Journal of Chromatographic Science 2012;50:373–379
doi:10.1093/chromsci/bms015 Advance Access publication March 5, 2012

Article

Capillary Electrophoresis for Simultaneous Analysis of Heparin, Chondroitin Sulfate and Hyaluronic Acid and its Application in Preparations and Synovial Fluid

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Received 19 May 2010; revised 20 April 2011

A simple and accurate capillary electrophoresis (CE) method was developed to simultaneously separate and quantify heparin, chondroitin sulfate and hyaluronic acid. The relative standard deviations (intra-day) of migration time, peak height and peak area for heparin, chondroitin sulfate and hyaluronic acid were lower than 1.11, 5.45 and 2.82%, respectively. The limits of detection of heparin, chondroitin sulfate and hyaluronic acid were 0.91, 0.12 and 9.04×10^{-3} mg/mL, respectively. The developed electrophoretic method was successfully applied to the analysis of commercial drug products and biological samples containing chondroitin sulfate and/or hyaluronic acid. The recoveries for chondroitin sulfate and hyaluronic acid were in the range of 95.9 ∼107.0%. This was the first time the content of hyaluronic acid in the synovial fluids from osteoarthritic rabbits was investigated by CE. The results suggested that hyaluronic acid in the synovial fluids from osteoarthritic rabbits may be further metabolized and the administration of chondroitin sulfate or hyaluronic acid could affect the content and metabolism of hyaluronic acid in the synovial fluids. The developed CE method was simple to implement without sample pretreatment such as depolymerisation, very repeatable and easily transferred from lab to lab.

Introduction

Glycosaminoglycan (GAG), which plays a variety of important roles in curing and preventing diseases, is one type of polysaccharide consisting of repeating disaccharide units of aminohexose and uronic acid. There are two major classes of GAG: (1) glucosaminoglycan, such as heparin, heparan sulfate, keratan sulfate and hyaluronic acid; (2) galactosaminoglycan, such as chondroitin sulfate and dermatan sulfate (1).

Heparin, a highly-sulfated GAG, has high negative charge density and a molecular weight range of 6,000 ∼ 20,000 (2). It is the first anticoagulant drug used for the treatment of blood clotting and cardiovascular diseases. It is also be used in cosmetics to improve subcutaneous microcirculation (2, 3). Chondroitin sulfate, extracted from cartilages of pigs, cattle or chicken, is one kind of sulfated GAG with a molecular weight range of 10,000 ∼ 50,000 (2). Chondroitin sulfate is used internationally for the treatment of arthritis; it does not cause toxicity or side-effects after long-term administration. Hyaluronic acid, also called hyaluronan, is an anionic and non-sulfated acidic GAG. Its molecular weight is very large, ranging from 200,000 to 7,000,000 (2). Hyaluronic acid is a homogeneous polymer that is generally used as viscoelastic agents, eye drops in pharmaceuticals and synovial fluids in the clinic (2). The structures of heparin, chondroitin sulfate and hyaluronic acid are shown in Figure 1. Currently, chondroitin sulfate and hyaluronic acid have been turned into a pharmaceutical product (Runjie Eye Drops) to alleviate eye fatigue. It is necessary to develop an analytical method that can separate these GAGs at the same time for quality control.

Many methods have been developed to analyze these complex polymers. Cellulose acetate membrane electrophoresis, which permits visualization by Alcian blue staining, was first used for the separation of different GAGs (4). Another method, polyacrylamide gel electrophoresis, has been proposed that made a greatly improved resolution of these compounds, often resulting in discrete banding of GAGs (5). However, both cellulose acetate membrane electrophoresis and polyacrylamide gel electrophoresis have relatively poor resolution capacity for structurally similar GAGs. Capillary electrophoresis (CE) has a high resolution capacity and its separation mechanism is based on molecular charge-to-size ratios, which is particularly useful for the analysis of polyanions. At present, many CE methods for GAG analysis just deal with the small oligosaccharides derived from chemical and/or enzymatic degradation of heparin, chondroitin sulfate or hyaluronic acid (6–12). These methods are rather laborious and difficult to use widely. However, for quality control of the drug substances, analysis of intact GAG is preferred.

