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Relative mobility values for the discrimination of endogenous and recombinant EPO on SDS gels

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Abstract

Erythropoietin (EPO) promotes the production of red blood cells, the key factor in the regulation of the oxygen transport, and has been abused by athletes for performance enhancement in endurance sports. Current methods to detect EPO misuse are based on isoelectric focussing (IEF), double blotting, and chemiluminescence detection.

Separation of endogenous and recombinant EPO by SDS-PAGE complements the current method and improves the detection of different recombinant erythropoietins. Two internal standards (Novel Erythropoiesis Stimulating Protein and recombinant rat EPO) are used for calculation of relative mobility values. Epoetin alfa, beta and delta can be discriminated from endogenous EPO and especially the detection of Epoetin delta is improved by the SDS method. A reference group of 53 healthy volunteers and samples originating from a Dynepo™ (epoetin delta) excretion study were analyzed and led to a significant discrimination of endogenous urinary and recombinant EPO. Another currently introduced EPO is the Continuous Erythropoiesis Receptor Activator (CERA) which is pegylated Epoetin beta and first results show a possible detection from urine by SDS as well as IEF.

Introduction

Erythropoietin (EPO) stimulates red blood cell production in the human body. Human EPO is a highly glycosylated protein with 165 amino acids and a molecular weight of approximately 30 kDa with a carbohydrate moiety accounting for ca. 40%[1]. Glycosylation of the protein core of EPO prolongs the hormone's half life in the circulation and is essential for biological activity[2, 3]. The glycosylation pattern strongly depends on the cells used for expression of the

protein. Consequently, endogenous EPO as produced in human kidney cells is differently modified compared to recombinant EPO, which is commonly produced in chinese hamster ovary (CHO), human fibrosarcoma or baby hamster kidney (BHK) cells. The sets of glycosylation enzymes of non-mammalian cells deviate considerably and are not capable of synthesising complex carbohydrate residues[4, 5].

Epoetin alpha, beta, delta and human EPO are composed by identical sets of amino acids and vary only slightly in their glycosylation pattern. In contrast, the primary structure of darbepoetin alpha differs at five positions, and the asparagine residues at positions 30 and 88 are additionally glycosylated, which results in a higher molecular weight but a maintained conformation[6, 7]. Epoetin delta (gene-activated erythropoietin, Dynepo^{TM}) is the first recombinant EPO that is produced in human cell lines and its glycosylation pattern is therefore supposed to be similar to that of natural human EPO, which should avoid immune reactions. The Continuous Erythropoietin Receptor Activator (CERA, Micera^{TM}) is a pegylated form of Epoetin beta and the pegylation is positioned predominanty at the N-terminus or lysine 45 or 52[8]. The pegylation considerably prolongs the half life in the circulation and increases the molecular weight. This may result in lower amount of the recombinant protein excreted into urine in comparison to other recombinant EPOs. Doping with recombinant EPO is currently detected by the method of Lasne et al.[9], which is based on isoelectric focussing (IEF) followed by double blotting[10] and chemiluminescent detection. Due to the differences in the glycoslation pattern, epoetin alpha and beta as well as darbepoetin alpha can be differentiated from endogenous human urinary EPO (huEPO). Complementary approaches use sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for discrimination of these recombinant variants[11-14]. In this work, an SDS-PAGE method is presented that allows the detection of Dynepo^{TM} by determination of relative mobilities as well as the discrimination of Epoetin alfa and beta from the endogenous hormone[15]. Furthermore, preliminary results on the detection of Micera^{TM} by SDS-PAGE as well as the IEF are provided.

Materials and Methods

Chemicals and reagents: The recombinant EPO mix, BRP, was from the European Pharmacopoeia Commission for the Biological Reference Preparation (BRP, Strasbourg, France) and contains epoetin alpha and beta in equimolar amounts. Further recombinant EPOs used were NESP (Aranesp^{TM}) from Amgen (Munich, Germany), epoetin delta (Dynepo^{TM}) from Shire (Cologne, Germany) and CERA (Micera^{TM}) from Roche (Mannheim, Germany).

