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Determination of dermatan sulfate and chondroitin sulfate as related substances in heparin by capillary electrophoresis

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ABSTRACT

Capillary electrophoresis (CE) was applied to the quantitation of dermatan sulfate (DS) and chondroitin sulfate (CS) as related substances in sodium heparin. The method is based on the selective digestion of either CS and DS contained in the main drug heparin, by using chondroitinase ABC (specific for both DS and CS) and chondroitinase AC (specific for only CS). The unsaturated disaccharides released after exhaustive digestion, can be separated by CE using a 110 mM phosphate buffer, pH 3.5 as the background electrolyte in a fused silica capillary (64.5 cm × 50 μm i.d.) at 40 °C and −30 kV. Since the level of each disaccharide released upon enzymatic digestion corresponds to its content in the native glycosaminoglycan, the amount of CS and DS was determined by proportion with the released disaccharides. Inparticular, ΔUA → GalNAc-4S Na_2 and ΔUA → GalNAc-6S Na_2 were selected for quantitation of CS and DS because of their significant response and short migration time (less than 7 min).The method was validated for linearity, accuracy, precision and it showed to be able in detecting selectively, DS and CS at impurity level (LOD 0.01%, w/w). The proposed CE approach was finally applied to real samples. The results obtained were found in excellent correlation with those achieved by the analysis of the same samples using the official USP method based on high performance anion exchange chromatography (HPAEC) with pulsed amperometric detector.

1. Introduction

Heparin, the widespread anticoagulant, has recently been the focus of attention of health authorities because of the significant increase of reports of serious adverse effects (allergic-type reaction). The cause has been correlated with the presence of oversulfated chondroitin sulfate (OSCS) as an impurity [1,2]. Starting from early 2008, the US Food and Drug Administration (FDA) and successively the United States Pharmacopeia (USP), devoted an intensive effort aiming to ensure the worldwide safe heparin supply. In particular official test methods based on nuclear magnetic resonance (NMR) and capillary electrophoresis (CE) were recommended for OSCS analysis in heparin [3,4]. The CE approach consists in a simple capillary zone separation (CZE) and allows the detection of the impurity OSCS in the presence of unfractionated heparin. The method is of practical application, but the reported limit of detection of the impurity is at the level of 1–5% (w/w) with respect to heparin because of partial co-migration of OSCS and heparin [5]. Furthermore the method did not consider the presence of other glycosaminoglycans (GAGs) such as dermatan sulfate (DS) and chondroitin sulfate (CS) as additional heparin impurities. Although the latter substances can be reasonably considered as common and safe components of therapeutic heparins, the restrictions recently adopted by many authorities have led to regard DS and CS as contaminants as well as OSCS. In particular, the European Directorate for the Quality of Medicine and HealthCare (EDQM), modified the word "*contaminant*" in "natural contaminants" or "related substances" and recommended a limit and specific tests for DS. Accordingly, in the Stage 2 revision, USP recommended to lower the limit of DS in therapeutic heparin to ≤1% and the method proposed for the analysis is based on high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection [6]. Undoubtedly, according to the current literature, CE plays an important role in analysis of GAGs. These biopolymers show favourable electrophoretic behaviour because of their high anionic charge [7]. Using acidic background electrolytes (BGEs) the EOF is strongly suppressed and the GAGs can be separated by reversed polarity. Patel et al., showed that low molecular

Abbreviations: BGE, background electrolyte; CS, chondroitin sulfate; CZE, capillary zone electrophoresis; DS, dermatan sulfate; EDQM, European Directorate for the Quality of Medicine and HealthCare; EMA, European Medicines Agency; EOF, electroosmotic flow; FDA, US Food and Drug Administration; GAG, glycosaminoglycan; HPAEC, high performance anion exchange chromatography; NMR, nuclear magnetic resonance; OSCS, oversulfated chondroitin sulfate; USP, United States Pharmacopeia.

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weight (LMWH) and unfractionated heparins can be quantified by CE using a 50 mM phosphate buffer (pH 3.5) [3]. Wielgos et al., improved the early USP CE method for the analysis of OSCS so much that its complete separation from heparin was achieved [8]. Under similar conditions, the quantitation of OSCS and, additionally, of DS was accomplished, in the presence of heparin, with LOQs of 0.05% and 0.5% (w/w), respectively [9]. These CE applications showed good analytical performance, however acceptable sensitivity was achieved only by injecting highly concentrated samples (50 mg/mL of heparin), which needed to be stacked using high molarity buffers (typically 850 mM). Narrow capillaries (internal diameter of 25 μm) were then necessary to reduce Joule heating. Under the described extreme conditions and mainly because of the polydispersity of the GAGs of interest, the corresponding electrophoretic peaks were broad and the critical separation of DS from CS was not considered.

In the present study, a novel CE approach is proposed for the selective quantification at impurity levels of CS and DS as related substances in heparin. The method is based on the specific digestion of either CS and DS in the presence of heparin, by using appropriate lyases, namely, chondroitinase ABC (specific for both DS and CS) and AC (specific for only CS). After exhaustive lyase digestion, the different species of GAGs generate unsaturated disaccharides, depending on their characteristic structure [10]. The analysis of the GAGs-derived unsaturated disaccharides can be performed by simple CZE because of their ionic character and relatively good UV absorbance [11–13].

The analysis of GAGs-derived disaccharides has been described in several studies dealing with structural elucidation and sequencing of the heparinoids [14–17], however there are no reports on its application in quantitation of DS and CS as heparin related substances. The proposed method was validated and applied to the analysis of real samples; the obtained results were finally compared with those obtained by the official "Limit of Galactosamine in Total Hexosamine", method recently proposed by the USP [6].

