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Detection of EPO-Fc fusion protein in human blood: Screening and confirmation protocols for sports drug testing

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The neonatal Fc receptor (FcRn) has been under investigation for several years as a pharmaceutical drug target. Clinical studies have shown that fusion proteins consisting of human recombinant erythropoietin (rhEPO) and the Fc-part of IgG can be transported after pulmonary administration via FcRn across the airway epithelium to the blood stream. So far, no clinically approved pharmaceutical formulation of EPO-Fc is available. Since various forms of recombinant erythropoietins have been frequently misused by athletes as performance-enhancing agents, EPO-Fc might play a similar role in sports in the future. In order to investigate the detectability of EPO-Fc in human blood, different strategies were tested and developed. Only two of them fulfilled the necessary requirements regarding sensitivity and specificity. A *rapid protocol useful for screening purposes* first enriches EPO-Fc from human serum via high capacity protein A beads and subsequently detects EPO-Fc in the eluate with a commercial EPO ELISA kit. The limit of detection (LOD) of the method is about *5 pg (45 amol)* EPO-Fc and is independent of the serum volume used. For *screening and/or confirmation purposes* a second protocol was evaluated, which consists of a fast EPO immunopurification step followed by sodium dodecyl sulfate or sarcosyl polyacrylamide gel electrophoresis (SDS-PAGE, SAR-PAGE) and Western double-blotting with chemiluminescence detection – a method already established in routine EPO anti-doping control. The latter strategy allows the detection of EPO-Fc in serum together with all other recombinant erythropoietins and with an identical LOD (5 pg/45 amol) as for the rapid screening protocol. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: EPO-Fc; fusion protein; recombinant erythropoietin Fc chimera; erythropoietin; SDS-PAGE; SARCOSYL-PAGE; SAR-PAGE; isoelectric focusing; IEF-PAGE; doping control

Introduction

In addition to its transport function for maternal immunoglobulin G (IgG) molecules to the fetus via interaction with the Fc (fragment crystallisable) region of IgG and transcytosis, the neonatal Fc receptor (FcRn) plays an important role in protecting serum albumin and IgG from degradation in adults. Throughout its lifetime, the receptor is expressed by various cells including alveolar macrophages and the airway epithelium.[1–3] The receptor specifically binds to the CH2-CH3 hinge region of IgG molecules and in a pH-dependant manner. Binding occurs only under acidic pH-conditions as encountered within endosomes (pH 6–6.4), but not under physiological pH of 7.4.[1] Hence, FcRn is ideally suited for mediating vesicular trafficking of its interaction partners. Erythropoietin (EPO), the protein hormone responsible for differentiation of bone marrow stem cells into erythrocytes, has been recombinantly produced since 1985 and was first approved for clinical application in anaemia treatment in 1989.[4,5] In order to prolong the serum half-life, EPO was structurally modified in the following years. Both the expression of additional N-glycans (Darbepoetin alfa (NESP)) and the chemical modification with polyethylene glycol (PEGylation; methoxy polyethylene glycol-epoetin beta (MIRCERA)) led to pharmaceuticals fulfilling this demand.[6,7] A prolongation of serum half-life can be also achieved by EPO-dimerization or by fusion of the EPO molecule with other proteins (e.g. albumin, IgG-Fc, granulocyte/monocyte colony stimulating factor (GM-CSF), hCG).[8–15] All of these modifications decrease the affinity of EPO to its receptor (EPO-R) and delay clearance of EPO from the blood stream due to less efficient glomerular filtration. Fusion proteins consisting of EPO and the Fc-part of human IgG have the additional advantage of opening the route of pulmonary administration via the neonatal Fc receptor.[9,10,16] Two prototypes of EPO-Fc molecules have been under clinical investigation so far. *EPO-Fc dimer* consists of two EPO molecules linked to the Fc-dimer (CH2-CH3 hinge region); the *EPO-Fc monomer* on the other hand contains only one EPO molecule attached to the dimeric Fc-component.[10,17,18] Administration studies with monkeys and inhaled EPO-Fc revealed higher bioavailability for the EPO-Fc monomer.[10,19] A study with humans, who inhaled different doses of EPO-Fc dimer (3, 10, 30 μg/kg), showed highest reticulocyte increase for the 30 μg/kg group. Maximum EPO-Fc serum concentrations for the three groups were 0.2, 1.2, and 7.1 ng/ml, terminal half-life time of the latter two groups was 14.4 and 15.7 h, respectively.[20] However, EPO-Fc is not restricted to pulmonary application and can also be used via the traditional subcutaneous or intravenous route.[10] So far, EPO-Fc has not been approved for clinical application, but is currently available for research purposes from

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several non-pharmaceutical companies (e.g. ProSpec, Cell Sciences, Neuromics, GenWay, Chimerigen). Doping with EPO-Fc is prohibited according to article S2 of the 2012 Prohibited List of the World Anti-Doping Agency (WADA).[21] Due to its increased molecular mass and serum half-life, blood (serum/plasma) will be the preferred matrix for EPO-Fc anti-doping testing.

