SEPARATION OF RECOMBINANT ERYTHROPOIETIN ISOFORMS BY CAPILLARY ELECTROPHORESIS

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TÓM TẮT

PHÂN TÁCH CÁC DẠNG ISOFORM CỦA ERYTHROPOIETIN BẰNG PHƯƠNG PHÁP ĐIỆN DI MAO QUẢN

Thành phần isoform của protein erythropoietin (EPO) tái tổ hợp dùng làm dược phẩm đã được nghiên cứu bằng kỹ thuật điện di mao quản (CE) với đầu dò UV. Quá trình phân tách các isoform EPO bằng CE được thực hiện trên cột silica sử dụng putrescine để biến tính bề mặt mao quản nhằm giảm sự hấp thụ protein EPO lên thành mao quản và tăng độ phân giải, dung dịch điện ly nền ngoài putrescine còn chứa urea, CH_3COONa và tricine. Sau khi tối ưu các yếu tố như thành phần dung dịch điện ly nền, thông số của thiết bị CE quy trình có khả năng phân tách hoàn toàn 7 isoform có trong protein EPO với độ phân giải và độ lập lại cao.

1. INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone which stimulates erythropoiesis produced from the kidneys. Recombinant erythropoietin is available as a drug in the clinical treatment of anaemia. The carbohydrates content of roughly 40% of total molecular mass of EPO and polypeptide chain contains 4 glycosylation sites with 3 N-linked and 1 O-linked [1]. Each carbohydrate chains may contain 2-4 branches, and each branch may terminate with a sialic acid, thus EPO isoforms possess different numbers of sialic acid. The biological activity of each isoform depends on the number of sialic acid residues [2]. The glycosylation of polypeptide chain is a post-translational process which depends on the type of cell in which the recombinant is synthesized and physical factors, such as culture conditions, and isolation procedures used in purification [3]. Therefore, the isoform profile of EPO decides biological activity of products and distinguish recombinant products from cell type.

Differences in sialic acid content of EPO isoforms EPO result in differences in

electric charge [4], therefore, capillary zone electrophoresis (CZE) is the best method used in separation of EPO isoforms. CE is a technique that separates molecules based on their different charge to mass ratio with high resolution [5].

There are some general considerations when working with proteins: (i) avoiding protein adsorption onto the capillary wall which could be done by capillary surface modification. (ii) denaturing proteins *ie*. breaking internal bonds in order to straighten the biomolecules for better separation. The complete separation is of utmost importance when a non-selective detector such as UV is employed. (iii) The buffer must be able to maintain constant charges of proteins and capillary surface.

Though there were few puplications dealing with the separation of EPO isoforms, the authors used rather complicated background electrolyte solutions without any explanation [2-4, 6-8]. Therefore, we carried out a study in order to have better understanding on the effects of each components, simplify the background electrolyte solution if possible, as well as optimize our CE system for this special application. In this study there were several important issues investigated namely, the process of dynamic coating of capillary surface with amines, denaturation of protein by urea, pH and composition of background electrolyte solution. In addition, instrumental parameters *eg*. voltage, capillary temperature, detection wavelength were also optimized.

2. EXPERIMENTAL

2.1. Chemicals and instruments

NaOH, NaCl, CH_3COONa, CH_3COOH, urea were purchased from Merck (Germany), tricine and putrescine were obtained from Sigma-Aldrich (USA). Protein erythropoietin (EPO) 800 µg/mL was a product of Nanogen Biopharmaceutical (Vietnam). Deionized water was obtained using a Mili-Q water purification system (Millipore, France).

CZE separations were carried out on CE instrument (Agilent Technologies, USA) with diode array detector. Uncoated fused-silica capillary with the length of 75 cm (68 cm effective length) and 50µm ID from Polymicro Technologies (USA) was used.

2.2. Separation procedures

Based on the puplished works and the experience on our CE system the initial conditions for separation EPO isoforms were set as follows:

The background electrolyte solution at pH 5,5 consisted of 6 M urea, 10 mM NaCl, 10 mM CH_3COONa, 10 mM tricine, 2,5 mM putrescine. Samples were injected at 50 mbar for 20 s, the voltage was set at + 20 kV and the analysis was conducted at a constant temperature of 35 ^oC. Detection was performed at 214 nm.