2. Materials and methods

2.1. Instrumentation

2.1.1. Capillary electrophoresis

The equipment for CE analysis consisted of a HP^{3D}CE Capillary Electrophoresis (Agilent Technologies, Waldbronn, Germany). The electrophoretic runs were carried out using extended path fused-silica capillaries by Agilent Technologies (total length 64.5 cm, effective length 56 cm, internal diameter 50 μm). The optimised separation conditions were the following: capillary temperature was constant at 40 °C; the voltage was set at −30 kV; the detection wavelength was 200 nm. The samples were injected hydrodynamically at 50 mbar for 10 s after a conditioning program consisting of a washing step with water (2 min) and an equilibration step with the BGE (2 min). New capillaries were activated by flushing in the order, 1 N sodium hydroxide, 0.1 N sodium hydroxide and water (each for 10 min). In order to obtain high reproducibility of migration time, the buffer reservoirs were changed every two runs.

2.1.2. High performance anion exchange chromatography

HPAEC analysis was carried out following the conditions reported by USP as "Limit of Galactosamine in Total Hexosamine" method [6]. In particular the HPAEC system was a DX-600 consisting of a GP50 gradient pump, equipped with ED50 electrochemical detector with Amperometry Cell and disposable gold working electrode; the reference electrode was Ag/AgCl. The system included the AS50 Autosampler. The separations were performed on a CarboPac PA20 analytical column (3 mm × 150 mm), a strong anion-exchange column providing high-resolution separation of underivatized mono- and disaccharides. A CarboPac PA20 guard column (3 mm × 30 mm) was also installed in front of the analytical column. All these equipments were from Dionex (Sunnyvale, CA, USA).

The HPAEC analysis was carried out by gradient elution under the following conditions: NaOH 14 mM (from 0 to 10 min); NaOH 100 mM (from 10 to 20 min); NaOH 14 mM (from 20 to 30 min). The flow rate was 0.5 mL/min and the separations were performed at the constant temperature of 30 °C. The injection volume was 10 μL. Pulsed amperometric detection was set to four waveform at the potential of +0.1, −2.0, +0.6 and −0.1 V according to the USP method [6].

2.2. Materials

Eight standard disaccharides (sodium salts) were from Dextra (Reading, UK): ΔUA → GalNAc Na (ΔDi-0S); ΔUA → GalNAc-4S Na_2 (ΔDi-4S); ΔUA → GalNAc-6S Na_2 (ΔDi-6S); ΔUA → GalNAc-4S, 6S Na_3 (ΔDi-diS_E); ΔUA-2S → GalNAc-4S Na_2 (ΔDi-diS_B); ΔUA-2S → GalNAc-6S Na_3 (ΔDi-diS_D); ΔUA-2S → GalNAc-4S-6S Na_4 (ΔDi-triS); ΔUA-2S → GalNAc Na_2 (ΔDi-UA2S). Heparin, dermatan sulfate (DS) and chondroitin sulfate (CS) were from Opocrin S.p.A. (Corlo di Formigine, Modena, Italy). Oversulfated chondroitin sulfate (OSCS) was purchased from USP. Sodium phosphate monobasic dihydrate, phosphoric acid, 5-sulfosalycilic acid (used as internal standard), chondroitinase AC (PN C8618) and chondroitinase ABC (PN C2905) were from Sigma–Aldrich (Milan, Italy). Tris(hydroxymethyl)aminomethane (Tris) and hydrochloric acid were from Carlo Erba Reagents (Milan, Italy).

The water used for all the preparations was deionized by MilliQ apparatus (Millipore, MA, USA). The samples and buffer solutions were filtered by 0.45 μm Phenex filters (Phenomenex, Castelmaggiore, Bologna, Italy), before the CE analysis.

2.3. Procedure

2.3.1. Background electrolyte preparation

The BGE was prepared by dissolving in MilliQ water the appropriate amount of sodium phosphate monobasic dihydrate; the pH was then adjusted to 3.5 by addition of 0.1 M phosphoric acid. In optimisation of CE separation conditions, phosphate electrolytes of different concentrations and pH values were prepared by adjustment of acidity using phosphoric acid or sodium hydroxyde (0.1 M).

2.3.2. Calibration with standard disaccharides

Individual stock solutions of disaccharides were prepared at concentrations ranging from 0.015 to 5.0 mg/mL for disaccharides ΔDi-0S, ΔDi-4S and ΔDi-6S and from 0.015 to 1.0 mg/mL for disaccharides ΔDi-diS_E, ΔDi-diS_B, ΔDi-diS_D, ΔDi-triS and ΔDi-UA2S. 5-Sulfosalycilic acid was used as internal standard at the final concentration of 0.025 mg/mL. The analysis was performed in triplicate under the optimised CE conditions. The peak area ratios (peak area of each disaccharide to that of the internal standard) were plotted versus the concentration (mg/mL) of the analytes to obtain the calibration curves.

2.3.3. Digestion with chondroitinases

Stock solutions of a heparin sample with a very low content of "related substances" (heparin reference solution, 120 mg/mL) and of DS and CS (50 mg/mL) were prepared by dissolution with Tris–HCl buffer 50 mM, pH 7.9. Mixtures of these solutions were used as reference samples for the preparation of the calibration curves of Section 2.3.4.

