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Development of a novel analytical method for determination of chondroitin sulfate using an in-capillary enzyme reaction

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Abstract

A novel analytical method for determination of total amount of chondroitin sulfate (CS) based on its conversion to desulfated chondro-disaccharide via an enzyme-catalyzed reaction, was developed. Using the in-capillary enzyme reaction, the method was also applied to the successful construction of an on-line analytical system. Within this system, electrophoretic migration was used to mix zones containing the enzyme mixture (chondroitinase ABC, chondro-4-sulfatase, chondro-6-sulfatase and 2-*o*-sulfatase) and the substrate (CS). The reaction was then allowed to proceed in the presence of a weak electric field and, finally, the product (desulfated chondro-disaccharide) of enzyme reaction migrated to the detector under the influence of an applied electric field. A polyvinyl alcohol-coated capillary was used to reduce protein adsorption. Desulfated chondro-disaccharide was successfully migrated toward the anode in 10 mM Tris–acetate buffer (pH 7.0) under reversed polarity and detected at 232 nm. The established method was validated and demonstrated to be applicable in the determination of total amount of CS in a commercial ophthalmic solution. No interference from the formulation excipients was observed. Good linearity was obtained, with correlation coefficients above 0.999. Recoveries and precisions ranged from 100.0 to 100.5%, and from 0.2 to 0.6% of the relative standard deviation, respectively. Good agreement was obtained between the established method and traditional photometric method based on carbazole reaction. In this study, application of the method to disaccharide compositional analysis was also performed.

Keywords: In-capillary enzyme reaction; Pharmaceutical analysis; Chondroitin sulfate; Glycosaminoglycans; Polysaccharides

1. Introduction

Chondroitin sulfate (CS) is one of the glycosaminoglycan (GAG) compounds that form main components of glycoconjugate found mainly in connective tissues, and is considered to contribute to the maintenance, reinforcement and retention of the flexibility of tissues [1–3]. Evidence derived from studies in recent years suggests that GAGs also play pivotal roles in cell proliferation, differentiation and adhesion [4]. The biological significance of these compounds have gradually become clear. GAGs, including CS, may be implicated in several diseases, such as arthritis, malignancy, diabetes, renal failure and emphysema [5]. CS is found in many pharmaceuticals, such as ophthalmic solutions and agents for arthralgia, cosmetics and health foods [6–9]. The widespread use of CS has stimulated research on accurate, efficient, and convenient methods of quality control. GAGs are linear polysaccharides composed of variable number of repeating disaccharide units. Each disaccharide consists of one hexosamine (D-galactosamine or D-glucosamine) and one uronic acid (D-glucuronic acid or D-iduronic acid) or hexose (D-galactose). The repeating unit of CS is D-glucuronic acid and *N*-acetylgalactosamine, which is then sulfated at the C-4 or C-6 positions of *N*-acetylgalactosamine. Other less-frequent *o*-sulfation occurs, e.g., at C-2 of D-glucuronic acid. A few of these positions remain unsulfated (Fig. 1).

For the determination of total amount of CS, several techniques have been developed, principally including the photometric method (based on carbazole reaction) [2,10,11], measurement after reaction with cationic dye (alcian blue [12], stains-all [13], etc.) and the photometric titration method using cetylpyridinium chloride [9]. The carbazole reaction method is the most commonly used analytical method for quantitative determination of total CS. However, the method is relatively time-consuming and demands a high level of skill because tightly controlled reaction conditions

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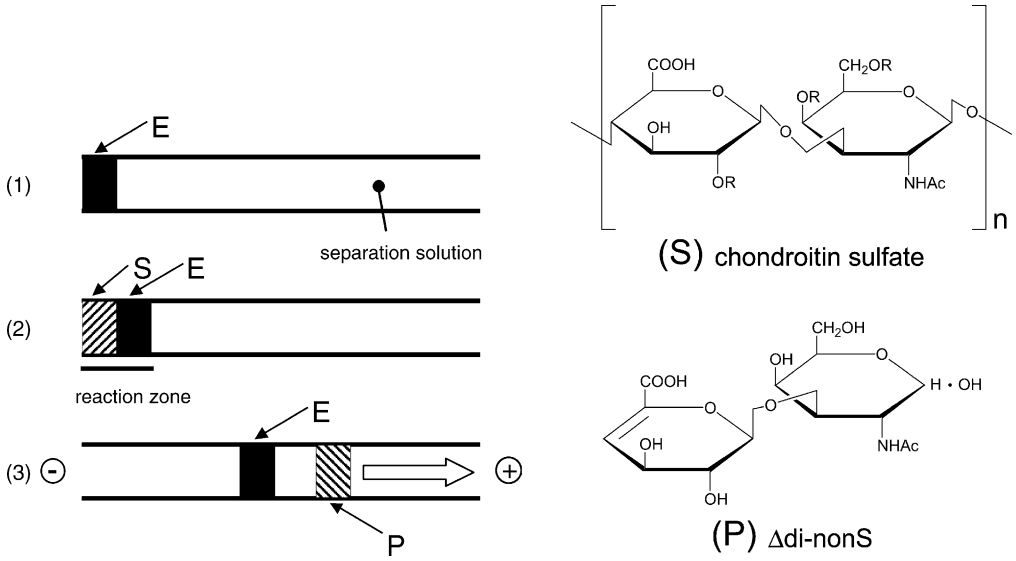

