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Doping control of biosimilar epoetin kappa and other recombinant erythropoietins after intravenous application

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Since the expiration of patent protection, a number of new recombinant erythropoietin (rEPO) biosimilars have appeared on the worldwide market. In 2010, epoetin kappa, which is biosimilar to epoetin alfa, was clinically approved in Japan. Currently, both isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) are approved by the World Anti-Doping Agency (WADA) for detection of rEPO doping. Because it was unclear whether epoetin kappa could be detected by WADA-accredited detection methods, intravenous administration studies of epoetin kappa, epoetin alfa, and epoetin beta were performed to test the applicability of these methods. The isoform bands of epoetin kappa expanded more widely towards the basic area and the profile appeared to be composed of at least eight bands, which were clearly different from those of other epoetins. The results showed that epoetin kappa also contains isoforms of higher molecular masses than those of originator epoetins on SDS-PAGE; the mass distribution was confirmed by electrospray ionization time-of-flight mass spectrometry. We clearly detected epoetin kappa after its administration up to 10 h by IEF-PAGE and 24 h by SDS-PAGE; the detection window of the SDS-PAGE is longer than that of the IEF-PAGE. SDS-PAGE compensates for the disadvantages of IEF-PAGE in detecting urinary epoetin kappa. We also concluded that athletes abusing rEPO might move to intravenous injections for shorter clearance times instead of subcutaneous injections. In conclusion, out-of-competition tests need to be applied more frequently to improve the effectiveness of the rEPO detection. Copyright © 2011 John Wiley & Sons, Ltd.

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Keywords: doping; erythropoietin; epoetin kappa; isoelectric focusing; SDS-PAGE; mass spectrometry; biosimilar

Introduction

Erythropoietin (EPO) is a hormone produced mainly by the kidneys. It acts on the bone marrow to stimulate the proliferation and differentiation of red blood cells.[1] Human EPO was purified from urine obtained from patients with aplastic anaemia in 1977;[2] it is approximately a 30kDa glycosylated protein composed of 165 amino acids, three N-linked glycans (Asn^{24}, Asn^{38} and Asn^{83}), and one O-linked glycan (Ser^{126}) as well as two disulfide bonds (Cys^{7}-Cys^{161} and Cys^{29}-Cys^{33}).[3]

In 1989, the first recombinant EPO (rEPO) preparation, so-called epoetin alfa (Epogen®, cell line: Chinese hamster ovary cell (CHO); Amgen), was approved by the US Food and Drug Administration for treatment of anaemia related to kidney disease.[4,5] Since then, several clinically approved rEPO preparations such as epoetin beta (Epogin®, cell line: CHO), epoetin delta (Dynepo®, cell line: human fibrosarcoma cell), epoetin omega (Epomax®, cell line: baby hamster kidney cell), epoetin alfa derivative darbepoetin alfa (Aranesp®) and epoetin beta derivative CERA (Mircera®, continuous erythropoiesis receptor activator), have been commercially produced. In addition, a number of new EPO-biosimilar products have appeared globally in the market since the recent expiration of the patent for epoetin alfa.[5,6]

EPO was listed on the prohibited list issued by the International Olympic Committee (IOC) to coincide with the release of rEPO in the early 1990s and the misuse of EPO by athletes has been prohibited by the World Anti-Doping Agency (WADA).[7] A test method for detection of rEPO abuse was developed in 2000 by Lasne and de Ceaurriz,[8] which relies on differentiation of endogenous EPO and exogenous EPO with several glycosylation isoforms by isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) analysis followed by double blotting.[9,10] The IEF-PAGE method is based on the fact that urinary isoelectric profiles of endogenous EPO are different from those of rEPOs with predominantly more basic patterns because of their differences in structure, such as the amount of antennary branching, sulphation, sialylation or fucosylation.[8,11–14]

Even when no rEPOs are administered, some urine samples contain isoform profiles shifted to the basic region in IEF-PAGE, so-called instable urine (active urine) and effort-type urine because of proteinuria caused by strenuous exercise.[15–17] Therefore, laboratories have to take into account the importance of the positivity criteria established to prevent any false-positive findings.[18] As another approach, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) has proved to be a useful tool for detecting Dynepo abuse and differentiation of instable/effort-type urine samples from rEPO-positive urine.[19–22]

This method is based on the fact that the molecular weight of endogenous EPO differs from that of rEPO.[11,13,14] However,

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these techniques do not provide structural information on the complete pattern of erythropoietin isoforms. Structural characterization of rEPO using time-of-flight mass spectrometry (TOFMS) has been performed using matrix-assisted laser desorption ionization (MALDI),[23–25] capillary electrophoresis electrospray ionization (CE-ESI) and nano-flow high performance liquid chromatography-chip electrospray ionization.[14,26–28] Unfortunately, these mass-spectrometry-based technologies for the detection of rEPO in human biological fluids need to be improved in terms of sensitivity. Currently only IEF-PAGE and SDS-PAGE methods are approved by WADA.[18]

In Japan, the first biosimilar product of epoetin alfa was approved as a therapeutic agent in 2010.[5] Its generic name is epoetin kappa (cell line: CHO) and it is produced by Japan Chemical Research Pharmaceuticals Co., Ltd (EPOETIN ALFA BS Injection). Epoetin kappa is a biopharmaceutical product based on serum-free media after master cell bank preparation.[29] However, it is unclear whether epoetin kappa could be detected by the WADA-approved urinary EPO detection methods, namely, IEF- and SDS-PAGE. Less information in the field of anti-doping analysis is available on the excretion study of intravenous injection of rEPOs than that of subcutaneous injections.

