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Capillary electrophoresis for the quality control of chondroitin sulfates in raw materials and formulations

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Abstract

Exogenous administration of chondroitin sulfate (CS) is widely practiced for the treatment of osteoarthritis, although the efficacy of this treatment has not been completely established by clinical studies. A reason for the inconsistency of the results may be the quality of the CS preparations, which are commercially available as dietary supplements. In this article, we describe the development of a new method of capillary electrophoresis (CE) for the quantification of CS concentrations, screening for other glycosaminoglycan or DNA impurities and determination of hyaluronan impurities in CS raw materials, tablets, hard capsules, and liquid formulations. Analysis is performed within 12 min in bare fused silica capillaries using reversed polarity and an operating phosphate buffer of low pH. The method has high sensitivity (lower limit of quantitation [LLOQ] values of 30.0 μg/ml for CS and 5.0 μg/ml for hyaluronan), high precision, and accuracy. Analysis of 11 commercially available products showed the presence of hyaluronan impurities in most of them (up to 1.5%). CE analysis of the samples after treatment with chondroitinase ABC and ACII, which depolymerize the chains to unsaturated disaccharides, with a previously described method (Karamanos et al., J. Chromatogr. A 696 (1995) 295–305) confirmed the results of hyaluronan determination and showed that the structural characteristics (i.e., disaccharide composition) of CS are very different, showing the different species or tissue origin and possibly affecting the therapeutic outcome.

Keywords: Chondroitin sulfate; Nutritional supplements; Analysis; Capillary electrophoresis; Hyaluronan impurities

Chondroitin sulfate (CS)[1] is an anionic glycosaminoglycan (GAG) consisting of repeating disaccharide units of [→4GlcAβ1→3GalNAcβ1→]. CS in mammals is commonly sulfated at the C-4 (CS-A) and/or C-6 (CS-C) of GalNAc [1]. Further modifications of GlcA (C-5 epimerization to iduronic acid and, less often, O-sulfation at C-2 of iduronic acid) lead to the formation of the dermatan sulfate (DS) chain (formerly known as CS-B), which has

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[1] *Abbreviations used:* CS, chondroitin sulfate; GAG, glycosaminoglycan; DS, dermatan sulfate; HA, hyaluronic acid; HPLC, high-performance liquid chromatography; FT–IR, Fourier transform–infrared; CE, capillary electrophoresis; ESI–MS, electrospray ionization–mass spectrometry; Δdi-di(2,6)S, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose; Δdi-di(4,6)S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose; Δdi-di(2,4)S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-4-*O*-sulfo-D-galactose; Δdi-mono6S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-4-*O*-sulfo-D-galactose; Δdi-mono6S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose; Δdi-nonScs, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose; Δdi-nonS_{HA}, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose; LLOQ, lower limit of quantitation; LOD, limit of detection; RSD, relative standard deviation; DMMB, dimethylmethylene blue; HARE, HA receptor for endocytosis.

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a hybrid copolymeric structure consisting of low modified (CS) and highly modified domains [2,3]. CS is covalently linked to proteins forming proteoglycans, which are present mainly in the extracellular matrix of cartilage and other connective tissues (e.g., cartilage, skin, tendon, bone, aorta). Structural studies of CS chains from vertebrate and especially mammalian species have shown the great heterogeneity of structural organization of the repeating disaccharide region [1,3]. Mounting evidence shows that the structure of CS depends on the animal species, the tissue, the physiological or pathological stimuli, and the proteoglycan source [4–7].