Heparin samples (125 mg/mL) and both chondroitinases (about 2.5 U/mL each) were dissolved with the same buffer.

Samples and reference solutions were incubated for the digestion in reaction vials for 4 h at 37 °C: each vial contained 10 mg of GAG mixture and about 0.125 units of chondroitinase. Digestion was stopped by immersion for 2 min in a boiling bath.

Digested samples were moved to Eppendorf tubes and centrifuged for 2 min at 12,000 rpm in a microcentrifuge. The scheme of digestion of DS and CS by chondroitinases is reported in Fig. 1 together with the structure of the released unsaturated disaccharides.

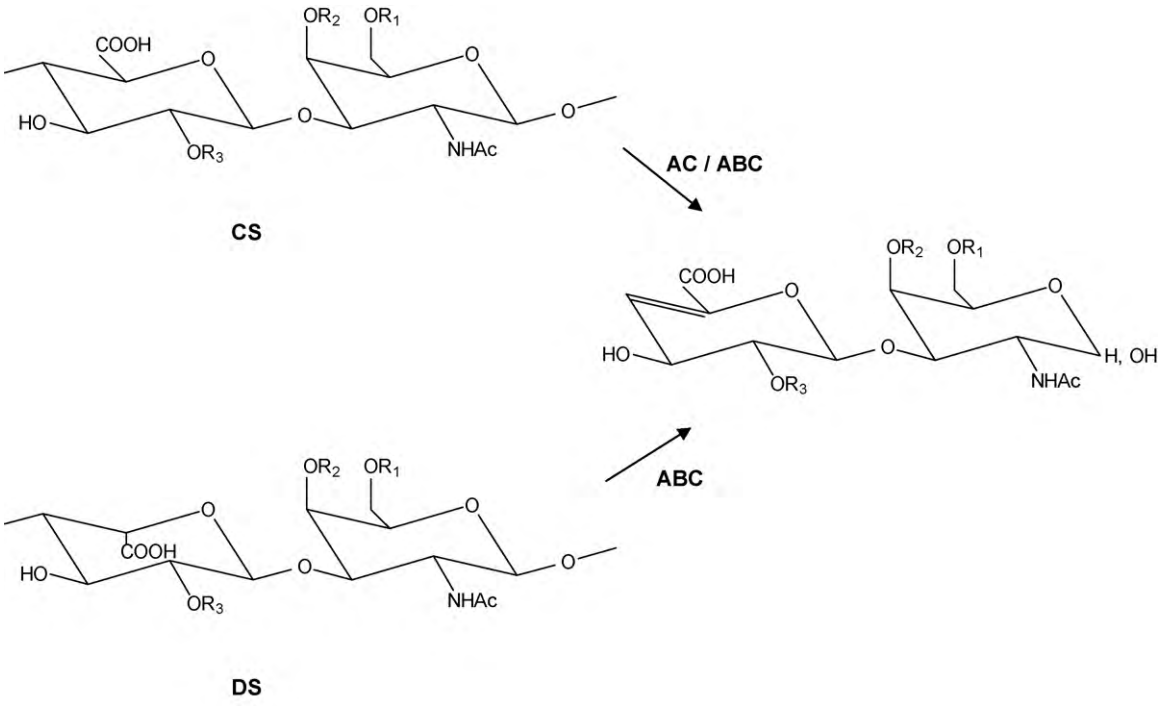
2.3.4. Calibration curves of disaccharides after chondroitinase digestion of DS and CS

The heparin reference stock solution was spiked with DS and CS stock solutions in order to obtain seven calibration points containing a fixed level of heparin (50 mg/mL) as the main drug, in the presence of DS and CS each ranging from 0.015 mg/mL to 1.0 mg/mL (corresponding to the range 0.03–2.0%, w/w, with respect to heparin). Blank samples (containing only heparin) were also prepared. All samples were digested under the conditions described above (Section 2.3.3) using either chondroitinase AC and chondroitinase ABC. After the digestion, the sample mixtures were diluted with water (1-to-10 fold) in the presence of internal standard at the concentration of 0.025 mg/mL and subjected to CE separation for the analysis of the released disaccharides. The peak area ratios (disaccharide-to-internal standard) referred to the two specific disaccharide ΔDi-4S and ΔDi-6S, released by chondroitinase AC digestion, were plotted versus the percentage of CS with respect to heparin content in the original mixtures. Similarly, the peak area ratios of ΔDi-4S and ΔDi-6S, released by chondroitinase ABC digestion, were plotted versus the sum of the percentages of DS and CS with respect to the heparin content.

2.3.5. Accuracy and precision

Accuracy was assessed on heparin samples (50 mg/mL) spiked with known amounts of DS and CS at the following levels: QC1 (1.5% DS + 0.5% CS); QC2 (0.75% DS + 0.25% CS) and QC3 (0.375% DS + 0.125% CS). All samples were digested using chondroitinases, as described above and analysed by CE after dilution and addition of internal standard.

The percentage of CS with respect to heparin content in QC samples was calculated by means of the calibration curve by considering the peak area ratios of disaccharides ΔDi-4S and ΔDi-6S, released after digestion using chondroitinase AC. Similarly, the percentage of CS plus DS with respect to heparin content in QC samples was calculated by means of the calibration curve by considering the peak area ratios of ΔDi-4S and ΔDi-6S, released after chondroitinase ABC digestion.



