

Improved detection of EPO in blood and urine based on novel Velum SAR precast horizontal gels optimized for routine analysis

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To enhance the endurance performance the misuse of erythropoietin (EPO) is currently detected by isoelectric focusing (IEF) based on the profile of altered isoforms of EPO. Changes within the new WADA technical document allows for the detection of the different erythropoietins by using the Sarcosyl-PAGE, a modified SDS-PAGE. The Sarcosyl-PAGE which separates by difference in the molecular mass of EPO isoforms introduces a number of advantages: clear evidence between endogenous and the new modified exogenous EPO even when applied in smaller quantities; extended detection time of exogenous EPO, and a more simplified analysis. For EPO routine analysis a protocol based on a new type of Sarcosyl precast gels was established. For the evaluation of this improved detection method blood and urine samples of a micro-dose study were investigated.

Introduction

The misuse of erythropoietin (EPO) is prohibited by the World Anti-Doping Code due to the performance-enhancing effect based on the increase of red blood cells production (1). Since the first published method (2) based on isoelectric focusing (IEF) the analysis had to be adapted in order to detect genetically modified erythropoietins like darbepoetin-alpha (3)). Due to further chemically modified erythropoietins, e.g. MIRCERA (a PEGylated epoetin beta), an additional method for the analysis the SAR-PAGE (Sodium N-lauroylsarcosinate („sarcosyl”) polyacrylamide gel electrophoresis) was established (7) . However, despite the clear discrimination of the modified erythropoietins with these new methods there are persistent concerns that a so called “micro-dose” misuse of EPO (4) will lead to a negative doping test.

Therefore, for the detection of genetically, chemically modified EPO and micro-dose EPO a further improved

detection method is required. In addition, for laboratories analyzing larger numbers of samples, the test should be easier in handling and offering a higher grade in automation.

Materials and methods

Materials

Pharmaceutical formulations of recombinant and biotechnologically as well as chemically modified erythropoietins were obtained from the manufacturers Roche (NeoRecormon, MIRCERA; Mannheim, Germany) and Amgen (NESP; Thousand Oaks, CA). The standard for human recombinant erythropoietin (rhEPO; BRP-EPO batch 3) were obtained from the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK). Blood was collected in Vacuette K2E tubes and Vacuette Z Serum Sep Clot Activator tubes, both purchased from Greiner Bio-One GmbH (Kremsmünster, Austria). Devices for microfiltration (Steriflip (0.2 µm), polyvinylidene difluoride (PVDF) membranes (Immobilon-P) and Immobilon Western HRP Substrat were purchased from Millipore (Billerica, USA). 10 x PBS-stock solution were from LONZA (Rockland, ME). Tris hydrochloride, Dithiothreitol (DTT), milk powder (blotting grade) were obtained from Roth (Karlsruhe, Germany). The primary antibody used, mouse anti-human EPO antibody (Clone AE7A5), was obtained from R&D Systems (Oxford, United Kingdom). The used secondary goat anti-mouse IgG (H+L), conjugated with a horseradish peroxidase was purchased from Pierce (Rockford, IL). The EPO purification kit containing anti-EPO monolith columns, UPD (Urine Precipitate Dissolution) buffer, sample dilution buffer, exposure aid, desorption buffer and adjustment buffer were obtained from MAIA Diagnostics (Uppsala, Sweden). Velum SAR gels (24, 30 and 40 slot wells, 10% T, 0.5 mm) were obtained from NH DyeAGNOSTICS

(Halle, Germany) in cooperation with Electrophoresis Development & Consulting (Tübingen, Germany). The isoelectric focusing (IEF) of all samples was performed by using a IEF-KIT from Serva (Heidelberg, Germany). The incubation and wash process was carried out with the BlotCycler™ (Mansfield, MA).

Samples

Serum and urine samples were obtained from four healthy volunteers who received single-dose subcutaneous applications of erythropoietin pharmaceuticals, i.e. 1000 international units (IU) of Epoetin alfa (Hexal, Holzkirchen, Germany) (11-16 IU/ kg Bodyweight). Urine samples were collected for 92 h. Blood samples were collected 5 h, 25 h and 75 h after injections. The EDTA blood samples were analyzed according to the WADA ABP protocol. The blood in the Vacuette Z tubes was allowed to coagulate for 30 min before centrifugation at 2000 rcf (15 min). The separated sera were aliquoted (1 ml) and then stored at – 20°C. All volunteers gave their written consent to using the anonymous samples for research purposes.

