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A simple capillary electrophoresis method for the rapid separation and determination of intact low molecular weight and unfractionated heparins

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Abstract

A simple, selective and accurate capillary electrophoresis (CE) method has been developed for the rapid separation and identification of various low molecular weight heparins (LMWHs) and unfractionated heparin. Separation and operational parameters were investigated using dalteparin sodium as the test LMWH. The developed method used a 70 cm fused silica capillary (50 μm i.d.) with a detection window 8.5 cm from the distal end. Phosphate electrolyte (pH 3.5; 50 mM), an applied voltage of −30 kV, UV detection at 230 nm and sample injection at 20 mbar for 5 s were used. The method performance was assessed in terms of linearity, selectivity, intra- and inter-day precision and accuracy. The method was successfully applied to the European Pharmacopeia LMWH standard, dalteparin sodium, enoxaparin sodium and heparin sodium with a significant reduction in the run time and increased resolution compared with previously reported CE methods. Different CE separation profiles were obtained for various LMWHs and unfractionated heparin showing significant structural diversity. The current methodology was sensitive enough to reveal minor constituent differences between two different batches of enoxaparin sodium. This CE method also clearly showed chemical changes that occurred to LMWHs under different stress conditions. The sensitivity, selectivity and simplicity of the developed method allow its application in research or manufacturing for the identification, stability analysis, characterization and monitoring of batch-to-batch consistency of different low molecular weight and unfractionated heparins.

Keywords: Low molecular weight heparin; Dalteparin; Heparin; Capillary electrophoresis; Batch-to-batch variations

1. Introduction

Heparin is an endogenously occurring glycosaminoglycan (GAG) extracted from mammalian tissues, consisting of complex linear polysaccharides [1]. The most important heparin derivatives from the viewpoint of clinical use as antithrombotic agents are the low molecular weight heparins (LMWHs) [2]. These are modified fractions of heparin, consisting of GAG chains of molecular weight ranging from 2000 to 8000 Da with an average of 5000 Da [3].

LMWHs are produced by fragmentation of native heparin using various techniques: β-elimination by enzymatic or chemical cleavage; deamination with nitrous acid or other nitrosating reagents such as isoamyl nitrate; or oxidation with hydrogen peroxide. Different LMWHs may show structural differences based on the fractionation method applied. The origin of the parent heparin may also introduce structural variability in the individual low molecular weight derivative, as heparin is obtained from different tissues and species. As a result LMWHs are more heterogeneous than unfractionated heparin [3].

Dalteparin is the sodium salt of a LMWH obtained by optimised nitrous acid depolymerisation (deaminative cleavage with nitrous acid) of heparin from porcine intestinal mucosa, followed by controlled fractionation to selectively eliminate most of the chains with molecular mass of more than 8000 Da [3].

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Nitrous acid deamination produces an anhydromanno group at the reducing end of the low molecular weight heparin chain [4]. Alternatively, production of LMWHs by the enzymatic cleavage of unfractionated heparin by heparinase I produces a double bond at the non-reducing end of the resultant LMWH [3] (for example tinzaparin). These minor structural differences between various LMWHs may differentiate them by their chemical profiles or their pharmacodynamic/pharmacokinetic properties [5]. As a result, each of these LMWHs should be identified as a separate entity. Identification of physicochemical properties of each LMWH is necessary. The methodology used must be capable of detecting minor differences between two different LMWHs or two different batches of the same LMWH. The European Pharmacopeia tests for LMWH analysis include chromogenic assays for AFXa and AFIIa activities and size exclusion chromatography for structural identification [6].

Various techniques have been applied to analyse and separate large anionic polysaccharides such as heparin and various LMWHs and their oligosaccharides and disaccharides. Polyacrylamide gel electrophoresis (PAGE) has been used for analysis of GAGs and oligosaccharides with the use of different cationic dyes for their visualization [7–11]. Strong anion exchange chromatography (SAX) and high performance liquid chromatography (HPLC) have been used successfully for the analysis of intact and enzymatically depolymerised GAGs [12–15].

