Research Article

Drug Testing and Analysis

Received: 3 October 2008 Revised: 2 November 2008 Accepted: 2 November 2008 Published online in Wiley Interscience:

(www.drugtestinganalysis.com) DOI 10.1002/dta.10

SDS-PAGE of recombinant and endogenous erythropoietins: benefits and limitations of the method for application in doping control

Christian Reichel,* Ronald Kulovics, Veronika Jordan, Martina Watzinger and Thomas Geisendorfer

Doping of athletes with recombinant and genetically modified erythropoietins (EPO) is currently detected by isoelectric focusing (IEF). The application of these drugs leads to a significant change in the isoform profile of endogenous urinary erythropoietin (uhEPO). Dynepo, MIRCERA, biosimilars with variable IEF-profiles as well as active urines and effort urines have made additional testing strategies necessary. The new generation of small molecule EPO-receptor stimulating agents like Hematide will also challenge the analytical concept of detecting the abuse of erythropoiesis stimulating agents (ESA). By determining their apparent molecular masses with SDS-PAGE a clear differentiation between endogenous and exogenous substances also concerning new EPO modifications is possible. Due to the orthogonal character of IEF- and SDS-PAGE both methods complement each other. The additional benefits of SDS-PAGE especially in relation to active and effort urines as well as the detection of Dynepo were investigated. Due to significant differences between the apparent molecular masses of uhEPO/serum EPO (shEPO) and recombinant, genetically or chemically modified erythropoietins the presence of active or effort urines was easily revealed. The characteristic band shape and apparent molecular mass of Dynepo on SDS-PAGE additionally evidenced the presence of this substance in urine. A protocol for the detection of EPO-doping in serum and plasma by SDS-PAGE was developed. Blood appears to be the ideal matrix for detecting all forms ESA-doping in the future.

Keywords: erythropoietin; doping control; SDS-PAGE; isoelectric focusing; biosimilars

Introduction

The misuse of erythropoietin (EPO) and other erythropoiesis-stimulating agents is prohibited according to the World Anti-Doping Code.[1] Their performance-enhancing effect is based on an increase in red blood cell production by stimulating the proliferation and maturation of precursor cells in the bone marrow.[2] EPO itself is mainly produced by the kidney in adults. It is a glycoprotein hormone consisting of a 165 amino acid polypeptide chain with three N- and one O-glycosylation sites. Due to slight differences in the glycan structure (for example, branching degree and complexity of N-glycans, content of sialic acids, sulfatation) EPO is no single molecule but is composed of a series of isoforms.[3,4] Their distribution is characteristic for each organism, tissue, body fluid and so forth.[5]

Control of doping with recombinant (for example, epoetin alpha, beta, delta) as well as genetically (for example, darbepoetin alpha) or chemically modified (for example, MIRCERA, a methoxy polyethylene glycol modified (PEGylated) epoetin beta) erythropoietins (EPO) is currently accomplished by isoelectric focusing (IEF) on polyacrylamide slab gels. The method is based on changes in the endogenous urinary EPO isoform profile, which are caused by the abuse of these drugs.[6,7] Typically, the most abundant isoforms of recombinant or modified erythropoietins are either more basic or more acidic than the endogenous urinary isoforms, hence EPO doping is detectable.[8] To rule out changes in IEF-profile induced by, for instance, enzymatic activity of the urine (for example, neuraminidases) an additional test has to be performed (stability test), which is also based on IEË-PAGE. Moreover, with the appearance of biosimilars and chemically modified epoetins further criteria or methods have to be defined that allow the detection of EPO abusers. Due to differences in the apparent molecular mass of recombinant and endogenuos erythropoietins EPO-doping can also be detected by SDS-PAGE.[9–11] A comparison of the SDS-PAGE and IEE-method is provided and the benefits and limitations of both methods are discussed.

Experimental Method

Materials

NuPAGE BisTris gels (10% T), lithium dodecyl sulphate (LDS) sample buffer, sample reducing agent, antioxidant, and 4-morpholinepropanesulfonic acid (MOPS) electrophoresis running buffer were from Invitrogen (Carlsbad, CA). Acrylamide/bisacrylamide solution for isoelectric focusing (PlusOne ReadySol IEF, 40% T, 3% C), ammonium peroxidisulphate (APS), N,N,N′,N′-tetramethylethylenediamine (TEMED), urea, Tris, and glycine were from GE Healthcare (Uppsala, Sweden). Servalyte carrier ampolytes (3–4, 4–5, 6–8) were obtained from Serva (Heidelberg, Germany). Millipore (Billerica, MA) provided all devices for filtration and Western blotting, namely for microfiltra-

* *Correspondence to: Christian Reichel, Doping Control Laboratory, Austrian Research Centers GmbH – ARC, A-2444 Seibersdorf, Austria. E-mail: christian.reichel@arcs.ac.at*

tion Steriflip filters (0.2 µm), for ultrafiltration Amicon Ultra-4, Amicon Ultra-15, and Microcon YM-30 filters (nominal molecular weight limit for all the three filter types 30 kDa), and for blotting polyvinylidene difluoride membranes (PVDF; Durapore, Immobilon-P). Methanol (HPLC grade), phosphoric acid (85%), and glacial acetic acid were from Merck (Darmstadt, Germany). Standards for human urinary erythropoietin (uhEPO; second international reference preparation) and human recombinant erythropoietin (rhEPO; BRP-EPO batch 3) were from the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK) and the European Directorate for the Quality of Medicines (Strasbourg, France), respectively. Pharmaceutical preparations of the following recombinant and modified EPOs were from following manufacturers: Amgen (NESP; Thousand Oaks, California, USA), Janssen-Cilag (Erypo; Vienna, Austria), Roche (NeoRecormon, MIRCERA; Mannheim, Germany), Shire (Dynepo; Hampshire, UK), Bioclones (Repotin; Cape Town, South Africa), Blausiegel (Alfaepoetina; Cotia, Brazil), Biosintetica (Hemax; Sao Paulo, Brazil), Biopharma (Epocrin; Kyiv, Ukraine), Microgen (Erythrostim, Moscow, Russia). Dithiothreitol (DTT) and phosphate buffered saline (PBS) tablets were from Sigma-Aldrich (St Louis, MO). For detecting erythropoietin on PVDF-membranes a monoclonal mouse antibody (clone AE7A5; R&D Systems, Minneapolis, MN) in combination with a biotinylated polyclonal antibody (ImmunoPure goat anti-mouse IgG (H + L); Pierce, Rockford, IL) and a streptavidin horseradish peroxidase complex (Biospa, Milano, Italy) was used. The substrate for enhanced chemiluminescence and Surfact-Amps 80 (a 10% solution of Tween-80) were from Pierce (West Pico; Rockford, Illinois, USA). Enzyme-linked immunosorbent assays (ELISA) for erythropoietin were acquired from R&D Systems (Minneapolis, MN) and StemCell Technologies (Vancouver, Canada). The Human 14 Multiple Affinity Removal System (MARS) column (10 × 100 mm) was from Agilent Technologies (Santa Clara, California, USA).

