Original Article

The matrix of SDS integrated with linear hydrophilic polymer for resolution of high- and low-molecular weight hyaluronic acids in MEKC

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ARTICLE INFO

ABSTRACT

Hyaluronic acid (HA), a multi-functional material, has a high dispersion in molecular weight, and the functions of HA are determined through the size. Nevertheless, hyaluronic acid mixtures are not easily separated due to their polydispersity. In this study, a capillary electrophoresis strategy was developed for resolution of different molecular-weight HA without enzymatic digestion. Here, hyaluronic acid mixtures with low molecular weight (380 kD; LHA) and high molecular weight (2180 kD; HHA) were successfully resolved by the SDS integrated with low molecular-weight polymer in capillary electrophoresis. By optimizing experimental conditions, the separation of LHA and HHA was completed within 14 min. The optimal conditions were as follows: the running buffer was 25 mM borate buffer (pH 9.75) containing 30 mM SDS and 10% polyethylene glycol (MW: 8000); applied voltage was 20 kV (detector at cathode side) and separation temperature was set at 25 °C. The data of method validation showed that calibration plots were linear ($r \geq 0.9977$) over a range of 10–50 μg/mL for LHA, and 40–200 μg/mL for HHA. In the evaluation of precision and accuracy for this method, the RSD and RE values were all less than 4.2%. This fascinating technique was successfully applied to the quality control of cosmetic and pharmaceutical containing different ratios of LHA and HHA, and it was feasible for serving as a tool to quantitatively analyze different sizes of HA for clinical survey.

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1. Introduction

Hyaluronic acid (HA) is a linear polysaccharide composing of a repeated disaccharide unit of β-1,4 linked D-glucuronic acid (GlcA) and β-1,3 linked N-acetyl-D-glucosamine (GlcNAc). Due to the random repeated disaccharide unit, HA has a high dispersion in molecular weight, and the functions of HA are determined through the size. In biology, HA is manufactured by fibroblasts or other specialized connective tissue cells and implicated in a variety of cellular processes such as migration, adhesion, tumor metastasis, cell morphogenesis and differentiation, inflammation, and wound repair etc [1,2]. In circulation, the HA levels are adjusted by an efficient receptor-dependent removal mechanism present in sinusoidal endothelial cells of the liver and by the enzymatic action of hyaluronidase [3]. The increased amount of HA in the connective tissue surrounding the joints reflects various inflammatory conditions, and thus can be used to monitor joint condition [4]. Various liver diseases may also increase serum levels of HA, due to decreased hepatic removal and/or increased hepatic production of HA during liver inflammation [5,6]. Additionally, HA could also serve as a useful tumor marker [7] and a wide variety of medical applications of HA ranging from osteoarthritis to vascular disease has been demonstrated [8].

Recently, HA has even been utilized as an excipient in pharmaceutics to increase drug stability and bioavailability [9]. It was used as a carrier or coating agent in the formulation to increase the drug bioavailability, due to the possibility to target cells overexpressing HA receptors, such as CD44 which is abundantly presented in various cancer cells [10]. Lee et al. utilized the dopa—HA conjugated calcium phosphate nanoparticles to stabilize the siRNA for targeting cancer cells [11]. Kechai et al. used the mixture of HA and liposomes for drug delivery [12]. Additionally, in order to understand the HA degradation, the activity of the hydrolysis enzyme, hyaluronidase, was also investigated [13]. However, the molecular weights of the HA utilized in these researches were different, and Almalik et al. demonstrated the molecular weights of HA coated on the nanoparticles would affect the stability of the formulation, the sensitivity of the target cells and the mobility of the HA-coated nanoparticles [14]. Therefore, it is very important for the identification of the molecular weights of the HA in the field of drug delivery. A lot of techniques were developed for the separation of HA with other materials, including fluorophore-assisted carbohydrate electrophoresis (FACE) [15,16], solid-phase assay [17], enzyme-linked immunosorbent assay (ELISA) [18,19], radioimmunoassay [20], high-performance liquid chromatography (HPLC) [21] or capillary electrophoresis (CE) [22—24]. However, all of the above techniques were developed for separation of one kind of HA from other materials. Although Hayase et al. tried to resolve three kinds of HA with different molecular weights, the method only could be applied to identify one kind of HA in once run [24]. If the three kinds of HA were mixed, they could not be separated simultaneously. Because previous techniques could not be applied to simultaneously recognize more than two kinds of HA with different molecular weights, thus, it is important to develop a technique to resolve the above problems.