Rat EPO from insect cells was purchased from Sigma (Deisendorf, Germany). The reference material of endogenous human EPO was from the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK). The protease inhibitor cocktail (Complete) was from Roche (Mannheim, Germany) and Steriflip microfiltration units (0.22 µM) as well as Centricon-plus 20 and Centricon YM 30 ultrafiltration units (both with a molecular weight cut-off of 30.000 Da) and Immobilon-P membranes were provided by Millipore (Billerica, USA). Dithiothreitol (DTT) and buffer ingredients were from Sigma (Deisendorf, Germany) and of analytical and electrophoresis grade, respectively. All buffers and solutions were prepared in MilliQ water (Water Lab System, Millipore). The anti-EPO ELISA kit with wells covered with an anti-EPO IgE/κ antibody (monoclonal, clone 26G9C10[16, 17]) was purchased from Stemcell (Vancouver, Canada). Bis-Tris SDS gels (10%), LDS sample buffer and MOPS running buffer were obtained from Invitrogen (Paisley, UK). Fat free milk was purchased from Regilait (Saint Martin, France), the primary antibody (IgG_{2A}, Clone AE7A5) was from R&D Systems (Minneapolis, USA) and the detection system Super Signal West Femto from Pierce (Rockford, USA). Proteins were detected using an Alpha Imager CCD camera from Biozym (Oldendorf, Germany). Ethanol was bought from VWR International (Darmstadt, Germany).

Concentration of urine samples: Urine samples were concentrated for analysis as described elsewhere[9]. Briefly, 20 mL of urine were filtered using Steriflip microfiltration units after addition of 2 mL of 3.75 M Tris (pH 7.4) and 0.4 mL of Complete. For concentration, centrifugation with Centricon-plus 20 filtration units was followed by further concentration using Centricon YM 30 ultrafiltration units. The resulting retentates of about 50 µL were stored at -20°C until analysis.

Separation and detection of EPO forms: Half of the retentate was incubated in 200 µL of phosphate-buffered saline (PBS, Buffer B of the ELISA-Kit) in the anti-EPO ELISA well plate over night at 4°C. Wells were washed with PBS, and EPO was eluted using 5 µL of lithium dodecyl sulfate (LDS) sample buffer. Proteins were reduced by addition of 2 µL of 1 M DTT to the wells and 0.1 ng of NESP and 0.5 ng of rat EPO were added as internal standards. Samples were heated for three minutes at 95°C and then applied to a 10% SDS gel (125 V, 90 min). After blotting to a PVDF membrane (200 mA, 45 min, blotting buffer: 39 mM glycine, 48 mM Tris, 0.0375% SDS, 20% ethanol) the membrane was blocked in 5% fat free milk in PBS for 1 h at room temperature. The primary antibody was diluted 1:1000 and incubated in 1% milk for one hour followed by washing three times 10 min with PBS and

incubating with the secondary antibody (diluted 1:5000 in PBS). Visualization of EPO was finally accomplished using chemiluminescent detection.

Isoelectric focussing, double blotting and immunostaining for preliminary Micera™ studies on IEF gels was carried out as described by Lasne et al. [9]. The SDS-PAGE analysis of samples containing Micera™ was carried out as for the other EPO variants.

Urine samples and excretion study for Dynepo™: Fifty three urine samples were obtained from healthy volunteers (30 males, 23 females; 20-46 years) that were not applying any medication or suffering from diseases that may influence the EPO production, metabolism or excretion. These urine specimens were analyzed to serve as reference population for the electrophoretic behaviour of huEPO on SDS gels.

A Dynepo™ excretion study was performed in agreement with the ethical commission of the German Sport University Cologne. Urine samples were continuously collected before and six days after subcutaneous injection of 50 U/kg body weight (4000 U) to a healthy male volunteer (56 years, 80 kg). All samples were frozen at –20°C until analysis.