Sample preparation

Affinity purification was performed according to the manufacturer's instructions (MAIIA AB). Briefly, 2 ml UPD-buffer was added to the urine samples, which were incubated for 10 min at RT and then heated on a simmering water bath for 10 min. After cooling, samples were diluted with 20 ml working sample buffer and filtered through Steriflip filters. Urines were passed through the monolith columns at 1 ml/min using a vacuum manifold at a pressure of -5 kPa. After washing, the isolated EPO was eluted with 40 µl desorption buffer into 4 µl adjustment buffer. The aliquots (20 µl) were at -20 °C until use. Serum (1-2 ml) was diluted 1:10 in working sample buffer, filtered through Steriflip filters (0.2 µm) and then affinity purified using the same columns and desorption buffers as for urine.

Sarcosyl PAGE

The Sarcosyl polyacrylamide gel electrophoreses (PAGE) were performed on precast Velum SAR gels (NH DyeAGNOSTICS, Germany). Each sample (6 µl affinity purified urine or sera samples or the mixture of EPO-pharmaceuticals) was diluted by 3.7 µl Sarcosyl sample buffer (500 µl SAR sample buffer delivered with the kit

and 115 µl freshly prepared 0.34 M DTT) for the gel size with 40 slots, for gels with 24 slots the sample volumes were doubled. The samples were denatured under reducing conditions by heating for 5 min at 95°C. After cooling the samples (8 µl res. 13 µl) were loaded onto the Velum SAR gels and the electrophoresis was performed at 15°C using the Orca Gel Electrophoresis System (NH DyeAGNOSTICS, Germany). The separation conditions were divided into a slow sample entrance (300 V, 20 mA, 10 W for 30 min) and the main electrophoresis (750 V, 35 mA, 35 W for 2 h). Compared to the previous used combination of NuPAGE gels and “Double-Blot” the total amount of EPO standards applied on the Velum gels was reduced (BRP-EPO 0.03 ng vs. 0.12 ng; NESP 0.015 ng vs. 0.05 ng; MIRCERA 0.03 ng vs. 0.025 ng). To monitor the separation process during the run and the efficiency of the capillary blot a pre-stained molecular weight marker (SeeBlue Plus2; Invitrogen, Carlsbad, CA) was applied on the first and last lane. An additional marker was applied in the middle of the gel to cut the membrane into two parts for the automated wash and incubation steps.

IEF

Isoelectric focusing on polyacrylamide slab gels (IEF-PAGE) was performed as described by Lasne et al. (5, 6) with some modifications. Briefly, pre-cast IEF gels were prepared as described by the manufacturer. All samples were affinity purified and were applied after heating (80 °C, 3 min, 16 µl) directly into the sample slots. Tween-80 was added to both samples and standards (1% (v/v) final concentration). The focusing conditions were exactly as described by Lasne et al. (5).

Capillary blot and chemiluminescence detection

After electrophoresis the Velum SAR gels were equilibrated in pure water for 15 min and then the proteins were blotted on an Immobilon-P membrane. The protein transfer was performed for 2 h by capillary blotting using the Beo Dry Blotter (NH DyeAGNOSTICS, Germany). The membrane was placed on top of the gel with additionally three sheets of wet and 12 sheets of dry blotting paper (Munktel, Falun, Sweden). The IEF gels were washed 5 min in 4 M Guanidinehydrochloride in 50 mM Tris hydrochloride pH 7.4. The same solution was used to wet the blotting paper. The protein transfer was performed as described for the Velum gels by capillary blotting using the Beo Dry Blotter but the transfer time was reduced to 1h. After transfer the membrane was

first incubated in 5 M DTT/PBS (45 min, RT), washed with PBS (3 x 1 min) and then placed in the trays of the BlotCycler. All subsequent steps were performed at 4°C to 8°C. After blocking in 5% milk/ PBS (70 min) the membranes were washed and incubated for 5 h in a solution of the primary antibody (mouse monoclonal anti-EPO antibody, clone AE7A5, 1 µg/ ml) in 1% milk/ PBS. The membranes were washed (PBS; 9 x 5 min) and incubated with secondary goat anti-mouse IgG (H+L), conjugated with a horseradish peroxidase (1:100.000, 1% milk/ PBS, 11 h). Immediately after washing with PBS (9 x 5 min) the membranes were incubated in chemiluminescence substrate (Immobilon Western HRP Substrate). Alternatively, these steps can be performed manually at room temperature with reduced incubation time for the secondary antibody. The images were acquired using a CCD camera (LAS-4000; Fujifilm, Tokyo, Japan). Finally, images were analysed using GASepo (version 2.1) software.