Fig. 1. An illustration of the conversion to chondroitin sulfate to desulfated chondro-disaccharide by the in-capillary enzyme reaction method. Plugs of (1) enzyme solution (E; chondroitinase ABC and chondrosulfatases) and (2) substrate solution (S; chondroitin sulfate) were introduced to the inlet of a capillary by pressure injection. After mixing enzyme and substrate, (3) reaction product (P; nonsulfated chondro-disaccharide) was subsequently determined by capillary electrophoresis. Chondroitin sulfate may be sulfated at one, two, or three of the positions marked with an "R".

are required to obtain consistent chromophore production and also because strong acid hydrolysis at elevated temperature must be regulated without excessive digestion of the uronic acid [2]. Moreover, if other carbohydrates and/or metals are present in the prescription, the determination of CS in multi-components pharmaceutical preparations could be affected [10,11]. In such cases, complicated pretreatment of the sample, e.g., liquid–liquid extraction or solid phase extraction, may be required. On the other hands, HPLC or CE with direct analysis of CS or detection of copper(II)–CS complexes have been reported [14–17]. However, the peak broadening was observed due to polydispersity and sequence heterogeneity, caused primarily by a variation in the degree and position of the sulfate groups of CS. Research is, therefore, needed for development of a new method that is more rapid, simpler, and precise.

Chromatographic procedures have been widely used for the disaccharide compositional analysis of GAGs in specific polysaccharide lyses digestion products [5,18–23]. The enzymatic digestion method provides a great deal of information on the structures and constituents of GAGs, which may in turn provide valuable insight into the nature of the biological process in which the GAGs are involved. The enzyme mainly used for the degradation of CS is chondroitinase ABC, which catalyzes the eliminative cleavage of *N*-acetylhexosaminide linkage in CS, yielding mainly disaccharides with Δ^4-hexuronate at the non-reducing ends [24] (Δdi-S, unsaturated chondro-disaccharides) (see Fig. 1). The unsaturated uronic acid strongly absorbs ultraviolet light at 232 nm and this wavelength is used for the quantification of the products. Each Δdi-S derived from CS shows good peak shape on the chromatogram because of its homogeneity. When total amount of CS is determined, however, identifying and evaluating all of the peaks becomes complicated, requiring preparation of a reference standard for each Δdi-S. Chondrosulfatases, i.e., chondro-4-sulfatase, chondro-6-sulfatase and 2-*o*-sulfatase, have been used to analyze sulfation profile of CS; in these cases they were used to release sulfate ester group(s) for the identification of each Δdi-S derived from CS [25–28]. When Δdi-S is desulfated completely by these chondrosulfatases, the total amount of CS can be determined from the yields of nonsulfated chondro-disaccharide [Δdi-nonS, 2-acetamido-2-deoxy-3-*o*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose] (Fig. 1). To date several related studies have been reported, in which the amount of CS product in biological samples was determined based on the Morgan–Elson reaction [26], HPLC [29] and CE [30] after combined enzyme treatment.

Generally, enzymes are expensive and sometimes only a very small amount of catalyst is available. Since the CE system requires only a small quantity of material, application of CE to an enzyme microreactor has evoked increased interest. Free solution analysis with CE allows in-situ enzyme reaction in a buffer inside a capillary. Recently, the CE system combined with the in-capillary enzyme reaction method has been applied to the evaluation of enzyme activity [31–36]. Differences in electrophoretic mobility among solutes have been widely applied in CE not only in separation but also in mixing. In the method, plugs of enzyme and substrate are first introduced into the capillary. After mixing substrate and enzyme, the amount of reaction product is subsequently determined by CE. Each analytical process, i.e., mixing, reaction, separation and detection, occurs within the capillary using commercial equipment without complicated modifications. This on-line

analytical system dramatically reduces the volume of the reaction solution compared to off-line batch operation. We have recently shown the applicability of the method to the determination of active ingredient in the pharmaceutical preparation [37]. The in-capillary enzyme reaction method was used to determine of the amounts of riboflavin phosphoric acid esters in a vitamin-enriched drink based on their conversion to riboflavin with alkaline phosphatase. The present study was performed to demonstrate the applicability of the in-capillary enzyme reaction method to analysis of CS in pharmaceutical preparations. The enzyme mixture, i.e., chondroitinase ABC, chondro-4-sulfatase, chondro-6-sulfatase and 2-*o*-sulfatase, was utilized to obtain complete conversion of CS to Δdi-nonS, and the in-capillary operation and the off-line batch operation was compared to determine the optimal enzyme reaction conditions. This method was validated for the determination of CS in a commercial preparation. Furthermore, attention was also focused on the applicability of the in-capillary enzyme reaction method to disaccharide compositional analysis.