To date, the CE method has already been used for the analysis of intact GAGs (13–15). Somsen *et al.* (15) analyzed the impurities (oversulfated chondroitin sulfate) and dermatan sulfate) in intact heparin by CE using *Tris* buffer with good results. There are two primary difficulties for the analysis of the intact GAGs. First, the GAG polymers have no characteristic maximum absorption; rather, they are detected by end absorption due to the *N*-acetyl function at 200 nm (16). The interference of background at this wavelength is large, resulting in a lower sensitivity. A large volume injection technique is usually adopted to improve the limit of detection (15). Secondly, the GAGs are polydisperse mixtures that have a wide range of molecular weights due to their heterogeneous nature, resulting in relatively broad peaks (15, 17, 18), which complicates the separation of the structurally similar GAGs.

In this study, we developed a CE method to analyze intact heparin, chondroitin sulfate and hyaluronic acid simultaneously without sample pretreatment such as derivatization, chemical degradation or enzymatic digestion. This work primarily focused on the applications of analyzing GAGs in commercial

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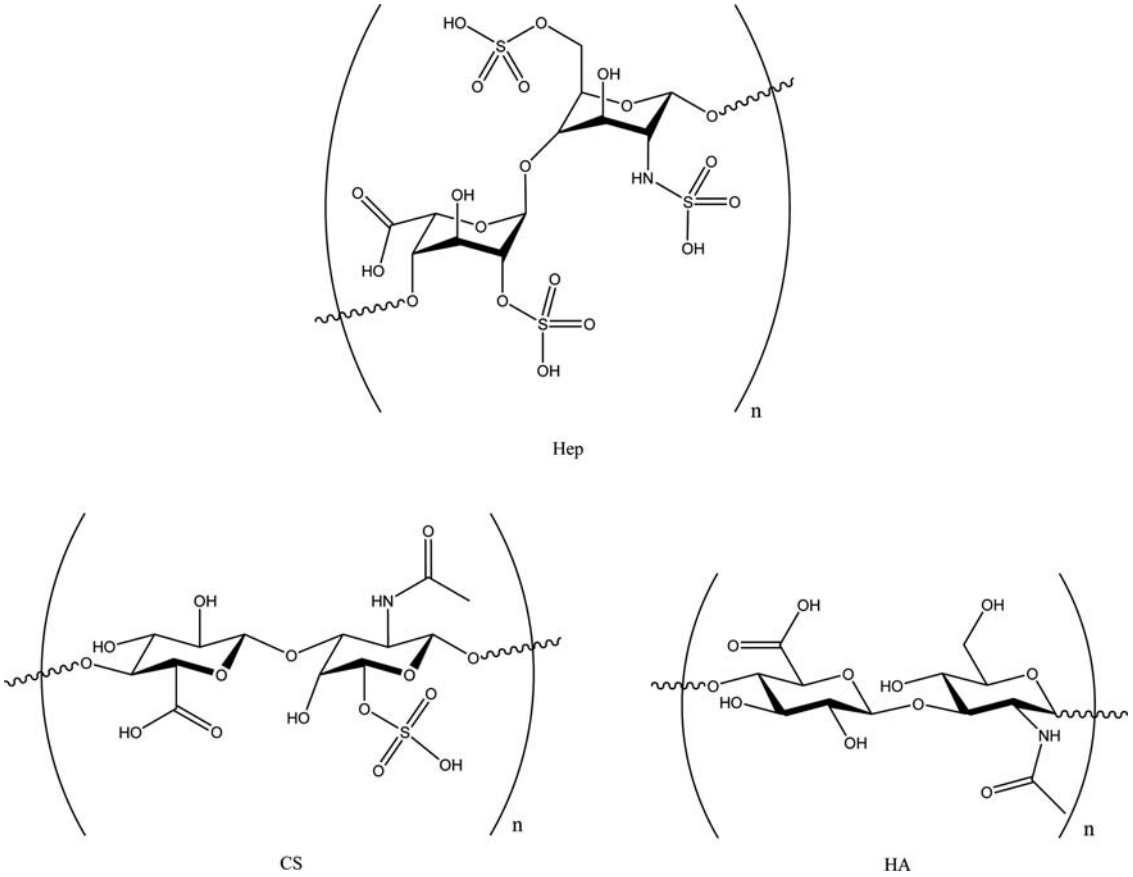

Figure 1. The structures of heparin (Hep), chondroitin sulfate (CS) and hyaluronic acid (HA).

drug products and synovial fluids. The content of hyaluronic acid in synovial fluids from osteoarthritic rabbits was investigated, which is significant for diagnosing the disease state of osteoarthritis. The orthogonal design was used for optimizing the separation conditions. The investigated variables included pH and concentration of background electrolyte, applied voltage, capillary temperature and injection volume. Several parameters, such as repeatability, linearity and limit of detection, were employed to validate the developed method.

