Development and Validation of a Simple and Rapid Capillary Zone Electrophoresis Method for Quantification of Heparin in a Pharmaceutical Product and Stability Studies

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Abstract
A simple and rapid capillary zone electrophoresis method has been developed and validated for determination of heparin in a pharmaceutical product and evaluation of heparin stability under different stress conditions. The best separation was achieved by a bare fused silica capillary (50 μm i.d.; 50 cm total and 41.5 cm effective length), phosphate buffer (pH=3.50, 72 mM) at 35°C, hydrodynamic injection at 50 mbar for 40 seconds and applied voltage of -30 kV. Heparin and force degradation products were detected by a photo diode array detector at 200 nm and 257 nm, respectively. The proposed method was validated in terms of linearity, accuracy, precision, limit of quantification (LOQ) and limit of detection (LOD). For evaluation of heparin stability, heparin solutions were subjected to thermal (90 ± 1°C), acidic (pH=2.00, 70 ± 1°C) and basic (pH=12.00, 70 ± 1°C) stress conditions, also sun light exposure (in the lab). The results indicated that, the method was linear in the range of 0.312 to 15.0 mg/ml with LOD of 0.078 mg/ml and LOQ of 0.312 mg/ml, accurate (between 97.27% and 101.0%) and precise (intra-day precision of 0.28 to 1.8 and inter-day precision of 0.78 to 3.2%). The migration time of heparin under optimized conditions, was 2.39 ± 0.03 minutes and force degradation products were separated within 7 minutes. Heparin content in the pharmaceutical product quantified by the method was 99.58 ± 0.70% of the label claim. Meanwhile the method could show the changes in the heparin electropherogram, also formation of degradation products under different stress conditions. Therefore the proposed method could be applied as a fast and suitable technique for determination of heparin in pharmaceutical dosage forms and stability studies.

Keywords: Capillary zone electrophoresis; Heparin; Pharmaceutical dosage form; Stability

Introduction
Heparin is one of the biological drugs which belongs to glycosaminoglycans family and consists of linear highly sulfated polysaccharide chains with average molecular weight of 12000 Da. It has antithrombic properties and isolated from mammalian tissues such as porcine intestine or porcine intestinal mucosa [1,2]. Heparin is one of the widespread anticoagulant medicines which was discovered by McLean in 1916, recognized as an anticoagulant in 1918 and has been used clinically since 1937 for prevention and treatment of thrombosis [3]. Also there are some events showed the biological role of heparin and heparin sulfate on cell differentiation and embryonic development, inflammation, infections and metabolism of lipoproteins [4,5].

Different analytical methods such as bioassay [6-9], electrochemical [10,11], spectroscopy [12-14], chromatography [15], flow injection [16], and electrophoresis [17-22] have been reported for determination of heparin in pharmaceutical and biological samples and in stability studies so far. In some studies especially in stability studies or monitoring of heparin level in plasma, bioassays such as coagulation tests or ELISA were applied [6-9]. Niu et al. developed voltammetric method for determination of heparin using malachite green as a bioprobe on a mercury working electrode [10]. In another work Niu and coworkers used acridine orange as an electrochemical probe to develop a voltammetric method for determination of heparin in injectable samples [11]. Jandik et al. studied accelerated stability of heparin with a UV-VIS spectrophotometry at wavelength of 232 nm. They found initial events of colored products with λ max of 210 and 280 nm in stressed samples [12]. In a different work, near infrared reflectance spectroscopy coupled with partial least square algorithm was used as a fast and non destructive tool for evaluation of heparin potency in heparin active ingredient and improving the quality of heparin products [13]. A spectrofluorimetric method reported for determination of heparin using a norflursaxin-mercury probe [14]. In the other study, Toida and coworkers described a HPLC method for pharmacokinetic study of heparin in rabbits, in which heparin was enzymatically depolymerized and the major disaccharide was analyzed using fluorometric post column derivatization [15].