Over the last two decades, CE has become a powerful analytical technique and applied to several fields due to its high-efficiency separation, low sample consumption, good resolution and its minimal solvent requirements. These advantages make CE become the trend for analysis of large or small molecules. However, poor sensitivity in CE is the major disadvantage because of the short optical path and small sample loading. Therefore, a lot of on-line sample stacking techniques had been established to improve the sensitivity, such as field-amplified sample injection (FASI) [25], large volume sample stacking (LVSS) [26] or micellar electrokinetic chromatography (MEKC) [27—29]. Among those techniques, MEKC, first introduced by Terabe et al., is one of the most well-known CE separation mode and could provide a great sensitivity enhancement [28—30]. In MEKC, various types of surfactants forming micelles as pseudostationary phases are utilized to interact with ionic or neutral substances for separation based on the different partitioning capacity. Nevertheless, only surfactant as the additive in MEKC may not be easy for resolution of analytes. Thus, in this study, a modified MEKC through the matrix of SDS integrated with low molecular-weight polymer was presented for resolution of low molecular-weight and high molecular-weight HA. The mechanism was shown in Fig. 1. First, the matrix of SDS integrated with polyethylene glycol (PEG, M.W. 8000) was prepared and injected into the capillary where the SDS micelles and sieving matrix of PEG would be formed. Subsequently, the mixture of low molecular-weight and high molecular-weight HA was hydrodynamically introduced into capillary. The low molecular-weight and high molecular-weight HA could be separated by the selective interaction with the SDS micelles, and the sieving networks of PEG polymer in the normal polarity. This separation technique was the first strategy for determination of different molecular-weights HA without enzyme digestion by using capillary electrophoresis. That is hoped to serve as a tool used to quantitatively analyze different sizes of HA for clinical survey.

2. Methods

2.1. Chemicals and drugs

All chemicals used were analytical-reagent grade. The low-density hyaluronic acid (LHA, 380 kD) and high-density hyaluronic acid (HHA, 2180 kD) were kindly provided from the BLOOMAGE FREDA BIOPHARM CO (Tianjin, China). Sulindac (IS) and linear polymer (polyethylene glycol, PEG, M.W. 8000) were purchased from Sigma—Aldrich (Sigma, St. Louis, MO, USA). Sodium tetraborate, phosphate (Na_2HPO_4), methanol (MeOH), sodium dodecyl sulfate (SDS), HCl and NaOH were purchased from Merck (Merck, Darmstadt, Germany). Milli-Q water (Millipore, Bedford, MA, USA) was used for preparation of the buffer and other aqueous solutions.