Based on this background information, intravenous administration studies of epoetin kappa, epoetin alfa and epoetin beta were performed to test the applicability of the current WADA-approved methods.

Experimental

Materials

ESPO® Injection Syringe (epoetin alfa 3000 IU/2 ml) and NESP® Injection Syringe (darbepoetin alfa) were purchased from Kyowa Hakko Kirin Co., Ltd (Tokyo, Japan). EPOGIN® (epoetin beta 3000 IU/0.5 ml) was from Chugai Pharmaceutical Co., Ltd (Tokyo, Japan). EPOETIN ALFA BS Injection (epoetin kappa 3000 IU/2 ml) was from Japan Chemical Research Pharmaceuticals Co., Ltd (Hyogo, Japan). DYNEPO® Injection (epoetin delta 3000 IU/0.3 ml, Shire Pharmaceuticals, Madrid, Spain) was obtained from WADA (Montreal, Canada). The mixture in equimolar amounts of epoetin alfa and epoetin beta was from the European Pharmacopoeia Commission for the Biological Reference Preparation (BRP, Strasbourg, France). Endogenous human urinary EPO (hEPO) was purchased from the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK). Rat EPO produced from insect cells (ref. E8905) was from Sigma (St Louis, MO, USA). Primary mouse monoclonal IgG antibody (Anti-human EPO, clone AE7A5) was purchased from R&D Systems (Minneapolis, MN, USA). Polymer-horseradish peroxidase (HRP)-labelled goat anti-mouse (EnVision™ K4001) as a secondary antibody was obtained from Dako (Glostrup, Denmark). CH_3CN and HCOOH were from Kokusan Chemical Co., Ltd (Tokyo, Japan). N,N,N′,N′-tetramethylethylenediamine, skim milk powder and HCl were from Wako Pure Chemical Industries, Ltd (Osaka, Japan). The chemiluminescent detection kit (CovaLight ref. opr0009) for SDS-PAGE was obtained from Covalab (Villeurbanne, France). The chemiluminescent detection kits (ECL Advance™ Western Blotting Detection Kit ref. RPN2135) for IEF-PAGE and blocking reagent (ECL Advance™) were from GE Healthcare Japan (Tokyo, Japan). Dithiothreitol (DTT), bovine serum albumin (RIA grade, ref. A-7888), pepstatine A (microbial source) and TRIZMA Base (Tris base, ref. T6066) were from Sigma. Urea and ammonium persulphate were from Bio-Rad Laboratories, Inc., (Tokyo, Japan). The protease inhibitor cocktail, Complete™, was from Roche Diagnostics (Indianapolis, IN, USA). Tween 80 Surfact-Amps® was from Pierce Chemical Co. (Rockford, IL, USA). Servalyte carrier ampholytes (2–4, 4–6, 6–8) were from Serva (Heidelberg, Germany). Acrylamide and N,N′-methylenediacrylamide were from Merck (Darmstadt, Germany). Ultra-pure water was prepared using a Milli-Q Ultra pure system (Millipore, Bedford, MA, USA). Microfiltration Steriflip filters (0.22 μm), Amicon Ultra-4 (cut-off 30 kDa), Amicon Ultra-15 (cut-off 30 kDa), Durapore membrane (0.65 μm), Centricon YM-10 (cut-off 10 kDa) and Immobilon-P membrane (PVDF membrane) were from Millipore. NuPAGE® Bis-Tris gel (10%, 1.5-mm 10-well), lithium dodecyl sulphate (LDS) sample buffer (4X), antioxidant, 4-morpholinepropanesulfonic acid (MOPS) running buffer and transfer buffer were from Invitrogen (Carlsbad, CA, USA). Human EPO enzyme-linked immunosorbent assay (ELISA) kit was from StemCell Technologies (Vancouver, Canada). Recombigen EPO radioimmunoassay kit was from Mitsubishi Chemical Medience Corporation (Tokyo, Japan).