Different capillary electrophoresis (CE) based methods were proposed for analysis and determination of heparin, its impurities and/or related substances. Ampofo and coworkers [17] used a capillary zone electrophoresis method for microanalysis of heparin and heparin sulphate by depolymerizing with enzymes. Then the disaccharide composition of each glycosaminoglycan was studied. Zhou et al. [18] reported a micellar electrokinetic capillary chromatography (MECC) for direct determination of free heparin in plasma. They suggested that the method could be used for determination of heparin in blood samples. In another work a simple CE method was developed for...
determination of intact low molecular weight and unfraccionated heparins in accelerated stability studies and monitoring of batch to batch consistency [19]. In the other work, a CE method was used for determination of intact heparin in infusion solutions and plasma with contactless conductivity detection [20]. CE was also applied for determination of impurities, contaminants and related compounds of heparin, for instance; Wielgos and coworkers [21] reported a CE method for determination of impurities, Volpi et al. [22] developed a CE method for determination of related substances, Bendazzoli et al. [23] presented a CE method for determination of a contaminant in heparin preparations and Liu and co workers [20] developed a rapid and simple CE method for simultaneously separation of heparin, chondroitin sulfate and hyaluronic acid in pharmaceutical preparations and synovial fluid.

The objective of the present study was the development and validation of a simple and rapid method for determination of heparin in pharmaceutical samples and evaluating the applicability of the developed method in accelerated stability studies of heparin in aqueous solutions under different stress conditions [24].

**Material and Methods**

**Materials**

Heparin (as a sodium salt) was purchased from Caspian Tamin Pharmaceutical Co. (Rasht, Iran) and heparin vials (Rotexmedica, (Trittau, Germany)) was purchased from a local pharmacy shop. According to the certificate of analysis, each mg of heparin powder is equal to 172 IU. Orthophosphoric acid 85%, sodium hydride and sodium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). All solutions were prepared by double distilled water (Millipore, Bedford, MA, USA) and filtered through a 0.2 μm pore size filter (Macherey–Nagel, Germany) before use.

**Apparatus**

A capillary electrophoresis instrument (Agilent Technologies 7100, (Waldbronn, Germany)) equipped with a photodiode array detector (PDA) with detection range from 190 to 600 nm was used for analysis. Agilent ChemStation software was applied for data analysis and instrument control. Bare fused silica capillaries were purchased from Agilent Technology. The detection window was located at 8.5 cm from the capillary end. A Metrohm pH meter (Herisau, Switzerland) was used for pH adjustments.

**CE analysis**

At the beginning of each working day capillary was flushed for 5 minutes with sodium hydride and 5 minutes with double distilled water, respectively, then capillary was conditioned by electrolyte solution for 20 minutes. Between each run capillary was flushed for 5 min with electrolyte. The samples were injected by hydrodynamic injection for 40 seconds at 50 mbar.

**Electrolyte preparation**

A stock solution of phosphate electrolyte (200 mM) was prepared by dissolving accurately weighted amount of sodium dihydrogen phosphate in double distilled water. For preparation of different concentrations of electrolyte, the appropriate volume of stock solution was diluted with double distilled water and its pH was adjusted by orthophosphoric acid or sodium hydride 5M.

**Method development**

Method development was carried out using heparin stock solution with concentration of 30 mg/ml which was diluted with double distilled water. For selecting the maximum absorbance wavelength, heparin and force degraded samples were scanned at the wavelength range of 190 to 400 nm by PDA. Fused silica capillaries of 50 μm i.d. (with total length of 40, 50, 64, 75, 80.5 cm), 75 μm i.d. (with total length of 50, 75 cm) and 50 μm i.d. bubble cell (with total length of 50 cm) and electrolyte with concentrations of 36, 50, 72 and 100 mM and pH of 2.50, 3.50 and 4.50 were used. Analyses were run using applied voltage of -10, -18.5 and -30 kV and at temperatures of 25, 35 and 45°C.

**Method validation**

Method validation was carried out according to ICH guideline [25]. Linearity of the method was investigated using peak area with repeat analysis (n=3) of 0.312, 0.625, 1.25, 2.50, 3.75, 5.00, 7.50, 10.0 and 15.0 mg/ml, which were serially diluted of stock solution with concentration of 30 mg/ml. Limit of quantification (LOQ) and limit of detection (LOD) were determined by injecting serially diluted samples (n=6). Intra and inter-day precision and accuracy of the method were determined at the lower, the middle and the upper level of the calibration range (at 0.625, 5.00 and 15.0 mg/ml). Precision was derived using calculated concentrations for peak area of repeated analyses (n=3) and accuracy was calculated as a ratio of observed concentration to real amount. The specificity of the method was investigated by determination of peak purity parameters of heparin samples using a PDA detector and ChemStation software. Due to the possible effect of storage conditions on concentration of heparin solutions, stability of them was studied in terms of short term temperature (lab temperature), stock solution (4°C) and freeze and thaw stability (−20°C) according to FDA guideline [26].