Experimental

Materials

Standard sodium heparin and oversulfated chondroitin sulphate-contaminated heparin were donated by Zaozhuang Sainuokang Biochemistry Co. (Zaozhuang, China). Chondroitin sulfate from shark cartilage was purchased from Chongqing Imperial Bio-Chemistry Co. (Chongqing, China). Hyaluronic acid was a gift from Shandong Freda Biochemistry Co. (Jinan, China). Phosphoric acid (85%) and sodium hydroxide were from Tianjin Second Reagent Factory (Tianjin, China). *Tris*, of analytical grade, was purchased from Shanghai Biochemistry Reagent Co. (Shanghai, China). Rellet Biologic Intelligent Lock-Moisture Lotion (RBILL) and Chondroitin Sulfate Eye Drops (Runjie Eye Drops) (RJED) commercial products were purchased from Shandong Freda Biochemistry Co. (Jinan, China). Physiological saline was from Lukang Pharmaceutical Co. (Jining, China). Papain and cysteine hydrochloride were from Shanghai Bioengineering(Shanghai, China). Adult New Zealand white rabbits (weighing approximately $2 \sim 3$ kg, of either sex) were obtained from the Shandong Institute Pharmaceutical Industry [License, SCXK (Lu), 20050017]. Synovial fluid was obtained from rabbit knees.

Apparatus

CE was performed on a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA) equipped with a photodiode array detector (PDA) (Beckman Coulter) operating at 200 nm (bandwidth, 10 nm). Samples were introduced from the cathode and detected at the anode under acidic conditions. Fused-silica capillary of 50 μm internal diameter (i.d.) with a total length of 60 cm (50 cm effective length) was from Yongnian Optical Fiber Factory (Baoding, China). The new capillary was first rinsed at 50 psi using the following reagents in sequence: water purified with a Milli-Q system (Millipore, Bedford, MA) for 10 min, 0.1M NaOH for 10 min and then purified water for 10 min followed by the running buffer for 10 min. Between runs, the capillary was washed at 50 psi with purified water (2 min), 0.1M NaOH (3 min), purified water (2 min) and fresh background electrolyte (3 min). The capillary was thermostated by liquid cooling. Electropherograms were acquired and analyzed using 32 Karat 7.0 software (Beckman Coulter).

Preparation of reagents and samples

The background electrolytes were prepared by dissolving *Tris* into the desired concentration and the pH was adjusted to 3.0 with phosphoric acid. Standard stock solutions of 50 mg/mL heparin, 60 mg/mL chondroitin sulfate and 4 mg/mL hyaluronic acid were prepared with purified water. Working standard solutions were then obtained by diluting the corresponding stock solutions to desired concentrations with purified water. Samples for RBILL and RJED were diluted with purified water to desired concentrations. The 2% (w/w) chondroitin sulfate and 1% (w/w) hyaluronic acid were prepared with physiological saline. Prior to analysis, all of the solutions were degassed using ultrasound and filtered through 0.45-μm cellulose acetate membrane filters.

Preparation of biological samples

Experimental rabbits were randomly divided into four groups: (1) healthy group ($n = 7$), (2) physiological saline group ($n = 7$), (3) hyaluronic acid group ($n = 7$) and (4) chondroitin sulfate group ($n = 7$). For the later three groups, 0.3 mL papain solution (1 mL physiological saline + 4 mg papain + 50 mg cysteine hydrochloride, was filtered through a 0.22-μm membrane filter under aseptic condition) was injected intra-articularly into knee joints of healthy rabbits to destroy the joint cartilage for constructing the osteoarthritic model (19). On the seventh day after inducing osteoarthritis, 0.3 mL physiological saline, 2% (w/w) chondroitin sulfate and 1% (w/w) hyaluronic acid were injected intra-articularly into knee joints of rabbits at the same place for physiological saline group, hyaluronic acid group and chondroitin sulfate group once every week for 5 weeks, respectively. On the seventh day after the last administration of the drug, 0.3 mL synovial fluids of rabbits were taken out and centrifuged at 5,000 r/min for 30 min. The supernatant was diluted to one tenth with physiological saline for CE analysis.