Between runs, the capillary was flused with the separation buffer for 10 minutes. Before use a new capillary had to be activated with 1 M NaOH for 30 minutes and then rinsed with water for 30 min.

3. RESULTS AND DISCUSSION

3.1. Dynamic coating

In this project, dynamic coating was used to prevent the adsorption of protein onto the inner surface of the capillary because of its simplicity and low cost in comparison to buying commercial capillaries whose

capillary walls are chemically modified for this specific application. Two agents namely, putrescine and ethylene diamine (EDA) were tested for this purpose [9, 10].

A small amount of dynamic coating agent was added to the background electrolyte solution to alter the capillary surface charge. The dynamic coating agents used in this study were diamines with pK_{a1} and pK_{a2} in the range of 7.6 – 10.8, so they carries +2 charge at pH 5.5. The electrostatic interaction between the protonated amines with the negatively charged SiO^- on capillary surface could prevent the interactions of EPO protein with the capillary surface. Another advantage of using the amines is to reduce the strength of the electroosmosis flow (EOF) and therefore, increasing the resolution of the isoforms. The results showed that both putresicne and EDA could be used interchangeable in separation of the EPO isoforms (data not shown). Nevertheless, the use of putrescine is more favorable than EDA because putrescine is solid and more stable while EDA must be re-distilled when stored for longer than a month.

To find suitable concentrations of putrescine it was varied from 2.5 to 15 mM while keeping other components of background electrolyte solution unchanged. It was found that there was a trend of increasing migration time (t_m) and resolutions between adjacent isoforms as concentration of putrescine increased [11](Fig. 1). However, at concentrations higher than 4 mM the repeatability became worse, unstable current, noisy electropherograms. As a result, putrescine concentration would be kept at 2.5 mM which gave high resolution and stability.

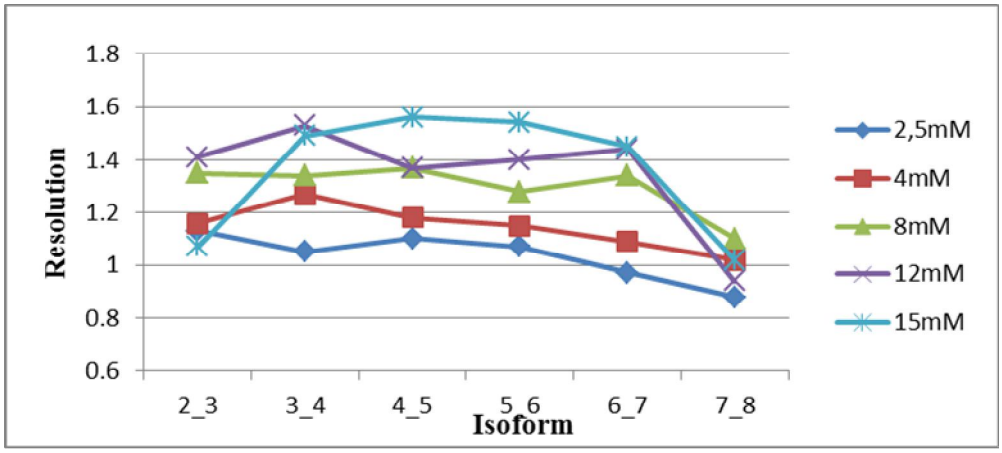

Figure 1. The effect of putrescine concentration on resolution between adjacent isoforms (The other parameters were as same as described in 2.2)

3.2 Denaturation of protein by urea

By denaturation proteins will be straightened, all acid or base groups are able to contact with the environment which lead to complete their dissociation or protonation. The differences in size and electrical charge of the isoforms increase, thereby the resolutions are getting better. Completely resolve all peaks of isoforms is

of utmost importance in quantitation when using non-selective UV detector.

Urea is one of the most popular denaturant for proteins because it is inexpensive and easy to use. Urea disrupts hydrogen bonds in molecules and bind to the polypeptide chain that stabilize the denatured state of protein (Fig. 2). However, urea cannot disrupt disulfur bond. Though the polypeptide chain cannot completely straight, but in many cases urea is sufficient to increase the difference of the isoforms to separate effectively.