Over the past two decades, capillary electrophoresis (CE) has been increasingly applied as a sensitive method of high resolving power for the analysis of complex mixtures of peptides, nucleotides and polysaccharides [16,17]. However, most of the polysaccharide work utilising CE involves the analysis of chemically or enzymatically depolymerised oligosaccharides [18–28]. Only a few applications of CE for the analysis of intact anionic polysaccharides have been documented [29–35]. Malsch et al. [30], Ramasamy et al. [35] and Toida and Linhardt [28] provide examples of the potential use of CE for the analysis of intact LMWHs, however these CE assays had limitations associated with resolution and run time. Ramasamy et al. [35] used an acidic copper sulfate buffer for the separation of LMWHs that resulted in a peak with a base width of nearly 30 min. Malsch et al. [30] used an acidic phosphate buffer for the analysis of heparin that resulted in a peak width of nearly 5 min. Similarly the acidic buffer containing copper employed by Toida and Linhardt [28] for heparin analysis resulted in a peak width of about 8 min.

Polyanions can be separated by CE with either high or low pH buffers, using normal or reverse polarity [26,30]. GAGs are negatively charged over a wide range of pH values and hence possess a favourable electrophoretic mobility for CE, due to the large number of sulfate groups. Under acidic conditions electroosmotic flow (EOF) is almost negligible due to protonation of silanol groups on the fused silica capillary surface. Samples introduced from the cathodic end migrate towards the anode without influence from the EOF. The separation of LMWHs is directly proportional to the average number of sulfate groups present in the repeating units [24]. In an alkaline environment the intrinsic mobility of the EOF is high due to dissociated silanol groups. Samples introduced from the anode possessing lower electrophoretic mobility than the EOF are swept towards the cathode [23,36,37].

CE is under-exploited for polysaccharide analysis. To the authors' knowledge, a versatile method with high resolution and a short run time for the separation and quantification of intact LMWHs for the evaluation of stability and batch-to-batch variation in pharmaceuticals is not available. Particular attention must be paid to the efficiency, precision, accuracy and selectivity of any analytical method used for pharmaceutical quality control. The LMWH dalteparin sodium was selected as a test analyte to develop a simple and rapid CE separation method. Several parameters were investigated such as buffer concentration, pH of the background electrolyte, different capillary lengths and diameters and the amount injected. The developed CE method was validated in terms of linearity, precision, accuracy and selectivity and results are presented as % R.S.D. (relative standard deviation). Furthermore, the developed CE method was applied to the characterization of structurally different LMWHs and different batches of the same LMWH, and chemically and thermally stressed enoxaparin sodium and dalteparin sodium to demonstrate its potential in applications for the analysis of intact LMWHs.

2. Materials and methods

2.1. Materials

The sodium salts of dalteparin (12,500 IU/ml) and heparin were purchased from Pharmacia (Rydalmere, NSW, Australia). The sodium salt of enoxaparin (10,000 IU/ml) was purchased from Aventis Pharma (Sydney, NSW, Australia). The European Pharmacopoeia LMWH standard was from Pharmacopée Européenne (Strasbourg, France). Orthophosphoric acid, sodium hydroxide, sodium chloride, hydrochloric acid and hydrogen peroxide were of analytical grade as purchased from Sigma–Aldrich (Castle Hill, NSW, Australia). Electrolytes and standard solutions were prepared from Milli-Q water and were filtered through a 0.22 μm pore size membrane filter prior to use.

2.2. Instrumentation

An Agilent^{3D} capillary electrophoresis instrument (Waldronn, Germany), equipped with a deuterium UV lamp and diode array detector (190–600 nm) was used for capillary electrophoresis investigations. Data acquisition and instrument control were carried out using Agilent Chemstation system software. Bare fused silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). A detection window was established through the polyimide coating at 8.5 cm from the capillary end using a butane torch. The detection wavelength and separation temperature were 230 nm and 25 °C, respectively.

2.3. CE analysis

Between each run, the capillary was flushed for 1 min with electrolyte. The samples were introduced into the cathodic end

of the capillary by hydrodynamic injection for 5 s at 20 mbar. The separation was performed in reverse polarity mode with a constant voltage of −30 kV. In order to ensure reproducible results of the CE method, the capillary was conditioned by applying a voltage of −30 kV with the electrolyte solution for 20 min at the start of each day. The capillary was flushed with Milli-Q water for 5 min followed by electrolyte for 2 min prior to running samples.

2.4. Electrolyte preparation

A stock solution of phosphate electrolyte (200 mM) was prepared by diluting the calculated weight of orthophosphoric acid with Milli-Q water. The pH was adjusted to 2.0 by careful addition of 1 M sodium hydroxide solution. Preparation of phosphate electrolytes of different concentrations and pH values was performed by appropriate dilution of electrolyte stock solution with Milli-Q water and adjustment of pH using 1 M sodium hydroxide solution.