Samples

The following matrices were studied by SDS-PAGE: urine (negative, positive, active, and effort profiles according to IEF-PAGE), serum, and plasma. Urine samples were either official samples taken from athletes for EPO-doping control or samples received from healthy volunteers. Blood samples were from a healthy volunteer who received a single subcutaneous dose of 5000 international units (IU; 66 IU/kg) of NeoRecormon. Another healthy volunteer provided urine samples after a single subcutaneous application of 3000 international units (35 I.U./kg) of Dynepo. Blood and urine samples were collected for 14 days and during the first five days on every day. Clot activator and K-EDTA tubes were used for obtaining serum and plasma, respectively (Vacuette system; Greiner Bio-One, Kremsmünster, Austria). Within 30 min after blood collection samples were centrifuged at 700–800 *rcf* (15 min) and the supernatant aliquoted (150 µL) and stored at −80 °C. Urine samples were stored at −20 °C (20 mL aliquots). Emphasis was put on the strategy of using SDS-PAGE as a supporting evidence for IEF-PAGE results[10,11] and on studying effort and active urine profiles according to their changes in the apparent molecular mass of the detected EPO. Blood samples were used with the purpose of developing an alternative strategy to detect rhEPO doping in blood instead of relying on urine. Athletes and volunteers gave their written consent to using the anonymous samples for research purposes. The project was approved by the local ethics committee.

Depletion of high abundant serum and plasma proteins

High abundant proteins in serum and plasma were removed by using a high capacity multi-immunoaffinity column (MARS column, 10 × 100 mm, Agilent Technologies). The column depleted 14 different proteins (albumin, IgGs, antitrypsin, IgAs, transferrin, haptoglobin, fibrinogen, alpha-2-macroglobulin, alpha-1-acid glycoprotein, IgMs, apolipoprotein AI, apolipoprotein AII, complement C3, transthyretin), which comprised a total of about 94% of the protein content of human serum and plasma samples. Depletion was performed exactly as described in the protocol of the manufacturer. Briefly, 200 µL of serum or plasma were diluted with 600 µL of buffer A (a salt-containing neutral buffer, pH 7.4; proprietary formulation), filtered through a 0.22 µm spin filter (16 000 *rcf*, 1 min), and then separated on the MARS column at a flow rate of 500 µL/min. UV-absorption was recorded at 280 nm and the flow-through fraction collected manually. In order to regenerate the column, the bound fraction was eluted with 100% of buffer B (a low-pH urea buffer; proprietary formulation) for 7.5 min and at a flow rate of 3 mL/min. Finally, buffer A was used to equilibrate the column at a flow rate of 3 mL/min and for 10 min. All steps were performed on a HPLC system (Dionex; Germering, Germany) consisting of a pump series P580, an AS-100 autosampler, and a UVD340U diode array detector. The flow-through fractions were concentrated by a two step ultrafiltration procedure (Amicon Ultra-4 at 4000 *rcf*/30 min, Microcon YM-30 at 14 000 *rcf*/*ca* 15 min) and stored at −80 °C until further use.

Isoelectric focusing

Isoelectric focusing on polyacrylamide slab gels (IEF-PAGE) was performed as described by Lasne *et al.*[12] and Lasne[13] with minor modifications. Briefly, pH 3 to 5 gradient gels (24 × 20 cm) were used with an interelectrode distance of 17 cm. The gels were either prepared according to Lasne *et al.*[12] (pH range 2 to 6 gels) or by using Servalytes 3–4 and 4–5 instead of Servalytes 2–4 and 4–6 (pH range 3 to 5 gels). Catholytes and anolytes (2% (w/v) Servalyte 6–8, 0.5 M phosphoric acid) and focusing conditions were as described by Reichel.[14] Samples (urinary retentates obtained after microfiltration and ultrafiltration of 20 mL of urine) and standards were applied on the gel using sample application pieces (made of blotting paper, GE Healthcare, Uppsala, Sweden). For microfiltration Steriflip units were used.[12] Ultrafiltration was performed in two consecutive steps and by using a combination of Amicon Ultra-15 (4000 *rcf*/30 min) and Amicon Ultra-4 (4000 *rcf*/30 to 60 min) or Microcon YM-30 filters (14,000 *rcf*/*ca* 15 min). The final volume of retentate was about 40 µL for each sample. Typically 20 µL of retentate and about 0.2 ng (or less) of erythropoietin standards were used. Tween-80 was added to both samples and standards (1% (v/v) final concentration). Retentates were heated at 80 °C for 3 min in a thermomixer (Eppendorf; Hamburg, Germany) for inactivating proteases and before performing IEF. All other steps (e.g. addition of protease inhibitors and of 3.75 M Tris-HCl buffer (pH 7.4), washing of retentates with 50 mM Tris-HCl (pH 7.4) buffer) were exactly as described by Lasne *et al.*[12]

Stability test for urine

According to the technical document on the 'Harmonization of the method for the identification of Epoetin alpha and beta (rEPO) and darbepoetin alpha (NESP) by IEF-double blotting and chemiluminescent detection' (TD2007EPO, World Anti-Doping Agency, 2007) suspicious samples have to be repeated by IEF-PAGE

and tested for their stability. Briefly, 500 µL of centrifuged (2700 *rcf*, 10 min) urine were supplemented with protease inhibitors (pepstatin A (Sigma-Aldrich; St. Louis, MO), Complete Protease Inhibitor Cocktail (Roche; Mannheim, Germany)) and diafiltrated against 50 mM acetate buffer (pH 5.0) using Microcon YM-30 ultrafilters. The retentate was recovered and the volume readjusted to 500 µL with acetate buffer. Again, protease inhibitors were added and then the sample was spiked with BRP-EPO and NESP. After an overnight incubation at 37 °C, subsequent heating at 80 °C for 3 min, and addition of Tween-80, the sample was separated on IEF-PAGE together with the suspicious or atypical profile. The aim of the stability test is to exclude the potential urinary enzymatic activity that might cause a shift in the IEF-profile.[8,15,16] Urine samples without activity show no changes in the isoform distribution of the standards.