2. Experimental

2.1. Equipment

CE was performed using an Agilent CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detection system operating at 232 nm. Polyvinyl alcohol-coated capillaries (50 μm i.d. × 375 μm o.d.; 56 cm in length to the detector and total length of 64.5 cm) were obtained from Agilent Technologies. A bubble cell capillary arrangement was employed to increase sensitivity. The capillary compartment temperature was maintained at 37 °C. Hydrodynamic injection (5 kPa) at the cathodic end of the capillary was used to introduce samples. The applied voltage was set at −30 kV. The capillary was equilibrated for 3 min between runs by flushing with the separation solution. All data were collected and analyzed using ChemStation software (Agilent Technologies).

2.2. Chemicals

CS-C sodium salt (grade SSG) from shark cartilage and CS-A sodium salt (grade SSG) from whale cartilage were purchased from Seikagaku (Tokyo, Japan). Chondroitinase ABC (EC 4.2.2.4) from *Proteus vulgaris*, chondro-4-sulfatase (EC 3.1.6.9) from *P. vulgaris*, and chondro-6-sulfatase (EC 3.1.6.10) from *P. vulgaris* were obtained from Seikagaku 2-*o*-sulfatase from *Flavobacterium heparinum* was purchased from Sigma–Aldrich (St. Louis, MO, USA). Unsaturated chondro-disaccharides (Δdi-nonS; Δdi-mono4S, 2-acetamido-2-deoxy-3-*o*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-4-*o*-sulpho-D-galactose; Δdi-mono6S, 2-acetamido-2-deoxy-3-*o*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*o*-sulpho-D-galactose; Δdi-di(2,6)S, 2-acetamido-2-deoxy-3-*o*-(2-*o*-sulpho-4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*o*-sulpho-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*-sulpho-D-galactose) and unsaturated hyaluronan disaccharide (Δdi-nonS_{HA}, 2-acetamido-2-deoxy-3-*o*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose) for identification of peaks on the electropherogram were obtained from Seikagaku Tris, potassium hydrogen phthalate, carbazole, sodium tetraborate, and sulfuric acid were obtained from Wako (Osaka, Japan). Acetic acid was obtained from Kokusan (Tokyo, Japan). All chemicals were of analytical grade. CS and enzyme mixture solution were each prepared by dissolving the saccharide and the lyophilized proteins in 10 mM Tris–acetate buffer (pH 7.0) in the same composition as the separation solution for electrophoresis. The solution was filtered through a polysulfone 0.45 μm membrane filter (Nihon Pall, Tokyo, Japan) prior to use.

2.3. Procedure for off-line enzyme reaction method

A 1 ml sample of the CS solution (1 mg ml^{-1}) was placed in a 10 ml volumetric flask. An internal standard solution was prepared by dissolving 20 mg of potassium hydrogen phthalate in 100 ml of water. A 1 ml volume of the internal standard solution was added to the solution in the volumetric flask and the mixture was diluted to the volume with 10 mM Tris–acetate buffer (pH 7.0). To 500 μl of the sample solution, 2 μl of chondroitinase ABC solution (12.5 units ml^{-1}) was added. The reaction was allowed to react at 37 °C for 60 min. A portion of the solution was analyzed by CE using the conditions described above. Hydrodynamic injection (5 kPa, 5 s) was utilized for sample introduction. To ensure that the reaction was complete, digestion was allowed to continue for an additional 60 min. When this time had elapsed, another sample of the digestion solution was removed and analyzed by CE. In each case, no increase in total amount of Δdi-S (the product of the digestion) was detected after longer digestion time. Once it was ensured that digestion was complete, a 2 μl solution of chondro-4-sulfatase (1.25 units ml^{-1}), a 2 μl solution of chondro-6-sulfatase (1.25 units ml^{-1}), and a 2 μl solution of 2-*o*-sulfatase (12.5 units ml^{-1}) were added to the 50 μl portion of the digestion solution. The reaction was allowed to react at 37 °C for 90 min. A portion of the solution was analyzed by CE. Each reaction tube was heated in a boiling water bath for 1 min to stop the reaction.

2.4. Procedure for in-capillary enzyme reaction method

An illustration of the conversion of CS to Δdi-nonS by the in-capillary enzyme reaction method is shown in Fig. 1. Plugs of enzyme solution (0.25 units ml^{-1} of chondroitinase ABC, 0.25 units ml^{-1} of chondro-4-sulfatase,

0.25 units ml^{-1} of chondro-6-sulfatase, 2.5 units ml^{-1} of 2-*o*-sulfatase, respectively) and substrate solution (0.1 mg ml^{-1} of CS) were introduced to the inlet of a capillary separately by pressure injection (5 kPa, 10 s for enzyme solution and 5 s for substrate solution). The reaction was then allowed to proceed, with the application of −0.2 kV for 20 min, and electrophoresis was finally performed, using the separation solution described above, with an applied −30 kV. Relatively high concentrations of the enzyme per substrate made it possible to complete reaction in short time without undesirable diffusion of reaction zone.