2.5. Method development

The method development was performed using dalteparin sodium solution diluted with Milli-Q water to a concentration of 5 mg/ml. Fused silica capillaries of 30, 50 or 70 cm length with 50 or 75 μm i.d. were used. Phosphate electrolyte (20, 50 or 100 mM) having a pH of 2, 3.5 or 5 was prepared from the stock solution. Analyses were run with an applied voltage of −10, −20 or −30 kV.

2.6. Assay performance

Intra- and inter-day (over five consecutive days) precision were investigated using peak area, with repeat analysis ($n = 6$) of 5 mg/ml dalteparin sodium solution. The intra-day precision of different concentrations of dalteparin (10, 20 and 50 mg/ml, $n = 6$) was also determined. Mean intra- and inter-day accuracy were calculated as follows; (observed concentration − expected concentration)/expected concentration × 100. The linearity of the method was investigated using 2.5, 5, 10, 20 and 50 mg/ml dalteparin sodium (estimated using correlation coefficient r^2) and peak retention time were obtained on each of the 5 days.

2.7. Degradation of dalteparin sodium

Dalteparin sodium and enoxaparin sodium solutions were subjected to chemical and thermal stresses in sealed N_2-filled glass ampoules. Dalteparin solutions were mixed with either 0.5 M hydrochloric acid or concentrated hydrogen peroxide and then heated at 100 °C for 30 min. Enoxaparin sodium solution was mixed with concentrated hydrogen peroxide and then heated at 100 °C for 30 min. A further sample of enoxaparin was heated at 70 °C for 2 h. CE analyses were performed on both stressed and unstressed samples for comparative purposes.

2.8. Preparation of unfractionated heparin and various LMWHs samples

Heparin sodium (5000 IU/ml), European Pharmacopoeia calibration standard for LMWHs, enoxaparin sodium (10,000 IU/ml) and dalteparin sodium (12,500 IU/ml) were diluted to 10 mg/ml with Milli-Q water before CE analysis. Six samples were analysed in all cases to test the reproducibility of the technique.

3. Results and discussion

3.1. Influence of separation parameters on dalteparin sodium analysis

Dalteparin sodium was selected as the test analyte because it gave a simple peak in its electropherogram, and its CE analysis has been demonstrated previously [35]. Efforts were made to improve the electrophoretic separation of intact dalteparin sodium. Molecular dispersion of polysaccharides is known to be influenced by the applied voltage and the distance they are required to migrate before the detection window [38]. The highest voltage tested, −30 kV, gave rapid migration with acceptable peak shape. Consequently, this voltage was chosen for further analyses. The effect of different length and inner diameter combinations of the fused silica capillary were investigated with respect to the separation efficiency that could be achieved. When using the 75 μm i.d. and 40 cm capillary, electrophoretic current was too high and current breakdown was observed, which had a negative impact on the separation. Decreasing the diameter of the capillary, increasing the length of the capillary or decreasing the ionic strength of the electrolyte has been shown to decrease the magnitude of the current generated [38]. This avoids the deleterious effects caused by boiling within the capillary. Increasing the length of the 75 μm i.d. capillary to 50 cm did not achieve better peak shape in the sample. Hence, a 50 μm i.d. capillary was utilised which allowed relatively higher ionic strength electrolyte solutions to be used. This aided in sample stacking, hence improving sensitivity and reduced the magnitude of current generation compared to using the same higher electrolyte concentration in a 75 μm i.d. capillary. Hence, the best separation conditions achieved were obtained using a capillary of reduced i.d. (50 μm) of greater length (70 cm) with the 50 mM phosphate electrolyte at pH 3.5.

3.2. Effect of electrolyte pH on the separation efficiency

It is established that at low pH oligosaccharides can be efficiently separated without complexation with borate. Operation at lower pH ensures that the cathodic EOF is negligible allowing migration of the analytes in the opposite direction towards the cathode. In order to maximise the electrophoretic mobility of the negatively charged polysaccharide dalteparin sodium, only acidic phosphate electrolytes (pH range 2–5) were investigated. The strength of the electrolyte was maintained at 50 mM at all the tested pH values.