2.2. Sample preparation

Stock solutions (10 mg/mL) of LHA and HHA were dissolved in the phosphate buffer solution (PBS, pH 6.8) which was prepared according to the procedures in USP. Subsequently, the
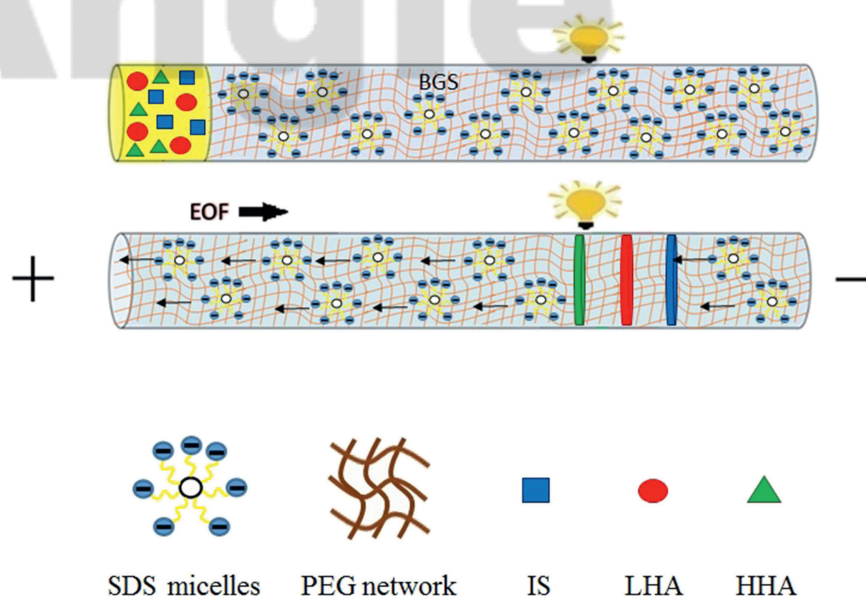
Fig. 1 — The diagrams of MEKC mode for resolution of LHA and HHA in the use of SDS micelles and PEG sieving matrix.

solutions of LHA and HHA were stirred at room temperature for 1 h in the speed of 1000 rpm, and appropriately diluted with PBS buffer (pH 6.8) for further utilization. The stock solution of sulindac (1 mg/mL) used as the internal standard (IS) was prepared in methanol and appropriately diluted with PBS buffer (pH 6.8) for further use. The calibration curves were established by mixing identical concentrations of LHA, HHA, and 5 μg/mL of IS. The ranges of calibration curves for LHA were over 10—50 μg/mL and HHA over 40—200 μg/mL.

2.3. The MEKC system

The CE separation of LHA and HHA was performed on the Beckman P/ACE MDQ system (Fullerton, CA, USA) equipped with UV detector and in an uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm internal diameter and 40 cm effective length (total length of 50.2 cm). The detector wavelength was set at 200 nm. The temperature was maintained at 25 °C. The matrix of SDS integrated with PEG (MW: 8000) was prepared by using 25 mM borate ($Na_2B_4O_7$, pH 9.75) containing 30 mM SDS and 10% PEG. Before CE separation, the matrix was filled in the capillary, and then the analytes were hydrodynamically injected into the capillary at 10 psi for 20 s. The CE separation was carried out at 20 kV in the normal polarity. Between runs, the capillary was washed with MeOH at 30 psi for 5 min, deionized water (dd-water) at 30 psi for 5 min, and then the matrix of SDS integrated with PEG at 30 psi for 10 min.

2.4. Calibration and method validation

Calibration curves were established by diluting the stock solution of LHA over a range of 10—50 μg/mL and HHA over a range of 40—200 μg/mL, Accuracy and precision were determined by using three different concentrations of 15, 25 and 45 μg/mL for LHA, and 50, 100 and 150 μg/mL for HHA in the successive 3 runs. The repeatability was evaluated by the RSD values of migration time and peak area in the successive 3 runs.

2.5. Pretreatment of cosmetic and pharmaceutical samples

The identical amount of LHA and HHA were mixed with the cosmetic matrix (olivem® 1000). Then, the 1 mL cosmetic mixture was collected and dissolved in the phosphate buffer solution (PBS, pH 6.8) by stirring at room temperature for 1 h in the speed of 1000 rpm to make 10 mL. The solution was centrifuged at 8000 rpm for 10 min, and then the supernatant was collected and filtered through 0.45 μm membrane. Finally, the filtrate was properly diluted and analyzed by using this MEKC method. The concentration of LHA and HHA were made to 20 μg/mL and 80 μg/mL for CE analysis, respectively.