Results and Discussion

Optimization of the separation conditions

Orthogonal design was used to optimize the separation conditions. The investigated factors included the buffer pH and concentration (C_{Tris}), applied voltage (V), capillary temperature (T), injection time (t) and injection pressure (P) at five levels. The orthogonal design table containing six factors and five levels is shown in Table I. A total of 25 experiments were involved in the orthogonal design matrix. *Tris* was used as buffer in this work because it can be used to increase the ionic strength while providing low conductivity and permits a high buffer concentration. Because the separation of heparin and chondroitin sulfate was difficult and the migration time of hyaluronic acid was lengthy, the resolution of heparin and chondroitin sulfate ($R_{Hep/CS}$), the migration time of hyaluronic acid (t_{HA}), the peak areas and peak shapes of the analytes were used as the chief optimization indexes. The mixed standard solution for optimization of the separation conditions included 20 mg/mL heparin, 10 mg/mL chondroitin sulfate and 1 mg/mL hyaluronic acid. The electric currents in Trials 4 and 5 were too large to work. The $R_{Hep/CS}$, t_{HA} and capillary currents observed during optimization (I) of other 23 trials are shown in Figure 2. As shown in Figure 2, Trial 15 obtained high $R_{Hep/CS}$, low t_{HA} and good peak shape. The optimum separation conditions were 600 mM *Tris* (pH 3.0), −20 kV separation voltage (reverse polarity, ramping time of 0.17 min), 35°C capillary temperature and injection for 12 s at 1.0 psi. Under the optimum conditions, the elution order of the analytes was heparin, chondroitin sulfate and hyaluronic acid.

Table I
The Orthogonal Design Table Containing Six Factors and Five Levels, L_{25} (5^6)

Exp.	pH	C_{Tris} (mM)	V (kv)	T (°C)	t (s)	P (psi)
1	2.0	200	19	20	12	0.3
2	2.0	300	20	25	16	0.5
3	2.0	400	21	30	20	1.0
4	2.0	500	22	35	24	1.5
5	2.0	600	23	40	28	2.0
6	2.5	200	20	30	24	2.0
7	2.5	300	21	35	28	0.3
8	2.5	400	22	40	12	0.5
9	2.5	500	23	20	16	1.0
10	2.5	600	19	25	20	1.5
11	3.0	200	21	40	16	1.5
12	3.0	300	22	20	20	2.0
13	3.0	400	23	25	24	0.3
14	3.0	500	19	30	28	0.5
15	3.0	600	20	35	12	1.0
16	3.5	200	22	25	28	1.0
17	3.5	300	23	30	12	1.5
18	3.5	400	19	40	16	2.0
19	3.5	500	20	40	20	0.3
20	3.5	600	21	20	24	1.0
21	4.0	200	23	35	20	0.5
22	4.0	300	19	40	24	1.0
23	4.0	400	20	20	28	1.5
24	4.0	500	21	25	12	2.0
25	4.0	600	22	30	16	0.3

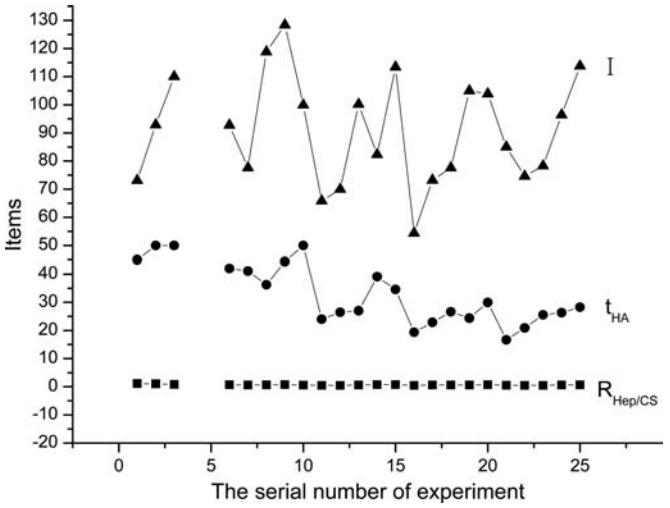

Figure 2. The $R_{Hep/CS}$, t_{HA} and capillary currents (I) of 23 entries.