**Preparing force degraded samples**

For evaluating the specificity of the proposed method and applicability for stability studies and separating the degraded forms, force degraded samples were required. For this reason heparin solution with concentration of 30 mg/ml in sealed vials and heparin pharmaceutical vials were subjected to the thermal stress at 90 ± 1°C. For acidic and basic accelerated degradation a solution of heparin with concentration of 30 mg/ml which was dissolved in hydrochloric acid 0.01 N and sodium hydroxide 0.01 N, respectively, were prepared. Then solutions were kept at 70 ± 1°C for 120 hours. Also a heparin vial was kept at room temperature and another vial was subjected to sun light exposure in the lab. Then sampling was done in definite intervals and the samples were diluted with double distilled water, and then analyzed by the proposed method.

**Results**

**Optimization of CE analysis**

As shown in Figure 1, maximum absorbance wavelengths of 200 nm and 257 nm were selected for detection of heparin and degradation products, respectively. According to the results, the best separation was achieved by a bare fused capillary of 50 μm i.d; 50 cm total and 41.5 cm effective length, voltage of -30 kV at 35°C, by hydrodynamic injection at 50 mbar for 40 seconds and phosphate buffer (72 mM, pH=3.50). Under optimized conditions, mean migration time of heparin was 2.39 ± 0.03 with intra-day and inter-day migration time RSD% of 1.3 (n=12) and 2.0 (n=24), respectively. An electropherogram of heparin standard solution (50 μg/ml) under these conditions is shown in Figure 2.
Validation of the proposed CE method

For evaluating the specificity of the method for determination of heparin, peak purity and similarity/threshold ratio of heparin in standard sample, and a pharmaceutical dosage form was calculated by ChemStation® software. Peak purity factor in all samples were between 999 and 1000 and the similarity/threshold ratio was under 1. The linearity parameters of the method were shown in Table 1. Concentration of 0.312 mg/ml was selected as LOQ of the method, due to the accuracy of 103.1%, precision of 4.0% and peak to noise ratio of more than 10, and concentration of 0.078 mg/ml was chosen as a LOD of the method because of the peak to noise ratio of 3. Using the bubble-cell capillary, the LOD of the method was decreased to 0.030 mg/ml. The results of accuracy and precision of the method were reported in Table 2.

Evaluating the stability of heparin solutions illustrated that, the assayed amount of freeze and thaw samples was 97.72 ± 4.73%, assayed

<table>
<thead>
<tr>
<th>Calibration range</th>
<th>Calibration equation</th>
<th>Regression coefficient</th>
<th>LOQ1</th>
<th>LOD2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.312–15.0 mg/ml</td>
<td>y=77.10x+0.189</td>
<td>0.998</td>
<td>0.312 mg/ml</td>
<td>0.078 mg/ml</td>
</tr>
</tbody>
</table>

* Separation conditions: capillary 50 μm i.d. (50 cm total and 41.5 cm effective length), buffer phosphate 72 mM, pH=3.50, temperature: 35°C, voltage: -30 kV.
1. Limit of quantification
2. Limit of detection

Table 1: Calibration range

<table>
<thead>
<tr>
<th>Conc. (mg/ml)</th>
<th>Accuracy (%)</th>
<th>Intra-day precision (RSD %)</th>
<th>Inter-day precision (RSD %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.625</td>
<td>97.46</td>
<td>1.8</td>
<td>2.3</td>
</tr>
<tr>
<td>5.00</td>
<td>101.0</td>
<td>1.7</td>
<td>0.78</td>
</tr>
<tr>
<td>15.0</td>
<td>97.27</td>
<td>0.28</td>
<td>3.2</td>
</tr>
</tbody>
</table>

* Separation conditions: capillary 50 μm i.d. (50 cm total and 41.5 cm effective length), buffer phosphate 72 mM, pH=3.50, temperature: 35°C, voltage: -30 kV.
1. Relative standard deviation

Table 2: Accuracy and precision of the method.
amount of short term temperature samples was 98.51 ± 0.19% and that of stock solution stability samples was 100.6 ± 0.46%.

Analysis of real samples

For determination of heparin content in the pharmaceutical product, 3 vials were analyzed by the developed method. One ml of each vial was diluted 5 fold with double distilled water, and then each sample was analyzed 3 times. The assayed amounts for three vials were 100.3 ± 0.03%, 98.94 ± 1.8% and 99.48 ± 0.26% of the label claim.