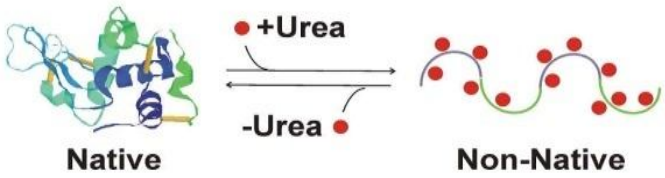

Figure 2. Denaturation of proteins by urea

Urea was added into the background electrolyte solution. At urea concentrations of 4, 5, 6, and 7 M experimental results showed no significant differences on resolution. The repeatability of peak area is low (% RSD ~ 20-30%) with low concentrations of urea (4-5 mM). When increasing concentrations of urea to 7 M, there were still good resolutions and good repeatability of peak areas, but system peak tended to overwhelm the first isoform peak. Additionally, migration times were longer because of high the viscosity of the solution. As a result, 6 M urea was chosen for the next experiments.

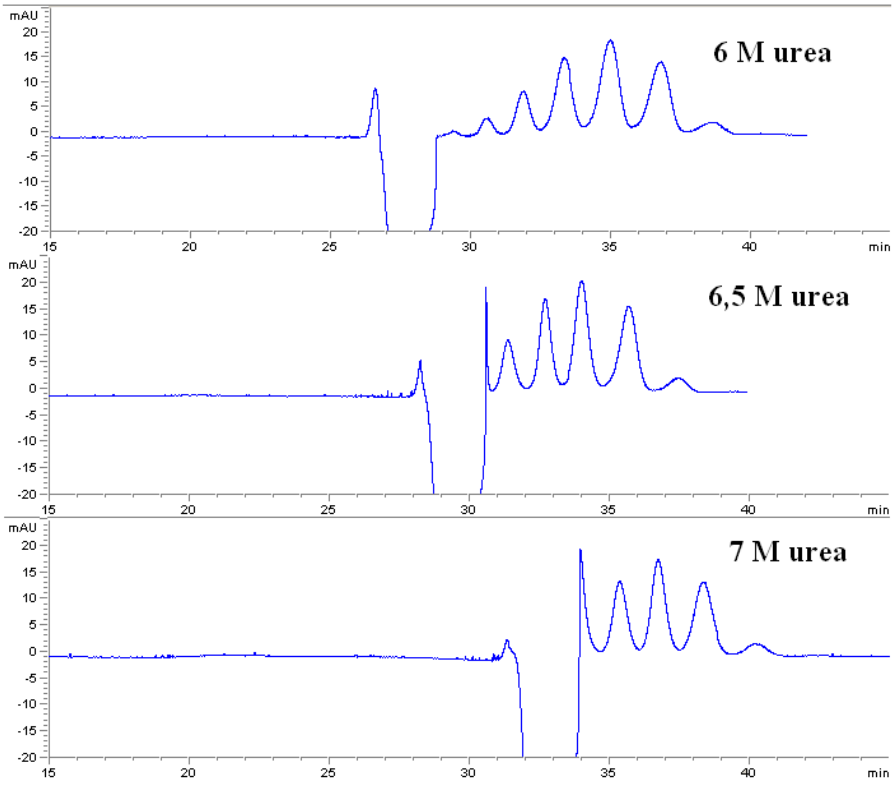

Figure 3. The effect of urea concentration on the separation of EPO isoforms

3.3. The electrolyte salt concentration and pH of background electrolyte solution

The effect of pH was studied from 4.3 to 6.0. At pH 4.3, isoform peaks were completely overlapped. When pH increased (4.8-6.0) the ability to separate isoforms initially increased, peaks of the isoforms tended to be away from the system peak but then isoforms co-migrated again at pH 6.0. It should be noted that pH has two reversed effect: (i) the higher pH, the faster EOF, the shorter migration time and therefore, the lower peak resolution is. (ii) the higher pH, the more negative charges of the isoforms which leads to bigger differences in their electromobility. Consequently, there is a small pH range that compromises these two effects. From experimental data, 5.0-5.5 was selected as the best pH range regarding the resolution (Fig. 4)