Figure 3A shows the electropherogram of a mixed standard solution obtained under optimum separation conditions. The first two wide peaks were heparin and chondroitin sulphate, respectively, due to their high heterogeneities. The average number of sulfate groups per repeating unit of disaccharides for heparin was bigger than that for chondroitin sulfate, so heparin migrated faster than chondroitin sulfate. The last and

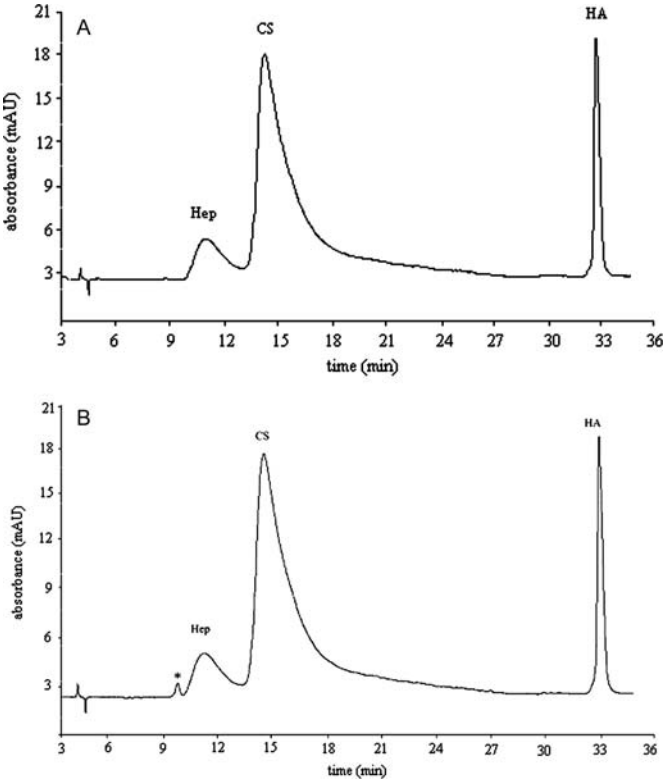

Figure 3. Electropherograms of standard mixtures: standard heparin (hep), chondroitin sulfate (CS) and hyaluronic acid (HA) (A); OSCS contaminated hep, CS and HA (B); OSCS peak (*); Sample concentrations: 20 mg/mL hep, 10 mg/mL CS and 1mg/mL HA; Experimental conditions: buffer, 600 mM *Tris* phosphate (pH 3.0); capillary, uncoated fused-silica capillaries of 50 μm i.d. and 60 cm total length (50 cm effective length); capillary temperature, 35°C; sample injection, 12 s at 1.0 psi; voltage, −20 kV; voltage ramping time, 0.17 min.

sharpest peak was assigned to hyaluronic acid because of its homogeneous property and low charge-to-mass ratio.

This study showed that a higher background electrolyte concentration and a lower pH might provide better separation for heparin and chondroitin sulfate. Furthermore, a higher background electrolyte concentration can reduce band broadening by electromigration dispersion (16). Although a capillary with an i.d. of 25 μm reduced the current because its big specific surface area dissipated Joule heat quickly, a capillary with a narrow inner diameter led to difficulty in detection and limited sample loading. As expected, a low pH and a high background electrolyte concentration had a relatively low electrophoretic mobility, resulting in a longer migration time. Increasing the temperature decreased the analysis time with a higher current and without the peak shape change, but resulted in a bad resolution of heparin and chondroitin sulfate. Detection sensitivity could be improved if relatively high concentrations and sample volumes were injected.

Method performance

The developed CE-UV system was further evaluated by testing the repeatability, linearity and limit of detection (LOD). The standard sample containing 20 mg/mL heparin, 10 mg/mL chondroitin sulfate and 1 mg/mL hyaluronic acid was repeatedly injected (12 s at 1.0 psi, $n = 5$) and analyzed. The results are summarized in Table II. Good linearity [correlation coefficient (R) > 0.9987] and high precision [relative standard deviation (RSD) < 7.64%] were obtained. The LODs based on a 3-to-1 signal-to-background noise ratio were as low as 1.20×10^{-1} mg/mL for chondroitin sulfate and 9.04×10^{-3} mg/mL for hyaluronic acid, respectively.