ELISA affinity extraction

Two commercial ELISA kits were used for the immunoaffinity extraction of EPO from urinary retentates and depleted serum/plasma samples, respectively. The protocols were independently developed from previously reported protocols[10,11] and were based on the instructions provided by the manufacturers of the kits (R&D Systems, StemCell Technologies). For the R&D Systems EPO-ELISA kit, 80 µL of assay diluent (as provided by the manufacturer) were transferred to each ELISA-well and mixed with 20 µL of urinary retentate (prepared as described for IEF-PAGE). The wells were covered with the supplied adhesive strip and incubated on a microplate shaker (*ca* 600 rpm) at least for 1 hour at room temperature or overnight in a coldroom. Then the solution was aspirated and the wells washed with PBS and blotted dry. The ELISA-wells were singularized and each well transferred into a 2.0 mL Eppendorf tube (Eppendorf; Hamburg, Germany). Elution of the bound proteins was performed by adding 30 µL of onefold concentrated (1×) LDS sample buffer to each well. The elution buffer was prepared by mixing, for example, 100 µL of LDS sample buffer (4×) with 40 µL of reducing agent (10×) and 260 µL of MQ-water. The tubes were inserted into a thermomixer and heated for 5 min at 95 °C (600 rpm). Subsequently, the tubes were cooled down on ice and all liquid spun down in a microcentrifuge (16.100 *rcf*, 1 min). The immunoaffinity extracts were then ready for application on SDS-PAGE.

A similar strategy was used for the StemCell ELISA kit. The ELISA wells were first separated from each other and inserted into a Microcon tube. Fifty µL of buffer A (as provided by the manufacturer) were pipetted into each well and mixed with 10 to 20 µL of the urinary retentates. Then the wells were covered with an adhesive strip and incubated either for 2 hours at 30 °C on a thermomixer (600 rpm) or overnight without shaking in the coldroom.[10,11] After removing all liquid the wells were washed five times (as described in the instructions of the ELISA kit) with 400 µL of PBS each. Phosphate buffered saline was used instead of the supplied washing buffer in reference to P. Desharnais (Laboratoire de contrôle du dopage, Québec, Canada; personal communication) and Kohler *et al*.[11] The ELISA wells were tapped dry on adsorbent paper to remove excess liquid. Elution was performed with LDS sample buffer (1×) as described above (30 µL buffer, 95 °C, 5 min, 1250 rpm). After cooling down on ice, the eluted fraction was ready for SDS-PAGE.

For the ELISA immunoaffinity purification of serum and plasma samples the retentates obtained after depletion on the MARS column and ultrafiltration of the flow-through fraction were used. All subsequent steps were identical to the protocols described for urinary retentates.

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on precast BisTris-gels (10% T, 1.5 mm, 10 wells, MOPS running buffer). Standards were heated at 95 °C for 5 min in LDS sample buffer and under reducing conditions. Electrophoresis was performed at constant voltage (200 V) and for

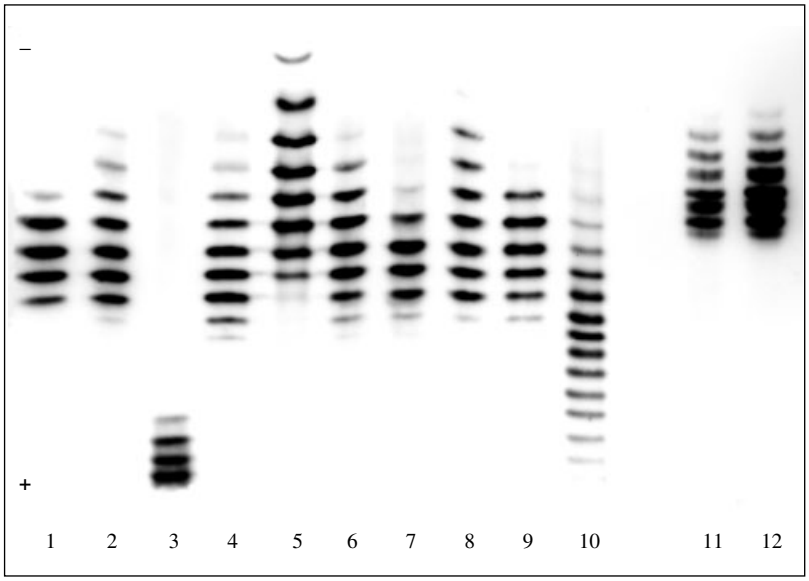

Figure 1. IEF-PAGE of various original and biosimilar erythropoietins. 1. Erypo, 2. NeoRecormon, 3. NESP, 4. Dynepo, 5. Repotin (South Africa), 6. Hemax (Brazil), 7. Alfaepoetina (Brazil), 8. Epocrin (Ukraine), 9. Erythrostim (Russia), 10. uhEPO (NIBSC), (0.2 ng each), 11. and 12. MIRCERA (0.5 ng and 1.0 ng, respectively). Isoelectric focusing was performed in the pH-range of 2 to 6 according to Lasne *et al*.[12] Dynepo (epoetin delta) showed a more intense band alpha than Erypo, NeoRecormon, and the four tested biosilmilars. The band distributions of Repotin and MIRCERA were significantly different from uhEPO and all the other epoetins.