	R_1	R_2	R_3
ΔDi-0S	H	H	H
ΔDi-4S	H	SO_3^-	H
ΔDi-6S	SO_3^-	H	H
ΔDi-UA2S	H	H	SO_3^-
ΔDi-diS_B	H	SO_3^-	SO_3^-
ΔDi-diS_D	SO_3^-	H	SO_3^-
ΔDi-diS_E	SO_3^-	SO_3^-	H
ΔDi-triS	SO_3^-	SO_3^-	SO_3^-

Fig. 1. Scheme of the enzymatic digestion of chondroitin sulfate (CS) and dermatan sulfate (DS) to the related Δ-disaccharides by using chondroitinase AC (AC) and chondrotinase ABC (ABC).

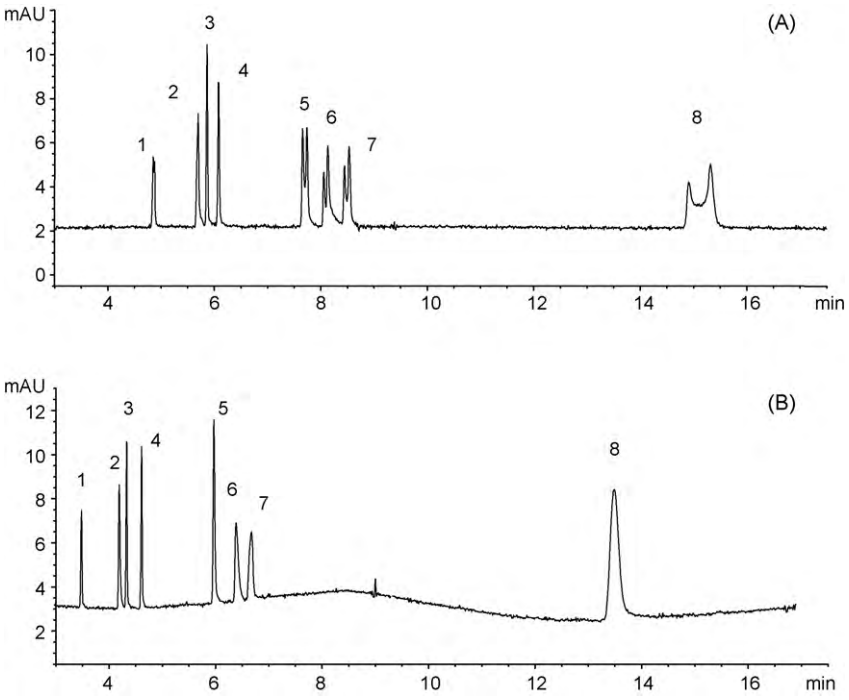


Fig. 2. Electropherograms of a standard mixture (concentration 50 μg/mL) of unsaturated disaccharides. CE conditions. (A) Fused-silica capillary (total length 64.5 cm; effective length 56 cm); i.d. 50 μm (extended path). BGE: sodium phosphate 50 mM, pH 3.50. Temperature: 25 °C; voltage: −30 kV. Injection: hydrodynamic at 50 mbar × 10 s. Detection: 200 nm. Labels: (1) ΔDi-trisS; (2) ΔDi-dis$_D$; (3) ΔDi-dis$_B$; (4) ΔDi-dis$_E$; (5) ΔDi-UA2S; (6) ΔDi-6S; (7) ΔDi-4S; (8) ΔDi-0S. (B) BGE: sodium phosphate 110 mM, pH 3.50. Temperature 40 °C. Others conditions and symbols as in caption (A).

2.3.6. Analysis of real samples

Sodium heparin samples from Opocrin S.p.A. were prepared at the concentration of 50 mg/mL and each of them underwent to the digestion with chondroitinase AC for the analysis of CS content and chondroitinase ABC for the analysis of both DS and CS content. Furthermore, blank samples (containing only heparin) were subjected to the same digestion conditions. The quantitation was performed by means of the calibration curve by considering the peak area ratio of ΔDi-4S and ΔDi-6S.

The determination of the content of DS and CS in real heparin samples was carried out also by applying the HPAEC method reported in the new heparin sodium monograph of USP [6].

3. Results and discussion

In the present study the quantitation of CS and DS as related substances in sodium heparin has been approached by means of the analysis of specific unsaturated disaccharides released after lyase digestion of the GAGs samples. As the first step of the investigation, the optimisation of CE separation of disaccharides derived from DS and CS was performed.

3.1. Optimisation of CE separation of GAGs-derived disaccharides

Capillary zone electrophoresis of GAGs-derived disaccharides can be favourably performed under reversed polarity conditions because of the high anionic character of the analytes. In this approach a low pH BGE is used to achieve a strong suppression of the EOF; this condition allows for the fast migration of anionic species toward the cathodic detection end [11–15]. Sodium orthophosphate buffer at pH 3.0 was reported as useful in the separation of a series of unsaturated disaccharides [18]. Ruiz-Calero et al. used volatile acidic buffers (formic acid–ammonium hydroxide) to achieve compatibility of the CE separation medium with MS detection [15] in analysis of heparin related disaccharides. In our experiments, being the UV detection chosen for CE analysis, a 50 mM phosphate buffer at pH 3.5 was initially used. As it can be seen in Fig. 2A, the complete separation of the eight considered standard disaccharides (molecular structures reported in Fig. 1) was obtained in about 15 min. However the peaks related to ΔDi-UA2S, ΔDi-6S, ΔDi-4S and ΔDi-0S resulted to be split and this situation, reported also in previous studies [18], was explained as the partial resolution of anomeric forms of the disaccharides. The observed peak split could be detrimental to the separation of complex mixtures, thus the initial CE conditions were modified in order to obtain single analytical peaks. A step-by-step optimisation of the separation conditions was approached by starting from the initially used 50 mM phosphate buffer at 25 °C. The pH value was varied within the range 2.5–4.5; at pH higher than 3.5, the co-migration of the anomers (single peaks for each of the studied compounds) was achieved but the separation of the couples ΔDi-dis$_D$/ΔDi-dis$_B$ and ΔDi-6S/ΔDi-4S was lost. On the other hand, at pH lower than 3.5, the peak split of ΔDi-UA2S, ΔDi-6S, ΔDi-4S and ΔDi-0S, increased; thus further experiments were carried out at pH 3.5. As expected, increased temperature improved the peak shape and by setting the separation temperature at 40 °C, single analytical peaks were obtained for all of the compounds with the exception of ΔDi-4S. Higher temperature was found to be detrimental for the separation of the couple ΔDi-dis$_D$/ΔDi-dis$_B$, thus further experiments were carried out at 40 °C.