Experimental

Materials

Reference standards for human urinary (uhEPO; second international reference preparation) and human recombinant erythropoietin (BRP-EPO batch 3) were obtained 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 human recombinant erythropoietins were from Janssen-Cilag (*Erypo (epoetin alpha)*; Vienna, Austria), Roche (*NeoRecormon (epoetin beta)*, MIRCERA; Mannheim, Germany), Amgen (*Aranesp (NESP)*; Thousand Oaks, CA, USA), Shire (*Dynepo (epoetin delta)*; Hampshire, UK), Hospira [distributor]/Stada Arzneimittel [manufacturer] (*Retacrit (epoetin zeta)*; Hoofddorp, Netherlands/Bad Vilbel, Germany), and CT Arzneimittel GmbH/Merckle Biotec GmbH (*Biopoin (epoetin theta)*; Berlin, Germany/Ulm, Germany). The standard for human *EPO-Fc* (recombinant human EPO-alpha/Fc chimera) was received from ProSpec (Ness-Ziona, Israel). Human serum (off the clot type) was bought from PAA Laboratories GmbH (Pasching, Austria).

Carrier ampholytes for isoelectric focusing (IEF)-polyacrylamide gel electrophoresis (PAGE) (Servalytes 2-4, 4-6, 6-8, 3-10 Iso-Dalt) were from Serva (Heidelberg, Germany). Urea (PlusOne), acrylamide/bisacrylamide solution (40% T, 3% C; PlusOne ReadySol IEF), N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium peroxidisulfate (APS), gel support film (GelBond PAGfilm, 124 × 258 mm), casting moulds (rubber U-framed, 125 × 260 × 1.0 mm), clamps (FlexiClamps), the flat-bed electrophoresis system (Multiphor II) and power supply (EPS 3500 XL) as well as sodium dodecyl sulfate (SDS; PlusOne), tris(hydroxymethyl)aminomethane (Tris; PlusOne), glycine (PlusOne), electrode strips and two types of blotting papers (NovaBlot, and Blotting Paper 21 × 26 cm) were from GE Healthcare (Uppsala, Sweden). The electric isolation tape (polyvinylchloride (PVC), *ca*. 15 mm width) was from Schuller Eh'klar GmbH (St Florian, Austria). Tween-20 and Tween-80 solutions (Surfact-Amps; 10%) were obtained from Thermo/Pierce (Rockford, IL, USA).

Precast gels performing SDS- and Sarcosyl (SAR)-PAGE (NuPAGE BisTris; 10% T/1.5 mm/10 wells and 4–12% ZOOM/1.0 mm/IPG well), 4-morpholinepropanesulfonic acid (MOPS) running buffer, lithium dodecyl sulfate (LDS) sample buffer, the Xcell SureLock Mini-Cell and PowerEase 500 power supply were bought from Invitrogen (Carlsbad, CA, USA). Extra thick blotting paper (BioRad, Hercules, CA, USA) was used for blotting both the SDS- and SAR-PAGE gels. Molecular weight marker proteins (SeeBlue Plus2, Mark12) were also from Invitrogen. Immobilized pH-gradient gel (IPG) strips (Immobiline DryStrip pH 3-10 linear, 7 cm), DeStreak rehydration solution, IPG buffer 3–10 linear, and the Ettan IPGphor II instrument were bought from GE Healthcare. Coomassie Brilliant Blue R-250 was obtained from Serva. Low melting point agarose was from Invitrogen.

A semi-dry blotter was employed for all Western blots (Trans-Blot SD; BioRad). Non-fat dry milk for preparing membrane blocking and washing solutions was from BioRad (Hercules, CA, USA) too. Antibodies were from R&D Systems (Minneapolis, MN, USA; cone AE7A5 anti-EPO antibody) and Thermo/Pierce (ImmunoPure biotinylated goat anti-mouse IgG (H + L)), respectively. The streptavidin horseradish peroxidase complex was bought from Biospa (Milano, Italy), the substrate for enhanced chemiluminescence detection (West Femto) was also from Thermo/Pierce. A Fujifilm LAS-4000 CCD-camera (Tokyo, Japan) was used for image acquisition and GASepo software (version 1.3b2; ARC; Seibersdorf, Austria) for analysing the membrane images.