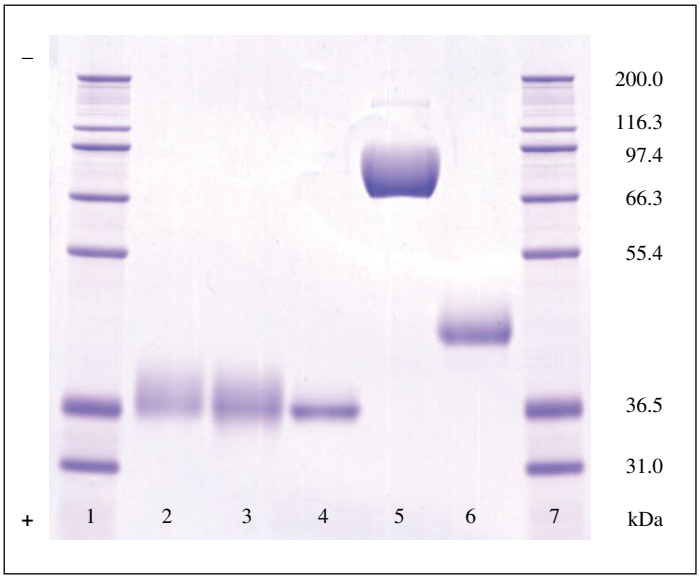

Figure 2. SDS-PAGE of three recombinant erythropoietins (Erypo, NeoRecormon), and one genetically (NESP) and one chemically modified erythropoietin (MIRCERA). 1. Mark12, 2. Erypo, 3. NeoRecormon, 4. Dynepo, 5. MIRCERA, 6. NESP, 7. Mark12. The amount of each pharmaceutical applied on the gel was between 1.6 to 3.3 µg. The gel was stained with Coomassie R-250. Compared to the other EPOs Dynepo showed a very characteristic thin band on SDS-PAGE.

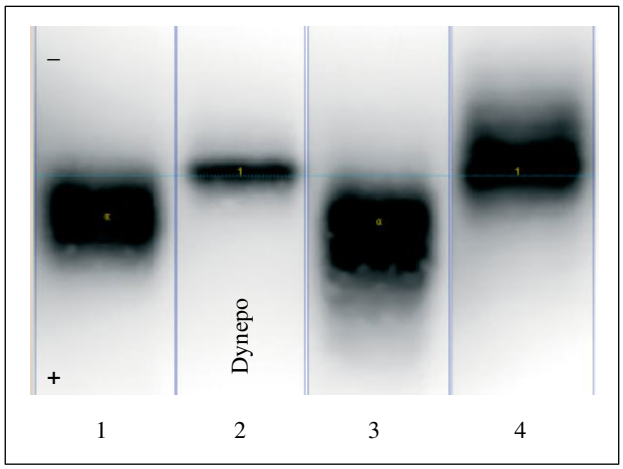

Figure 3. Comparison of uhEPO and Repotin on SDS-PAGE with Western blotting and chemiluminescence detection. 1. uhEPO (NIBSC standard), 2. Dynepo, 3. Repotin, 4. NeoRecormon (0.2 ng each). The characteristic band shape of Dynepo clearly distinguishes this type of rhEPO from all other EPOs.

ca 55 min. The absolute amount of EPO standards applied on SDS-PAGE gels was either equal to the amount applied on IEF gels (*ca* 0.2 ng, which corresponds to *ca* 6 to 8 fmol), lower (NESP), or higher (for example, MIRCERA) or between 1.6 to 3.3 µg for Coomassie R250 stained gels. Pre-stained molecular weight markers (SeeBlue Plus2; Invitrogen, Carlsbad, CA) were applied on the first and last lanes of each gel in order to control the separation during the run and the Western blotting transfer efficiency. Additional unstained molecular weight markers (Mark12; Invitrogen, Carlsbad, CA) were used for Coomassie stained gels. Eluates received after ELISA immunoaffinity purification of urinary retentates or depleted serum and plasma samples were applied on the gel immediately after cooling down and adjusting to room temperature.

Coomassie R-250 stain

SDS-PAGE gels were fixed for 60 min in a solution containing 7.5% acetic acid and 10% methanol. Subsequently, gels were stained with Coomassie R-250 overnight and then destained for several hours in a solution consisting of 10% acetic acid and 20% methanol. The staining solution was prepared by dissolving four tablets of Serva Blue R (Serva; Heidelberg, Germany) in 100 mL of a solvent containing 10% acetic acid and 45% methanol. The solution was freshly prepared and filtered (595 1/2 fold filters; Whatman; Dassel, Germany) immediately before usage.

Western blotting and chemiluminescence detection

After focusing, IEF-PAGE gels were equilibrated for 4 min in modified Towbin buffer (25 mM Tris, 192 mM glycine, no methanol) and blotted (1 mA/cm^2, 30 min) on PVDF membranes (Immobilon-P) as described by Lasne.[12] Four sheets of thick blotting paper (GE Healthcare, Uppsala, Sweden) were used on each side of the blotting sandwich. After the transfer the membrane was incubated in a solution of 5 mM DTT in PBS (37 °C, 60 min) in order to increase the sensitivity of the method. Subsequently, the membrane was blocked in 5% non-fat milk (BioRad, Hercules, CA) in PBS (60 min), incubated in a solution of anti-EPO antibody (clone AE7A5) in 1% non-fat milk (1 µg/mL; usually overnight at 4 to 8 °C), washed in 0.5% non-fat milk in PBS (3 × 10 min), and double-blotted (0.8 mA/cm^2, 10 min) using 0.7% acetic acid according to Lasne.[13] After another blocking step (5% non-fat milk in PBS, 60 min) the membrane was incubated with the biotinylated goat anti-mouse IgG (H+L) antibody (1 : 2000 dilution in 1% non-fat milk, 60 min) and washed for 3 × 10 min in 0.5% non-fat milk. Finally, the membrane was transferred to a solution of streptavidin horseradish peroxidase (HRP) in 1% non-fat milk (1 : 2000, 60 min) and then washed with PBS (3 × 10 min). Enhanced chemiluminescence was achieved by incubation of the blot in a luminol based substrate (West Pico). Images were acquired using a CCD camera (LAS-4000; Fujifilm, Tokyo, Japan). The exposure

time was adjusted according to the initial signal intensity (for example, 1 min to 30 min). For all blotting steps a semi-dry blotter was used (Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell; BioRad, Hercules, CA).