Analysis of force degraded samples

The proposed method could show the changes in the heparin electropherogram, and also formation of degradation products (Figures 3 and 4) under different stress conditions. The results of thermal stress studies indicated that, after 24 hours of the beginning of the test, shape of heparin peak started getting wide, after 96 hours colored products formed (Figure 5) and peaks of major degradation products became appeared at 2.99 ± 0.05 minutes and 3.45 ± 0.07 minutes, then heparin peak was degraded extremely after 412 hours and three other products with migration times of 3.22 ± 0.05 minutes, 3.70 ± 0.07 minutes and 4.05 ± 0.02 minutes became detectable after 600 hours. Then insoluble products began to form after 720 hours (therefore after 720 hours samples were centrifuged and supernatants were analyzed by the proposed method). After 2000 hours of the beginning of the test, a peak appeared around 6 minutes, in addition to the same peaks at wavelength of 200 and 257 nm. Further studies on samples of different intervals showed that, by reducing in heparin concentration with time, peak area of all degradation products, especially products with migration time of 2.99 ± 0.05 minutes and 3.45 ± 0.07 minutes, increased. Correlating stress time and peak area of two major degradation products formed under thermal stress, illustrated that, there was a linear relationship between logarithm of peak area of two major degradation products and time (during 96 to 720 hours) (Figure 6).

The results of acidic stress studies showed that, the peak area of heparin decreased but peak shape became wider and a additional peak appeared at 3.74 ± 0.13 minutes after 24 hours, that its area increased with time, then heparin peak was degraded dramatically and another additional peak

Figure 5: Formation of colored products and insoluble residue under thermal stress in heparin aqueous solution (samples of 0 to 1000 hours) and powder (a) standard heparin powder, (b) heparin powder exposed to thermal stress for 1000 hours. (Colored products formed in powder, too).

Figure 6: Correlation between time and peak area of 2 major degradation products formed under thermal stress (during 96 to 720 hours). A: exponential relationship between time and peak area. B: linear relationship between time and logarithm of peak area. 1: product with.
appeared at 4.94 ± 0.11 minutes and colored products began to form after 48 hours and after 96 hours insoluble residue appeared.

Stability studies under basic stress demonstrated that, after 24 hours the peak area decreased but peak shape became wider and an additional peak appeared at 4.98 ± 0.07 minutes. After 72 hours another peak became appeared at 3.72 ± 0.12 minutes and colored products began to form and after 120 hours peak shape was degraded and insoluble residue appeared after 600 hours.

Finally the samples which were kept at sun light exposure and room temperature conditions were analyzed. The results showed that, in both samples heparin peak became wider but peak area decreased slowly with time without any additional peaks, so that after 3000 hours of beginning of the test, 84.95% and 87.31% of heparin remained at sun light exposure and room temperature, respectively.

**Discussion**

**Optimization of the CE method**

For detection of heparin and force degraded samples 200 and 257 nm were chosen, respectively, in order to achieve the highest sensitivity. Evaluating the effect of capillary diameter on separation showed that, the capillary of 75 μm i.d. gave slower migrations and wider peaks in comparison with the capillary of 50 μm i.d. For obtaining the better peak shapes and shorter migration times, the total length of the capillary was reduced, but when the length of capillary was decreased to less than 50 cm, the current became too high and current breakdown was observed. Therefore the capillary of 50 μm i.d. (with 50 cm total length and 41.5 cm effective length) was chosen for further analysis. For improving the LOD of the method, a bubble-cell capillary (50 μm i.d.; 50 cm total and 41.5 cm effective length) was used. In comparison with the straight capillary, bubble-cell capillary gave wider peaks and longer migration times, but the sensitivity was increased and LOD of the method was decreased to 0.03 mg/ml. However the straight capillary was used for further analyses, because it provided acceptable results. The voltage of -30 kV was chosen for determinations, because it gave rapid migration and good peak shapes and also acceptable separation of degradation products. The reported pH for determination of heparin was between 2.00 to 5.00 (21-23) and the best separation was achieved at pH of 3.50. The effect of electrolyte concentration was investigated and the results showed that by the enhancement in the concentration, the resolution and the separation were increased, but in concentration of 100 mM peak shapes were degraded extremely, therefore the electrolyte with morality of 72 mM was applied for analyses, also in this concentration force degradation products could be completely separated at 257 nm. Evaluating the effect of cassette temperature on separation showed that, enhancement in cassette temperature improved the peak shapes and migration times and the best peak shape and migration time were achieved at 35°C, but when the temperature was increased more than 35°C, the peak shape got worse and at 40°C the current breakdown was observed, so the cassette temperature was set at 35°C. Evaluating the effect of injection method on separation showed that, hydrodynamic injection gave better results and the best results were obtained by hydrodynamic injection at 50 mbar for 40 seconds.