The powder of LHA and HHA were weighed and homogenously mixed with the formulation excipient, including corn starch, lactose and magnesium stearate. Subsequently, the powder was dissolved in the phosphate buffer solution (PBS, pH 6.8) by stirring at room temperature for 1 h in the speed of 1000 rpm for dissolution. Finally, after centrifuged at 8000 rpm for 10 min and filtered through 0.45 μm membrane, the solution was properly diluted and analyzed by using this MEKC method. The concentration of LHA and HHA were made to 20 μg/mL and 80 μg/mL, respectively.

3. Results

3.1. Optimization of MEKC conditions

In this study, the matrix of SDS integrated with PEG was utilized for first resolution of different molecular-weight HA in MEKC. In order to obtain the optimal condition for resolution

of LHA and HHA, a lot of CE conditions were evaluated, including buffer pH values, buffer concentration, the content of PEG polymer and the concentration of SDS. In order to know the separation efficiency, the value of resolution calculated from equation (1) between LHA and HHA was utilized. The equation was as followed:

$$Rs = 2(t_B\text{-}t_A)/W_A + W_B \tag{1}$$

Controlling pH value not only affects the EOF but also changes the charge of the HA that would improve the interaction between HA and the SDS micelles. In order to maintain the ionic mobility of HA, four basic pH values of the 25 mM borate buffer (9.25, 9.50, 9.75, and 10.00) containing 10% PEG and 30 mM SDS were chosen to investigate. The data was shown in Fig. S1. When the pH value was lower than 9.25, the LHA and HHA could not be separated (Rs = 0). Nevertheless, LHA and HHA could be well-separated in the pH value higher than 9.50 (Rs = 0.93 at pH 9.50; Rs = 1.19 at pH 9.75; Rs = 1.07 at pH 10.00). That was supported the EOF under pH 9.25 was smaller than that under 9.5, and the EOF force against the migration of the SDS micelles would affect the SDS micelles to separate LHA and HHA. Because the Rs of LHA and HHA at pH 9.75 was the highest, thus, the separation of LHA and HHA was performed at pH 9.75. The LHA and HHA were dissolved in the phosphate buffer solution (PBS, pH 6.8) closed to neutral just for dissolution. However, those were separated at high pH value (pH 9.75) in this MEKC strategy because the high EOF against the migration of the SDS micelles would be helpful for resolution of LHA and HHA.

When raising the buffer concentration, the ionic strength would increase and electrical double layer would be compressed and results in a smaller EOF. However, the change of EOF and ionic strength would affect the separation of LHA and HHA. Therefore, the concentrations of borate (Na$_2$B$_4$O$_7$) buffer was investigated from 20 to 35 mM. When 20 mM borate buffer were used, the LHA and HHA would not be base-line separated (Rs = 0.86; as shown in Fig. S2). As soon as the concentration is higher than 25 mM, the LHA and HHA could be resolved (Rs = 1.21 in 25 mM borate; Rs = 1.17 in 30 mM borate; Rs = 1.27 in 35 mM borate). However, the peak of LHA would be interfered with the peak of IS in 30 and 35 mM borate solution. That was supported the higher concentration buffer would decrease the EOF resulting in the slow migration of IS which would interfere with the separation of LHA. Therefore, 25 mM borate was chosen as the optimal condition for resolution of LHA and HHA.

Polymer utilized in CE system could not only adsorb on the surface of capillary to reduce the EOF but also form the network for sieving large molecules. In this study, a linear polymer, PEG (M.W. 8000), was utilized to form the network for separation of LHA and HHA. The effect of the PEG content in the SDS matrix from 8 to 11% was evaluated (Rs = 0.82 in 8% PEG; Rs = 0.90 in 9% PEG; Rs = 1.20 in 10% PEG; Rs = 1.17 in 11% PEG). The data was shown in Fig. S3. Among all ratios of PEG, LHA and HHA could be resolved, but the peak shape of LHA was poor under 8 and 9% PEG. That was supported the sieving matrix under such concentration was not good enough to resolve LHA and HHA well. As considering the resolution between LHA and HHA, the separation was carried out under addition of 10% PEG in the SDS matrix.