Data analysis: Images of SDS-PAGE gels were integrated and converted into ASCII-data by vendor-provided software (Alpha Ease FC 3.0, Biozym, Oldendorf, Germany).

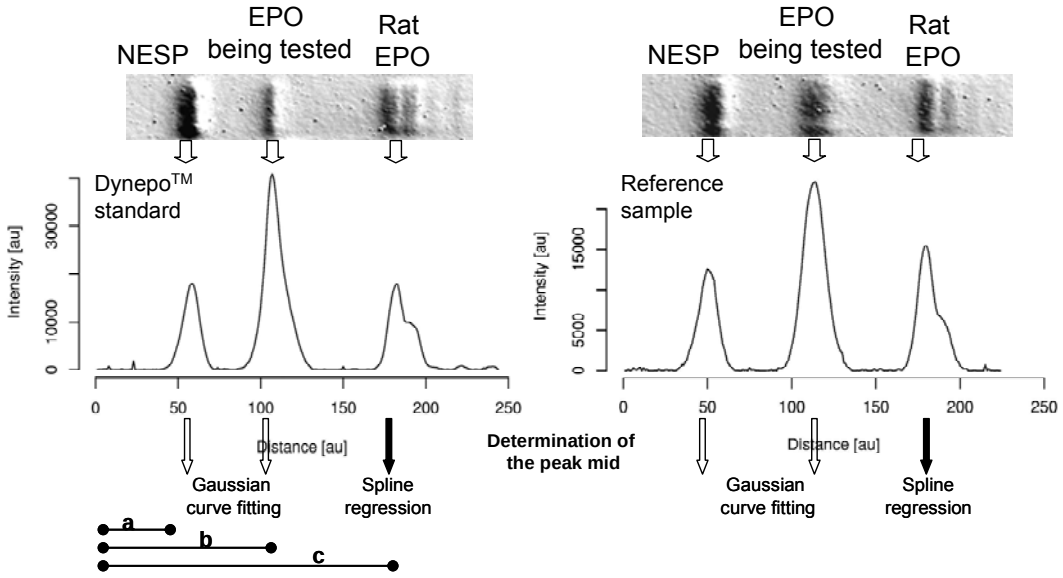


Figure 1 Illustration of the transformation of in-gel protein bands to distances. Distance values a-c were used for mobility calculations of the analytes. Left: Dynepo™ standard, right: sample from reference group.

Analyte mobility = 1- (b-a)/(c-a)

Peak mids for the respective compounds were calculated by curve fitting in order to obtain objective measures for these parameters. Gauss curves were fitted to the NESP signal and to the signals of human EPO, epoetin alpha, beta, and delta. Non-linear least squares methods

were applied for this purpose [18, 19]. Assumption of Gaussianity was inappropriate with respect to the rat EPO signal, which exhibited two unresolved bands. Cubic spline regression was performed instead [20], and the resulting maximum of the first band was used for further calculations.

Values for the relative mobility of the analytes in-gel were calculated as the ratio of the distances between NESP and the analyte and NESP and rat EPO as demonstrated in Figure 1:

Parametric reference limits at the 99.9% confidence level were calculated for the relative mobilities of huEpo in the reference population[21]. The parametric approach was considered justified, as no significant deviation from Gaussianity was found in the corresponding distribution (Shapiro-Wilks test, $p \sim 0.2$). All calculations were performed using R-2.5.1 software[22].

Urine samples and excretion study for Micera™: For the Micera™ excretion study, 50 µg of Micera™ were subcoutaneously injected by one male volunteer (56 years, 80 kg). Urine samples were collected especially immediately prior to and after an anaerobic cycling exercise[23] and frozen until analysis.