**Validation of the method**

The method validation results illustrated that the method was linear in concentration range of 0.312 mg/ml to 15 mg/ml, also accurate and precise in all concentrations, which met the guideline on validation of analytical methods by ICH [25]. Aside the method was specific for determination of heparin in pharmaceutical samples because of the purity factor of more than 999 and similarity/threshold ratio of less than 1 for heparin peak in presence of possible exipients in a pharmaceutical product, so the heparin peaks were pure and there were not any comigrate peaks with them [27].

According to FDA guideline [26], solutions were stable in stock solution and under short term temperature owing to the recovery of more than 98%, therefore heparin solutions were stored at 4°C before analysis and analyzed within 24 hours of preparation or sampling.

**Analysis of real samples**

The validated method was successfully used for quantification of heparin in 3 vials of a pharmaceutical product. The method was very simple and rapid so that a sample could be quantified in short time without any organic modifier or specific sample preparation procedure.

**Analysis of force degraded samples**

The results of force degradation studies showed that, the peak area of all degradation products were increased with time. According to the migration time of force degradation products, the same products have been formed under acidic and basic conditions. Also the same product with migration time of 3.70 ± 0.07 minutes has been formed under thermal, acidic and basic conditions.

More studies on heparin degradation products formed under thermal stress indicated that, there was a linear relationship between the logarithm of the peak area of two major degradation products with migration time of 2.99 ± 0.05 minutes and 3.45 ± 0.07 minutes and stress time. Assuming the linear relationship between concentration of degradation products and peak area reveals that, degradation products would form by the first order kinetic. For estimating this hypothesis, access to degradation products is necessary, but, this method could not provide any information about structure or concentration of degradation products, therefore further studies are suggested for identifying and quantitative determination of these products.

**Conclusion**

A simple and rapid CE method has been developed and validated for determination of heparin in a pharmaceutical product, and evaluation of heparin stability under different stress conditions. Using the optimized method, migration time of heparin was reduced to 2.39 ± 0.03 minutes and degradation products were separated within 7 minutes. In comparison with the other CE methods reported for analysis of heparin (Table 3), the developed method is fast, more accurate and precise and

**Table 3:** Comparison of the proposed method with other CE methods reported for determination of heparin.

<table>
<thead>
<tr>
<th>Method</th>
<th>Linearity range</th>
<th>Correlation coefficient (r)</th>
<th>Precision (RSD %)</th>
<th>Accuracy (%)</th>
<th>LOD4</th>
<th>Migration time (min)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MECC1</td>
<td>0.08-7.0 U/ml</td>
<td>0.998</td>
<td>1.5-4.4</td>
<td>95.6-98.7</td>
<td></td>
<td>2.58 ± 0.02</td>
<td>(18)</td>
</tr>
<tr>
<td>CZE2</td>
<td>2.5-50 mg/ml</td>
<td>0.998</td>
<td>2.3-3.8</td>
<td>104.0-105.5</td>
<td></td>
<td>5.69 (RSD% of 0.57 to 1.9)</td>
<td>(19)</td>
</tr>
<tr>
<td>CZE</td>
<td>4.0-40 mg/ml</td>
<td>0.998</td>
<td>1.3-2.6 (for peak area)</td>
<td>-</td>
<td>0.91 mg/ml</td>
<td>Less than 12 (RSD% of 0.92 to 1.2)</td>
<td>(20)</td>
</tr>
<tr>
<td>CZE5</td>
<td>0.312-15.0 mg/ml</td>
<td>0.998</td>
<td>0.28-3.2</td>
<td>97.27-101.0</td>
<td>0.078 mg/ml</td>
<td>2.39 ± 0.03 (RSD % of 1.3 to 2.0)</td>
<td>This work</td>
</tr>
</tbody>
</table>

1. Micellar electrokinetic capillary chromatography; 2. Capillary zone electrophoresis; 3. Relative standard deviation; 4. Limit of detection
has a lower LOQ, but the main advantage of the proposed method is its ability to show the changes in heparin electropherograms and formation of degradation products under different stress conditions. At the end, it could be said that, the proposed method could be used as a fast and simple method in quality control laboratories for evaluating the quantity and stability of heparin products under different conditions.

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References