In the mode of MEKC, the addition of surfactant plays an important role in separation. SDS, an anionic surfactant, is the most commonly used in MEKC. Here, different concentrations of SDS from 15 to 35 mM was mixed with PEG to form the micelles for the separation of LHA and HHA (Rs = 0 in 15 mM SDS; Rs = 0 in 20 mM SDS; Rs = 1.20 in 25 mM SDS; Rs = 1.18 in 30 mM SDS; Rs = 1.14 in 35 mM SDS, as shown in Fig. 2). The addition of different concentrations of SDS would greatly affect the peak shapes of LHA and HHA. Although the critical micellar concentration of SDS in water is about 8 mM, the SDS was mixed with the PEG for formation of SDS micelles in this study, and the concentration of SDS higher than 8 mM was required. Thus, when the SDS concentration was too low (15 mM and 20 mM), the SDS micelles may not form well for resolution of LHA and HHA resulting in the overlap of two HAs, as shown in Fig. 2A and B. Finally, 30 mM SDS was mixed with 10% PEG in borate buffer (25 mM, pH 9.75) to form the SDS micelles and PEG sieving matrix for resolution of LHA and HHA. Additionally, the PEG in the buffer would form the sieving matrix resulting in the separation order is, LHA and HHA in the MEKC technique.

3.2. *Method validation*

In this experiment, different molecular-weight HA, LHA and HHA, were simultaneously determined by using sulindac (5 μg/mL) as the internal standard (IS). The calibration curves were linear over the range of 10—50 μg/mL for LHA and 40—200 μg/mL for HHA. The electropherograms of different
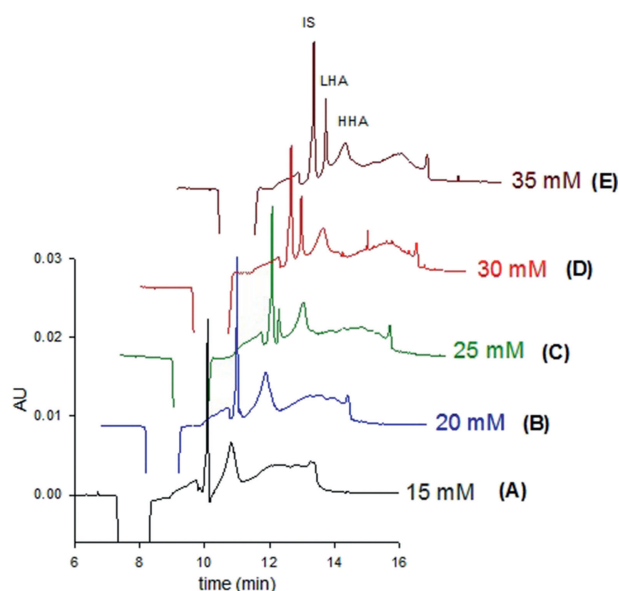

Fig. 2 — **Effect of different amount of SDS contained in borate buffer for the separation of LHA and HHA. Other CE conditions: separation buffer: 25 mM borate (pH 9.75) containing different concentrations of SDS and 10% PEG; pressure injection: 2 psi, 99.9s; voltage: 20 kV; uncoated fused silica capillary, 50 cm (effective length) × 50 μm I.D.; wavelength: 200 nm. The concentration of LHA was 50 μg/mL, HHA was 200 μg/mL and IS (sulindac) was 5 μg/mL.**

concentrations of LHA and HHA for the calibration curves were shown in Fig. 3A. The equations of calibration curves of LHA and HHA were y = 0.0020× - 0.0022 and y = 0.0008x + 0.0028, respectively, where the x is the concentration of analyte and y is the peak area ratio (Fig. 3B). Correlation coefficients (r) of the calibration curves were above 0.9977. For the assays of precision and accuracy, three different concentrations of LHA (15, 25 and 45 μg/mL) and HHA (50, 100 and 150 μg/mL) were analyzed in 3 successive times (n = 3), respectively. The data was shown in Table 1. All of the absolute values of relative standard deviation (R.S.D.) and relative errors (R.E.) were below 4.2%. The RSD values of the migration time and peak area were below 3.2 and 4.8, respectively. All of the data implied good precision, accuracy and repeatability of this MEKC method for the analysis of LHA and HHA. The limits of detection defined as the peak height of analytes is three folds higher than the noise (LOD, S/N = 3) for LHA and HHA in this MEKC were 2 μg/mL and 10 μg/mL, respectively.