Protein A and G beads (POROS A, 20 μm; POROS G, 20 μm; POROS MabCapture A, 45 μm particle size) were purchased from Applied Biosystems (Foster City, CA, USA)/ Invitrogen. The rotator was from Stuart (Model SB3) and the high capacity immunoaffinity depletion column (Human 14 Multiple Affinity Removal System (MARS), 10 × 100 mm) from Agilent Technologies (Santa Clara, CA, USA). EPO-specific enrichment was achieved with an anti-EPO antibody-based purification kit from MAIIA Diagnostics (Uppsala, Sweden) or an EPO ELISA kit from StemCell Technologies (Vancouver, Canada). A QIAvac 24 Plus system (QIAGEN; Hilden, Germany) was used in combination with the MAIIA purification kit. Quantifications of human serum EPO (shEPO) and EPO-Fc were done with an EPO ELISA kit from R&D Systems and a microplate reader from Perkin Elmer (1420 Multilabel Counter Victor^{3}V).

All micro- (Steriflip, Ultrafree-MC centrifugal filters; 0.2 μm) and ultrafiltration devices (Amicon Ultra-0.5, Ultra-4, Ultra-15; nominal molecular weight limit (NMWL) 30 kDa) as well as the membranes for blotting (Durapore, Immobilon-P) were purchased from Millipore (Billerica, MA). All buffers and solutions with exception of eluents for liquid chromatography were prepared with MilliQ (MQ) water (Millipore). GELoader tips, protein low-bind sample tubes (0.5 and 1.5 ml), Thermomixer, and microcentrifuge were from Eppendorf (Hamburg, Germany).

Sequencing grade modified trypsin was received from Promega (Madison, WI). The incubator for enzymatic digests and the Megafuge 1.0 centrifuge were from Heraeus (Hanau, Germany). All mass spectrometric analyses were performed on an LTQ-Orbitrap mass spectrometer (MS) (Thermo Electron; Bremen, Germany) which was coupled via a nano-ESI source (Proxeon; Odense, Denmark) and MicroCross (Upchurch Scientific; Oak Harbor, WA, USA) to an Ultimate 3000 nano-liquid chromatograpghy (nano-LC) system from Dionex (Sunnyvale, CA, USA). NanoViper trap (75 μm i.d. × 2 cm; Acclaim PepMap 100 C18, 3 μm particle size, 100 Å pore size) and analytical columns (75 μm i.d. × 15 cm; Acclaim PepMap 100 C18, 3 μm particle size, 100 Å pore size) were also from Dionex. Uncoated glass emitters (360 μm OD, 75 μm i.d., 15 μm tip i.d.) were purchased from New Objective (Woburn, MA, USA). The mass spectrometric calibration solution containing caffeine, MRFA tetrapeptide, and Ultramark 1621 was from Thermo/Pierce. BioWorks 3.3 (Thermo Electron; San José, CA, USA) and the UniProt data base (updated September 2011)[22] were used for identification and evaluation of the experimentally obtained MS/MS peptide spectra.

DL-dithiothreitol (DTT), sucrose (electrophoresis grade), L-aspartic acid, L-glutamic acid, citric acid, methyl red, decane, sodium metabisulfite ($Na_2S_2O_5$), sodium chloride (NaCl), bovine serum albumin (BSA; ELISA grade), ammonium bicarbonate (NH_4HCO_3), trifluoroethanol (TFE), iodoacetamide, Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), sodium N-lauroylsarcosinate (sarcosyl), ethylenediaminetetraacetic acid (EDTA, free acid), glycerol, phenol red, sodium metabisulfite, phosphate buffered saline (PBS) tablets, and Tris buffered saline (TBS) were bought from Sigma-Aldrich (St Louis, MO, USA). Sodium hydroxide (NaOH; 1 N, Titrisol), glacial acetic acid (p.a.), phosphoric acid (p.a.), and methanol (LiChrosolv,

gradient grade) were obtained from Merck (Darmstadt, Germany). Acetonitrile (ACN), water, formic acid (FA), and trifluoroacetic acid (TFA) (all ultra liquid chromatography/mass spectrometry (ULC/MS) grade) were from Biosolve (Valkenswaard, the Netherlands).