Results and Discussion

Horizontal SAR-PAGE and IEF for control samples

To compare the sensitivity of the Velum SAR and NuPAGE gels for the detection of the different amounts of recombinant erythropoietins a serial dilution was used (Figure 1). The bands of NESP and BRP-EPO decrease on NuPAGE gels as well as on Velum SAR gels in a linear manner. In addition the bands of a serial dilution of MIRCERA also decrease in a linear manner on Velum SAR gels, which could not be observed for NuPAGE gels (7). Furthermore, there is also no negative effect for the MIRCERA signal on Velum SAR gels by reducing the Albumin concentration from 0.1% (w/v) to 0.005% in contrast to the NuPAGE gels which show a drastic signal loss (data not shown).

The main focus of the present study was to establish the SAR-PAGE as a routine method according the new technical document WADA-TD2014EPO. With the clear band characteristics of MIRCERA and NESP the objective is to detect the mixed band of endogenous EPO and rEPO especially the diffuse area as described in section 4.2.2.2 WADA-TD2014EPO. This is even of higher importance since there are persistent concerns that athletes have learned to use rEPO by using micro doses which may lead to a negative doping test result. That is with such low doses the endogenous production

will not be suppressed (4) and the IEF profiles of doping analysis will show endogenous isoforms during the application period. In our study we therefore reduced the doses of rEPO to an absolute minimum, yielded in a very low hematological response.

In order to evaluate the performance of the EPO analysis based on the Velum SAR precast gels positive and negative controls as well as a suspicious rhEPO and an ultra-low dose rhEPO were analyzed by IEF-PAGE and the novel Velum SAR PAGE. These four samples served as a control for the IEF method. Furthermore, the profiles of positive and negative controls serve as a reference in order to screen for suspicious profiles (Figure 4).

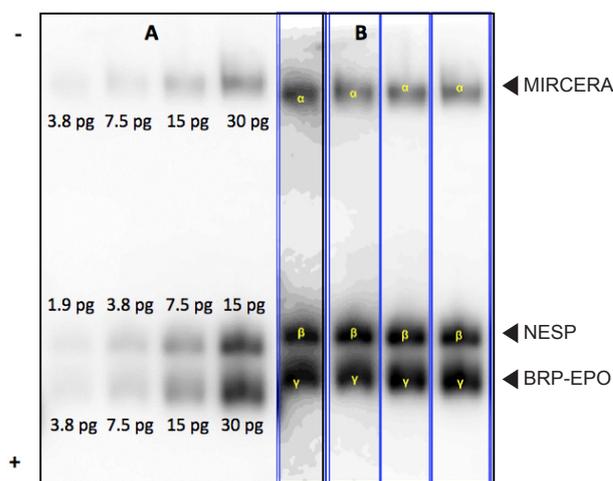


Figure 1. A: Dilution series of the standard preparation used for Velum SAR PAGE. The highest concentration (MIRCERA 30 pg; NESP 15 pg and BRP-EPO 30 pg) was used as standard concentration for the 40 slot gel. B: lanes with individually optimized contrast.

The comparison of the results obtained from SAR-PAGE (Figure 2) and IEF (Figure 4) show that the negative and the positive control can clearly be allocated. However, when using the IEF method for EPO analysis the suspicious sample (Figure 4: lane 4) and especially the ultra-low dose sample (Figure 4: lane 5) do not fulfill the criteria for an adverse analytical finding as given by the technical document WADA-TD2014EPO. As a consequence, these samples would not be further investigated if the initial testing procedure was performed by IEF.

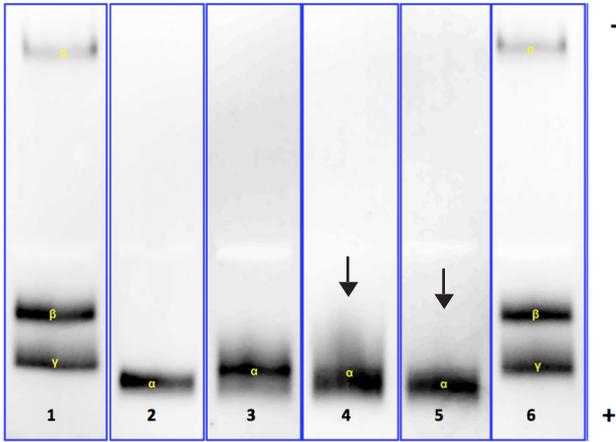


Figure 2. Velum SAR PAGE of positive and negative controls used previously for IEF. Lanes: 1. and 6. MIRCERA (0.03 ng), NESP (0.015 ng), BRP-EPO (0.03 ng); 2. negative control; 3. positive control rhEPO; 4. suspicious sample rhEPO; 5. ultra-low dose sample rhEPO.