IEF-PAGE, double blotting and chemiluminescent detection

Reference standards and urine samples were analyzed by IEF-PAGE as described previously with minor modifications.[10,18] In brief, 20–30 ml of urine samples fortified with 600 μl of protease inhibitor cocktail (pH 7.1–8.3 with Tris-HCl), were micro-filtrated using Steriflip (0.22 μm) and two consecutive ultra-filtrations were performed using Amicon Ultra-15 and Amicon Ultra-4. The retentate was heated (80 °C, 3 min) in 1% (*v/v*) Tween 80 before IEF separation. The polyacrylamide slab gels (pH gradient 2–6, 250 mm x 120 mm x 1 mm) were prepared using Servalyts 2–4 and 4–6. After pre-focusing (250 V, 30 min, 8 °C), the gel was focused for approximately 3 h (3600 Vh with maximum setting of 2000 V, 50 mA and 30 W). Catholyte and anolyte were 2% Servalyt 6–8 and 1 M phosphoric acid, respectively. The focused gel was equilibrated in buffer (25 mM Tris-192 mM glycine, 2 min) and blotted (0.8 mA/cm^2, 30 min) on PVDF membrane using the NovaBlot semi-dry electrophoretic transfer system (GE Healthcare, Japan). Three sheets of blotting paper (ref. 3030917, Whatman, Kent, UK) were used on each side of the gel. After the first blot, the PVDF membrane was incubated (37 °C, 60 min) in 5 mM DTT in phosphate-buffered saline (PBS). Subsequently, the membrane was blocked in 5% skim milk in PBS and incubated (4 °C, overnight) in primary antibody solution (1 μg/ml anti-EPO antibody AE7A5 in 1% skim milk in PBS). The second blotting was performed by capillary pressure blotting using 0.1 M glycine/HCl (pH 2.5).[9] After this double blotting step, the PVDF membrane was blocked using blocking reagent (ECL Advance™), incubated with EnVision™/HRP anti-mouse antibody (in 2% ECL Advance™ blocking reagent, 60 min) and washed with PBS. Chemiluminescence was achieved using ECL Advance™ detection kit. Images were acquired using a CCD camera LAS-3000 (Fujifilm, Tokyo, Japan) and were analyzed using GASepo version 1.3b2 (Seibersdorf, Austria) and Image Gauge version 4.0 (Fujifilm). An in-house method was validated, which was included in the scope of the ISO/IEC17025 accreditation.

SDS-PAGE, western blotting and chemiluminescent detection

In order to evaluate the benefit of a SDS-PAGE analysis, reference standards and urine samples were analyzed by SDS-PAGE as described previously with minor modifications.[18,20,21] After

concentration of urine samples as described above, the retentates were incubated (4 °C, overnight) using an immunoaffinity ELISA well plate (StemCell Technologies) and they were washed with PBS. Internal standard (1.7 ng of rat EPO and 0.1 ng of NESP), LDS sample buffer and DTT as protein-reducing agent were added and heated for 5 min at 95 °C. Samples were applied to a NuPAGE precast 10% Bis-Tris gel (MOPS running buffer) and electrophoresis was performed for 55 min at 200 V. After semi-wet blotting (400 mA, 60 min, Xcell II semi-wet blot module, Life Technologies Japan, Tokyo, Japan) on PVDF membrane in NuPAGE transfer buffer (incl. antioxidant and methanol), the blotted membrane was blocked (ambient temperature, 60 min) using 5% skim milk in PBS. Two incubation steps with the primary antibody and secondary antibody were performed as described for the IEF method above. Chemiluminescence was achieved using a CovaLight (ref. opr0009) detection kit. Images were acquired using a CCD camera LAS-3000 (Fujifilm) and analyzed using Image Gauge version 4.0 (Fujifilm). Images were integrated and converted into ASCII-data by Image Gauge. Peak mids of the respective bands were calculated. The relative mobility of EPOs was calculated based on the following equation: relative mobility = 1 − (b − a)/(c − a), where b is the distance of analyte, c is the distance of rat EPO and a is the distance of NESP.[20]

Time-of-flight mass spectrometry

Reference materials for epoetin alfa, epoetin beta, darbepoetin alfa, epoetin delta and epoetin kappa were obtained after ultrafiltration (Centricon YM-10) of injectable solutions and were analyzed using electrospray ionization (ESI)-TOFMS. The precise mass determination of intact rEPOs was conducted after deconvolution of the multiply charged ions using the MaxEnt1 algorithm (MassLynx version 4.1). The ultra-performance liquid chromatography (UPLC)/TOFMS system was an Acquity UPLC/Synapt G2 HDMS from Waters Corporation (Milford, MA, USA). The analytical column was a Waters MassPREP™ Micro-desalting column (2.1 x 5 mm) and the mobile phases used were 0.1% HCOOH in water (mobile phase A) and 0.1% HCOOH in CH_3CN (mobile phase B). The column oven temperature was 80 °C. Gradient elution was as follows: 5% B for 0.5 min (0.5 ml/min), linear increase to 90% B in 4.0 min (0.2 ml/min), hold in 90% B for 2.0 min, followed by a decrease to 5% B in 0.1 min (0.5 ml/min). Finally, the column was equilibrated for 2 min (0.5 ml/min). The sample temperature and injection volume were 10 °C and 10 µl, respectively. Ionization was accomplished using ESI in positive ion mode. The ionspray and desolvation temperatures were 120 °C and 450 °C, respectively, and the capillary voltage was set at 3.0 kV. Nitrogen was employed as the cone gas (at a flow rate of 50 L/h) and the desolvation gas (at a flow rate of 600 L/h). The cone voltage was 40 V. The TOFMS range was scanned from *m/z* 100 to 5000 (scan time: 1 s). The mass resolution was approximately 18000 (resolution mode). Daily calibration was performed prior to analysis using sodium iodide. The lock mass mode was employed using [Glu1]-fibrinopeptide B (GFP, *m/z* 785.8426 $[M+2H]^{2+}$, Sigma, St Louis, MO, USA) and the capillary voltage and the flow rate of lock spray were 3.0 kV and 20 µl/min, respectively.