SDS-PAGE gels were blotted in a similar way as IEF-PAGE gels. Equilibration was performed for 3 × 6 min in Bjerrum[17] buffer (48mM Tris, 39mM glycine, 1.3mM SDS, 20% methanol). No Durapore separating membrane was used between gel and Immobilon-P membrane. One sheet of extra-thick blotting paper was used on each side of the sandwich. Membrane and blotting paper were equilibrated in Bjerrum buffer, too. First blots were performed on a semi-dry blotter (Hoefer TE 77; GE Healthcare, Uppsala, Sweden) at constant current (1.5 mA/cm^2), and second blots on the Trans-Blot SD blotter (0.8 mA/cm^2). All other steps were identical to the IEF blots. All subsequent steps were exactly as described for IEF-PAGE gels.

Images were analysed using two different types of software (GASepo, version 1.3b2; ARC, Seibersdorf, Austria; AIDA, version 3.52, Raytest, Straubenhardt, Germany).

Results and Discussion

SDS-PAGE of EPO standards

Recombinant as well as genetically and chemically modified erythropoietins were separated both on SDS-PAGE and IEF-PAGE. While epoetin alpha (Erypo), beta (NeoRecormon) and delta (Dynepo) pharmaceuticals showed slight but subtle differences in their isoform profiles (Fig. 1) the average apparent molecular masses of all of these epoetins were very similar on SDS-PAGE (10% T, MOPS running buffer, Coomassie R-250 stained gels), i.e. Erypo *ca* 36.9 kDa, NeoRecormon *ca* 36.6 kDa, Dynepo *ca* 36.2 kDa (Fig. 2). Similar results were obtained for biosimilar erythropoietins (Alfaepoetina, Hemax, Epocrin, Erythrostim) – not necessarily completely identical IEF-profiles if compared with the original products[18] but almost identical apparent molecular masses on SDS-PAGE. However, due to the glycoprotein nature of EPO and the resulting decrease in SDS-binding capacity of the molecule, the molecular masses of epoetins are typically higher on SDS-PAGE ('apparent' molecular mass) than measured by mass spectrometry.[19]

Among these pharmaceuticals Dynepo showed a very characteristic band shape on SDS-PAGE gels, which made differentiation from epoetin alpha and beta products very easy. Characteristic average apparent molecular masses were also received for darbepoetin alpha (NESP; *ca* 44.2 kDa) and MIRCERA (*ca* 70.2 kDa). However, both proteins again showed broader bands than Dynepo (Fig. 2, lane 4).

The SDS-PAGE experiments were repeated using Western blotting and chemiluminescence detection. Identical results were obtained for the recombinant proteins if compared with the Coomassie R-250 stained gels. Contrary to that the apparent molecular mass of uhEPO (NIBSC standard; Fig. 3, lane 1) was lower (*ca* 34 kDa) than the masses of all recombinant or modified epoetins and the band was again broader than for Dynepo (using identical absolute amounts on gel). Repotin (a baby hamster kidney (BHK) cell line produced rhEPO comparable to epoetin omega) was the only recombinant epoetin that showed an average apparent molecular mass which was even lower than the mass of uhEPO (Fig. 3, lane 3). The band shape was comparable to uhEPO. However, there are significant differences between BHK- and Chinese hamster ovary (CHO)-cell produced epoetins in the

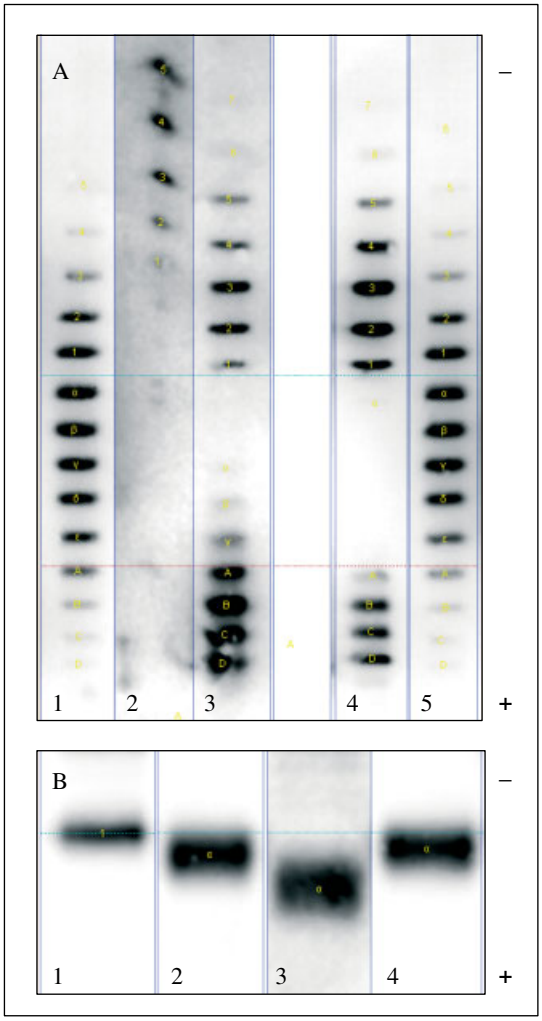

Figure 4. Comparison of an active urine on IEF- and SDS-PAGE. IEF-PAGE (Fig. 4A): 1. uhEPO (NIBSC), 2. active urine, 3. stability test, 4. BRP-EPO/NESP, 5. uhEPO (NIBSC); SDS-PAGE (Fig. 4B): 1. Dynepo, 2. uhEPO (NIBSC), 3. active urine, 4. rhEPO-negative control urine. The EPO IEF-profile was shifted towards the basic side of the gel (lane 2, Fig. 4A); the stability test indicated an instable urine (lane 3, Fig. 4A). The same urine was also analysed by SDS-PAGE (lane 3, Fig. 4B). A significant decrease in apparent molecular mass was observed when the sample was compared with the uhEPO standard (lane 2, Fig. 4B) or an rhEPO-negative control urine (lane 4, Fig. 4B).

structure of the glycans.[20] The majority of erythropoietin-based originator (for example, epoetin alpha/beta/delta, darbepoetin alpha, MIRCERA) and biosimilar pharmaceuticals, which are worldwide available, are non-BHK-EPOs.

SDS-PAGE of urine samples

In order to evaluate the additional benefit of SDS-PAGE for doping control purposes a series of urine samples with exceptional IEF-profiles were analysed by SDS-PAGE. All samples were first immunoaffinity purified by ELISA and then separated on 10% T BisTris gels. In these experiments/applications the efficiency of the ELISA immunoaffinity extraction method appeared to be higher for the StemCell Technologies than the R&D Systems ELISA kit (data not shown).