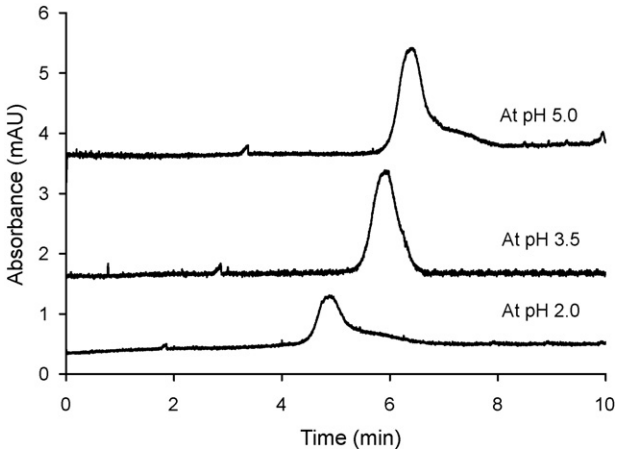

Fig. 1. Effect of the buffer pH on the peak shape and migration time of the dalteparin peak (5 mg/ml): fused silica capillary of total length of 70 cm with 50 μm i.d; background electrolyte 50 mM phosphate buffer at pH 3.5; applied voltage of −30 kV; detection at 230 nm, injection at 20 mbar for 5 s.

The electrolyte pH was found to greatly influence the CE separation. Migration times varied significantly across the tested pH range, with an increasing trend with increase in pH. As seen in Fig. 1, at pH 5.0 a very broad peak was obtained with longer migration time. This is because an increase in pH increases the EOF, whose direction is towards the inlet electrode. Consequently the decreased net mobility of the solutes tends to increase the migration time, leading to higher dispersion of the solute in the capillary. At pH 2.0, the sample migrated earlier past the detector but with a poor peak shape. The peak height and area were also less at pH 2.0 compared with results obtained using electrolyte of higher pH values. This could be explained by the partial degradation of dalteparin sodium in very acidic conditions [39]. The best efficiency for dalteparin sodium separation was achieved at pH 3.5. However, the electrolyte required replacement with freshly titrated electrolyte after every 100 min due to the lack of buffering capacity of phosphate at this pH value. This time interval allowed the running of 10 sample analyses without any observed change in electrophoretic separation.

3.3. Influence of the electrolyte concentration

The effect of increasing the electrolyte strength from 20 to 200 mM was investigated, since increasing the electrolyte strength increases beneficial sample stacking, which improves separation efficiency. Increasing the concentration of electrolyte also increases the current during electrophoresis. Potential Joule heating effects limit the electrolyte concentration used. At a concentration of 100 mM phosphate electrolyte the peak became broad with tailing and at 200 mM the dalteparin sodium peak shape degraded dramatically. The 50 mM electrolyte gave a sharp, well-resolved and represented peak with a good compromise between current and peak shape. Highly negatively charged large molecules, including polysaccharides such as heparins, tend to become more compact in higher strength solutions due to charge neutralisation that effectively decreases the intra molecular repulsive forces from the negatively charged sulfate groups. This change in conformation also benefits electrophoretic separation, as has been observed in the CE separation of highly sulfated carrageenans [40].

3.4. Assay performance

The intra- and inter-day precision R.S.D. was 3.2% ($n = 6$) and 4.4% ($n = 5$), respectively at the level of 5 mg/ml dalteparin sodium. The intra- and inter-day accuracy was +4.0% and +5.5%, respectively at 5 mg/ml dalteparin sodium. The intra-day precision R.S.D. at the level of 10, 20 and 50 mg/ml of dalteparin sodium ($n = 6$) was 3.8%, 4.4% and 2.3%, respectively. Linearity estimated by correlation coefficient r^2 was greater than 0.997 with each of five different concentrations of dalteparin sodium (2.5, 5, 10, 20 and 50 mg/ml) over 5 days. Mean migration time of dalteparin sodium was 5.69 min with an intra-day and inter-day migration time R.S.D. of 0.57% ($n = 6$) and 1.99% ($n = 24$), respectively.

3.5. Analysis of stressed LMWHs

The CE profiles for degraded and non-degraded dalteparin and enoxaparin sodium samples, using the method developed in this work are presented in Fig. 2. With acid stressed dalteparin sodium, a degradation product migrated past the detector after 3.2 min. The same peak was increased in height and area following peroxide degradation. The peak area of the main dalteparin sodium peak with a migration time of 5.64 min was reduced more than five times after acid stress. Two very small peaks were obtained instead of a single dalteparin sodium peak after treatment with H_2O_2.