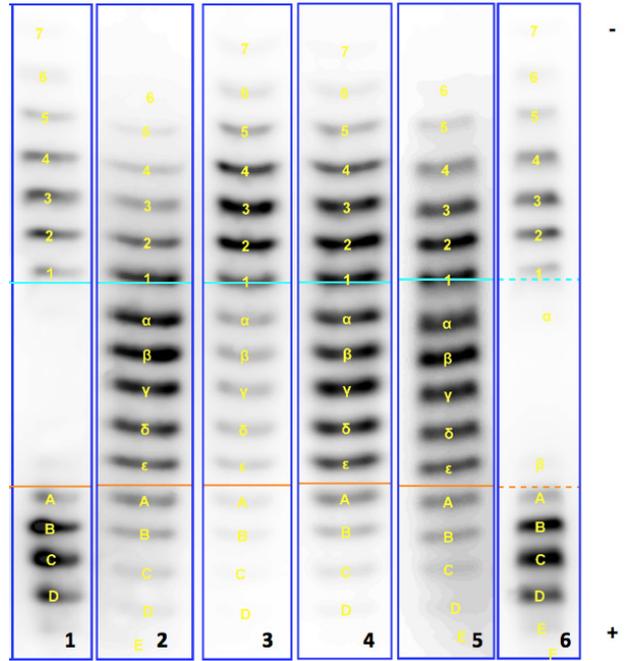


Figure 4. IEF-PAGE of positive and negative controls corresponding to the Velum SAR PAGE analyzed samples from Figure 2, lanes: 1. and 6. BRP-EPO (0.09 ng); NESP (0.08 ng), 2. negative control; 3. positive control rhEPO; 4. suspicious sample rhEPO; 5. ultra-low dose sample rhEPO.

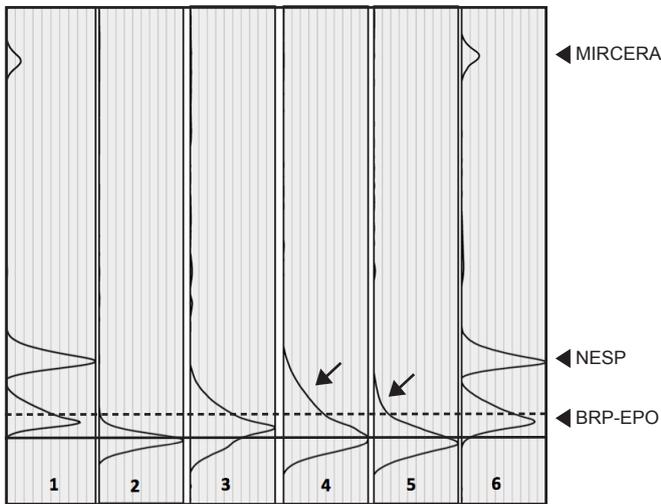


Figure 3. Profile of the lanes from the SAR-PAGE shown in Figure 2, the black dotted line indicates the end of the endogenous EPO band, the black continuous line indicates the peak position of endogenous EPO, faint areas are indicated by arrows.

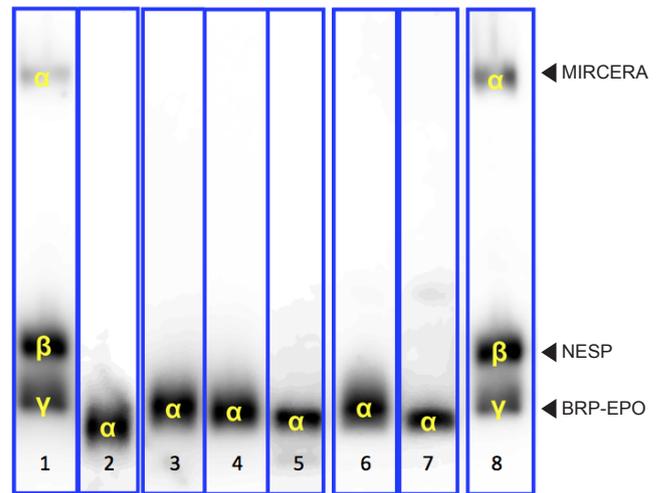


Figure 6. Velum SAR PAGE of urine sample from the micro-dose application study: lane 1 and 8 - MIRCERA (0.03 ng), NESP (0.015 ng), BRP-EPO (0.03 ng); lane 7: negative control; lane 6: positive control rhEPO; lane 5 - 2: volunteer 1 – base, 5, 25 and 75 h after application.