To determine the amount of CS in a commercial ophthalmic solution, a 2 ml sample of the preparation was placed in a 10 ml volumetric flask. A 2 ml volume of the internal standard solution was added to the solution in the volumetric flask and the mixture was diluted to the volume with 10 mM Tris–acetate buffer (pH 7.0). For the preparation of standard solution, CS was weighed and diluted in Tris–acetate buffer (pH 7.0) to concentration similar to that of the prepared sample solution. The same volume of internal standard solution was also added to the standard solution. Each solution was digested and desulfated in capillary under the same condition. All test solutions were passed through a polysulfone 0.45 μm membrane filter (Nihon Pall).

2.5. Procedure for photometric method based on carbazole reaction

The amount of CS in a commercial ophthalmic solution was determined by photometric method based on carbazole reaction, in accordance with Bitter and Muir [11]. To eliminate interference to the carbazole reaction by the formulation excipients, solid phase extraction (Sep-Pak Plus C_{18}, Waters, Milford, MA, USA) was performed prior to the reaction. The reaction product of hydrolyzed uronic acid with carbazole was measured at 525 nm. U-3000 spectrophotometer (Hitachi, Tokyo, Japan) was used to measure the absorption.

3. Results and discussion

3.1. Separation of unsaturated chondro-disaccharides

Separation conditions of Δdi-S were optimized prior to perform the in-capillary enzyme reaction. Because the separation solution for electrophoresis also served as an enzyme reaction solution, the buffer determined to be most efficient for all enzyme reactions was selected for the solution. Tris–HCl and sodium acetate buffer could not obtain sufficient reaction. Accordingly, a buffer of 10 mM Tris–acetate (pH 7.0) was selected as optimal, both for the enzyme reactions and for the separation. Polyvinyl alcohol-coated capillary was utilized to reduce protein adsorption. Since Δdi-S may become negatively charged and attracted to the anode under these conditions, disaccharides analysis

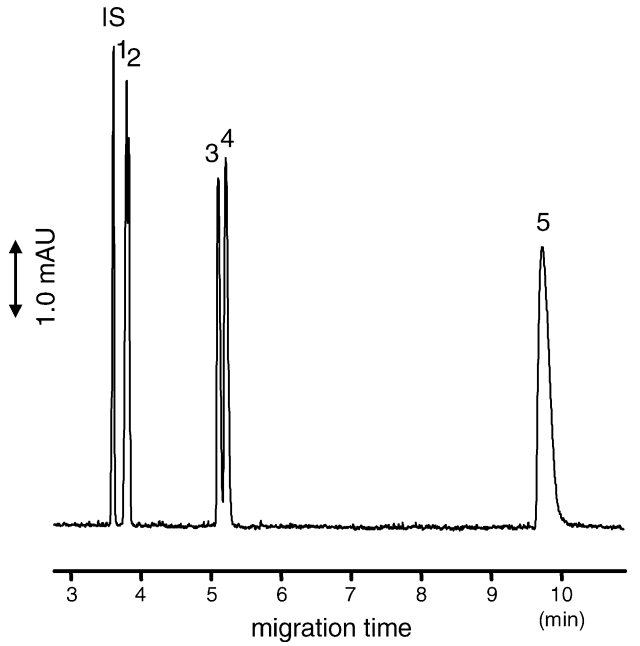

Fig. 2. An electropherogram of standard unsaturated chondro-disaccharides. Peak identification: (1) Δdi-di(2,6) S; (2) Δdi-di(4,6)S; (3) Δdi-mono4S; (4) Δdi-mono6S; (5) Δdi-nonS. IS: internal standard (potassium hydrogen phthalate).

was performed under reversed polarity. A typical electropherogram is shown in Fig. 2. Separation of Δdi-di(2,6)S and Δdi-di(4,6)S was insufficient; however, this was not relevant, because the aim of the procedure was to analyze Δdi-nonS completely converted from CS. In the established condition, Δdi-nonS could not be separated from hyaluronan-derived disaccharide (Δdi-nonS_{HA}) (data not shown).

3.2. Optimization of in-capillary enzyme reaction method

In the in-capillary enzyme reaction method, different electrophoretic mobilities of enzyme and substrate are used to merge each zone. To initiate the reaction, the fast migrating plug is caused to traverse across the slow migrating plug under an electric field. In order to obtain complete conversion of CS to Δdi-nonS, attention was focused on the order of introduction of elements. The efficient order of introduction into capillary was as follows: first, a plug of enzyme solution was introduced, followed by substrate solution (CS). The rates of conversion (compared to off-line enzyme reaction) in the established order and in the reversed order were 48.3 and 6.8%, respectively. These examinations revealed that the first migrating CS plug traversed across the slow migrating enzymes to initiate the reaction. Furthermore, attempts to obtain complete reaction resulted in a 99.2% rate of conversion by increasing the volume of enzyme used and lengthening mixing time. A typical electropherogram is shown in Fig. 3. Baseline drift observed on the electropherogram of in-capillary enzyme reaction method was due