3.3. Applications

HA is mostly used in the cosmetic products as the moisturizing agent. Therefore, the LHA and HHA were added into the cosmetic matrix (olivem® 1000) to investigate the applicability of the CE strategy for separation of LHA and HHA in commercial products. After pretreatment of the cosmetic sample, the LHA and HHA were well-separated in this CE method (as shown in Fig. 4A). The recoveries of LHA and HHA (20 μg/mL and 80 μg/mL) in this cosmetic matrix were 91.5% and 102.6%, respectively. Additionally, HA has also been frequently utilized as an excipient in the pharmaceutical production to increase drug bioavailability. In order to demonstrate the applicability of this method in the drug formulation, the power of LHA and HHA were homogeneously mixed with the formulation excipient, including corn starch, lactose and magnesium stearate. And then, the powder was dissolved in the phosphate buffer solution (PBS, pH 6.8). After centrifuged and filtered through 0.45 μm membrane, the supernatant was
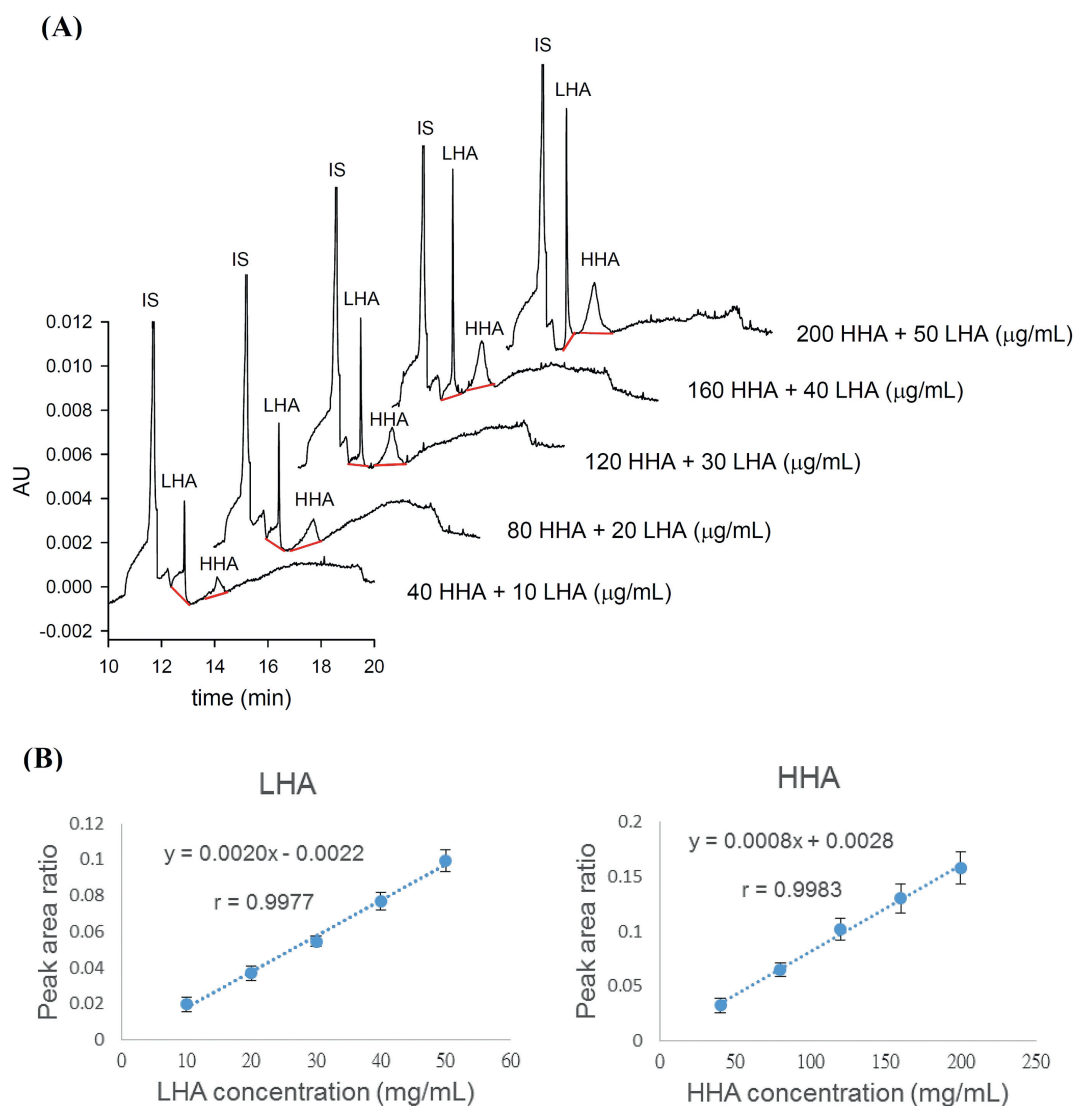