Results and Discussion

SDS-PAGE for separation of EPO forms:

Figure 2 shows a typical SDS gel with samples containing recombinant or endogenous urinary EPO. The highest band in each sample represents NESP (0.1 ng per sample), the lowest band recombinant rat EPO (0.5 ng per sample). The multiple bands for rat EPO may result from differently low-level glycosylated forms. Bands located in the middle of the two internal standards represent huEPO or a recombinant epoetin. Epoetin alpha/beta as well as epoetin delta possess higher molecular weights than endogenous urinary EPO and, thus, a different electrophoretic mobility. The higher molecular weight of recombinant EPO from CHO cells (epoetin alpha and beta) is due to a higher amount of oligosaccharides with N-acetyllactosamine repeating units[24]. For epoetin delta, a lower heterogeneity was detected with mainly tetraantennary structures without N-acetyllactosamine repeats and differences in the O-glycosylation in comparison to CHO derived EPO were found[25]. In Figure 2, the different mobilities are illustrated with different reference population samples with huEPO (lanes 2, 4 and 8), a BRP standard (lane 1), and a urine sample spiked with 30 mU of BRP prior to concentration (lane 3). Lane 7 shows a specimen collected 12 h post administration of Dynepo™. Lane 5 represents a Dynepo™ reference standard (15 mU), and lane 6 a negative standard consisting of 5 mU of endogenous EPO (NIBSC).

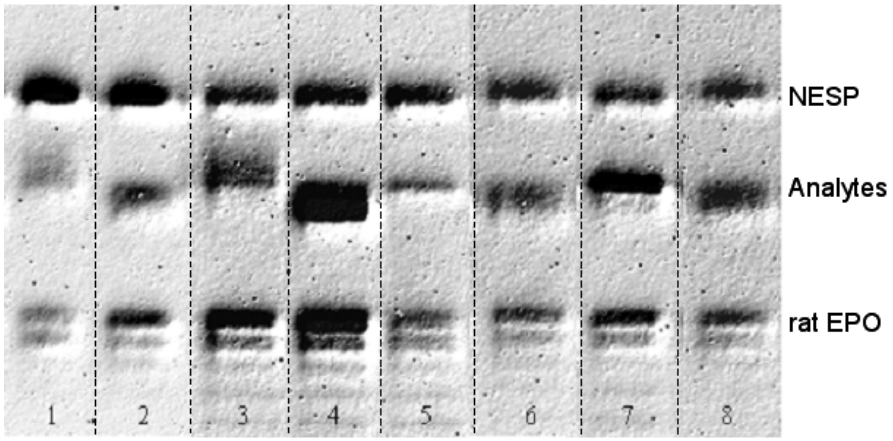


Figure 2 SDS gel of different standards and urine specimens. ***1****: BRP standard (15 mU),* ***2****,****4****,****8****: reference population samples,* ***3****: BRP spiked urine sample,* ***5****: Dynepo™ standard (20 mU),* ***6****: NIBSC standard,* ***7****: Dynepo™ excretion study sample (12 h post administration).*

In contrast to epoetin alpha and beta as well as huEPO, Dynepo™ appears as a much sharper band. This supports the findings that Dynepo™ has a more homogeneous structure[25] and allows for a visual distinction between BRP and Dynepo™.

Calculation of the different migration behaviours of endogenous urinary and recombinant EPO:

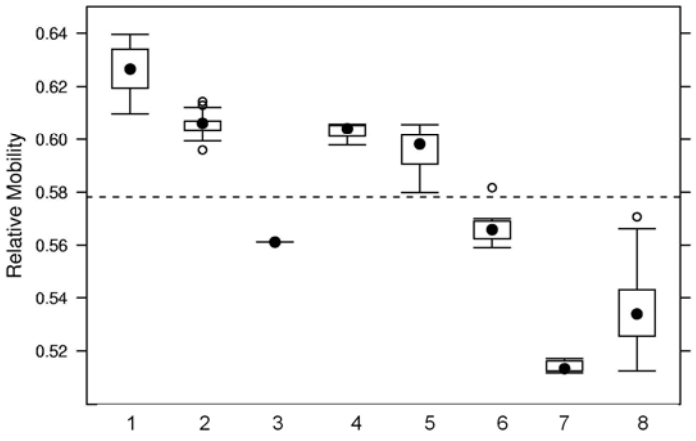


*Figure 3 Comparison of mobility values of positive and negative samples.****1****: BRP standards,* ***2****: Dynepo standards* ***3:*** *blank sample prior to Dynepo™ excretion study,* ***4:*** *samples from Dynepo™ excretion study days 1-2,* ***5:*** *samples from Dynepo™ excretion study days 3-4,* ***6****: samples from Dynepo™ excretion study days 5-6,* ***7****: NIBSC standards,* ***8****: reference samples. The horizontal line indicates the 99.9% confidence level of the reference samples.*