Methods

In-solution tryptic digestion of EPO-Fc

For protein denaturation, the content of one vial EPO-Fc (10 μg) was dissolved in 25 μl ammonium bicarbonate buffer (100 mM) and 25 μl TFE. Subsequently, disulfide bridges were cleaved by supplementing the mixture with 1 μl of a 200 mM DTT solution (water) and incubation for 60 min at 60°C (400 rpm; Thermomixer). Then 4 μl of a 200 mM iodoacetamide solution (water) were added (25 °C, 60 min, 400 rpm; light-protected) and then again 1 μl of the 200 mM DTT solution (25 °C, 60 min, 400 rpm; light-protected). For enzymatic digestion the reduced and alkylated protein solution was diluted with 300 μl ULC/MS grade water and 100 μl 100 mM ammonium bicarbonate buffer and then 2 μl of a trypsin stock solution (0.1 μg/μl; in 100 mM NH_4HCO_3 buffer) was added. The reaction mixture was incubated overnight at 37°C (Heraeus incubator) and either immediately analysed by mass spectrometry or stored in aliquots at -80 °C (protein low-bind sample tubes).

Mass spectrometric characterization of EPO-Fc

For mass spectrometric characterization of EPO-Fc 2.5 μl of the tryptic digest were diluted with 22.5 μl of 0.05% TFE and then 19 μl of this solution were preconcentrated on the C18 trap column of the Ultimate 3000 system (12 min, 2 μl/min; loading solvent: 10% methanol/0.05% TFA). Peptides were then separated on the C18 analytical column using a linear gradient from 2% to 55% B (90 min, 300 nl/min; solvent A: 0.1% FA in water, solvent B: 0.1% FA in ACN). Column oven temperature was 40 °C and the temperature of the heated capillary of the mass spectrometer was 160 °C. Positive ion mode and a spray voltage of 2 kV were used; the spray voltage was applied via liquid junction and MicroCross connector. The LTQ-Orbitrap mass spectrometer was operated in data-dependent acquisition (DDA) mode using a survey scan in the FT-analyzer at resolution R = 60 000 followed by up to three MS/MS (CID) scans of the three most abundant ions in the ion-trap (dynamic exclusion: 30 s). Mass range was from *m/z* 300–2000. For CID fragmentation following settings were used: default charge state 2, normalized collision energy 35, activation Q 0.25, and activation time 35 ms. The mass spectrometer was externally calibrated using the caffeine/MRFA tetrapeptide/Ultramark 1621 calibration solution and then online recalibrated with the polysiloxane *m/z* 445.120025 ion as lock mass.[23] Peptide spectra were searched against UniProt database with BioWorks 3.3.1 search engine using following settings: trypsin (fully enzymatic, cleaves at both ends), no missed cleavage sites, peptide mass tolerance 10 ppm, monoisotopic precursor mass, 0.5 amu fragment ion tolerance, oxidized methionine (variable modification), carbamidomethylated cysteine (fixed modification).

Selective enrichment of EPO-Fc using Protein A and G beads

Four different types of experiments were performed in order to evaluate the performance characteristics of POROS Protein A and G media for enrichment of EPO-Fc from human serum.

Selection of the most suitable type of beads. POROS beads (POROS A, POROS G, POROS MabCapture A) were washed with PBS for removing the storage solvent (20% ethanol). One part bead slurry was mixed with four parts PBS, centrifuged at 2300 rpm for 2 min (Megafuge 1.0), and then the supernatant was removed. The procedure was repeated three more times and the final bead volume was adjusted with PBS to the starting volume of the slurry. Human serum was microfiltered (Steriflip) and then used for preparing a dilution series containing 0.0/0.1/0.25/0.5/1.0 ng/ml EPO-Fc. Fifty μl of each sample (resembling absolute EPO-Fc amounts of 0.0/5.0/12.5/25/50 pg) were mixed with 450 μl PBS/0.1% Tween-20 and 100 μl of washed POROS beads (1.5 ml Eppendorf tubes). The tubes were rotated for 60 min at room temperature (Stuart rotator; 40 rpm) and then each sample was transferred to an Ultrafree-MC centrifugal filter for separating the beads from the liquid (2000 *rcf*/2 min). The filtrate was discarded and the beads were washed two times with 500 μl PBS/Tween-20. For eluting the bound EPO-Fc together with the also captured serum IgG antibodies, beads were treated two times with 100 μl Citrate buffer (100 mM, pH 2.5, 0.1% Tween-20) under rotation (40 rpm, 5 min, room temperature). After each elution step the filtrate was collected (2000 *rcf*/2 min) in a protein low-bind tube, neutralized with 20 μl 3.75 M Tris-HCl buffer (pH 7.8) and combined. Filtrates were concentrated using Amicon Ultra-0.5 filters (14000 *rcf*/15 min) and washed with 500 μl PBS/Tween-20 (14000 *rcf*/15 min). Retentates were recovered by centrifugation of the inverted filters (2000 *rcf*/2 min) and were then adjusted to 100 μl with PBS/Tween-20. Subsequently, the EPO concentration of retentates was measured with an EPO ELISA kit from R&D Systems (*vide infra*).