Fig. 3 – (A) The electropherograms of different concentration of LHA and HHA by the MEKC method (B) The calibration curves of LHA for 10–50 μg/mL and HHA for 40–200 μg/mL.

Table 1 — The precision, accuracy and repeatability assays in this MEKE technique for separation of LHA and HHA.

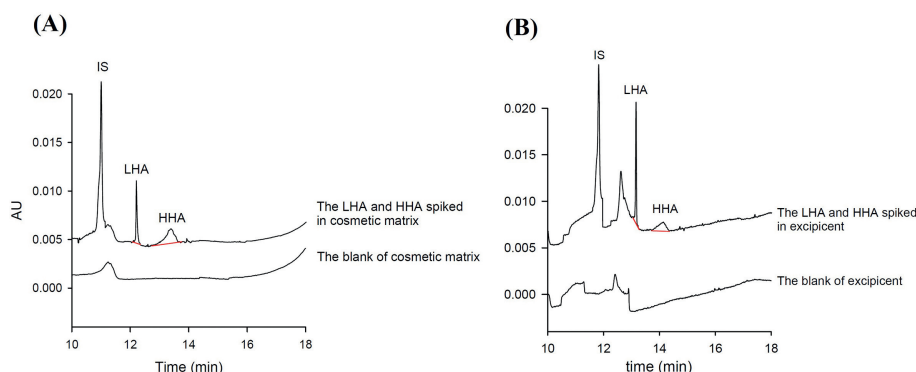| Analyte	Concentration spiked (μg/mL)	Concentration found (μg/mL)	RSD (%)	RE (%)	RSD of migration time (%)	RSD of peak area (%)
LHA	15.0	14.7 ± 0.2	1.4	−2.0	1.7	3.5
	25.0	24.5 ± 1.0	4.1	−2.0	1.5	4.8
	45.0	45.6 ± 1.9	4.2	1.3	1.8	2.8
HHA	50.0	50.6 ± 1.7	3.4	1.2	3.2	2.5
	100.0	102.3 ± 2.1	2.1	2.3	2.8	0.9
	150.0	148.9 ± 3.8	2.6	0.7	2.3	1.9

Fig. 4 — The application of the MEKC method for analysis of LHA and HHA spoiled in (A) cosmetic matrix (olivem® 1000) and (B) the formulation excipient, including corn starch, lactose and magnesium stearate. The concentrations of LHA and HHA in the cosmetic matrix and formulation excipient were all made to 20 μg/mL and 80 μg/mL, respectively. The recoveries of LHA and HHA in cosmetic matrix were 91.5% and 102.6%, and in formulation excipient were 98.2% and 95.3%, respectively.

collected for analysis by using this MEKC method. The data was shown in Fig. 4B. The LHA and HHA could be resolved in the presence of the interferences existed in the pharmaceuticals. The recovery of the LHA and HHA (20 μg/mL and 80 μg/mL) were 98.2% and 95.3%, respectively. The result demonstrated this strategy was applicable for separation of LHA and HHA in different matrix, including the cosmetic products and drug formulations.

