedures should be enforced for the control of high-quality products and applied by quality control laboratories to confirm the purity and label claims of chondroitin sulfate in raw materials and nutraceuticals. Additionally, there are no definite regulations governing the origin of the ingredients in these nutraceuticals and yet the origin of the ingredients in natural products is the most important factor ensuring quality, and thus safety and efficacy. In fact, due to the extractive origin of chondroitin sulfate from several animal sources, there is a possible risk of specific species diseases, such as bovine spongiform encephalopathy.

Figure 5  Agarose-gel electrophoresis of chondroitin sulfate in several European, Czech Republic, nutraceutical samples (A–L). Chondroitin sulfate was separated on an agarose-gel having a thickness of about 4–5 mm and, after migration, the plate was stained with toluidine blue. The migration of the European Standard chondroitin sulfate reference standard (St) is also illustrated. Modified from Volpi and Maccari.[23]
(BSE), foot-and-mouth disease, influenza spread in birds, and other animal diseases. This risk is practically absent in pharmaceutical-grade chondroitin sulfate thanks to the choice of selected raw material and to specific chemical treatment capable of inactivating prion agents\textsuperscript{41,42} as established and controlled by the various national health institutes. In the case of chondroitin sulfate in nutraceuticals, no indication of the origin is given on the label.

Stricter regulations for the production of chondroitin sulfate and evaluation of its properties are necessary right now. As a consequence, until stricter regulations regarding quality control are available, pharmaceutical-grade chondroitin sulfate formulations approved by local national health institutes are strongly recommended instead of chondroitin sulfate contained in general food supplements.

Declarations
Conflict of interest
The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding
This review received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.
Various unsaturated disulfated disaccharides are also produced by the action of chondroitinase ABC. J Biol Chem 1994; 269: 3957–3962.

Figure 7 Strong-anion exchange-HPLC separation of the disaccharides from the chondroitin sulfate samples of two European food supplements (Sample E and G) produced by the action of chondroitinase ABC. Sample E: ΔDi-0s, ΔHexA-GalNAc; ΔDi-4s, ΔHexA-GalNAc (6-OSO3); ΔDi-2,6dis, ΔHexA-GalNAc (4-OSO3). Various unsaturated disulfated disaccharides are also separated in sample E. Modified from Volpi and Maccari.[23]

References


42. Note for Guidance on Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products. EMEA/410/01.