The BGE concentration was found to affect the peak shape of the studied disaccharides; in particular, increasing concentrations of phosphate buffer inhibited the separation of the anomeric forms. Actually, starting from phosphate 50 mM (pH 3.5), the concentration was progressively increased with a general improvement of the peaks shape. Using 110 mM phosphate buffer (pH 3.5) in a fused silica capillary (64.5 cm of total length) at constant temperature of 40 °C and voltage of −30 kV (Fig. 2B) the separation of the studied analytes was considered satisfactory for the relatively short analysis time, good peaks shape and baseline resolutions.

Table 1
Linearity data[a] in CE analysis of unsaturated disaccharides.

Disaccharide	Calibration range (mg/mL)	Linear regression $Y = ax + b$	r^2
ΔDi-0S	0.015–5.00	$Y = 8.291x + 0.299$	0.998
ΔDi-4S	0.015–5.00	$Y = 8.161x - 0.270$	0.999
ΔDi-6S	0.015–5.00	$Y = 8.456x - 0.249$	0.998
ΔDi-dis_E	0.015–1.00	$Y = 8.434x - 0.112$	0.998
ΔDi-dis_B	0.015–1.00	$Y = 8.927x - 0.118$	0.998
ΔDi-dis_D	0.015–1.00	$Y = 8.728x - 0.0960$	0.999
ΔDi-triS	0.015–1.00	$Y = 6.489x - 0.0830$	0.998
ΔDi-UA2S	0.015–1.00	$Y = 11.205x - 0.153$	0.998

[a] Regression curve data for five calibration points, $Y = ax + b$, where Y is the relative peak area of analyte to internal standard, x is the analyte concentration, a is the slope, b is the intercept and r^2 is the correlation coefficient.

3.2. Reproducibility, linearity and sensitivity in analysis of disaccharides

The analytical performances of the CE separation were tested by means of a preliminary validation of reproducibility of migration time and linearity of the UV response.

Repeated injections of a standard mixture of the eight analysed unsaturated disaccharides (50 μg/mL each) allowed the reproducibility of migration time to be evaluated. The RSD intraday ($n = 3$) was less than 2.0% and the RSD on 3 consecutive days ($n = 9$) was less than 3.5%. The reported values suggest the high reproducibility of the CE separation and the opportunity for the unambiguous identification of the disaccharides by comparison of the migration time.

The linearity of the response to the detector (at 200 nm) was evaluated for each of the disaccharides in the presence of 5-sulfosalicylic acid as the internal standard. In Table 1 are reported the parameters of the calibration graphs obtained by linear regression analysis of the peak area ratio (peak area of the analytes versus the peak area of the internal standard) against the concentration (mg/mL) of the analytes. The sensitivity data LOQ and LOD were obtained by diluting standard solutions till a signal-to-noise ratio of 10:1 and 3:1, respectively, was achieved. Although it is well known that unsaturated disaccharides show good UV absorption at 232 nm, the wavelength of 200 nm was preferred for all the measurements in order to achieve higher sensitivity. In particular, under the described conditions, LOD and LOQ were found to be 3 μg/mL and 8 μg/mL, respectively.

3.3. CE analysis of disaccharides after lyase digestion

The polysaccharides CS and DS are constituted of repeating disaccharides units consisting in the hexosamine *N*-acetyl-D-galactosamine (GalNAc), common to both CS and DS, and one uronic acid as D-glucuronic acid (GlcA) or L-iduronic acid (IdoA). The essential difference between CS and DS is the nature of the uronic acid, being IdoA present only in DS [19]. Chondroitin lyase are eliminases converting the terminal uronic acid residue of the GAGs to Δ4,5-unsaturated uronic acid. In particular, chondroitinase ABC cleaves the glycosidic linkages between D-glucuronic acid (GlcA) or L-iduronic acid (IdoA) and D-galactosamine (GalN) either in CS and DS. Differently, chondroitinase AC degrades only the linkages of GlcA thus only CS can be depolymerised (Fig. 1). Since the repeating disaccharide units in heparin are distinctly different from those of CS and DS, it remains essentially intact upon digestion using both chondroitinases ABC and AC [10,20,21].

Our interest was firstly focused on the optimisation of the digestion conditions providing the enzymatic depolymerization of DS and CS. In particular two different levels (0.625 U/mL and 1.25 U/mL) of chondroitinase ABC, were incubated with a solution of DS at 5.0 mg/mL. The same experiment was carried out using chondroitinase AC (0.625 U/mL and 1.25 U/mL) incubated with CS. The mixtures, after proper dilution with water were then subjected to CE analysis. The peak area of the unsaturated disaccharides obtained after digestion with lyase at 0.625 U/mL and 1.25 U/mL did not differ significantly (data not shown). The further experiments were thus performed using chondroitinases (either ABC or AC) at 0.625 U/mL level.

