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Characteristics of IEF Patterns and SDS-PAGE Results of Korean EPO Biosimilars

Min-Jung Kang,[*] Sang Mi Shin,[†] Hey Hyun Yoo,[‡] Oh-Seung Kwon,[†] and Changbae Jin

Integrated Omics Center, Korea Institute of Science and Technology, P.O. BOX 131, Cheongryang, Seoul 130-650, Korea
[*]E-mail: mjkang1@kist.re.kr
[†]Doping Control Center, Korea Institute of Science and Technology, P.O. BOX 131, Cheongryang, Seoul 130-650, Korea
[‡]Department of Pharmacy, College of Pharmacy, Hanyang University, Ansan, Gyeonggi-do 426-791, Korea
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Erythropoietin (EPO) is mainly produced in kidney and stimulates erythropoiesis. The use of recombinant EPOs for doping is prohibited because of its performance enhancing effect. This study investigated whether biosimilar EPOs could be differentiated from endogenous one by iso-electro-focusing plus double blotting and SDS-PAGE for anti-doping analysis. The established method was validated with positive control urine. The band patterns were reproducible and meet the criteria, which was made by world anti doping agency (WADA). Isoelectric focusing was conducted in pH range 2 to 6. Recormon (La Roche), Aropotin (Kunwha), Epokine (CJ Pharm Co.), Eporon (Dong-A), Espogen (LG Life Sciences), and Dynepo (Shire Pharmaceuticals) were detected in basic region. All biosimilars showed discriminative isoelectric profiles from endogenous EPO profiles, but they showed different band patterns with the reference one except Epokine (CJ Pharm Co.). Next, SDS-PAGE of biosimilar EPOs resulted in different molecular weight patterns which were distributed higher than endogenous EPO. Commercial immune assay kit as an immune affinity purification tool and immobilized antibody coated magnetic bead were tested for the purification and concentration of EPO from urinary matrix. The antibody-coated magnetic bead gave better purification yield. The IEF plus double blotting and SDS-PAGE with immunoaffinity purification method established can be used to discriminate biosimilar EPOs from endogenous EPO.

Key Words: Erythropoietin, Biosimilars, Isoelectric focusing, SDS-PAGE, Doping control

Introduction

Erythropoietin (EPO) is a glycoprotein hormone with a molecular mass of 39000 Da, which is synthesized predominantly in the kidneys. EPO stimulates proliferation and final maturation of red blood cell precursors in bone marrow.[1-2] The history of EPO detection in sports started in 1988 at the same time EPO was prohibited by sports authorities. In 2000, Francoise Lasne *et al*. developed a recombinant EPO (rEPO) detection method and published in Nature.[3] This developed method employs isoelectric focusing and double blotting for microheterogenious EPO separation and chemiluminescent detection. She showed that administered Epoetin α (or β) could be differentiated from endogenous EPO and it is excreted in urine without noticeable change in its isoelectric profile.[3-6] There have been an another detection method developed in 2000 by Allan G. Hahn,[7] which employs an indirect method to measure blood markers of altered erythropoiesis. Seoul doping control laboratory has been adopted this indirect method for analysis of world cup game samples in 2002.

UCLA Laboratory[8] and Barcelona Laboratory[9] have established isoelectric focusing and double blotting method for analysis of rHuEPO and EPO mimetics. During these times, synthetic erythropoiesis (SEP) was synthesized[10] and various kinds of EPO mimetics were produced in commercially. Therefore detection of EPO abuse has become more challenging. 2DE (two-dimensional gel electrophoresis) method was developed by Alamgir Khan in 2005 to overcome overlapping problem of rHuEPO and HuEPO.[11] However, a question about the current WADA-adapted method arises very frequently. For example, the nonspecific binding problem of AE7A5 antibody was posed by the Wener W. Franke and Hans Heid in 2006.[12] There was a big discussion about the WADA-adapted protocol worldwide, but evidences to support the reliability of WADA-adapted protocol were published.