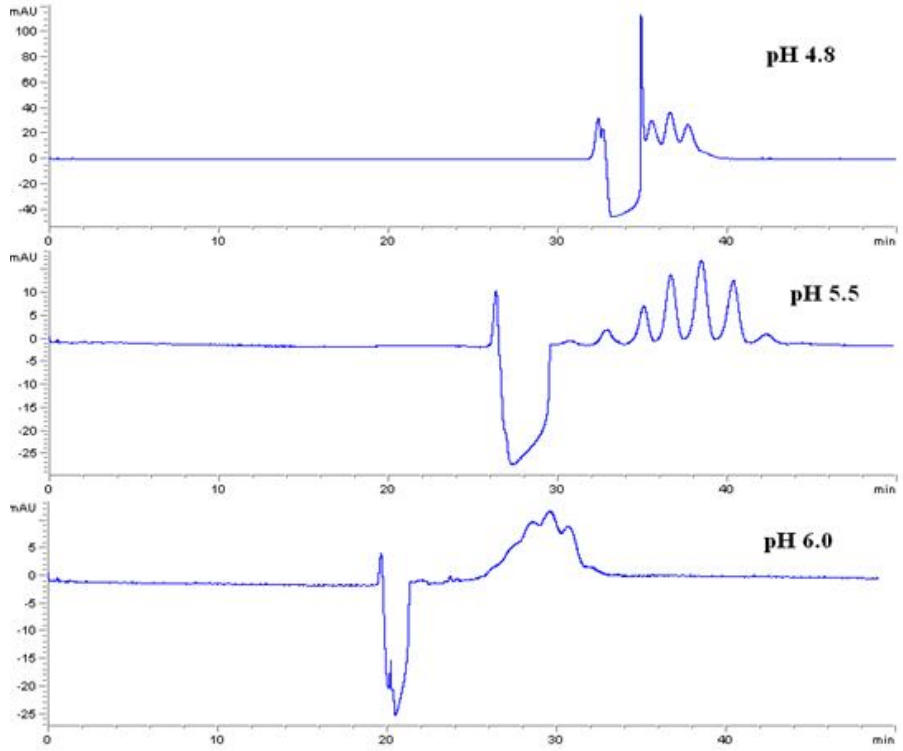

Figure 4. The effect of pH on the separation of EPO isoforms

The last thing we concerned was that whether it is possible to reduce the number of electrolyte components. It should be kept in mind that high electrolyte concentration is usually necessary for stacking phenomenon and low risk of protein adsorption on the capillary wall. The electrolyte, however, should not be added too much to avoid Joule heating.

In each subsequent experiment at pH 5.5 only one salt was used, either NaCl, CH_3COONa, or tricine. Resulted data showed that it was able to well separate all isoforms using only one of these salts (data not shown), however NaCl gave the worst repeatability due to unstable current. The

% RSD of the CE current observed for NaCl, CH_3COONa, and tricine were of 25, 11, and 7 %, respectively. It is obvious that NaCl has no pH buffer ability. Acetate sodium and tricine (zwitterion) were finally used as the only two electrolytes in the solution thanks to their buffer ability and low level of Joule heating..

3.4. The instrumental parameters of CE system

In this part instrumental parameters of our CE system were optimized for the best sensitivity and resolution (data not shown). The optimal values of these parameters as well as the background electrolyte composition are summarized in Table 1.

Table 1: Optimal paremeters of CE system and background electrolyte composition for separation of EPO isomers

Voltage	+25kV	Injection pressure	50 mbar
Temperature of capillary	25°C	Injection time	20 s
Wavelength for detection	214 nm		
Background electrolyte solution: 6 M urea + 2,5 mM putrescine + 10 mM CH_3COONa + 10 mM Tricine, pH 5,5			

3.5. Repeatability and sensitivity of the analytical method

The EPO pharmaceutical products from Nanogen Biopharmaceutical were analyzed to determine the isoform patterns *ie*. the number and relative concentrations of isoforms using the conditions found in this study (Table 1). Using the 6^{th} isoform as internal standard we obtained very good repeatability for both migration time (%RSD ~ 0,14-0,55 %) and peak area (%RSD ~ 0,5-4,1 %) and the LODs were of 7-16 ppm.

4. CONCLUSIONS

The analytical procedure for separation of EPO isoforms by capillary electrophoresis with UV detector had good resolution, completely separated 7 isoforms existing in the EPO products of Nanogen. This study revealed the effect of factors involved in the separation including the composition of background electrolyte solution and instrumental parameters, which will become the foundation for other applications in the field. The analytical method had high repeatability and sensitivity for quality control in pharmaceutical industry.

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