This CE method demonstrated changes in the electropheric profile of enoxaparin sodium heated at 70 °C for 2 h, as shown in Fig. 2B. It is evident from the electropherograms that major changes have occurred during the relatively mild heating of enoxaparin sodium. The H_2O_2 stressed sample produced a number of new peaks accompanied by complete loss of the original enoxaparin sodium peaks. This accurate, fast and highly specific technique offers a method suitable for stability studies of various LMWHs.

3.6. CE analysis of different LMWHs and unfractionated heparin

The electropherograms of the European Pharmacopeia LMWH standard, two different batches of enoxaparin sodium and heparin sodium are shown in Fig. 3. The electrophoretic profiles of various LMWHs differed from the European Pharmacopeia calibration standard. The CE method detected major structural differences between dalteparin sodium and enoxaparin sodium. The mean migration time of the principle peak of LMWHs and unfractionated heparin ($n = 6$) were 5.23 min (0.96% R.S.D.; European Pharmacopeia LMWH standard), 5.68 min (0.74% R.S.D. enoxaparin sodium) and 4.87 min (0.63% R.S.D.; heparin sodium). The intra-day precision R.S.D. of the principle peak of European Pharmacopeia LMWH standard, enoxaparin sodium and heparin sodium ($n = 6$) were 3.5%,

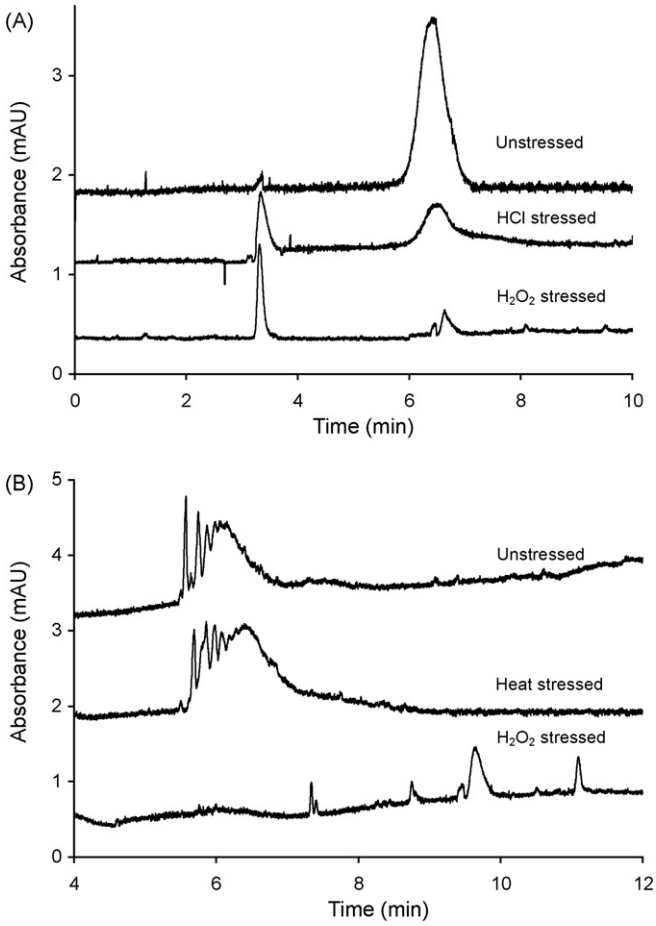

Fig. 2. Electropherograms of dalteparin sodium (5 mg/ml) before and after stressing under oxidative or acidic conditions (A) and enoxaparin sodium before and after stressing at 70 °C for 2 h or under acidic conditions (B). CE conditions are the same as those described for Fig. 1.

4.0% and 3.2%, respectively. The electrophoretic profile of dalteparin sodium (Fig. 2) was more similar to its parent compound heparin compared with enoxaparin sodium or the European Pharmacopeia LMWH standard.