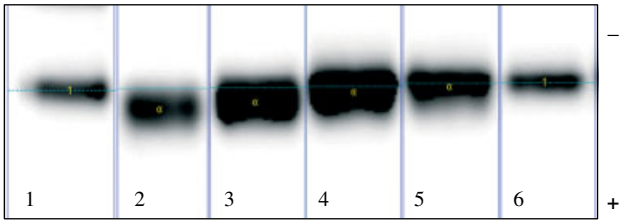

Figure 5. SDS-PAGE of urine specimens of a Dynepo application study. After the application of 3000 IU Dynepo a continuous change from the apparent molecular mass of uhEPO to the higher mass of Dynepo was observed. Also note the characteristic change from the broad band shape of uhEPO to the sharp band shape of Dynepo. Lanes: 1. Dynepo (0.2 ng), 2. uhEPO (NIBSC; 0.2 ng), 3. immediately before application, and 4. 8.4 hours, 5. 13.3 hours, 6. 21.5 hours after application, respectively.

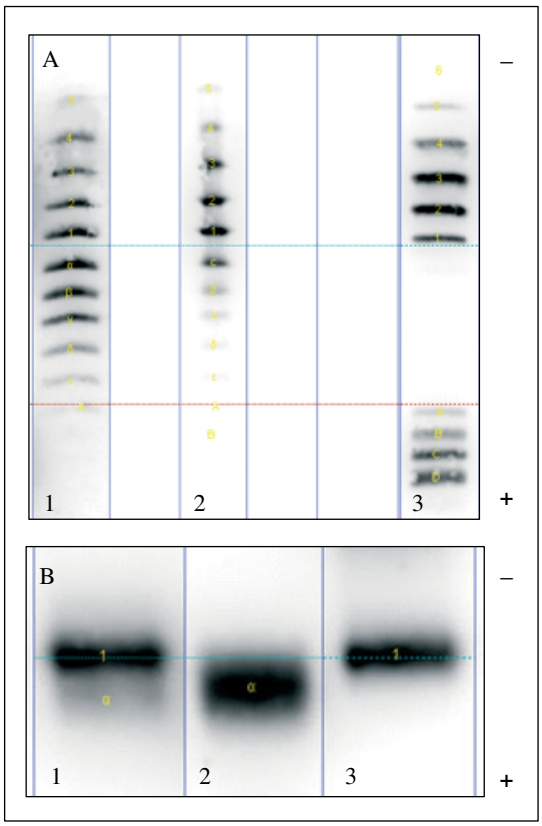

Figure 6. Comparison of IEF-PAGE (*Fig. 6A*) and SDS-PAGE (*Fig. 6B*) of a urinary sample suspicious for recombinant EPO. SDS-PAGE directly evidenced the presence of Dynepo. Lanes: 1. uhEPO (NIBSC), 2. suspicious sample, 3. BRP-EPO/NESP (*Fig. 6A*); 1. suspicious sample, 2. rhEPO-negative control urine, 3. Dynepo (*Fig. 6B*).

Example 1: active urine samples

Doping control samples that showed suspicious or atypical profiles on IEF-PAGE were repeated and tested for stability in accordance with the regulations of the technical document TDEPO2007.[8] Lane 2 in Fig. 4A shows the IEF-profile of a urine sample that was shifted towards the basic region of the gel. The stability test (Fig. 4A, lane 3) indicated a slight activity due to the occurrence of additional bands on the anodic side of the gel (NESP). Subsequently, an SDS-PAGE of the ELISA immunoaffinty purified urinary retentate and Western double blot were performed (Fig. 4B). Compared to the standard of uhEPO (Fig. 4B, lane 2) and the negative control urine (Fig. 4B, lane 4) the atypical sample (Fig. 4B, lane 3) produced a band with a much lower apparent molecular mass on SDS-PAGE, indicating a degradation process of the urinary erythropoietin in the sample. Typically, losses of terminal sialic acids of EPO cause comparable shifts in apparent molecular mass on SDS-PAGE.[21–23] The maximum number of terminal sialic acids is 14 for uhEPO and rhEPO. The mass N-acetylneuraminic acid is 309.3 Da, so a complete loss of the sialic acids of the EPO molecule would result in a decrease in the apparent molecular mass of *ca* 4 kDa (asialoerythropoietin). Losses of other charged groups (such as phosphate or sulphate groups) would not lead to similar decreases in mass due to the lower apparent molecular mass of these groups. Consequently, SDS-PAGE added additional value to the data obtained by IEF-PAGE and the stability test. However, the sample was clearly negative on both IEF-PAGE and SDS-PAGE.

Example 2: presence of Dynepo in urine samples

Urine samples of a Dynepo application study were analysed by both IEF-PAGE and SDS-PAGE. During the first five days at least two or three urine samples were collected per day. The samples were not pooled in order to be able to investigate time-dependent changes in the EPO profiles as detailed as possible. Changes in the IEF-profiles were comparable to data published elsewhere, for example by Breidbach *et al.*[24] However, due to the higher intensity of band alpha of Dynepo in comparison to band alpha of Erypo or NeoRecormon (see also Figs 1 and 6) the evaluation of the IEF-profiles was greatly simplified by using SDS-PAGE as additional evidence for the presence of Dynepo. Since Dynepo produced a very sharp and hence characteristic band on SDS-PAGE, both methods perfectly complemented each other. Typically, erythropoietins run as broad bands on SDS-PAGE due to the highly glycosylated nature of these proteins (see Fig. 2). The results of the application study also confirmed the usefulness of the unique nature of the Dynepo band. Due to an increasing suppression of the production of endogenous EPO only Dynepo's characteristic band was seen on the second day (Fig. 5, lane 6). The suppression effect lasted for at least two days.

Figure 6A (lane 2) shows the IEF-profile of a urine sample fulfilling the criteria of TD2007EPO. After ELISA immunoaffinity extraction of the urinary retentate an SDS-PAGE analysis was performed (Fig. 6B, lane 1). It revealed the presence of Dynepo due to the characteristic band shape and higher apparent molecular mass of the EPO contained in the sample.