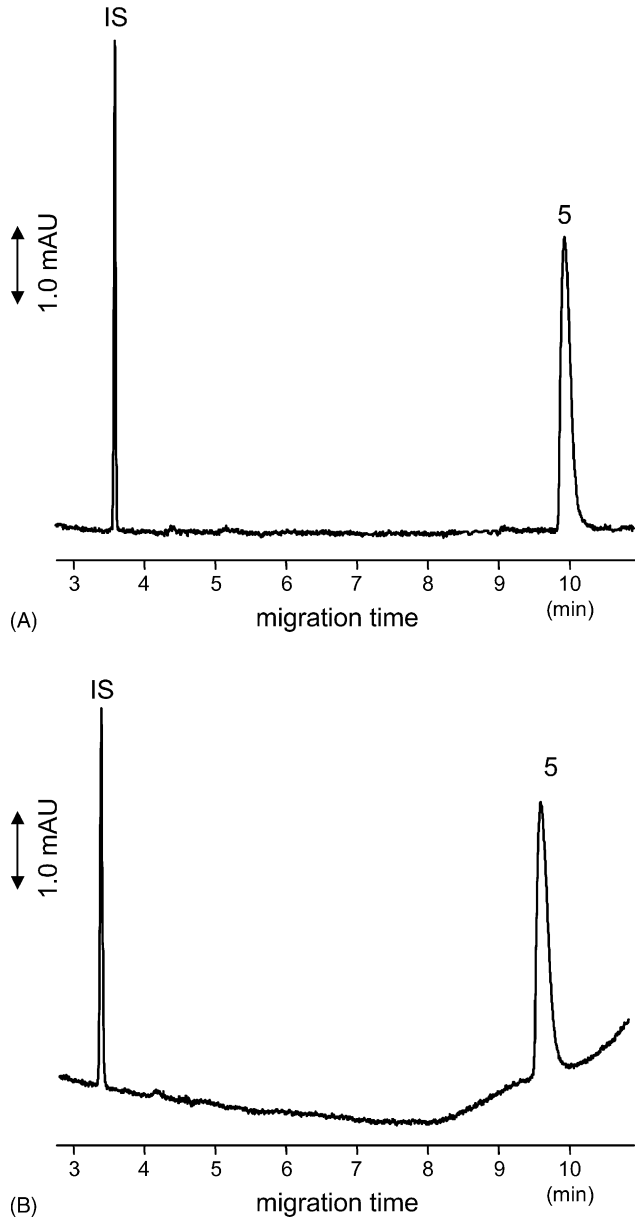

Fig. 3. Comparison of the conversion of chondroitin sulfate to desulfated chondro-disaccharide by (A) the off-line batch operation and (B) the in-capillary enzyme reaction method. Injection of enzyme mixture, 30 s; mixing time, −0.2 kV for 30 min. Other conditions were as described in text. Solutes were as noted in Fig. 2.

to bovine serum albumin added as a stabilizer in the introduced enzyme solution.

3.3. Analysis of various CS samples

To demonstrate the usefulness of the methodology, various CS samples were examined following chondroitinase ABC digestion and subsequent chondrosulfatases desulfation. The results are shown in Fig. 4.

First, digestion of each CS by only chondroitinase ABC in the in-capillary enzyme reaction was performed; representative electropherograms of each sample are shown in Figs. 4A and B. CS-C from shark cartilage was found to comprise mainly Δdi-mono6S, with several Δdi-mono4S and Δdi-di(2,6)S (Fig. 4A). CS-A from whale cartilage was found to contain mainly Δdi-mono4S, with several Δdi-mono6S (Fig. 4B). The above demonstrates that proposed procedure in case of using chondroitinase can be effectively used as a definitive analytical technique for structural studies of CS, to obtain information on disaccharide composition and sulfate content.

Second, simultaneous digestion and desulfation of each CS using both chondroitinase ABC and chondrosulfatases on the in-capillary enzyme reaction was performed; representative electropherograms of each sample are shown in Fig. 4C and D. Each CS sample completely converted to Δdi-nonS; therefore, the proposed procedure in case of using both chondroitinase ABC and chondrosulfatases may be applicable to the determination of total amount of CS. Only to exchange the vials of chondroitinase solution for that of mixed enzyme solution enabled simultaneous structural analysis and total-amount analysis for CS with low sample consumption.

3.4. Determination of chondroitin sulfate in a commercial pharmaceutical

3.4.1. Validation of the method

The established technique was applied and validated as a means of determination of CS in a commercial preparation.

