Separation and detection of erythropoietin by CE and CE–MS

Bing Yu, Hailin Cong, Huwei Liu, Yuanzong Li, Feng Liu

For doping control, the standard method of testing recombinant human erythropoietin (rhEPO) is a combination of a blood test and a urine test. The blood test is based primarily on changes in blood parameters, and the urine test employs an immunoblotting assay that includes one isoelectric focusing (IEF) separation step, two blotting steps and luminescence detection, so the whole testing procedure is time-consuming and expensive. The present review summarizes the separation and the detection of rhEPO by capillary electrophoresis (CE) as well as by CE on-line coupled with electrospray ionization mass spectrometry (CE–ESI-MS). We demonstrate the usefulness of these approaches.

Keywords: Capillary electrophoresis; Detection; Erythropoietin; Mass spectrometry; Review

Abbreviations: CE; Capillary electrophoresis; CHO; Chinese hamster ovary; CZE; Capillary zone electrophoresis; DAD; Diode array detector; EM; Electrophoretic mobility; EOF; Electroosmotic flow; ESI; Electrospray ionization; EPO; Erythropoietin; FQ; 3-(2-Furoyl) quinoline-2-carboxaldehyde; GC-MS; Gas chromatography-mass spectrometry; hGH; Human growth hormone; HPLC; High-performance liquid chromatography; HPMC; Hydroxypropylmethylcellulose; IEF; Isoelectric focusing; IOC; International Olympic Committee; LIF; Laser-induced fluorescence; LOD; Limit of detection; MALDI-TOF-MS; Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MS; Mass spectrometry; MS–MS; Tandem MS; PEI; Polyethyl enimine; rhEPO, Recombinant human erythropoietin; uEPO; Human urinary erythropoietin; UV; Ultraviolet

Bing Yu, Hailin Cong, Huwei Liu *, Yuanzong Li, Feng Liu
Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, PR China

*Corresponding author.
Tel.: +86 10 6275 4976;
Fax: +86 10 6275 1708;
E-mail: hwliu@pku.edu.cn.

1. Introduction

1.1. Physiological and biochemical properties of EPO

Erythropoietin (EPO) is a glycoprotein hormone produced by the kidney in adult humans [1–4]. It stimulates the production of red blood cells by promoting the proliferation and the differentiation of erythroid-progenitor cells. Human EPO consists of a 166-amino acid polypeptide chain (p*I* 4.5–5.0) with disulfide bonds (between 7 and 161, between 29 and 33) and four heavily polysaccharide chains (one *O*-linked at Ser126 and three *N*-linked at Asn24, Asn38 and Asn83), as shown in Fig. 1 [5,6]. The molecular mass of EPO is 30–34 kDa, but carbohydrate chains amount to about 40% of the molecular mass [3,7,8]. The carbohydrate moiety is of great importance for the biological activity of EPO [9–13].

Since 1985, rhEPO derived from Chinese hamster ovary cells (CHO) has been available for therapeutic use in certain forms of anemia [1]. However, this hormone quickly became misused as a doping agent for endurance athletes to improve aerobic performances, and the International Olympic Committee (IOC) officially prohibited its use in 1990 [14]. Because endogenous EPO and rhEPO possess nearly identical biochemical and immunological properties [15–17], detection of illegal use of rhEPO becomes difficult [18].

However, it is well known that both the natural and the recombinant forms of EPO present extensive microheterogeneity in relation to post-translational modifications in the proteic moiety. Some of these post-translational events are influenced by the nature and the environmental conditions of the cell that produces the protein [12,13]. Since human natural and recombinant EPO are synthesized in human kidney and Chinese hamster ovary cells, respectively, some of these modifications may be different in the two hormones.

Furthermore, several studies have demonstrated that the microheterogeneity is associated with the presence or the absence of terminal *N*-acetyl neuraminic acid residues with varying amounts of acetylation and the presence or the absence of *N*-acetyl lactosamine extensions. The degree of sialylation of polysaccharidic chains therefore strongly influences the electrophoretic mobility (EM) and the isoelectric point (p*I*) of the molecule [12,16]. Differences in their

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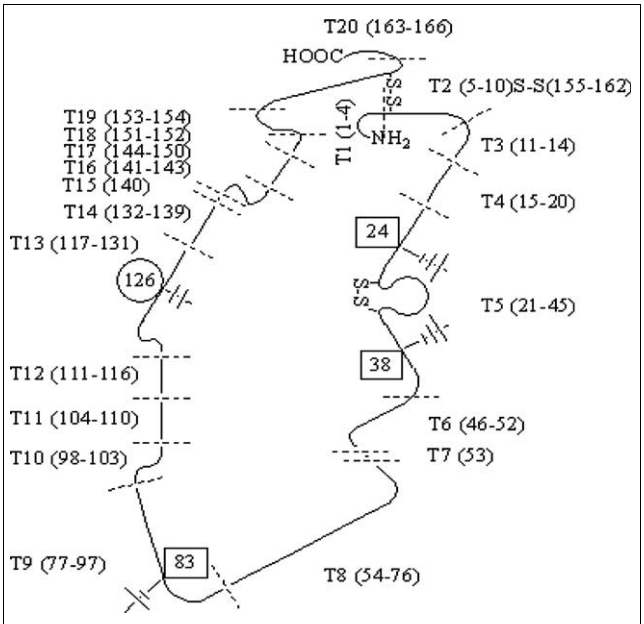