Table II
Analytical Performance of CE for Heparin, Chondroitin Sulfate and Hyaluronic Acid ($n = 5$)

		Heparin	Chondroitin Sulfate	Hyaluronic Acid
LOD (S/N = 3) (mg/mL)		0.91	0.12	9.04×10^{-3}
Migration time RSD (%)	intra-day	0.92	0.96	1.11
	inter-day	1.18	1.05	1.26
Peak height RSD (%)	intra-day	4.50	5.45	4.60
	inter-day	5.84	5.79	6.29
Peak area RSD (%)	intra-day	1.38	2.82	1.56
	inter-day	2.70	2.85	3.78
Peak width RSD (%)	intra-day	2.50	3.42	3.95
	inter-day	3.58	7.38	7.64
Regression equation[1]				
a		7645	59583	163641
b		11508	4608	486
Correlation coefficient		0.9987	0.9993	0.9997
Linear range(mg/mL)		4.0–40	0.5–50	0.02–3.0

[1] y = a+b x; y, peak area; x, standard concentration (mg/mL).

Table III
Determination Results of Chondroitin Sulfate and Hyaluronic Acid in Commercial Products and Biological Samples ($n = 4$)

Preparations and samples	Ingredient	Found content (mg/mL)	Recovery (%)	Average (%)
RJED	chondroitin sulfate (Labeled content: 1mg/mL)	1.05	105.2 (1 mg)[a] 106.0 (10.8 mg) 109.9 (20 mg)	107.0
	Hyaluronic acid	1.21	102.4 (0.5 mg) 96.0 (1 mg) 103.3 (1.5 mg)	100.6
RBILL	Hyaluronic acid	12.67	94.3 (1.0 mg) 96.2 (1.5 mg) 104.2 (2.0 mg)	98.2
SF[b] (No.1)	Hyaluronic acid	0.69	97.1 (0.5 mg)	95.9
SF (No.2)	Hyaluronic acid	0.66	93.8 (1.0 mg)	
SF (No.3)	Hyaluronic acid	0.75	96.9 (1.5 mg)	

[a] The amounts added of standard substances.
[b] synovial fluid.

Serious adverse reactions to oversulfated chondroitin sulfate (OSCS)-contaminated heparin were first discovered in 2008. To investigate the applicability of the developed method for the analysis of OSCS-contaminated heparin sample, the heparin in the standard mixture was replaced by OSCS-contaminated heparin. Figure 3B shows the CE-ultraviolet (UV) analysis for OSCS-contaminated heparin, chondroitin sulfate and hyaluronic acid. In this figure, a new peak was detected before the peak of heparin and a baseline separation was achieved between the new peak and heparin. The new peak might be the OSCS peak, according to the reference (16). Because OSCS standard was not available, the qualitative and quantitative analysis of OSCS will be studied in the future. The results indicate that the developed method may be used for the determination of OSCS contaminant in heparin.