During recent years, there has been a rise of pharmaceutical formulations and dietary supplements on the market containing CS manufactured from various animal sources (shark/bovine/avian cartilage or bovine trachea). Supplements of CS are used mainly for improving symptoms and arresting or reversing the degenerative process occurring in the development of osteoarthrosis [8,9]. Besides, CS sometimes is used for ophthalmologic diseases, and there is preliminary evidence that CS may help in the treatment of psoriasis [10]. The effectiveness of oral administration of CS, however, is still questioned, due in part to doubts concerning the absorption/metabolism of such a macromolecule. Pharmacokinetic studies in experimental animals have shown that administration of highly modified CS (i.e., DS or highly sulfated CS) results in negligible absorption and a dependency of pharmacokinetic characteristics on molecular mass [11–14]. Furthermore, results of clinical trials have shown that the outcome of treatment is directly related to the quality of CS supplements used [8]. Indeed, it has been shown that many of the dietary supplements marketed might not provide high-quality material or meet labeled quantities because the requirements for quality manufacturing are not as strict as those for pharmaceuticals [15–17]. In a study of CS content, only 5 of 32 products contained the labeled amount of CS within the acceptable 10% variation, whereas 17 of 32 contained less than 40% of the label claim [15]. Furthermore, because CS is extracted from animal tissues, skepticism toward these products arises from the fact that impurities may cause allergic or other side effects or may transmit epidemics such as bovine spongiform encephalopathy and the avian flu. The presence of impurities such as hyaluronic acid (HA), another GAG commonly found in cartilage not efficiently removed during the extraction process, has been shown in variable amounts in dietary supplements [18].

Analytical methods for the determination of the CS content, the presence of other GAG or non-GAG impurities, and the disaccharide composition and origin of the raw material are necessary for the quality control of both the nutraceutical and pharmaceutical raw materials and the marketed products. The methods used so far for the analysis of the CS content involve the colorimetric measurement of uronic acid [19], photometric titration with cetylpyridinium chloride [20], dye binding assays, high-performance size exclusion chromatography [21], high-performance liquid chromatography (HPLC) on C18 columns and detection at 195 nm [22], and agarose gel electrophoresis [23]. However, these methods do not provide an acceptable specificity for CS, and most of them determine total GAGs. More specific methods involve Fourier transform–infrared (FT–IR) using a chemometric pattern of recognition [24]; solid phase assays [25]; the combined use of specific bacterial lyases (i.e., chondroitinase ABC), which depolymerize the CS chain to unsaturated disaccharides (these absorb strongly at 232 nm), and/or derivatization with fluorescent molecules; and separation with HPLC [1,16,26], gel electrophoresis [27,28], or capillary electrophoresis (CE) [14,29,30]. Further miniaturization was accomplished with capillary electrophoretic determination using in-capillary enzymic conversion of CS to nonsulfated disaccharides [31]; however, the disaccharide composition could not be determined. Some of the enzymes (e.g., chondroitinase ABC) that depolymerize CS also depolymerize HA; thus, the unsaturated HA disaccharide has been used as a measure of HA amounts [1,29,32]. Saad and coworkers [18] developed a procedure for the determination of HA impurities in CS preparations involving treatment with specific hyaluronidase, centrifugal filtration for the removal of undigested CS, and electrospray ionization–mass spectrometry (ESI–MS) of the HA disaccharides. All of these methods are rather laborious and expensive for routine analysis of CS content in the CS materials and preparations.

In this article, we present a simple strategy for quality control of CS raw materials and formulations using CE. More specifically, we developed a CE method for analysis of the preparations in a low-phosphate buffer, reversed polarity and detection at 200 nm directly after their dissolvement (e.g., powders, tablets, hard capsules) or dilution in H_2O. This method was completely validated and can be used for the quantitation of CS content with specificity, accuracy, and precision; identification of other GAG or DNA impurities; and determination of HA impurities, which our random screening of 11 commercially available preparations showed to be common. Furthermore, our results showed that this method can give an estimation of the charge density of CS. More precise data on the charge density and on the origin of the CS polysaccharides can be achieved if the preparation is treated with chondroitinase ABC and AC II (a 3-h procedure) and the previously developed [29] CE method for the analysis of all differently sulfated CS-derived disaccharides is performed in parallel.

Materials and methods

Materials

CS from shark cartilage, CS-B (DS) from porcine intestinal mucosa, heparan sulfate from bovine intestinal mucosa, and keratan sulfate from bovine cornea were obtained from Sigma–Aldrich (Steinheim, Germany). Low-molecular-weight heparin (Innohep) was supplied by Pharmion (Boulder, CO, USA). Avian CS (average molec-