Finally, Oliver P. Ravin, a member of WADA, has suggested with France, Switzerland, Belgium and Spain laboratory that 2D method does not highlight any issue in relation to the 1D method used for anti-doping controls[13] and the current adapted WADA protocol was fixed as a standard protocol. Although the 1D isoelectric focusing plus double blotting was fixed as a standard protocol by WADA, SDS-PAGE detection method was developed and successfully applicated to EPO samples as an ancillary tools.[14] Recently, Korea, China, and India EPO products were compared with US Epoetin alfa manufactured by Amgen.[15] Different biophysical properties were shown as different patterns of iso-electro-focus gel with western blots. Now, a new detection technique called "MAIIA 2" was commercialized and used widely in Europe.[16]

In this work, we have established a current WADA-adapted EPO detection method and validated this method with positive control sample. Additionally, we have analyzed Korea EPO products by SDS-PAGE and iso-electro-focus gel with double blots. Only three Korean products were analyzed by iso-electro-focus gel with double blots and previously published. We have analyzed 5 different Epoetin α or, and β with darbepoetin α and Mircera using not only iso-electro-focus gel with double blots but also SDS-PAGE.

Experimental

Materials. The rhEPO (EPO BRP) was purchased by Council

of Europe European Pharmacopoeia, and NIBSC (endogenous hEPO) was provided by National Institute for Biological Standards and Control. Sucrose and phosphoric acid were purchased by Yakuri pure Chemicals Co., Ltd., (Japan), Ampholytes, Servalyt 2 - 4, 4 - 6, and 6 - 8 were purchased by Serva (Germany). Phosphate-buffered saline (PBS) containing 0.01 M sodium and potassium phosphate buffer, 2.7 mM potassium chloride, 0.137 M sodium chloride, and *N*-acetyl-D-glucosamine were purchased by Sigma (USA). Purified Tween 80 was provided by Pierce (USA). The protease inhibitor cocktail, Complete, and pepstatin were purchased by Roche. Steriflip microfiltration (0.22 μm) units, Centricon-plus 20, Centricon YM 30 ultra filtration (molecular weight cutoff (MWCO) 30,000 Da) units, Durapore (0.65 μm), and Immobilon-P (0.45 μm) membranes were provided by Millipore (USA). Urea Plus one was purchased by GE Healthcare (USA). The primary antibody purified mouse monoclonal IgG (Anti-hEPO clone AE7A5) and secondary antibody Anti mouse IgG (biotinnylated mouse IgG affinity purified goat IgG) were provided by R&D systems (USA). Primary antibody Anti-human EPO (E0271) from sigma, secondary antibody, Goat Anti-Rabbit HRP conjugate, chemiluminescent substrate and 30% acrylimide/Bis-solution, (29:1) from BioRad (USA). Streptavidin:HRP conjugate from Zymax (USA), Nonfat dry milk from Seoul Skim milk (Korea) were provided. Glycine, Tris, Ammonium per sulphate (APS), TEMED and Dithiothritol (DTT) were purchased by USB (USA). Two EPO ELISA kits were purchased from R&D Systems (USA). LDS was purchased from Invitrogen (USA). BisTris and MOPS were purchased from Sigma (USA). Eporon inj. Prefilled syringe was presented from Dong-A pharmaceutical. Epokine inj. Prefilled syringe was purchased from CJ pharma. Espogen inj. Prefilled syringe was purchased from LG Life Sciences. Aropotin inj. Prefilled syringe was purchased from Kunwha pharmaceuticals Co. Ltd. Recormon inj. Prefilled syringe was purchased from Choongwae pharma Co. manufactured by Hoffman-La Roche Ltd. Aranesp® prefilled syringe was presented from Jeil-kirin pharm. Inc. manufactured by Amgen manufacturing.

Stock solution preparation. In case of Aranesp, 24 ng/mL of was prepared by diluting 50 ug/0.5 mL of solution with 0.1% bovine serum albumin (BSA)/50 mM Tris-HCl pH 7.4 (solution A). The rHuEPO standard solutions were prepared in solution A at a final concentration of 1800 IU/L. In case of Epokine, 3333 IU/L was prepared by diluting 6000 IU/ 0.6 mL solution with solution A. In case of Espogen, 3333 IU/L was prepared by diluting 10000 IU/mL solution with solution A. In case of Eporon, 4000 IU/L was prepared by diluting 4000 IU/mL solution with solution A. In case of Recormon, 3333 IU/L was prepared by diluting 6000 IU/0.3 mL solution with solution A. In case of Aropotin, 4000 IU/L was prepared by diluting 4000 IU/0.5 mL solution with solution A. All samples were aliquoted at 20 μL each and freezed at −20 °C.