In order to simulate conditions in which heparin is the main drug and DS and CS the potential related substances, a heparin sample with a very low content of such GAGs was spiked with impurity levels of DS and CS. The mixtures were then subjected to exhaustive digestion with chondroitinases. Before CE analysis, the digested samples were diluted (1-to-10 fold) with water in order to avoid matrix effect due to the high concentration of protein (enzymes) and salts in the reaction mixture. The injection of diluted samples allowed for high reproducibility of migration time without any evidence of protein adsorption on the capillary wall; furthermore, 5-sulfosalicylic acid was introduced as internal standard. The electropherograms of a sample mixture (heparin, DS and CS) digested either with chondroitinase AC and ABC are reported in Fig. 3A and B, respectively. In Fig. 3C is shown the electropherogram of the sample mixture digested with chondrotinase ABC recorded at the detection wavelength of 232 nm. Under this condition, disaccharides ΔDi-dis_D, ΔDi-dis_B and ΔDi-dis_E, released by lyases from DS and CS, can be detected without interference from undigested heparin. However, the depolymerization of CS and DS produces mainly ΔDi-4S and ΔDi-6S (monosulfated at C4 and C6, respectively) [22] whose determination can be conveniently performed at 200 nm.

The used chondroitinases, because of their high specificity, are unable to digest the semisynthetic OSCS [23] which can also be present as a heparin impurity. In our experiments, the CE analysis of digested heparin samples contaminated with OSCS, besides DS and CS, showed the peak of the intact OSCS as a band partially overlapped to the front of the heparin peak without interference to the determination of the disaccharides. Thus, the level of each disaccharide released upon the enzymatic digestion, selectively corresponds to its content in the native GAG and the related substances, CS and DS, can be accurately quantified by proportion with the released disaccharides. According to Fig. 3, both ΔDi-4S and ΔDi-6S can be chosen for quantitation of CS and DS, because of their significant response reflecting the high content in the parent GAGs. Conversely, ΔDi-0S was not considered because of the low response and the relatively long migration time.

3.4. Validation of the CE method for quantitation of CS and DS as impurities in heparin

3.4.1. Linearity and sensitivity

Mixtures of a fixed amount of heparin (50 mg/mL) and containing both CS and DS in the range 0.03–2.00% (w/w) were depolymerised under the described conditions with chondroitinase ABC, to digest simultaneously both CS and DS. The same mixtures were also digested using chondroitinase AC to obtain the depolymerization of CS only. Furthermore, also blank samples (containing only heparin with a very low content of related substances) were digested with both chondroitinases AC and ABC.

The CE analysis of the digested mixtures was carried out in order to evaluate the linearity of the response of the peak area ratio (peak area of disaccharide to that of internal standard) against the percentage of the GAG in the original mixtures. Precisely, the linearity was evaluated for each of the disaccharides individually (peak area of ΔDi-4S and of ΔDi-6S) and for their sum (peak area of ΔDi-4S and ΔDi-6S). By regression analysis, linear relationships were obtained as shown in Tables 2a and 2b. Interestingly, the digestion of the blank samples using chondroitinase AC did not provide any significant peak (neither ΔDi-4S nor ΔDi-6S), whereas the