To assess specificity, a standard, a sample of a commercial ophthalmic solution, and a placebo mixture (prepared in the absence of CS in an ophthalmic solution base) were digested and desulfated according to the established in-capillary enzyme reaction method. Analysis was performed by an internal standard method. A representative electropherogram of sample solution is shown in Fig. 5. Several observed peaks, except Δdi-nonS and IS, were the results of formulation excipients and other active ingredients. No interference with the formulation excipients was detected at the migration times of the Δdi-nonS. Furthermore, no reaction was occurred when sample solution was treated with Tris–acetate solution instead of enzyme solution in the in-capillary enzyme reaction method. No remaining enzyme was observed after a series of in-capillary reactions, and the polyvinyl alcohol-coated capillary can be used as long as normal CE mode.

To estimate the stability of analytical solutions, the sample, standard, and enzyme solutions were stayed in a light-resistant container at room temperature and evaluated base on changes in obtained peak area of Δdi-nonS. Analytical conditions were stable for at least 24 h.

The detection limit, observed as a peak with a signal-to-noise ratio of 3, was found to be 4 $\mu g\,ml^{-1}$ of CS solution; this limit was determined by injecting sample solution with a known low concentration of analyte.

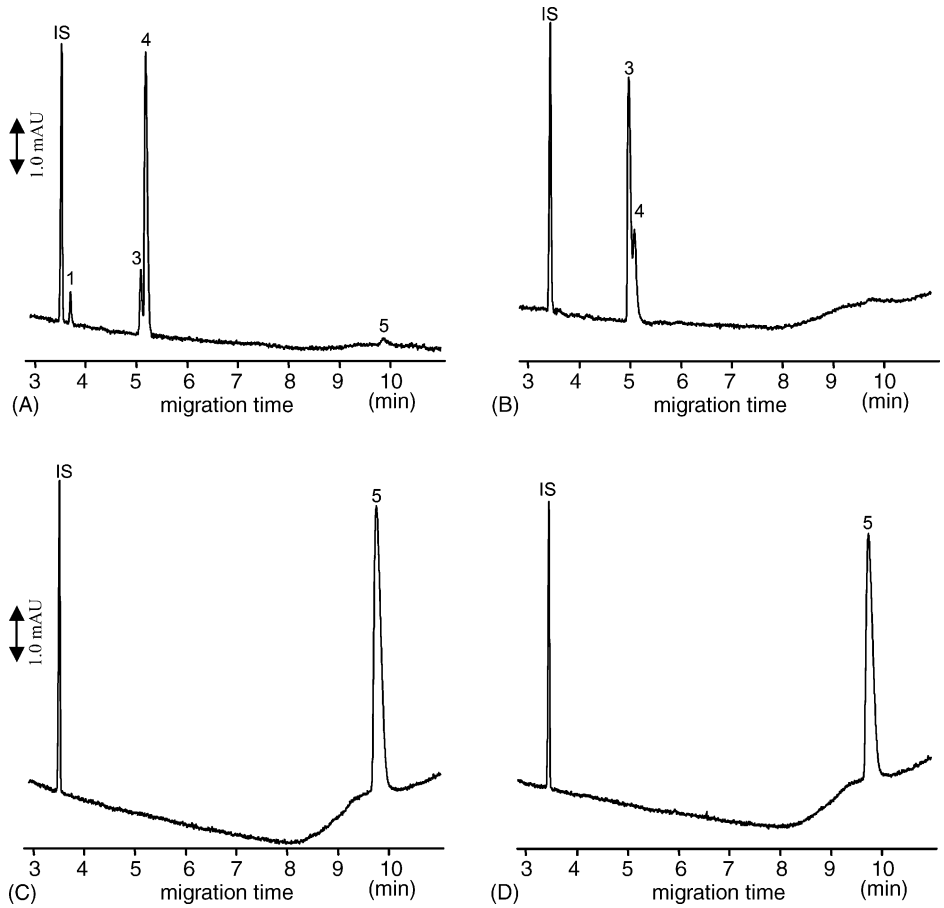

Fig. 4. In-capillary enzymatic analysis of various chondroitin sulfates. Chondroitin sulfate C from shark cartilage (A and C), chondroitin sulfate A from whale cartilage (B and D) were digested with chondroitinase ABC (A and B) or simultaneously digested and desulfated with chondroitinase ABC, chondro-4-sulfatase, chondro-6-sulfatase, and 2-*o*-sulfatase (C and D), respectively. Analytical conditions were as noted in Fig. 3. Solutes were as noted in Fig. 2.