Determination of serum EPO level by immunoradiometric assay (IRMA)

Serum EPO concentration was determined using a commercially available radioimmunoassay kit (Recombigen EPO Kit). Epoetin alfa was used as the tracer and the standard. Radioactivity was measured using an automated gamma counter (ARC-950, Aloka, Tokyo, Japan). The detection method was validated and included in the scope of the ISO15189 accreditation for clinical testing.

Human subjects

The administration study was reviewed and approved by the Ethical Review Board of Mitsubishi Chemical Medience Corporation from the viewpoint of ethical, scientific and medical validity and the study was conducted on an open-label basis (Rin A11-06). The study was performed with nine healthy Japanese male volunteers. Written consent was obtained from all participating volunteers. The volunteers refrained from ingesting alcohol, caffeine, grapefruit, any supplements or medicines and did not perform exercise during the studies. The time courses of urine sample-collection were 0 h (pre-administration), 2 h, 4 h, 6 h, 8 h, 10 h, 13 h, 24 h, 26 h, 28 h, 30 h, 32 h, 34 h, 37 h, and 48 h. Also those of serum sample collection were −24 h, −22 h, −18 h, −14 h, 0 h (pre-administration), 2 h, 6 h, 10 h, 24 h, 26 h, 30 h, 34 h, and 48 h. For safety, vital signs (blood pressure, body temperature and pulse rate) were monitored during administration studies, heart rate was measured prior to administration, and laboratory clinical tests of haematology and blood chemistry were performed prior to and after administration at a clinical facility. Epoetin alfa (ESPO®, 3000 IU) was administered intravenously after overnight fasting (n = 3, 21–35 years old, body mass index (BMI): 20.8 ± 1.4, body weight: 55.5 to 65.5 kg). Similarly, Epoetin beta (EPOGIN®, 3000 IU) was injected intravenously (n = 3, 22–36 years old, BMI: 20.2 ± 0.7, body weight: 58.8 to 68.6 kg). The other three volunteers (28–32 years old, BMI: 21.2 ± 1.1, body weight: 56.3 to 69 kg) received Epoetin kappa (EPOETIN ALFA BS, 3000 IU) intravenously after overnight fasting. A typical effort-type urine sample was a doping control sample collected from an athlete (competing in an endurance sport) during in-competition testing. The sample was anonymous and no individual information on the athlete except gender was included. The athlete agreed that the sample could be used for research purposes and written consent was obtained on a doping control form.

Results and discussion

IEF-PAGE, double blotting and chemiluminescent detection

As shown in Figure 1, the isoform bands of epoetin kappa (lane 1) expanded more widely towards the basic area and the profile appeared to be composed of at least eight bands, which were clearly different from those of other epoetins. Reichel *et al.* demonstrated that various biosimilar EPOs of epoetin alfa have isoforms with the bands shifted to the basic region in IEF-PAGE and epoetin kappa also showed similar properties.[21] These variations of isoelectric points (pI) might be attributable mainly to micro heterogeneity of the glycan structures.

Figure 2 shows the typical IEF-profile of urinary EPO after intravenous administration of epoetin kappa. After administration of epoetin kappa, the IEF profiles were similar to that of epoetin beta and at least one basic band (lanes 3 and 4 in Figure 2) could not be detected in urine samples. Nevertheless, epoetin kappa doping was clearly detected up to 10 h after intravenous administration by applying the identification criteria in WADA TD2009EPO (Table 1).[18] Regarding the post-24-h sample (lane 5 in Figure 2), the isoelectric patterns met

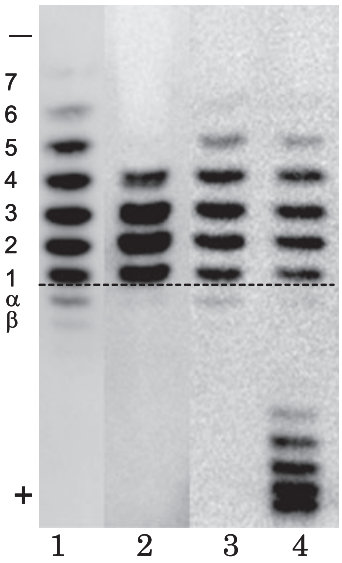

Figure 1. Immunoblot of recombinant EPOs separated by IEF-PAGE. 1: epoetin kappa, 2: epoetin alfa, 3: epoetin beta, 4: BRP/NESP.

the three criteria mandatory for reporting an adverse analytical finding;[18] however, the sum of intensity of all bands in the basic area was 61.7% (<85%) of the total intensity of the bands within the window of the sample lane. Although the profile of post-32-h urine did not fulfill the positivity criteria,[18] each of the two most intense bands in the basic region was slightly more intense than any other bands in the endogenous region (lane 6 in Figure 2). In such a case, additional scientific evidence would be needed to arrive at a definitive conclusion.

On the other hand, we could detect epoetin alfa and epoetin beta up to 24 h after the intravenous administration (data not shown). This seems to be because of the more intense alfa band of epoetin kappa compared with the alfa band of the two other epoetins.