Calculation of the analyte mobility of huEPO and the three epoetins in the gel enabled a clear differentiation of recombinant and endogenous urinary EPO. Calculated values in relation to NESP and rat EPO are depicted in Figure 3. The horizontal dashed line in Figure 3 illustrates the value for the 99.9% confidence level. Neither the BRP standard (Epoetin alpha/beta) nor the epoetin delta standard or the epoetin delta values from the first four days of the excretion study fall below this threshold, which demonstrates that positive urine samples can significantly be distinguished from the reference group. The negative standard (NIBSC, Figure 3, box 7) was reproducible with a relative standard deviation of only 0.4% (n=10) and showed even lower values than the reference group (box 8). Samples originating from the Dynepo^{TM} excretion study are summarized in 48h periods. Box 4 comprises the samples collected on days one and two, box 5 those from days three and four and box 6 summarizes the last two sampling days. Box 2 represents Dynepo^{TM} reference standards only. As seen by the reference confidence level, Dynepo^{TM} samples of the first four days are clearly separated from the reference population while the samples from days five and six are in the range of the reference group and comparable to the blank sample (box 3), which was taken prior to the application of Dynepo^{TM}.

Dynepo excretion study:

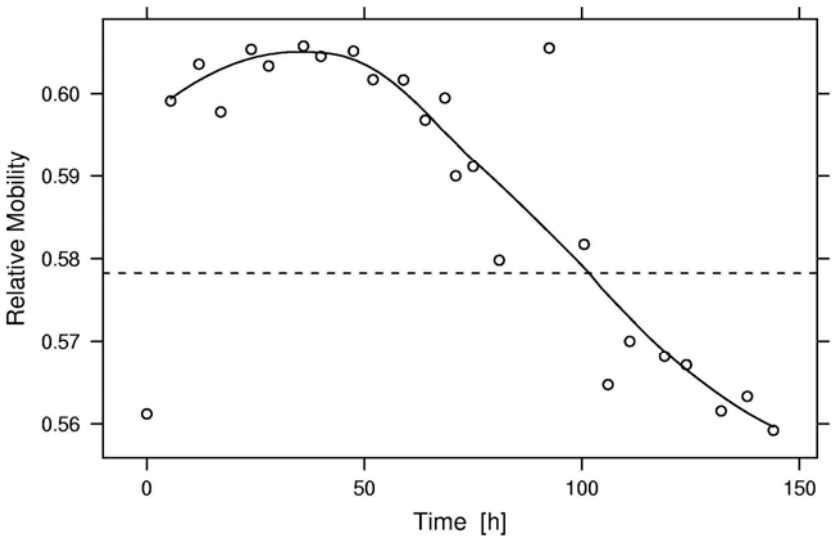


Figure 4 Mobility values for EPO in urine samples measured after subcoutaneous injection of Dynepo^{TM}. The horizontal line indicates the 99.9% confidence level.

In Figure 4, the qualitative evaluation of different forms of EPO in the urine samples over the period of the Dynepo^{TM} excretion study is presented. The first sample at t=0 h is a blank specimen taken before *s.c* injection of 4000 U of Dynepo^{TM}.

With the first sample collected after administration (5.5 hours), an abundant band representing Dynepo™ was detected, which demonstrated a rapid admission into the circulation and subsequent urinary elimination after subcutaneous injection. Recombinant EPO was detected and differentiated from huEPO for 4 days before the mobility values of the analytes returned to the reference range (see also Figure 3).