Example 3: effort urine samples with IEF-profiles shifted to the basic region

Isoelectric focusing profiles of effort urines (urine specimens with an isoform distribution that is comparable to uhEPO but which is, in its entirety, slightly more basic than uhEPO) were further

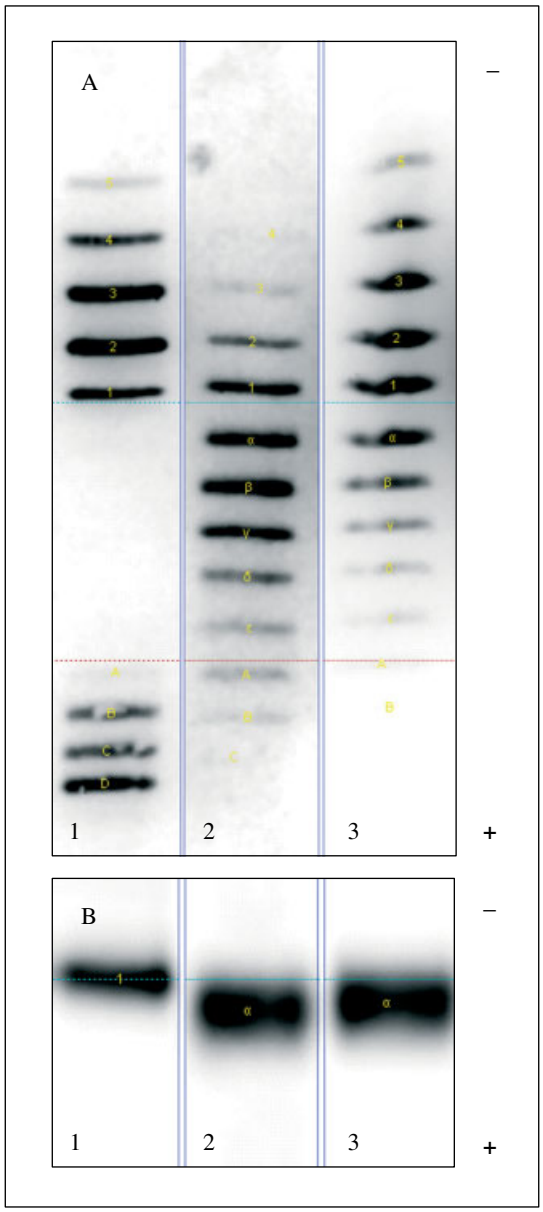

Figure 7. IEF-PAGE of a urine with a profile shifted to the basic area of the gel (Fig. 7A): 1. BRP-EPO/NESP, 2. uhEPO (NIBSC), 3. sample. The sample did not fulfil the criteria of positivity (TD2007EPO). SDS-PAGE confirmed that the shift was not caused by the abuse of recombinant EPO or an instable urine (Fig. 7B): 1. Dynepo, 2. uhEPO (NIBSC), 3. sample.

analysed by SDS-PAGE. Again, the urinary retentates were first purified by ELISA immunoaffinity extraction and then separated according to their apparent molecular mass on SDS-PAGE gels (10% T, MOPS running buffer, 1.5 mm). Figure 7A (lane 3) shows an IEF-PAGE profile of a urine, which was shifted to the basic area of the gel. However, no suppression effect caused by the application of exogenous EPO was observed. The sample was also clearly negative according to the criteria of TD2007EPO.[8] On SDS-PAGE only one band which was corresponding to the band of the uhEPO standard could be observed (Fig. 7B, lane 3). Both uhEPO and human serum EPO (shEPO) show similar apparent molecular masses on SDS-PAGE[9,25] so it was very likely that the shift in the IEF profile was caused by human serum EPO.[26] No decrease

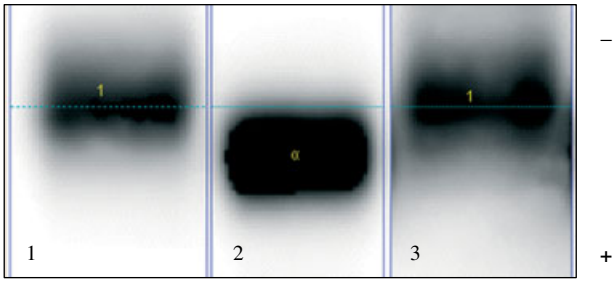

Figure 8. SDS-PAGE of human serum after a single dose application of NeoRecormon (5000 IU): 1. BRP-EPO, 2. uhEPO (NIBSC), 3. sample. The sample (200 µL) was depleted, ultrafiltrated, purified by EPO-ELISA, and then separated by electrophoresis. Due to its higher apparent molecular mass rhEPO could be easily detected. The masses of shEPO and uhEPO are comparable on SDS-PAGE.[9].

in mass was observed, so SDS-PAGE also confirmed the negative stability test of this sample (IEF data not shown).

SDS-PAGE of serum and plasma samples

The EPO concentrations of the serum and plasma samples of the NeoRecormon application study were measured by ELISA (R&D Systems). The highest amount of EPO was observed on the first day after the application (56 mIU/mL serum; data not shown). Due to the high protein content the samples (200 µL aliquots) had to be depleted before SDS-PAGE could be performed. A high-capacity multi-affinity column (MARS system) directed against 14 high-abundant plasma proteins was used. The non-bound fraction was collected and concentrated by ultrafiltration (filters with a nominal molecular weight limit of 30 kDa were used). In order to further reduce interfering proteins EPO was subsequently enriched by ELISA immunoaffinity purification of the retentate (StemCell Technologies). After elution (LDS sample buffer (1×), 95 °C, 5 min) the bound fraction was separated on SDS-PAGE (10% T, MOPS running buffer, 1.5 mm) and EPO detected by Western double blotting and chemiluminescence reaction (Fig. 8, lane 3).

The applied enrichment strategy allowed the identification of rhEPO in serum and plasma samples. Since the apparent molecular masses of human serum and urinary EPO were comparable on SDS-PAGE[9,25] the application of rhEPO could be detected. So far, no attempt was made to figure out the detection window of the method and to compare it with the detection window of the IEF-PAGE method.