Lane 2 in Figure 3 is a doping control urine sample with an IEF-profile shifted toward the basic region (effort-type urine). The sum of the intensity of all bands in the basic area was 85.6% (>85%) of the total intensity of the bands within the window of the sample lane, which fulfilled the positivity criteria of recombinant epoetins in accordance with WADA TD2009EPO.[18] The three intense bands corresponding to

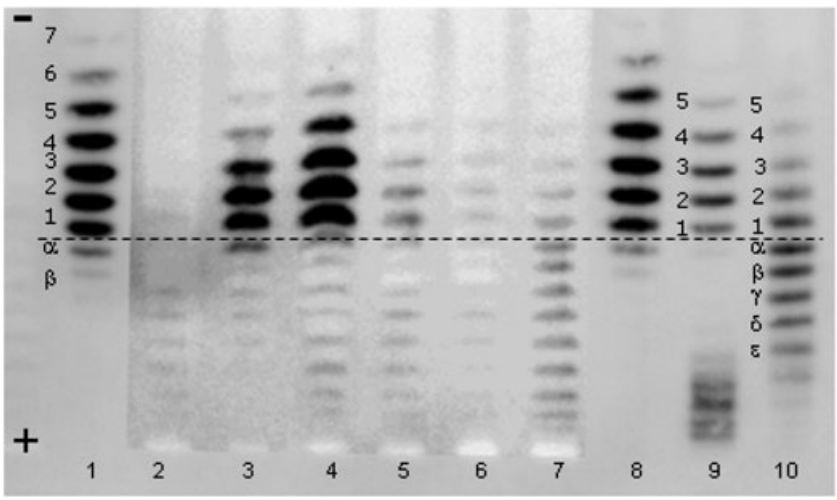

Figure 2. Immunoblot of urine samples after intravenous administration of epoetin kappa separated by IEF-PAGE. 1: epoetin kappa, 2: pre-administration, 3: 4 h, 4: 10 h, 5: 24 h, 6: 32 h, 7: 48 h, 8: epoetin kappa, 9: BRP/NESP, 10: hEPO (NIBSC).

Table 1. Urinary EPO isoform band area measured by IEF-PAGE in epoetin kappa-treated subject.

Lane No		% of basic isoforms	Most intense band in recombinant region	Second most intense band in recombinant region	Most intense band ratio (recombinant to endogenous)	Second most intense band ratio (recombinant to endogenous)	Presence of rEPO
1	epoetin kappa	94.2	3	2	6.6	5.6	Yes
2	pre	33.0	1	2	1.1	0.9	No
3	4 h	80.5	2	1	2.9	2.3	Yes
4	10 h	89.2	2	3	6.7	5.4	Yes
5	24 h	61.7	1	2	2.4	2.2	Suspicious for α,β rEPO
6	32 h	47.9	1	2	1.5	1.3	Suspicious for α,β rEPO
7	48 h	40.6	1	2	1.1	1.0	No
10	hEPO (NIBSC)	47.6	1	2	1.1	1.0	No

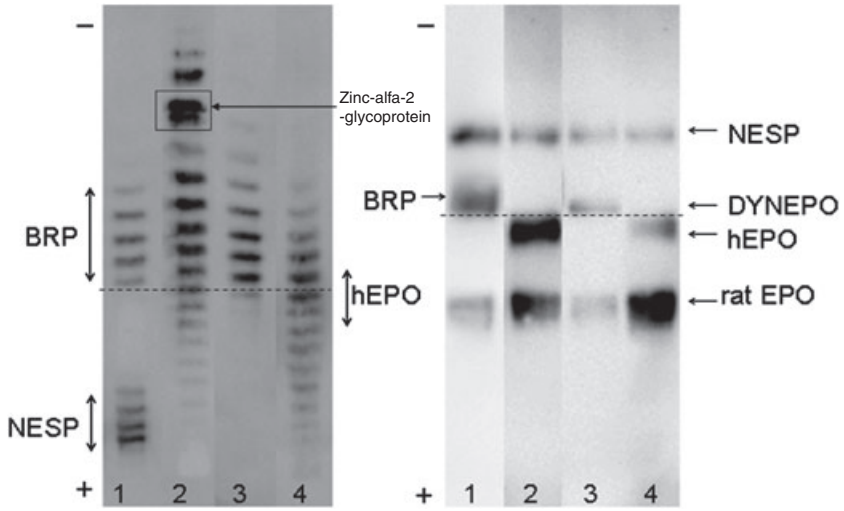

Figure 3. Immunoblots of effort-type urine separated by IEF-PAGE (left) and SDS-PAGE (right). 1: NESP/BRP, 2: effort-type urine, 3: epoetin delta (DYNEPO), 4: urinary EPO (NIBSC). The IEF-profile in lane 2 was shifted to the basic region, but the band as observed on the SDS-PAGE gel corresponded to the band of urinary EPO (NIBSC).