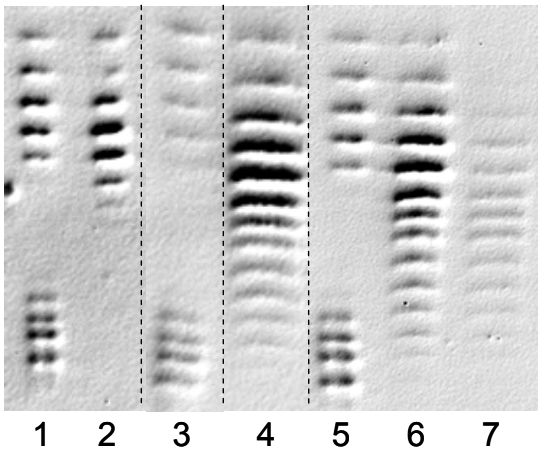


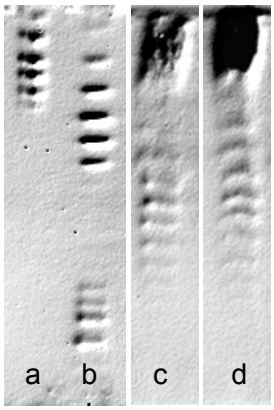
Figure 5 Dynepo™ on an IEF gel. While a Dynepo™ standard clearly fulfils WADA criteria, excretion study samples do not. 1,3,5: BRP standard, 2: Dynepo™ standard, 4: 12 h p.a. of Dynepo™, 6: 40 h p.a., 7: blank, prior to administration

Interestingly, the study of Smith et al.[26] showed that the amount of EPO in serum reaches normal physiological levels after 48 hours after single subcutaneous injection of 40 as well as 100 U/kg body weight. This period is much shorter than the time-frame the recombinant EPO dominated the endogenously produced one in urine samples of our study (Figure 4). Conventional methods based on IEF[9] led to suspicious but ambiguous results for the excretion study samples of this volunteer within the first four days after application and did not fulfil the criteria of the World Anti-Doping Agency for adverse analytical findings of recombinant EPO as demonstrated in Figure 5.

Preliminary results on the detection of Micera™ excretion study samples:

Figures 6 and 7 show first results of CERA detection on IEF and SDS gels. After IEF the CERA standard is clearly visible and shows several bands as it is expected due to the similarity to Epoetin beta (Figure 5 a). In comparison to the BRP standard (Figure 5 b) the bands are shifted to higher pH values. After application of 50 µg of Micera™, it is visible in the basic area but without separated bands. This problem may be solved by the use of a bigger

gel. The bigger gel may also control the problem of a possible overlay of Micera™ and the cross reacting zinc-alfa-2-glycoprotein[12].



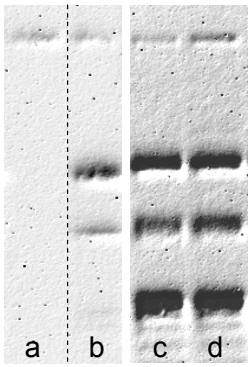


Figure 6 (left) and 7 (right) Samples after Micera™ application analyzed by IEF (Figure 6) and SDS-PAGE (Figure 7) 6a: Micera™ standard 0.08 ng, 7a: Micera standard 0.7 ng, 6b: BRP/NESP standard 7b: Micera™/NESP/Dynepo™/rat EPO standard, 6/7c: 79 h p.a., 6/7d: 80 h p.a. after exercise

The SDS-PAGE can also be used for detection of CERA although the sensitivity is much lower. CERA is visible on SDS gels at much higher molecular weight. Analysis with both gels indicated an increased excretion of CERA after anaerobic exercise.

In conclusion, epoetins alpha, beta, and delta were distinguished from natural EPO in urine employing SDS-PAGE and the calculation of relative mobility values. SDS-PAGE is therefore useful as a screening method in doping control or as complementary confirmation method to the established IEF based assay. A novel EPO variant, Micera™, can be detected by SDS or IEF.

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