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Title: Validated High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection Method for the Determination of residual Keratan Sulfate and other glucosamine impurities in Sodium Chondroitin Sulfate

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1 **Validated High-Performance Anion-Exchange Chromatography with Pulsed Amperometric**  
2 **Detection Method for the Determination of residual Keratan Sulfate and other glucosamine**  
3 **impurities in Sodium Chondroitin Sulfate**

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12 Abbreviations: CS(s), sodium chondroitin sulfate(s); Eur, European; EP, European Pharmacopoeia;  
13 GAG(s), glycosaminoglycan(s); Gal, galactose; GalN, Galactosamine; GalNAc, N-acetylgalactosamine;  
14 GlcA, glucuronic acid; GlcN, Glucosamine; GlcNAc, N-acetylglucosamine; HPAEC-PAD, high-  
15 performance anion exchange chromatography with pulsed amperometric detection; KS, keratan sulfate;  
16 Mw, Molecular Weight; NSC, Chondroitin non sulfated; USP, United States Pharmacopoeia.

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18 **Abstract:** An efficient and sensitive analytical method based on high-performance anion exchange  
19 chromatography with pulsed amperometric detection (HPAEC-PAD) was devised for the determination of  
20 glucosamine (GlcN) in Sodium Chondroitin Sulfate (CS). Glucosamine (GlcN) is intended as marker of  
21 residual Keratan Sulfate (KS) and other impurities generating glucosamine by acidic hydrolyzation. The  
22 latter brings CS and KS to their respective monomers. Since GlcN is present only in KS we developed a  
23 method that separates GlcN from GalN, the principal hydrolytic product of CS, and then we validated it in  
24 order to quantify GlcN. Method validation was performed by spiking CS raw material with known amounts  
25 of KS. Detection limits was 0.5% of KS in CS (corresponding to 0.1 µg/ml), and the linear range was 0.5%  
26 to 5% of KS in CS (corresponding to 0.1µg/ml-1 µg/ml). The optimized analysis was carried out on an ICS-  
27 5000 system (Dionex, Sunnyvale, CA, USA) equipped with a Dionex Amino Trap guard column (3 x 30  
28 mm), Dionex CarboPac-PA20 (3 x 30 mm) and a Dionex CarboPac-PA20 analytical column (3 x 150 mm)  
29 using gradient elution at a 0.5 mL/min flow rate. Regression equations revealed good linear relationship ( $R^2$   
30 = 0.99, n = 5) within the test ranges. Quality parameters, including precision and accuracy, were fully  
31 validated and found to be satisfactory. The fully validated HPAEC-PAD method was readily applied for the  
32 quantification of residual KS in CS in several raw materials and USP/EP reference substance. Results  
33 confirmed that the HPAEC-PAD method is more specific than the electrophoretic method for related  
34 substance reported in EP and provides sensitive determination of KS in acid-hydrolyzed CS samples,  
35 enabling the quantitation of KS and other impurities (generating glucosamine) in CS.

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37 **Keywords:** HPAEC-PAD; Method Validation; Keratan Sulfate; Chondroitin Sulfate

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41 their profitable suggestions and for the fine purification of keratan sulfate reference substance.

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43 **1. Introduction**

44 Sodium chondroitin sulfate (CS) is a glycosaminoglycan (GAG) composed of disaccharide units containing  
45 N-acetylgalactosamine (GalNAc) and a glucuronic acid joined by (1•4) [1]; [2]. Inadequate purification  
46 process can lead to residual presence of other GAGs. Keratan sulfate is one of the most common residual  
47 GAG likely to be found in large-scale production of chondroitin sulfates by raw purification procedures [3].  
48 The KS can be selectively distinguished from CS owing to its disaccharide unit containing GlcNAc. The  
49 HPAEC-PAD method that we developed and validated, provides sensitive and accurate determination of  
50 glucosamine in acid-hydrolyzed sodium CS samples, enabling the identification of CS that has been  
51 contaminated with keratan sulfate (KS) and other glucosamine compounds. Furthermore, as already  
52 observed in literature [4]; [5], a more specific method for determination of residual KS is needed. We  
53 demonstrated that this method for related substance is more specific and accurate than the one reported in  
54 both USP [6] and EP [7] monographs. Briefly, after acidic hydrolyzation, CS is converted to its monomers  
55 Galactosamine and Glucuronic Acid, while KS is reduced to Galactose and Glucosamine. During method  
56 development we demonstrated (data not shown) that Galactose and Glucuronic Acid degrade during

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