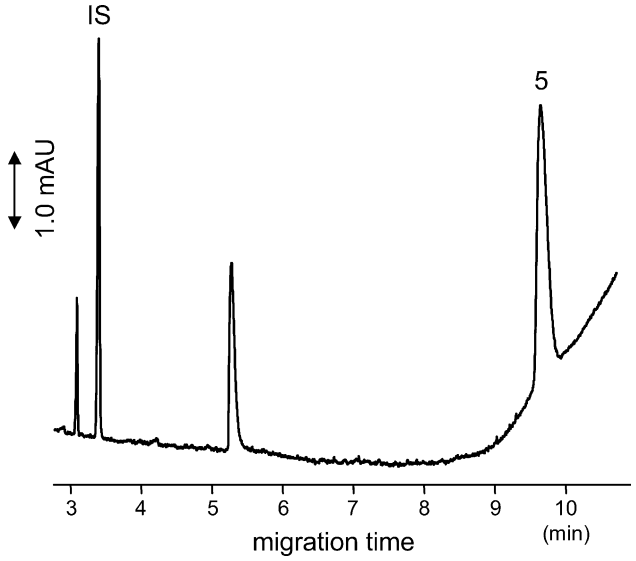

Fig. 5. A typical electropherogram in the assay of a commercial ophthalmic solution. 1.25 units ml^{-1} of chondroitinase ABC was used for complete digestion in the assay of a commercial ophthalmic solution. Other conditions were as Fig. 3. Solutes were as noted in Fig. 2.

The quantitation linearity of CS in standard solution was examined at five concentration levels in the range from 25 to 150 $\mu g\,ml^{-1}$. The relationship between relative corrected peak area of Δdi-nonS obtained from the in-capillary reaction and concentration of CS was calculated. A straight regression line ($y = 0.01186x - 0.0139$) with a correlation coefficient $r = 0.9998$ was obtained. The intercept value was statistically insignificant (95% confidence).

Accuracy was assessed over the entire concentration range (80, 100, and 120% of the normal concentration in the preparation) by analyzing placebos spiked with CS at three concentration levels. The solutions were replicated three times each, and the amounts determined were compared to the theoretical amounts. The average recoveries for triplicate determinations at 80, 100 and 120% levels were 100.5, 100.0 and 100.3%, respectively. Results were adequate in terms of recovery.

Precision was determined by measuring ($n = 3$) Δdi-nonS in spiked placebos at the three concentration levels. RSD for triplicate determinations at 80, 100 and 120% levels were 0.4, 0.2 and 0.6%, respectively. Precision was determined to be good.

3.4.2. Quantitative analysis of chondroitin sulfate in a commercial ophthalmic solution

The established in-capillary enzyme reaction method was applied to analyze CS in a commercial ophthalmic solution. For comparison, the same sample was analyzed using the traditional photometric method based on carbazole reaction. To prevent the formulation excipients from interfering with the carbazole reaction, solid phase extraction was performed prior to the reaction. The results generated by the in-capillary enzyme reaction method and photometric method correlate very well, 100.5 ± 0.9 mg per 100 ml, 99.7 ± 2.2 mg per 100 ml, respectively. In terms of precision, S.D. of the established method was lower than that of the photometric method. Moreover, the major advantage of the in-capillary enzyme reaction method over photometric method is to quickly analyze large amount of samples without complicated pretreatment. In this study, analysis time for the established method is less than 60 min per sample as compared with 3 h per sample for the traditional method. It may therefore be concluded that the in-capillary enzyme reaction method may be applied as an alternative technique of pharmaceutical quality control.

4. Conclusion

We developed the first-ever analytical method for determination of CS amounts based on conversion to Δdi-nonS via in-capillary enzyme reaction. The established method offers numerous advantages over the traditional photometric method in terms of improved precision, simplicity, safety, rapidity, and automation of handling. The method is also superior not only in terms of automation but also in terms of the reduction of enzyme consumption compared with the off-line batch reaction followed by HPLC or CE analysis. In the present system, a vial of enzyme solution can be used repeatedly; however, sample solutions must be prepared in microliter amounts, due to the structure of the instrumentation. Attempt to reduce costs may be accomplished by using a microchip enzyme reactor [38–40].

The result also indicates that the proposed procedure may be useful in structural studies of CS. Analyses of both of total amount and the chemical structure of CS are required for the diagnosis of the pathophysiological status of a specific tissue or of a patient. In particular, an increase in CS with a concomitant increase in Δdi-nonS and Δdi-mono6S are found in most cancers [5,41,42], and therefore analysis of these substances may be useful in follow-up monitoring of patients after treatment. When the separation between Δdi-nonS and hyaluronan-derived disaccharide is achieved, the method will widely extend its application area to various biological samples. The choice between total-amount analysis and structural analysis can be based on the information that is required. The established method can be applied for the analysis of samples available in very small amounts, such as tissue biopsies. Preparing only two vials of enzyme solution will enable simultaneous structural analysis and total-amount analysis for CS with low sample consumption. Therefore the in-capillary enzyme reaction method can promise wide application not only in the field of pharmaceutical quality control but also in medical diagnosis.