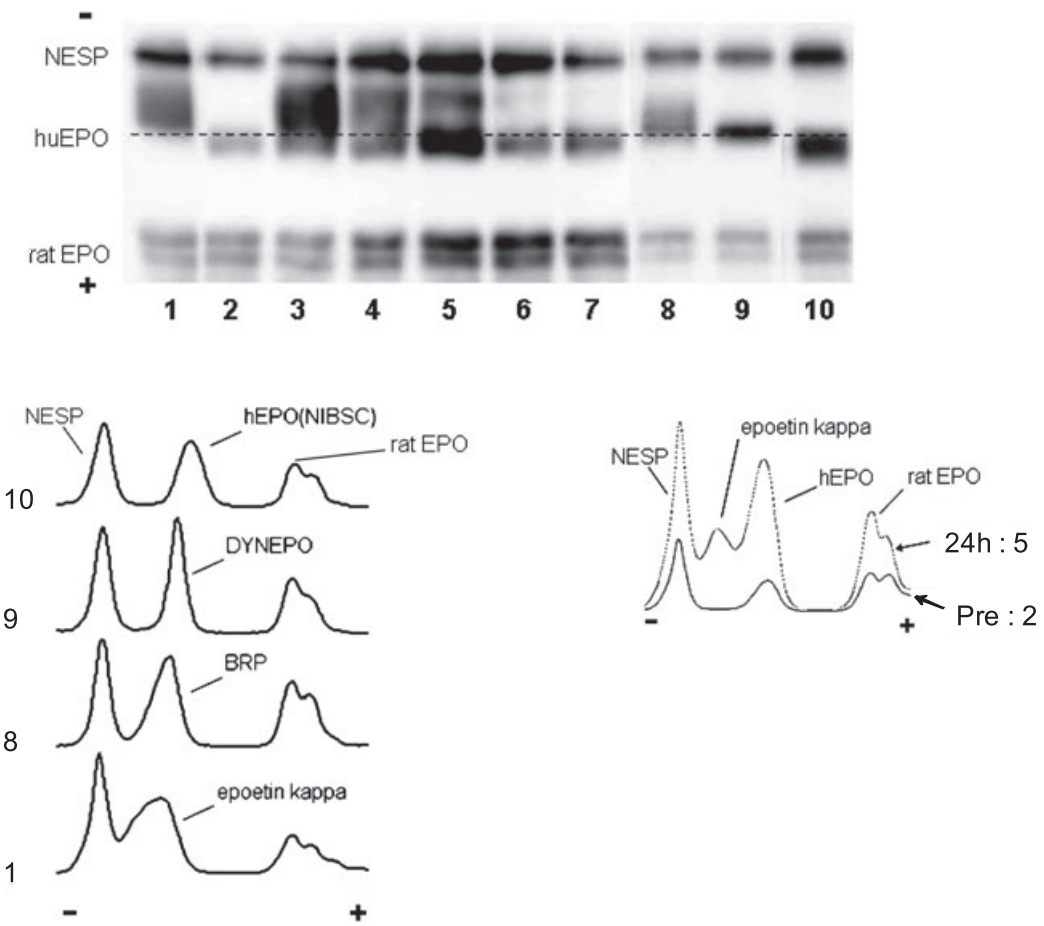

Figure 4. Immunoblot of urine samples after intravenous administration of epoetin kappa separated by SDS-PAGE (upper). 1: epoetin kappa, 2: pre-administration, 3: 4 h, 4: 10 h, 5: 24 h, 6: 32 h, 7: 48 h, 8: BRP, 9: epoetin delta (DYNEPO), 10: hEPO (NIBSC). Images of immunoblot are integrated and converted into ASCII-data by Image Gauge (lower).

zinc-alfa-2-glycoprotein (ZAG) were also observed outside the used pI range.[30] The isoelectric profile of epoetin kappa standard was distinctly different from that of effort-type urine. However, post-32-h urine sample (lane 6 in Figure 2) could not be easily discriminated from that of effort-type urine. Thus, the risk of making a wrong decision exists (e.g. reporting a