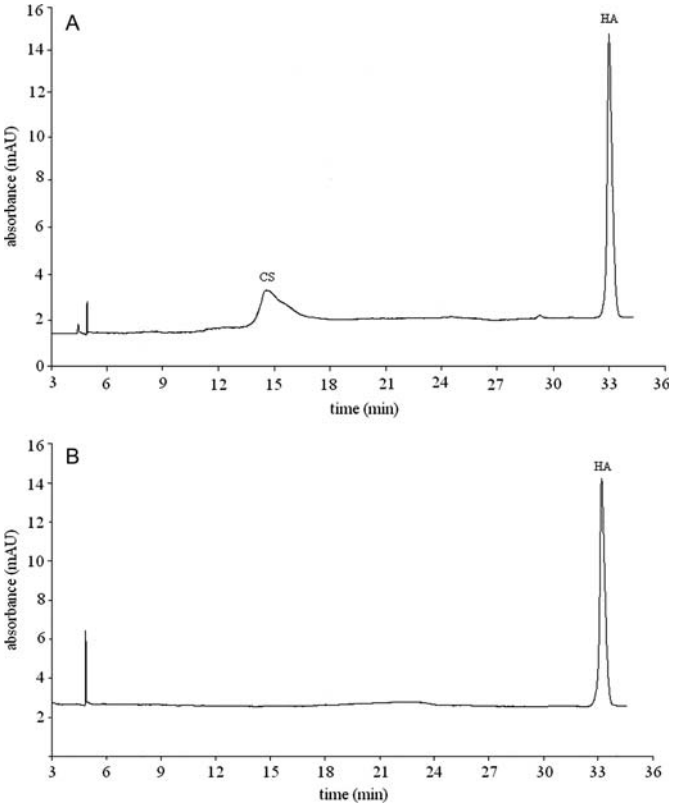

Figure 4. Electropherograms of commercial products containing chondroitin sulfate (CS) and hyaluronic acid (HA): RJED (A); RBILL (B); the experimental conditions were shown in Figure 3.

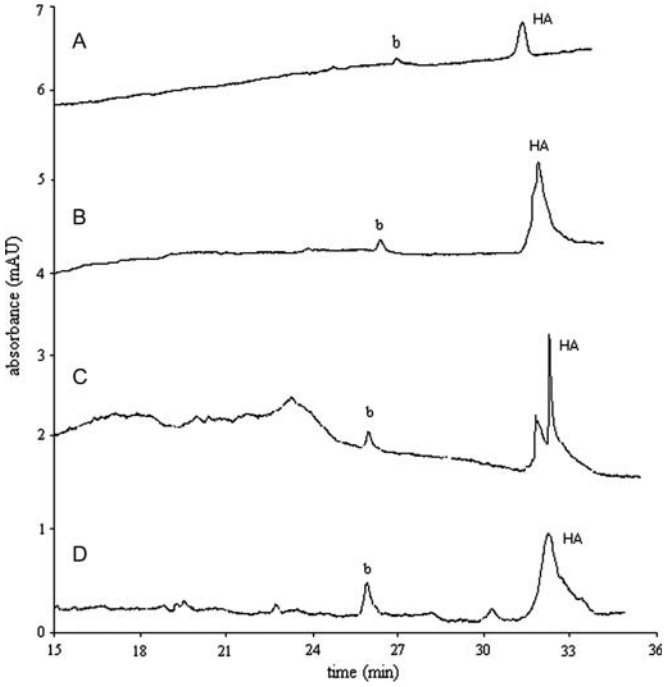

Figure 5. Electropherograms of synovial fluid samples: synovial fluid from healthy rabbits (A); synovial fluid from osteoarthritic rabbits administrated with physiological saline (B); synovial fluid from osteoarthritic rabbits administrated with chondroitin sulphate (C); synovial fluid from osteoarthritic rabbits administrated with hyaluronic acid (D); The experimental conditions were shown in Figure 3B, unknown component.

Application

Analysis of commercial preparations

The CE-UV method was applied to analyze commercial products RBILL and RJED. The contents of analytes found in different preparations are given in Table III. Figures 4A and 4B show the typical electropherograms of RJED and RBILL. The standard addition method was used to identify the peaks. The accuracy of the method and the potential matrix effects were established by analyzing the spike samples. The standard substance amounts added and the results are shown in Table III. The recoveries of the spiked analytes for these products were satisfactory, and the method was applicable for the quantification of chondroitin sulfate and hyaluronic acid with high accuracy, precision and repeatability.