IEF and double blotting. Sample preparation and iso-electro-focus (IEF) plus double blotting were performed as described previously.[5] Briefly, 20 mL of urine samples were ultra filtrated using Centricon Plus-20 and Centricon YM-30. The retentate was subjected to the IEF gel (pH 2 - 6) and focused. The separated isoforms were double-blotted and visualized by chemiluminescence. The emitted light was taken pictures with a ChemiDoc (Bio-Rad).

Immunoaffinity purification and SDS-PAGE. Samples were purified using immunoaffinity ELISA kit and submitted to Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for separation as described previously.[14] In brief, two commercial ELISA kits were used for immunoaffinity extraction of EPO according to the previously reported protocols.[17-18] The eluted fractions were then applicated to the SDS-PAGE, which was performed on manually prepared 1.5 mm gels. The electrophoresis was performed at constant voltage (120 V). After finishing separation, rEPO and standards were blotted to PVDF membrane via 100 V for 100 minutes. The transferred EPOs were detected using ChemiDoc (Bio-Rad).

Results and Discussion

Validation of iso-electro-focus and double blotting method. Iso-electro-focus method was established followed by double blotting for the EPO drug abuse. The established method was validated by the positive control urine provided by WADA (world anti-doping analysis). According to the WADA criteria described in TD2009EPO,[19] First all the bands should satisfy acceptance criteria. Spots, smears, areas of excessive background or absent signal in a lane invalidate the bands. The positive sample or athlete's samples should be assigned with comparison of reference standard bands. Secondly, each band profile meets the identification criteria. In case of Epoetin, there should be at least three acceptable and consecutive bands in the basic area. The two most intense bands measured by densitometry should exist in basic area and be consecutive. Each of the two most intense bands in the basic area must be more intense than any other bands in the endogenous area. The established method clearly differentiated recombinant EPO (rEPO) from

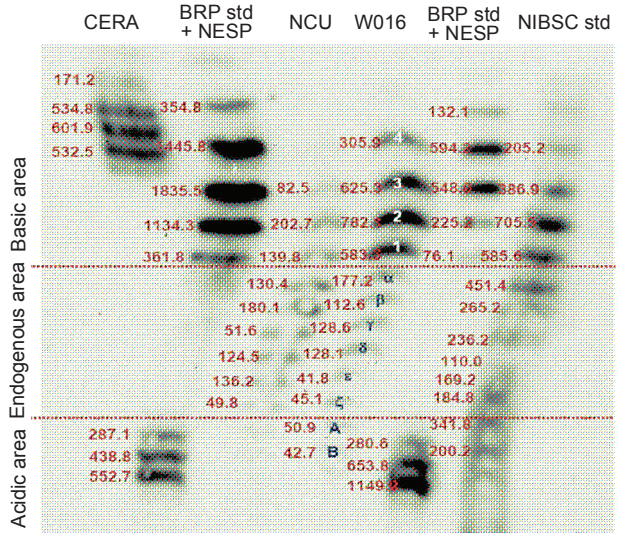

Figure 1. Validation of established EPO IEF plus Double-Blotting method using positive control urine. Lane 1: CERA, Lane2: BRP standard + NESP, Lane 3: NCU, Lane 4: PCU (W016), Lane5: BRP standard + NESP, Lane 6: NCU, Lane 7: NIBSC standard. Basic area bands were labeled 1, 2, 3, 4 and acidic area bands were labeled A, B, C, and D. The endogenous area bands were labeled with α, β, γ, δ, ε, η.

Table 1. Band area of erythropoietin samples measured by initial test and confirmation test

	Initial test	Confirmation
% of basic isoforms	76	57
Intensity of bands		
Area of height	Area	Area
Most intense band in recombinant region	782.1	2493.0
Second most intense band in recombinant region	625.3	2418.7
Most intense band in endogenous region	177.2	1179.0
Second most intense band in endogenous region	128.6	1117.4
Final results		
Stability test		
Conclusion (if conducted)		Stable
Presence of rEPO	○	○
Absence of rEPO		

endogenous EPO meeting all criteria.