4. Discussion

In previous researches for analysis of HA, one kind of HA with fixed molecular weight was utilized [15–24]. Table 2 showed the comparisons of this study and the previous researches utilized for separation of HA or with other materials. Kakehi et al. utilized the capillary electrophoresis strategy to separate

Table 2 — The comparison of this study and previous researches for separation of HA.

Analytes	Methods	Linearity	LOD	Ref.
Nonsulfated hyaluronic acid (HA) oligosaccharides and chondroitin sulfate	Fluorophore-assisted carbohydrate electrophoresis	none	none	[15]
Hyaluronic acid (HA)	Solid-phase assay (covalent immobilization of HA)	0.1–0.7 μg/mL	none	[17]
hyaluronic acid (HA) in urine	Competitive enzyme-linked immunosorbent assay	400–1200 ng/mL	none	[19]
Hyaluronate in biological sample	^{125}I-labeled radioassay	40–1000 ng	none	[20]
Hyaluronic acid (HA), chondroitin sulfate (CS), dermatan sulfate (DS), heparin (HP)	HPLC-MS/MS	0.01–1.0 mg/mL	none	[21]
N-acetylneuraminic acid, hyaluronic acid (HA)	Capillary gel electrophoresis-UV detector	none	none	[22]
Heparin, chondroitin sulfate, hyaluronic acid (HA)	Capillary gel electrophoresis-LIF detector	0.02–3.0 mg/mL	9.04×10^{-3} mg/mL	[23]
One kind of HA once	Capillary electrophoresis-UV detector	0.01–3.3 mg/ml	1 μg/mL	[24]
Low-MW HA (LHA), high MW HA (HHA)	MEKC-UV detector	LHA: 10–50 μg/mL HHA: 40–200 μg/mL	LHA: 2 μg/mL HHA: 10 μg/mL	this

different size of HA, but the HA should be pretreated with enzyme to form the various small size of HA [23]. Additionally, Hayase et al. tried to resolve three kinds of HA with different molecular weights. However, the method only could be applied to identify one kind of HA in once run, and three kinds of HA could not be separated simultaneously [24]. Because HA has been demonstrated the various functions according to its molecular size [1,2], and thus resolution of high-dispersion HA is wonder tool for clinical survey of the HA. In this research, a capillary electrophoresis technique, MEKC, was presented for simultaneous determination of two kinds of HA with different molecular weights. The mixture of HA with low molecular weight (380 kD; LHA) and high molecular weight (2180 kD; HHA) were successfully resolved by the SDS micelles and PEG seeing matrix in capillary electrophoresis. In comparison of only SDS, the combination of SDS and low molecular-weight polymer, PEG, could form the SDS micelles and sieving matrix that are helpful for resolution of highly dispersed HA. After optimizing experimental conditions, the LHA and HHA was base-line separated within 14 min. In the method validation, the calibration curves were linear over a range of 10–50 μg/mL for LHA, and 40–200 μg/mL for HHA (r ≥ 0.9977). Good precision and accuracy was presented for this method through the RSD and RE values were all less than 4.2%, and good repeatability was demonstrated through the RSD values of migration time and peak area were all less than 4.8%. This fascinating technique was successfully applied for quantity control of cosmetic matrix and pharmaceutical excipients containing different ratios of LHA and HHA, and hence it was feasible for serving as a tool for quantitatively analyzing different sizes of HA for further estimating.

Declaration of Competing Interest

All authors declare that they have no conflict of interest.

Acknowledgement

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfda.2019.10.005.

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