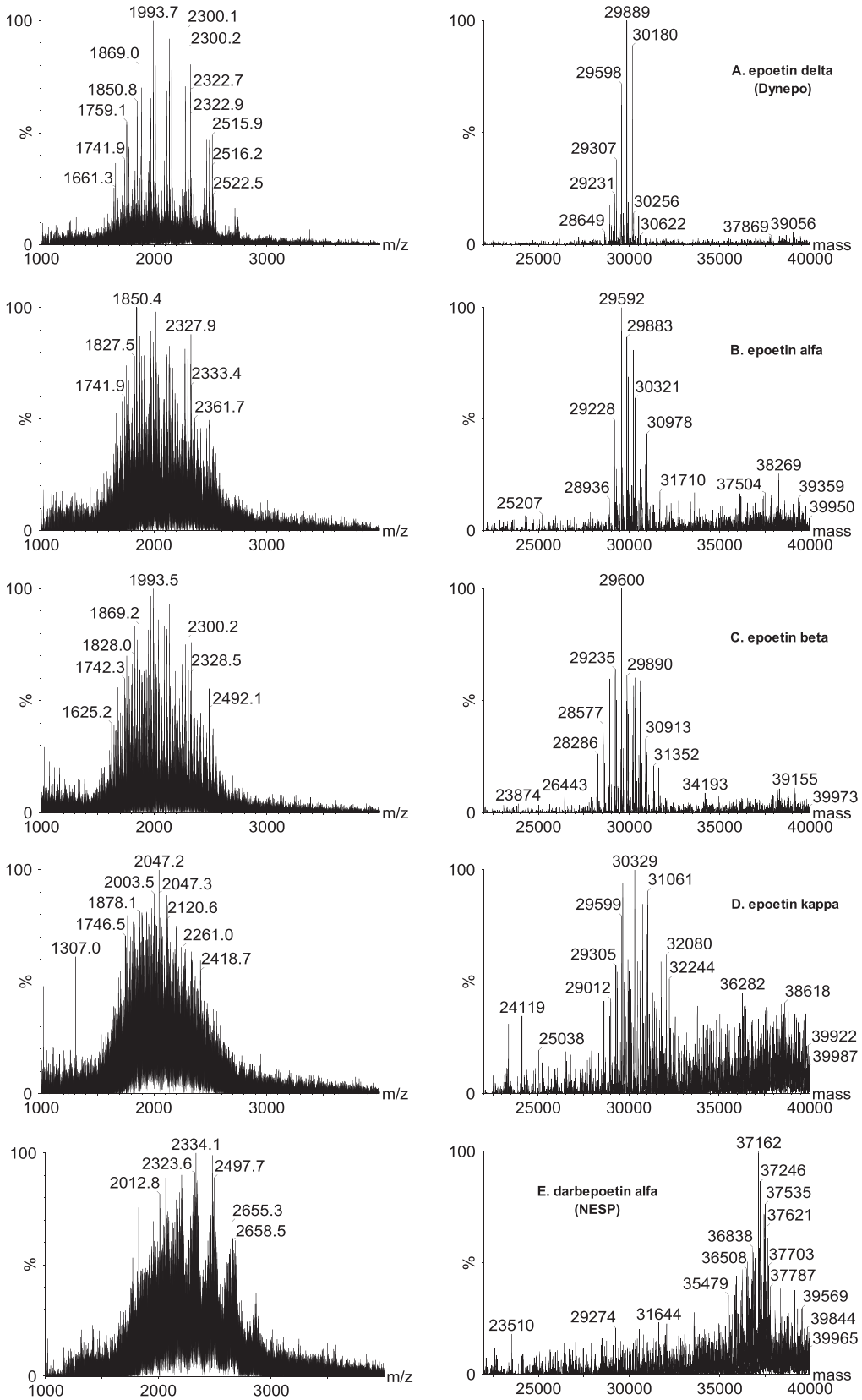

Figure 5. ESI-TOF mass spectra of intact rEPOs (A: epoetin delta, B: epoetin alfa, C: epoetin beta, D: epoetin kappa, E: darbepoetin alfa). (left: raw mass spectra, right: deconvoluted mass spectra).

false-positive result) if only the IEF-PAGE method is conducted. Although Lamon *et al.* demonstrated that it may be possible to use the urinary retinol-binding protein (RBP) level as a marker to identify effort-type urine,[16] it is unclear whether the amount of ZAG can be a marker of the total amount of protein in the urine. Hence an additional SDS-PAGE analysis has to be performed (Figure 3).

SDS-PAGE, western blotting and chemiluminescent detection

The relative mobilities of hEPO (NIBSC), epoetin delta, BRP and epoetin kappa were 0.547, 0.605, 0.639 and 0.680, respectively. As shown in Figure 4, the results indicated that epoetin kappa (lane 1) contains isoforms with a higher molecular mass than those of BRP (lane 8) and epoetin delta (lane 9) on SDS-PAGE. No bands of epoetin kappa could be detected in the lower mass region. We recognize that the loss of charged groups (terminal sialic acids) from intact epoetins results in a shift to the basic area on IEF-PAGE and a migration to the lower mass region on SDS-PAGE.[14,21,31] Hence the basic bands of epoetin kappa on IEF-PAGE were not suggestive of degradation products such as desialylated epoetin alfa, but rather isoforms with a different glycan structure.

The higher molecular weight of rEPOs from CHO cells is because of a greater amount of oligosaccharides with N-linked tetra-antennary acetyllactosamine,[11,32] indicating that the glycans from rEPO contained more glycan structures with higher molecular mass than serum EPO.[11,13] However, urinary EPO isoforms were detected in a more acidic region than those of rEPO on IEF-PAGE. These facts show that the acidities of glycans from intact glycoproteins could not be directly compared with the charge pattern of the intact glycoprotein and they are consistent in supporting the argument that the high molecular weight of epoetin kappa may be because of a large amount of higher-molecular-mass glycans such as tetra-sialic-N-glycans.

Thus, epoetin kappa was distinguished from epoetin alfa, epoetin beta and epoetin delta by employing SDS-PAGE. The IEF-PAGE method could detect epoetin kappa up to 10 h after intravenous administration, but epoetin kappa was clearly detected up to 24 h using the SDS-PAGE method.

As shown in Figure 3, the IEF-profile in lane 2 was shifted to the basic region, but on SDS-PAGE the respective band was corresponding to the band of urinary endogenous EPO. No decrease or increase in apparent molecular mass was observed and we could clearly confirm that this rEPO suspicious urine sample in the IEF method was effort-type urine. Thus, SDS-PAGE is sensitive for detecting epoetin kappa and useful as a complementary confirmation method of the screening result using IEF-PAGE.