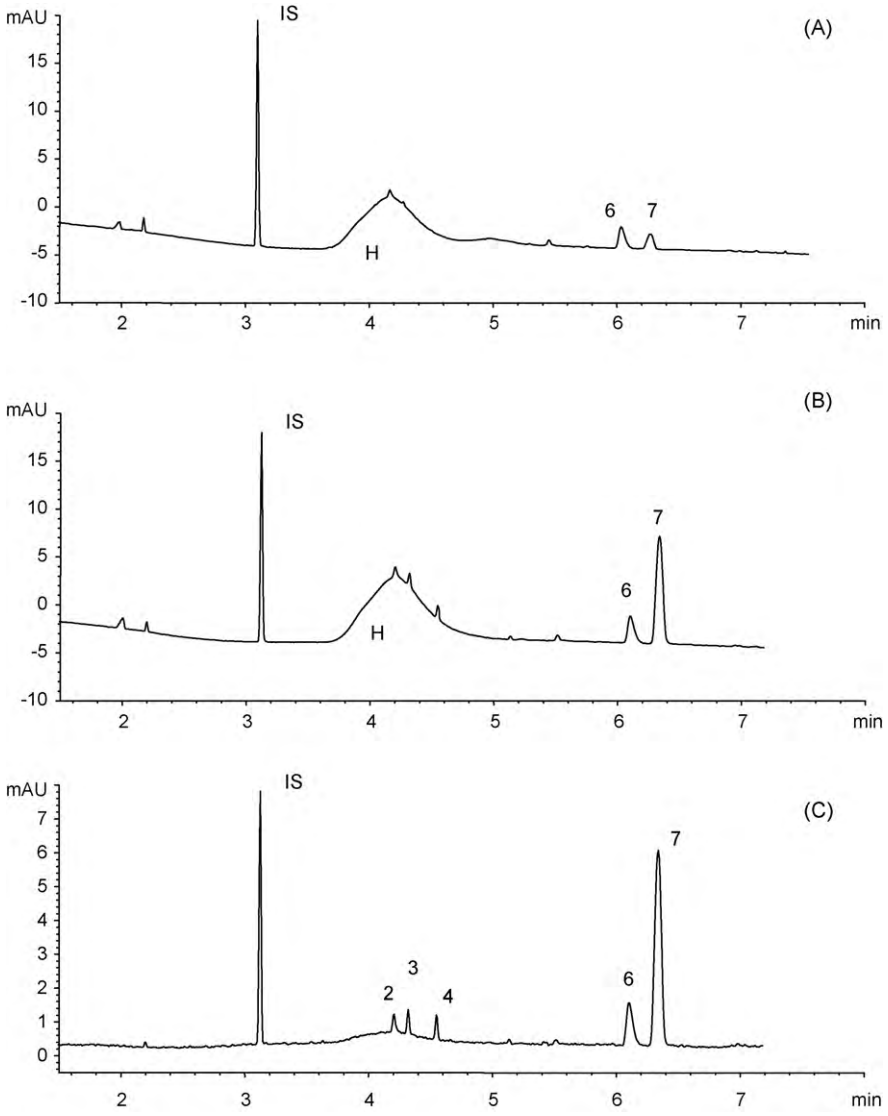


Fig. 3. Electropherograms of a sample mixture of heparin (H) 50 mg/mL containing DS at 1.5% (w/w) and CS at 0.5% (w/w) after depolymerization with chondroitinase AC (electropherogram (A)) and chondroitinase ABC (electropherogram (B) at the wavelength of 200 nm, and electropherogram (C) at the wavelength of 232 nm). CE conditions and symbols as in Fig. 1B. IS (internal standard) 5-sulfosalycilic acid at the final concentration of 25 μg/mL.

digestion of blanks using chondroitinase ABC originated a small but detectable peak (ΔDi-6S) whose area ratio was considered in the evaluation of linearity.

The sensitivity of the method was determined simultaneously to the linearity study. As the digestion using chondroitinase AC selectively depolymerises CS, the response in term of disaccharide peak area is lower than that obtained after digestion using chondroitinase ABC (depolymerization of both CS and DS). Accordingly, the LOQ of the method was reasonably assumed as the lowest CS concentration releasing, after digestion with chondroitinase AC, an accurately measurable peak area (signal-to-noise of 10:1) of ΔDi-4S and ΔDi-6S. It was found that this condition (peak area RSD% <2.5%, $n = 3$) was obtained at the CS concentration level of about 0.03% (w/w) with respect to heparin, thus corresponding to the lowest calibration point. By means of progressive dilution of this

Table 2a
Linearity data of the selected disaccharides released after digestion of CS (range 0.03–2.00%, w/w with respect to heparin) using chondroitinase AC.[a].

Selected disaccharide	Linear regression	r^2
ΔDi-4S[b]	$Y = 0.291x + 0.0217$	0.998
ΔDi-6S[b]	$Y = 0.486x + 0.0217$	0.993
ΔDi-4S + ΔDi-6S[c]	$Y = 0.777x + 0.0437$	0.996

[a] Only CS is considered being the digestion using chondroitinase AC, selective for this GAG.
[b] The response is the peak area ratio (the single disaccharide to the internal standard).
[c] The response is the sum of the peak area (ΔDi-4S + ΔDi-6S) ratio to the internal standard.

Table 2b
Linearity data of the selected disaccharides released after digestion of CS and DS (range 0.03[a]–2.00%, w/w each with respect to heparin) using chondroitinase ABC.

Selected disaccharide	Linear regression	r^2
ΔDi-4S[b]	$Y = 0.669x + 0.124$	0.999
ΔDi-6S[b]	$Y = 0.322x - 0.0002$	0.998
ΔDi-4S + ΔDi-6S[c]	$Y = 0.994x + 0.113$	0.999

[a] Blank sample is subjected to digestion with chondroitinase ABC and the provided response is plotted as the calibration point related to the GAG concentration equal to 0.
[b] The response is the peak area ratio (the single disaccharide to the internal standard).
[c] The response is the sum of the peak area (ΔDi-4S + ΔDi-6S) ratio to the internal standard.

Table 3
Accuracy and precision data (RSD%, $n = 3$) in analysis of CS and DS as related substances in sodium heparin after chondroitinases digestion and CE analysis.

Related substance (GAG)	Added (%)[a]	Found (%)	Recovery (%)	RSD%
	0.150	0.164	109.3	4.6
CS	0.299	0.289	96.6	8.0
	0.591	0.622	105.3	5.9
	1.965	1.990	101.3	5.5
Total CS + DS[b]	0.992	0.917	92.4	8.0
	0.499	0.450	90.2	10.5

[a] % w/w of the GAG spiked to heparin as reported in experimental Section 2.3.5.
[b] The amount of DS can be determined as the difference "Total% – CS%".

solution it was found that the LOD (signal-to-noise ratio of 3) was about 0.01% (w/w).