Figure 1 shows the isoprofile pattern of BRP standard, NESP standard, negative control urine (NCU) and positive control urine (PCU). BRP standard showed 1, 2, 3, and 4 bands in the basic area. NESP standard showed A, B, C, and D bands in the acidic area. Band pattern of positive samples meets the criteria of acceptance and identification criteria, which showed 1, 2, 3, and 4 bands co-localized with BRP standard bands. The band 2 and 3 were the most intense of other bands in the endogenous area and the acidic area. In case of CERA (methoxy polyethylene glycol epoetin beta), there must be at least 4 consecutive bands in the basic area corresponding with CERA reference substance. As it is shown in Figure 1, CERA bands are located in the highly basic area. Both of PCU and CERA were discriminated from NCU pattern and endogenous EPO (NIBSC) standard. NCU and NIBSC standard bands don't meet the identification criteria.

The established method could reliably differentiate PCU from NCU. Table 1 showed the bands intensity and stability test results of PCU samples.

Analysis of Korean EPO products. Four different Korean EPO products and one Roche product were analyzed together by using the validated method. The results are shown in Figure 2.

Lane A, B, D, and I are the reference standard bands. Recormon (Roche manufactured) showed a different band pattern with Korean products. Band 2 and 3 were especially thicker than the other bands. Epokine showed the most similar band pattern with Recormon among Korean products. Band 2 and 3 were the most intense of the other bands. Aropotin from Kunwha showed a different band pattern with typical Amgen product and standard. Band 3 and 4 were the most intense of the other bands. Eporon from Dong-A showed an extremely interesting band pattern. Band 3, 4, and 5 were most intense of the other bands. Especially, band number "5" was the most intense of the other bands. In case of Espogen from LG life Sciences, band "2", "3", and "4" showed a similar band intensity.

Figure 3 shows the IEF patterns of Cera, Dynepo compared with endogenous urine and BRP plus NESP standards. Cera showed 6 bands in the highly basic area. Dynepo band pattern showed a larger band gap than other Epoetins and showed 8 bands. Band 1, 2, and 3 showed a similar band intensity.

Figure 4 shows SDS-PAGE pictures of Korean EPO biosimilars compared to BRP plus NESP standards. Dynepo and Cera were also analyzed together.

As it is shown in Figure 4, NESP (lane C) and Cera (lane J) have much higher molecular weight and therefore it could be easily discriminated from endogenous urinary EPO (NIBSC, Lane B). The other epoetins have a little higher molecular weight and different band shape. Band width of other epoetins is larger than NIBSC, Dynepo and BRP standard. Recormon (La Roche,

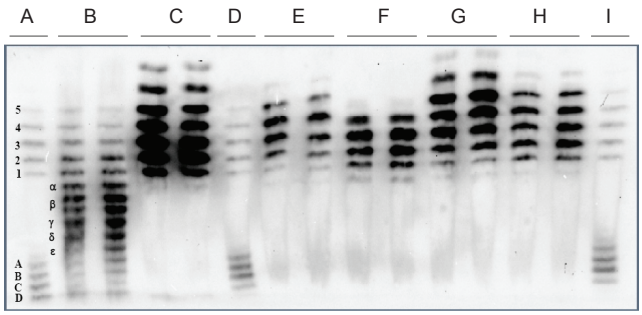

Figure 2. IEF pattern of various EPO biosimilars. Lane A: BRP + NESP standards, Lane B: NIBSC standard, Lane C: Recormon (Choongwae imported, Roche manufactured), Lane D: BRP + NESP standards, Lane E: Aropotin (Kunwha), Lane F: Epokine (CJ pharma), Lane G: Eporon (Dong-A), Lane H: Espogen (LG Life Sciences), Lane I: BRP + NESP standards.

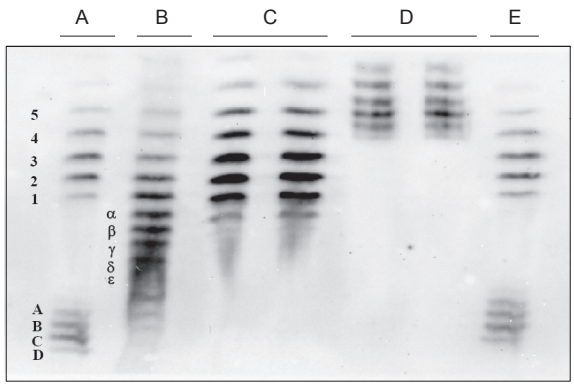

Figure 3. IEF pattern of Dynepo and Cera. Lane A: BRP + NESP standards, Lane B: NIBSC standard, Lane C: Dynepo, Lane D: Cera, Lane E: BRP + NESP standards.