The CE method described here has several advantages over previously reported CE methods for intact LMW and unfractionated heparin. Electrophoretic separation can be performed in less than 10 min for LMWHs which compares with more than 20 and 40 min for previous CE methods [28,35]. The resolution of peaks was excellent, with more than eight peaks evident in the electropherograms of enoxaparin sodium and the European Pharmacopeia LMWH standard compared with only a few peaks evident in electropherograms of the same LMWHs using the method described by Ramasamy et al. [35]. In contrast with previously reported methods [28,30,35] by the method reported here, each LMWH showed distinctive electropherogram features with narrow peak width and characteristic migration times and each LMWH could readily be distinguished from other LMWHs and unfractionated heparin. This method could be applied to further characterize different LMWH constituents by comparison of results with CE analyses of oligosaccharide standards of known molecular weight and polydispersity. This method allows the analysis of individual molecular components of LMW and unfractionated heparins which is not possible following enzymatic or chemical digestion procedures prior to CE analysis [22–26]. This CE method was so sensitive that it clearly revealed differences in six different regions of the electropherograms from two different batches of enoxaparin sodium (Fig. 3B). As enoxaparin sodium is used for wide ranges of different indications, the observed compositional differences in two different batches of enoxaparin sodium may have significant clinical implications. As a dose of enoxaparin sodium varies with different indications, minor compositional differences may lead biological or clinical variability [3]. These results demonstrate that this method would be suitable for analysis of batch-to-batch variation in pharmaceutical LMWHs or for "fingerprinting" batches of LMWHs. These compositional differences may be due to the inherent structural variability of the precursor heparin from which the LMWH was derived or due to differences in the fractionation process.

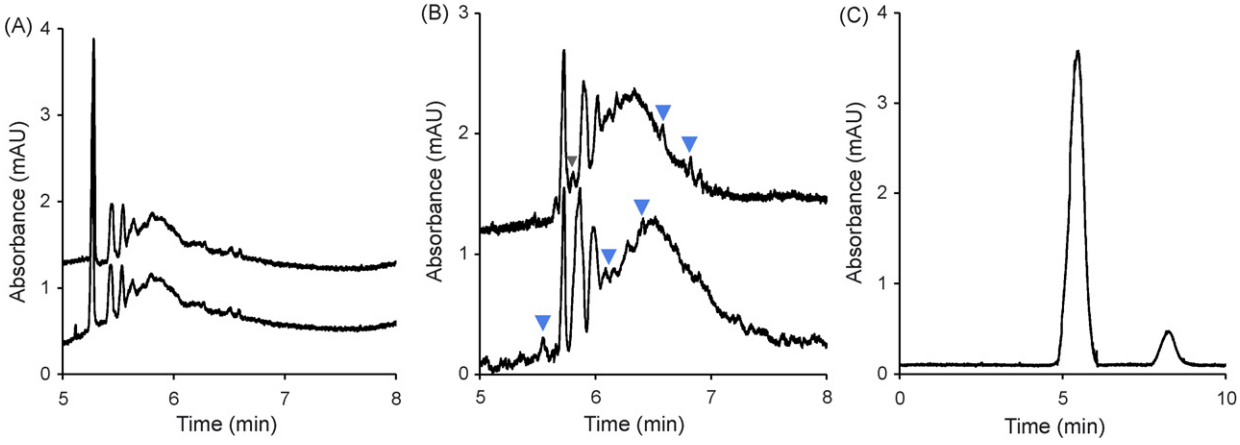

Fig. 3. Electropherograms of European Pharmacopoeia LMWH standard (A), two different batches of enoxaparin sodium (B) with differences highlighted by the arrows and heparin sodium (C). Each sample was diluted with Milli-Q water to achieve a concentration of 5 mg/ml. CE conditions are the same as those described for Fig. 1.

4. Conclusion

This work has demonstrated the advantages of the developed CE method, with specified length and diameter of capillary, applied voltage, ionic strength and pH of background electrolyte, to achieve efficient separation of LMW and unfractionated heparins. The CE assay performance was assessed in terms of linearity, accuracy, precision, selectivity and gave acceptable performance and reproducibility. This method is simple, quick to perform and gives higher resolution than other reported CE methodologies for intact LMWHs. This versatile method was able to clearly differentiate three different LMWHs and unfractionated heparin, and each giving electropherograms with sharp peaks at consistent migration time. This method is capable of detecting compositional differences between batches of the same LMWH and able to show degradation products of LMWHs after chemical or mild thermal stress conditions. This CE method potentially offers a simple and rapid analytical technique to study of batch-to-batch variation, the characterization and stability testing of pharmaceutical LMW and unfractionated heparins.

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