3.4.2. Accuracy, precision and robustness

The accuracy was assessed on heparin samples spiked with known amounts of DS and CS, the QC samples, as described in Section 2.3.5, covering the calibration curve range. These control samples were analysed after digestion with chondroitinases and the quantitation of CS and DS was achieved by the calibration curve. The obtained results were compared with the nominal values and are reported in Table 3. The shown data, which meet the requirements of accuracy and precision recommended by the guideline on validation of bioanalytical methods by the European Medicines Agency (EMA) [24], can be considered satisfactory taking into account the number of steps involved in the analytical procedure. Furthermore, the electrophoretic method was tested for robustness by evaluating its capability to remain unaffected by small variations of the optimised parameters. In particular, buffer concentration and pH as well as the separation temperature were considered as the factors which have to be studied because of their influence on the separation of the critical couple of analytical peaks ΔDi-6S/ΔDi-4S. The investigation was carried out following the "one-by-one" approach, where individual operating parameters were separately changed over a predetermined range around the optimised values. In particular, the BGE concentration was varied in the range 110 ± 5 mM; the pH was varied in the range 3.5 ± 0.2 pH and the separation temperature was varied in the range $40 \pm 2\,^{\circ}C$. The response chosen to evaluate the robustness was the mean resolution (R_s) between ΔDi-6S/ΔDi-4S calculated from three replicate ($n = 3$) injections of each set of conditions. The found R_s values ranged within 2.00–2.55 (RSD% <1.40), thus confirming that the baseline separation of the critical couple of analytes ΔDi-6S/ΔDi-4S, is maintained under the applied experimental conditions.

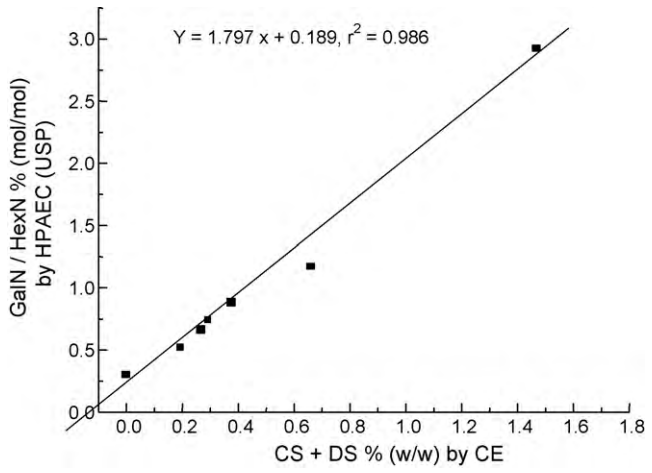


Fig. 4. Correlation between the data obtained in analysis of DS and CS as related substances in real sodium heparin samples using the official USP method (HPAEC with pulsed amperometric detection) and the proposed CE method. The data reported are the mean of three determinations.

Table 4
Analysis of GAGs as related substances in real sodium heparin samples by CE method[a] and HPAEC-USP method.[b].

Sample	CS% (RSD%)[a]	(DS + CS)% (RSD%)[a]	GalN/HexN%[b]
C1	–	0.195 (7.6)	0.52
C2	0.097 (9.7)	0.660 (2.5)	1.17
C3	–	0.292 (4.5)	0.74
C4	–	0.378 (3.7)	0.88
C5	–	0.268 (4.9)	0.66
C6	0.05 (10.5)	1.47 (1.5)	2.92
C7	–	–	0.30

[a] The CE method provides the amount of GAGs as % (w/w) in heparin.
[b] The HPAEC method provides the amount of GAGs (DS + CS) referred as % (mol/mol) of galactosamine (GalN) in total hexosamine (HexN). See text for precision data of the USP method.

3.4.3. Analysis of real samples

Seven real samples of sodium heparin from Opocrin S.p.A. were analysed for the presence of DS and CS, after selective digestion with chondroitinases, by the developed CE method. The quantitation of CS and DS was obtained by means of the calibration curve and the results are reported in Table 4. The same samples were also analysed by the USP official method [6]; this approach measures the content of galactosamine (GalN) from DS and CS, in comparison with the total amount of hexosamines (HexN) from heparin, DS and CS. The measurements were carried out by pulsed amperometric detection with HPAEC separation, after acid hydrolysis of the samples. This analysis provides the content % (mol/mol) of the total amount of GAGs (CS plus DS) in heparin, whereas the CE method is able to provide the amount of the single GAG (CS and DS) referred as % (w/w) in heparin.

The repeatability of the HPAEC method was evaluated at three concentration levels of galactosamine, namely 0.15, 0.9 and 2.5%, of the total hexosamine. The obtained RSD% ($n = 6$) values, were 3.6, 1.0 and 2.6%, respectively; the intermediate precision, evaluated at the same GalN concentration levels was found to correspond to RSD% ($n = 8$) of 3.6, 2.3 and 2.4%, respectively.

The correlation of the results obtained by the two methods is shown in the graph of Fig. 4. By means of CE analysis, the sample C7 (see Table 4) resulted to be free from GAGs, whereas by the HPAEC analysis, the same sample was found to contain a small amount (0.30% of GalN/HexN) of total galactosamine containing impurities. Notwithstanding the difference in sensitivity, a linear relationship was found between the total amounts of GAGs (CS plus DS) determined by the two methods, thus confirming the reliability of the novel CE approach.

4. Conclusion

Dermatan sulfate and chondroitin sulfate can be considered as safe components of therapeutic heparin, however the restrictions recently adopted by many authorities have led to regard them as contaminants.

The present study is the first showing the usefulness of CE in the selective quantitation of DS and CS as related substances in heparin. Differently from the previous electrokinetic methods, the proposed CE approach is based on the analysis of the unsaturated disaccharides released after specific chondroitinase digestion of CS and DS. The achieved sensitivity (LOD) was found to be 0.01% (w/w), thus more than one order higher than that reported by previous methods based on the direct CE-UV analysis of the intact GAGs. The present method can be a useful alternative to the HPAEC recommended by USP, which only provides the unselective estimation of the whole

organic impurities (DS and other galactosamine containing impurities) in heparin.

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