Conclusion

SDS-PAGE and IEF-PAGE are two orthogonal separation methods that complement each other in the detection of doping with recombinant erythropoietins. While IEF separates proteins according to charge, SDS-PAGE separates them according to their apparent molecular mass. Due to the lower mass of uhEPO and shEPO compared to most rhEPOs (such as epoetins alpha, beta, and delta) and genetically (darbepoetin alpha) or chemically (MIRCERA) modified EPOs a differentiation between endogenous and exogenous erythropoietins is possible by SDS-PAGE.[9–11,14,19] Despite the fact that Dynepo is produced in a human cell line (human fibrosarcoma cell line HT-1080) instead of a Chinese hamster ovary (CHO) or baby hamster kidney (BHK) cell line, the IEF-profile is not identical to human endogenous EPO.[27] On SDS-PAGE epoetin delta (Dynepo) produced a very sharp band, which was unusual when

compared to epoetin alpha, beta, omega, darbepoetion alpha, PEGylated epoetin beta (MIRCERA), biosimilars, and human urinary and serum EPO.[9] Due to this very characteristic band shape of Dynepo on SDS-PAGE on the one hand and a relatively intense alpha band on IEF-PAGE on the other hand the detection of doping with Dynepo appeared to be more simple by SDS-PAGE. The latter method also revealed additional information about the nature of active urines and atypical or effort-type IEF-profiles. Preferably, the amount of EPO applied on an SDS-PAGE gel should be equal for each lane. This can be accomplished by measuring the EPO concentration of the urinary retentates with a commercial ELISA kit before the immunoaffinity extraction. However, due to the limited mass resolution of SDS-PAGE bands of uhEPO and epoetin alpha or beta were slightly overlapping, but nevertheless with a clear difference in the average apparent molecular mass. Perfect separation was obtained for NESP and MIRCERA. Blood (serum, plasma) in combination with immunoaffinity purification and SDS-PAGE appears to be a very promising strategy for detecting EPO doping with most types of erythropoietins (including biosimilars) and the latest generation of PEGylated erythropoiesis-stimulating agents (for example, MIRCERA, Hematide).

References

[1] World Anti-Doping Agency (WADA) **2008**, Available at http://www.wada-ama.org/rtecontent/document/2008_List_En.pdf, accessed 15 November 2008.
[2] W. Jelkmann, *Medicina Sportiva* **2007**, *11*(2), 32.
[3] W. Jelkmann, *Br. J. Haematol.* **2008**, *141*(3), 287.
[4] C. Neusüss, U. Demelbauer, M. Pelzing, *Electrophoresis* **2005**, *26*(7–8), 1442.
[5] R. C. Tam, S. L. Coleman, R. J. Tiplady, P. L. Storring, P. M. Cotes, *Br. J. Haematol.* **1991**, *79*(3), 504.
[6] F. Lasne, J. de Ceaurriz, *Nature* **2000**, *405*, 35.
[7] F. Lasne, *J. Immunol. Methods* **2001**, *253*(1–2), 125.
[8] World Anti-Doping Agency (WADA), **2007**, Available at www.wada-ama.org/rtecontent/document/td2007epo_en.pdf, accessed 15 November 2008.
[9] V. Skibeli, G. Nissen-Lie, P. Torjesen, *Blood* **2001**, *98*(13), 3626.
[10] P. Desharnais, C. Ayotte, in *Recent Advances in Doping Analysis (15)*, (Eds: W. Schänzer, H. Geyer, A. Gotzmann, U. Marek), Sportverlag Strauss: Cologne, **2007**, p. 303.
[11] M. Kohler, C. Ayotte, P. Desharnais, U. Flenker, S. Lüdke, M. Thevis, E. Völker-Schänzer, W. Schänzer, *Int. J. Sports Med.* **2008**, *29*(1), 1.
[12] F. Lasne, L. Martin, N. Crepin, J. de Ceaurriz, *Anal. Biochem.* **2002**, *311*(2), 119.
[13] F. Lasne, *J. Immunol. Methods* **2003**, *276*(1–2), 223.
[14] C. Reichel, *J. Mass. Spectrom.* **2008**, *43*(7), 916.
[15] N. Imai, A. Kawamura, M. Higuchi, M. Oh-eda, T. Orita, T. Kawaguchi, N. Ochi, *J. Biochem.* **1990**, *107*(3), 352.
[16] V. Belalcazar, R. Gutiérrez Gallego, E. Llop, J. Segura, J. A. Pascual, *Electrophoresis* **2006**, *27*(22), 4387.
[17] O. J. Bjerrum, C. Schafer-Nielsen, in *Electrophoresis '86*, (Ed: M. J. Dunn), Wiley-VCH: Weinheim, **1986**, pp. 315.
[18] H. Schellekens, *EHLP Journal* **2004**, *3*, 43.
[19] G. Stübiger, M. Marchetti, M. Nagano, C. Reichel, G. Gmeiner, G. Allmaier, *Rapid Commun. Mass Spectrom.* **2005**, *19*, 728.
[20] M. Nimtz, V. Wray, A. Rüdiger, H. S. Conradt, *FEBS Lett.* **1995**, *365*(2–3), 203.
[21] P. E. Groleau, P. Desharnais, L. Coté, C. Ayotte, *J. Mass. Spectrom.* **2008**, *43*(7), 924.
[22] N. Inoue, M. Wada, M. Takeuchi, *Biol. Pharm. Bull.* **1994**, *17*(2), 180.
[23] O. Sánchez, R. Montesino, J. R. Toledo, E. Rodríguez, D. Díaz, L. Royle, P. M. Rudd, R. A. Dwek, G. J. Gerwig, J. P. Kamerling, D. J. Harvey, J. A. Cremata, *Arch. Biochem. Biophys.* **2007**, *464*(2), 322.
[24] A. Breidbach, D. H. Catlin, G. A. Green, I. Tregub, H. Truong, J. Gorzek, *Clin. Chem.* **2003**, *49*(6), (Pt 1), 901.
[25] S. Masuda, M. Okano, K. Yamagishi, M. Nagao, M. Ueda, R. Sasaki, *J. Biol. Chem.* **1994**, *269*(30), 19488.
[26] F. Lasne, L. Martin, J. A. Martin, J. de Ceaurriz, *Int. J. Biol. Macromol.* **2007**, *41*, 354.
[27] V. Belalcazar, R. Ventura, J. Segura, J. A. Pascual, *Am. J. Hematol.* **2008**, *83*(9), 754.