Mass spectrometry

ESI coupled with high-resolution TOFMS allows the precise mass determination of intact proteins after deconvolution of the charge state distribution. Because the injectable solution contained additives such as salt, tonicity agent and emulsifier, the reference materials of epoetin alfa, epoetin beta, darbepoetin alfa, epoetin delta and epoetin kappa after filtration were analyzed using a desalting column coupled with ESI-TOFMS. All rEPOs eluted at the same retention time of 2.2 min. When the eluents were collected at the expected retention time (2–2.5 min) and analyzed by the IEF method mentioned above, we could confirm that analytes were successfully isolated and purified during column separation (data not shown). The raw and deconvoluted mass spectra obtained from epoetin alfa, epoetin beta, epoetin kappa, epoetin delta and darbepoetin alfa are shown in Figure 5. The main molecular masses of epoetin delta, epoetin alfa, epoetin beta, epoetin kappa and darbepoetin alfa were 29889 Da, 29592 Da, 29600 Da, 30329 Da and 37162 Da, respectively. Other molecular masses were also observed corresponding to other overlapping glycoforms differing in hexose-N-acetyl-hexosamine (HexHex-NAc, 365 Da) or N-acetylneuramic acid (NeuAc, sialic acid, 291 Da) units.

The mass spectrometrically determined molecular weights of intact epoetin alfa and epoetin beta were reported to be approximately 29.9 kDa using CE-ESI-TOFMS and 29.3–30.1 kDa using MALDI-TOFMS and those of darbepoetin alfa and epoetin delta were 36.4–37.4 kDa and 29.9 kDa, respectively.[20,24,26] Our results were in close agreement with these previous studies. The mass spectrum of epoetin delta showed a distribution of isoforms that was narrower than those of other epoetins and this provided clear evidence to support the characteristic sharp band shape and apparent molecular mass of epoetin delta on SDS-PAGE. Moreover, the mass range of epoetin kappa was more widely distributed and slightly shifted towards the high-mass region and this result agreed with the results of SDS-PAGE.

Serum EPO levels after intravenous administration of rEPOs

Serum EPO levels were measured for epoetin kappa, epoetin alfa and epoetin beta, to evaluate their evolution after intravenous administration. The measured serum EPO levels are plotted in Figure 6. After a single injection of epoetin alfa, average serum EPO concentrations were 15.7 mIU/ml (prior to injection), 967.3 mIU/ml (2 h) and 285.7 mIU/ml (10 h) and then they returned to baseline concentrations by the 48th h (17.6 mIU/ml). Those of epoetin beta were 25.8 mIU/ml (prior to injection), 1286.7 mIU/ml (2 h) and 379.3 mIU/ml (10 h), and then returned to baseline concentrations by the 48th h (32.0 mIU/ml). Similarly, those of epoetin kappa were 38.7 mIU/ml (prior to injection), 751.0 mIU/ml (2 h) and 256.7 mIU/ml (10 h) and then returned to the baseline by the 48th h (40.1 mIU/ml). It is recognized that carbohydrates

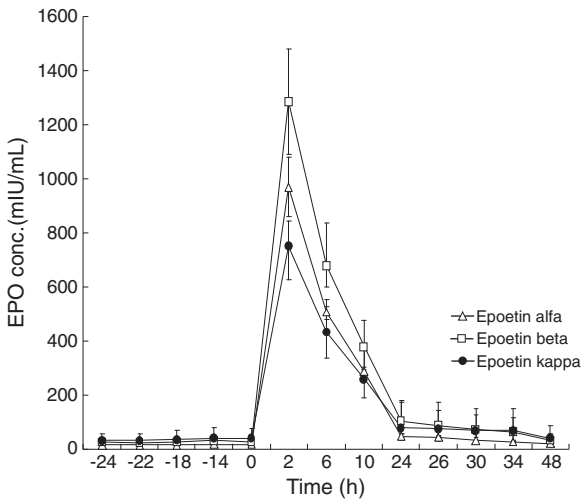

Figure 6. Change in serum concentrations of EPO after intravenous administration of epoetin alfa, epoetin beta and epoetin kappa (Mean of n = 3). The whisker plots indicate maximums and minimums of the measured values.

with a higher level of sialic acid attached to the protein core have a prolonged serum half-life.[33] Darbepoetin alfa has five N-linked carbohydrate chains and up to 22 sialic acid residues.[34] The increase sialic acid content of darbepoetin alfa results in a longer circulating half-life than epoetins. Structural differences between epoetin kappa and other rEPOs were observed (see above). However, serum EPO levels after injection of epoetin kappa showed similar behaviour to those of epoetin alfa and beta. Hence there does not seem to be a large difference in glycans between epoetin kappa and other epoetins in terms of their influence on the half-life in circulation, as compared to, for example, darbepoetin alfa.

Conclusions

Although the profiles of epoetin kappa on TOF mass spectrometry, IEF-PAGE and SDS-PAGE are different from those of other rEPOs and urinary EPO, urinary epoetin kappa can be detected using the current WADA-approved method. We clearly detected epoetin kappa after its intravenous administration up to 10 h by IEF-PAGE and up to 24 h by SDS-PAGE. Hence, the detection window of the SDS-PAGE method is longer than that of the IEF method. On the basis of these findings, we concluded that SDS-PAGE is useful as a complementary confirmation method of screening by IEF-PAGE. Despite structural differences between epoetin kappa and other rEPOs were observed, serum EPO levels after intravenous injection of epoetin kappa showed properties similar to those of epoetins alfa and beta and serum half-lives were rather short. Therefore, it is feared that athletes abusing EPO will move to intravenous injections for shorter clearance times instead of subcutaneous injections. To improve the effectiveness of the EPO detection approach, more frequent, unannounced, out-of-competition tests for EPO doping need to be applied.

Supporting information

Supporting information can be found in the online version of this article.

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