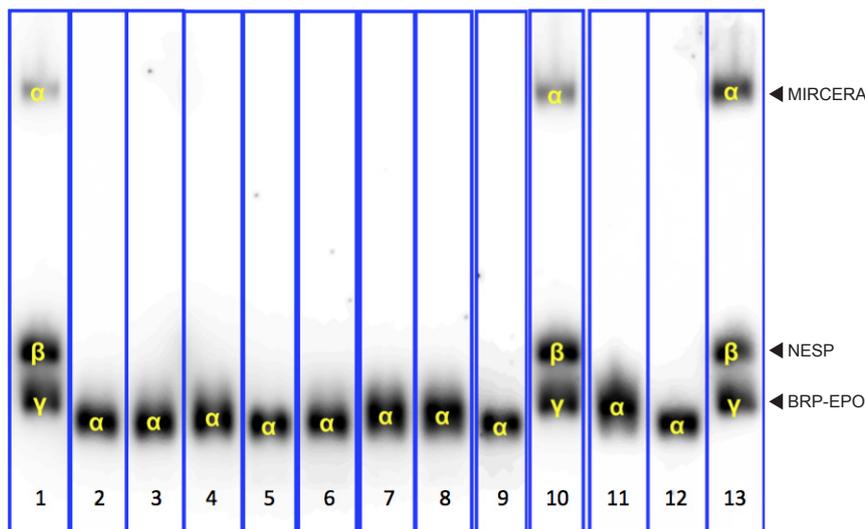


Figure 5. Velum SAR PAGE of sera samples from the micro-dose application study: lane 1, 10 and 13 - MIRCERA (0.03 ng), NESP (0.015 ng), BRP-EPO (0.03 ng); lane 12: negative control; lane 11: positive control rhEPO; lane 9 - 6: volunteer 1 – base, 5, 25 and 75 h after application; lane 5 - 2: volunteer 4 – base, 5, 25 and 75 h after application.

Using Velum SAR PAGE for the initial testing both the suspicious sample (Figure 2: lane 4) and the ultra-low dose sample (Figure 2: lane 5) show faint areas as indicated by arrows (Figure 2) which are absent in the negative control but present in the positive control. Especially the profiles of these samples (Figure 3) allowed the detection of the exogenous proteins. Even if the suspicious sample and the ultra-low dose sample (Figure 3: lanes 4,5) have the same molecular weight maximum as the negative control (black continuous line) the diffuse area of these bands above the corresponding endogenous band (black dotted line) indicate the presence of rEPO. These findings then lead to a re-analysis in order to confirm the results.

Serum and Urine sample: application for doping control

Velum SAR PAGE gels were taken to analyze samples from the micro-dose study. Due to the low used doses (11-16 IU/kg BW) and the low hematological response it was surprising that even the production of endogenous erythropoietin was not suppressed for all volunteers the recombinant erythropoietin could be detected at least 24 hours for all in blood and urine samples (Figure 5: lanes 3,7; Figure 6: lane 3). For urine samples the individual response for each yielded in an detection window up to 72 hours using the IEF method (data not shown) but was prolonged to 85 hours using the SAR-PAGE (Figure 5: lanes 2, 6). The results show that - due to the excretion of the recombinant protein - urine samples are the preferred matrix for recombinant EPO analysis.

However, in our study we observed that for one volunteer (volunteer 1), the detection window in urine was shorter compared to the corresponding blood sample (Figure 5 and Figure 6: volunteer 1). Again, it has to be emphasized that only a single application of 1000 IU rEpo was given to the volunteers.

Conclusions

According to the WADA Technical Document 2014EPO the initial testing procedure for the detection of doping should be done by IEF or SAR-PAGE.

Compared to the IEF method the detection windows were up to 12 hours longer, with a very individual response. Especially the detection of mixed bands consisting of endogenous EPO and rEPO using Velum SAR PAGE shows a much better performance for detecting low dose doping than the IEF method does.

With the adoption of the horizontal SAR-PAGE in combination with the precast film-supported Velum SAR gels the discriminatory capacity of micro-dose application of rEPO was significantly enhanced. The additional benefits of the used method were the easier handling of the Velum gels compared to the NuPage gels and, in combination with the BlotCycler, the automating of the time consuming washing and incubation process.

References

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