Analysis of biological samples

Hyaluronic acid is a major constituent of synovial fluid, and the level of hyaluronic acid in the synovial fluid shows a high correlation with the disease state of the joint (20). At present, chondroitin sulfate and hyaluronic acid have been widely used for treatment of osteoarthritic disease. Thus, an effort has been made to define the changes of these polymers, which occurs with the onset of arthritic disease (8). In this study, hyaluronic acids in the synovial fluids from healthy and osteoarthritic rabbits were determined by the CE method. The qualitative analysis of hyaluronic acid was determined by the standard addition method. The results are shown in Table III. A typical electropherogram of the synovial fluid from a healthy rabbit is shown in Figure 5A. The figure shows that the matrix in the synovial fluid did not affect the determination of hyaluronic acid; the content of hyaluronic acid in the synovial fluid of healthy rabbit was approximately 0.70 mg/mL. After constructing the osteoarthritic model, the synovial fluid samples administrated with different drugs were analyzed. The results are shown in Figures 5B–D. The electropherogram (Figure 5B) shows that hyaluronic acid in the synovial fluid from osteoarthritic rabbits may be further metabolized (21) and become more heterogeneous, resulting in peak shape deterioration of hyaluronic acid. The content of hyaluronic acid in the synovial fluid from osteoarthritic rabbit administrated with physiological saline was approximately 0.53 mg/mL. An electropherogram of the synovial fluid from an osteoarthritic rabbit after administration of chondroitin sulfate standard is shown in Figure 5C. Although chondroitin sulfate can accelerate the production of new cartilage and improve the capability for producing hyaluronic acid, the neonatal hyaluronic acid and original hyaluronic acid may have large differences in mass-to-charge ratio, resulting in a bifurcation peak of hyaluronic acid, which leads to the imprecise quantification of hyaluronic acid in synovial fluid. The content of hyaluronic acid in the synovial fluid from an osteoarthritic rabbit administrated with chondroitin sulfate was approximately 1.26 mg/mL. The CE analysis of the synovial fluid from an osteoarthritic rabbit administrated with hyaluronic acid (Figure 5D) showed that the injection of hyaluronic acid may decrease the metabolism of hyaluronic acid

Table IV
The Comparison of Contents of Hyaluronic Acid in Normal Rabbit Synovial Fluids Obtained by Different Methods

Sample	Method	Content ($\bar{x} \pm s$, μg/mL)	Reference
Knee joint synovial fluid from male Nihon white rabbit	HPLC(detection, refractive index)	121.9 ± 21.8	[22]
Knee joint synovial fluid from male New Zealand white rabbit	enzyme linked immunosorbent assay (ELISA)	401.28 ± 67.82	[23]
	radioimmunology	242 ± 46	[24]
	CE (wavelength of detection, 200 nm)	700 ± 46	

and increase the content of hyaluronic acid in synovial fluid (0.97mg/mL), resulting in a relatively better peak shape. Compared with the electropherogram in Figure 4, the peak shape of hyaluronic acid in synovial fluid became broader, and there was a migration time difference for hyaluronic acid in Figures 4 and 5, which may be a result of the interaction between matrix and hyaluronic acid in synovial fluid (12) or the metabolism of hyaluronic acid in vivo. Compared with the electropherogram in Figure 5A, an unknown component (b) eluted at 26 min in Figure 5 (B–D) is present in a greater amount in the fluid from osteoarthritic joints than in the healthy control samples, which may be associated with osteoarthritic disease. This study will be further evaluated in the next work.

Table IV shows the contents of hyaluronic acid in normal rabbit synovial fluids obtained by different methods. The results suggest that there is a large difference in the content of hyaluronic acid among different methods and different samples. The content of hyaluronic acid obtained by CE was higher than those from other methods, which may be the result of the background interference (wavelength of detection, 200 nm), resulting in an imprecise quantification of hyaluronic acid. However, the background interference has little effect on the investigation of the change in content of hyaluronic acid in synovial fluid.

Conclusions

In this work, a CE method was developed for simultaneous analysis of heparin, chondroitin sulfate and hyaluronic acid, simultaneously enhancing quality control of three analytes for corporations. The developed CE method was also suitable to analyze OSCS in heparin samples. The optimum separation conditions were obtained by orthogonal design. The synovial fluids were investigated by CE for the first time after administration of chondroitin sulfate and hyaluronic acid into the knee joints of osteoarthritic rabbits. The results suggested that the hyaluronic acid in synovial fluid from osteoarthritic rabbits may be metabolized and the administration of chondroitin sulfate or hyaluronic acid can affect the content and metabolic profile of hyaluronic acid in synovial fluid. Although the method developed by Grimshaw *et al.* (12) obtained a high accuracy for the analysis of hyaluronic acid after hyaluronidase treatment, their method procedures were complicated. Our developed CE method was very repeatable, simple to implement without sample pretreatment such as depolymerization and easily transferred from lab to lab. Conclusively, the presented methodology showed a good potential to be used for quality control of heparin, chondroitin sulfate and hyaluronic acid or their mixtures.

Acknowledgments

We are grateful for financial support from the Postdoctoral Innovation Fund of Shandong Province (No. 200802035).

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