Figure 1. Predicted structure of rhEPO [5,6]. (T1--T20 are peptides generated by tryptic; the amino acid residue numbers are indicated in parentheses; the *N*-linked saccharides are located at the amino acid residues 24, 38 and 83; whereas *O*-linked saccharide is located at the residue 126.)

isoelectric profiles thus seem to be a potential means of differentiating between natural and recombinant EPO.

1.2. The EPO epidemic and testing in sport

Doping control on athletes has been done for over 30 years, and is increasingly becoming an integral part of sports competitions. With the development of new technologies, the testing of traditional banned drugs, such as stimulates, narcotic analgesics, anabolic steroids, β-blockers and diuretics, has become rife. But effective detection of endogenous drugs, such as human growth hormone (hGH) and EPO, needs still more effort.

EPO boosts athletic performance by stimulating the production of red blood cells, which will transport increasing amounts of oxygen to muscles. Consequently, over the past decade, the performance of about two dozen European professional and amateur cyclists has been attributed to illicit use of rhEPO [8,9]. In particular, the 1998 Tour de France and the 2001 World Nordic Ski Championships made the detection of rhEPO really urgent [8–13].

After these disastrous drug scandals, scientists from the Australian Sports Drug Testing Laboratory (ASDTL) and the Australian Institute of Sport (AIS), together with collaborators from France, Canada and Norway, undertook a major study to validate a test for detecting the use of rhEPO by athletes. The EPO test used for the first time at the Sydney 2000 Olympic Games was a combination of a blood test and a urine test.

The blood test, as one of the indirect biomarkers, mainly detects the following changes in blood parameters after administration of EPO:

- serum EPO level;
- serum soluble transferrin receptor;
- hematocrit;
- the percentage of macrocytes (large red blood cells); and,
- the number and size of reticulocytes.

Among these parameters, hemoglobin, hematocrit and % reticulocytes were measured using a commercial hematology analyzer supplied by Bayer. The blood was analyzed by spectrometry, where light is shone through the sample and hematocrit was determined by the absorbance. Immunoassays for soluble transferrin receptor (sTfr) and EPO in the serum were also performed using proprietary analyzers [19].

The urine test was developed in the IOC-accredited laboratory in Paris and used an IEF technique to separate the different glycoforms of EPO in urine, and then the EPO was detected by the double-blotting techniques. The pattern of isoforms in rhEPO can be distinguished from the pattern observed with endogenous EPO found in urine (as shown in Fig. 2) [19].

2. Immunoassays for the detection of rhEPO

Since uEPO and rhEPO possess nearly identical properties, most classical analytical techniques, such as HPLC, GC-MS and spectroscopy, are essentially ineffective in distinguishing rhEPO from endogenous EPO. Accordingly, few publications have been reported for the analysis of EPO by these methods.

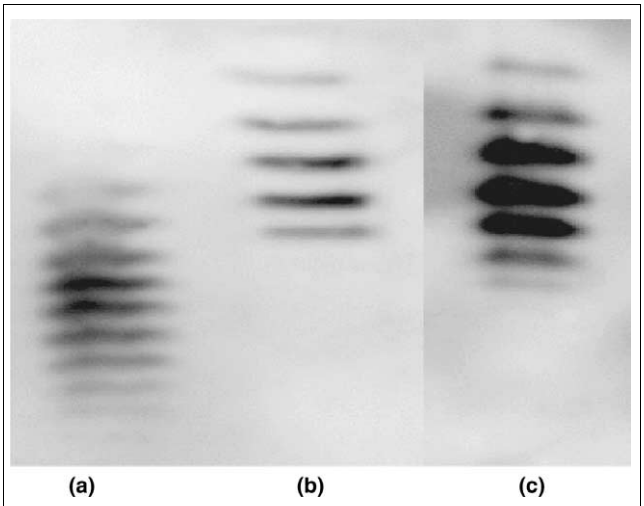

Figure 2. Western blotting of (a) normal urinary EPO, (b) recombinant EPO, and (c) EPO recovered from the urine of a subject after recombinant EPO injections (cathode top of gel and anode bottom of the gel) [19].