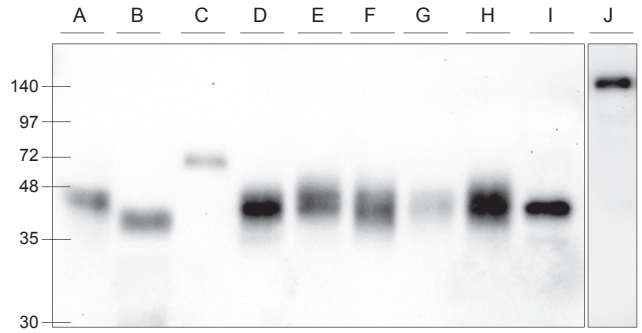

Figure 4. SDS-PAGE of various EPO biosimilars. Lane A: BRP standard, Lane B: NIBSC standard, Lane C: NESP standard, Lane D: Recormon, Lane E: Epokine, Lane F: Eporon, Lane G: Espogen, Lane H: Aropotin, Lane I: Dynepo, Lane J: Cera.

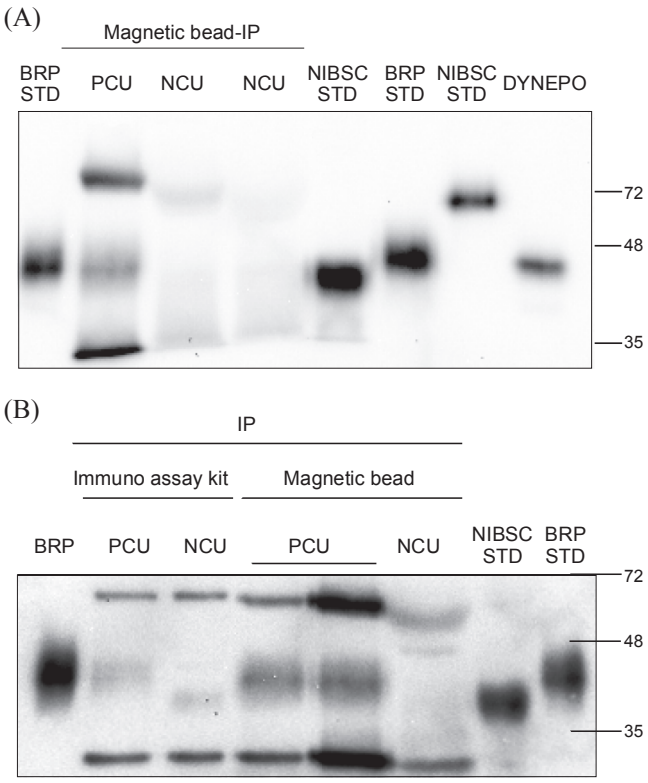

Figure 5. Comparison of the immunoaffinity purification method using immuno assay kit or magnetic bead.

lane D), and Eporon (Dong-A, lane F) bands were located a little bit lower than the other epoetins, therefore it is difficult to discriminate Recormon and Eporon from NIBSC. Dynepo band has a specific character of focusing than other bands. Epoetins showed a smear band pattern except Dynepo.

Comparison of immunopurification methods. The magnetic bead method and immunoassay kit method were compared for the purification degree shown in Figure 5.

For the analysis of real urinary samples, a sample purification method was developed. Commercial immune assay kit as an immune affinity purification tool and magnetic bead immobilized antibody were tested for the purification and concentration of EPO from urinary matrix. As shown in Figure 5, Immunoprecipitation by magnetic bead was successfully applied to the PCU and standard spiked urines (Figure 5A). From the PCU, rEPO and NESP were detected, which are not detected from NCU. Figure 5B shows the comparison result of magnetic bead method and Immunoaffinity purification using ELISA kit. Magnetic bead method gave better concentration effect. We suggest to use antibody immobilized magnetic bead as a purification and concentration tool for EPO analysis on the purpose of doping control.

Conclusions

For the anti-doping purposes, an iso-electro-focus plus double blotting method and SDS-PAGE method were established and sample concentration method was optimized for the analysis of EPO from urine samples. As more biosimilars of EPO are produced, it becomes more important to know similarities and differences of biosimilars compared to reference product. Among Korean EPO products, Epokine (CJ Pharm Co.) only showed a similar band pattern with the reference one. The other epoetins showed different band patterns from the reference one. A combined method of IEF plus double blotting and SDS-PAGE will provide a better analytical tool for EPO abuse.

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