References

[1] N. Sharon, Complex Carbohydrates: Their Chemistry, Biosynthesis and Functions, Addison-Wesley, Reading, MA, 1975.
[2] N.B. Beaty, R.J. Mello, J. Chromatogr. 418 (1987) 187.
[3] C. Kodama, T. Kodama, Z. Yoshizawa, J. Chromatogr. 429 (1988) 293.
[4] K. Sugahara, H. Kitagawa, Curr. Opin. Struct. Biol. 10 (2000) 518.
[5] D.H. Vynios, N.K. Karamanos, C.P. Tsiganos, J. Chromatogr. B 781 (2002) 21.
[6] T. Liesegang, J. Surv. Ophthalmol. 34 (1990) 268.
[7] A. Das, T.A. Hammad, Osteoarthritis Cartilage 8 (2000) 343.
[8] G. Ronca, A. Conte, Int. J. Clin. Pharm. Res. XIII (Suppl.) (1993) 27.
[9] Z. Liang, C. Bonneville, T. Senez, T. Henderson, J. Pharm. Biomed. Anal. 28 (2002) 245.
[10] Z. Dische, J. Biol. Chem. 167 (1947) 189.
[11] T. Bitter, H.M. Muir, Anal. Biochem. 4 (1962) 330.
[12] B. Fellström, B.G. Danielson, E. Lind, S. Ljunghall, B. Wikström, Eur. J. Clin. Invest. 16 (1986) 292.
[13] K.A. Homer, L. Denbow, D. Beighton, Anal. Biochem. 214 (1993) 435.
[14] T. Tyler, B. Khandelwal, D. Norden, F.R. Rolle, J. AOAC Int. 85 (2002) 567.
[15] D.W. Choi, M.J. Kim, H.S. Kim, S.H. Chang, G.S. Jung, K.Y. Shin, S.Y.J. Chang, J. Pharm. Biomed. Anal. 31 (2003) 1229.
[16] T. Toida, R.J. Linhardt, Electrophoresis 17 (1996) 341.
[17] T. Toida, M. Shima, S. Azumaya, T. Maruyama, H. Toyoda, T. Imanari, R.J. Linhardt, J. Chromatogr. A 787 (1997) 266.
[18] T. Imanari, T. Toida, I. Koshiishi, H. Toyoda, J. Chromatogr. A 720 (1996) 275.
[19] R.J. Linhardt, A. Pervin, J. Chromatogr. A 720 (1996) 323.
[20] J. Grimshaw, Electrophoresis 18 (1997) 2408.
[21] A. Hjerpe, C.A. Antonopoulos, B. Engfeldt, J. Chromatogr. 171 (1979) 339.
[22] A. Al-Hakim, R.J. Linhardt, Anal. Biochem. 195 (1991) 68.
[23] S. L Carney, D.J. Osborne, Anal. Biochem. 195 (1991) 132.
[24] R.J. Linhardt, P.M. Galliher, C.L. Cooney, Appl. Biochem. Biotechnol. 12 (1986) 135.
[25] T. Yamagata, H. Saito, O. Habuchi, S. Suzuki, J. Biol. Chem. 243 (1968) 1523.
[26] H. Saito, T. Yamagata, S. Suzuki, J. Biol. Chem. 243 (1968) 1536.
[27] M.W. McLean, J.S. Bruce, W.F. Long, F.B. Williamson, Eur. J. Biochem. 145 (1984) 607.
[28] K. Murata, Y. Yokoyama, K. Yoshida, J. Biochem. Biophys. Methods 15 (1987) 23.
[29] F. Lamari, J. Katsimpris, S. Gartaganis, N.K. Karamanos, J. Chromatogr. B 709 (1998) 173.
[30] N.K. Karamanos, S. Axelsson, P. Vanky, G.N. Tzanakakis, A. Hjerpe, J. Chromatogr. A 696 (1995) 295.
[31] J. Bao, F.E. Regnier, J. Chromatogr. 608 (1992) 217.
[32] D. Wu, F.E. Regnier, Anal. Chem. 65 (1993) 2029.
[33] B.J. Harmon, D.H. Patterson, F.H. Regnier, Anal. Chem. 65 (1993) 2655.
[34] Y. Xu, X. Liu, M.P.C. Ip, J. Liq. Chromatogr. Rel. Technol. 21 (1998) 2781.
[35] D.S. Zhao, F.A. Gomez, Chromatographia 44 (1997) 514.

[36] T. Watanabe, A. Yamamoto, S. Nagai, S. Terabe, Electrophoresis 19 (1998) 2331.
[37] H. Okamoto, T. Nakajima, Y. Ito, J. Chromatogr. A 986 (2003) 153.
[38] Y. Tanaka, M.N. Slyadnev, A. Hibara, M. Tokeshi, T. Kitamori, J. Chromatogr. A 894 (2000) 45.
[39] A.G. Hadd, S.C. Jacobson, J.M. Ramsey, Anal. Chem. 71 (1999) 5206.
[40] K. Sakai-Kato, M. Kato, T. Toyo'oka, Anal. Chem. 75 (2003) 388.
[41] J.R. Martins, M.E. Gadelha, S.M. Fonseca, L.O. Sampaio, P.A. Pontes, C.P. Dietrich, H.B. Nader, Otolaryngol. Head Neck Surg. 122 (2000) 115.
[42] A.D. Theocharis, M.E. Tsara, N. Papageorgacopoulou, D.D. Karavias, D.A. Theocharis, Biochim. Biophys. Acta 1502 (2000) 201.