Determination of the required bead and serum amounts and the detection limit. POROS MabCapture A beads (100 μl) were washed as described before and then incubated with either 50 μl or 200 μl of human serum containing absolute EPO-Fc amounts of 0.0/5.0/12.5/25/50 pg. All other steps remained unchanged.

Dependence of the absolute detection limit on the serum EPO-Fc concentration. POROS MabCapture A beads were prepared as described before. Absolute amounts of 5 pg EPO-Fc were added to 50, 100, and 250 μl of serum corresponding to 0.1, 0.05, and 0.02 ng/ml EPO-Fc. Bead amounts were increased accordingly, i.e. 100, 200, and 500 μl for 50, 100, and 250 μl of serum. Also citrate buffer elution volumes were adapted. The remaining steps were unaltered.

Determination of the EPO-Fc concentration in bead eluates by ELISA. In order to determine whether EPO-Fc was indeed captured by POROS A and G beads an EPO ELISA kit from R&D Systems was used. The instructions of the kit were followed closely. In brief, 100 μl assay diluent were added to the wells and mixed with 100 μl of volume-adjusted eluate (600 rpm, 60 min). Wells were wiped dry and incubated with 200 μl of conjugate (600 rpm, 60 min). Subsequently, wells were rinsed four times with 400 μl washing buffer, wiped dry again, and incubated with 200 μl of freshly prepared substrate solution. After 25 min 100 μl of stop solution were added and the absorption measured with an ELISA reader (450 nm, 600 nm reference wavelength). The EPO ELISA kit was also used for clarifying whether shEPO is non-specifically bound to the POROS beads or not.

Selective enrichment of EPO-Fc using ACN precipitation

For evaluating whether EPO-Fc can be selectively enriched by acetonitrile precipitation of high abundant serum proteins as shown recently for MIRCERA,[24] a serial dilution of EPO-Fc in serum was prepared (0.0/0.25/0.5/1.0/1.5/2.0/2.5/3.0 ng/ml). Two hundred μl of each sample were mixed with 200 μl MQ water

and 400 µl ACN, centrifuged at 13000 *rcf* (10 min, 20 °C), and then the supernatant was transferred to an Amicon Ultra-4 filter containing 1 ml of water (4000 *rcf*, 20 °C, 25 min). The retentate was washed with 1 ml of 50 mM Tris-HCl buffer (pH 7.4; 4000 *rcf*, 20 °C, 25 min). Then the EPO content was determined by ELISA (R&D Systems; *vide supra*) and compared with the corresponding non-precipitated samples.

Immunoaffinity purification of EPO-Fc

Two immunoaffinity purification strategies were evaluated regarding their recoveries for EPO-Fc (EPO purification kit by MAIIA Diagnostics, EPO ELISA kit by StemCell Technologies). A dilution series of EPO-Fc in serum was prepared containing 0.0/20/50/100/200 pg of EPO-Fc in 200 µl serum. Samples were purified with the MAIIA Diagnostics kit as described earlier.[25,26] Briefly, serum samples were diluted with TBS/0.1% Tween-20 (1:10) and then passed through anti-EPO columns (pre-equilibrated in washing buffer) at a flow rate of *ca* 1 ml/min (QIAvac system). After another washing step the columns were centrifuged (1000 *rcf*/1 min) and bound EPO-Fc and shEPO were spin-eluted with 100 µl of desorption buffer containing 1% Tween-20 (1000 *rcf*/1 min). After neutralization with 10 µl adjustment buffer component A (supplied with the kit) eluates were concentrated by ultrafiltration (Amicon Ultra-0.5; 14000 *rcf*/15 min) and washed with 500 µl PBS containing 0.1% Tween-20 (14000 *rcf*/15 min). Retentates (2000 *rcf*/2 min) were subsequently analysed by SDS-PAGE and Western double-blotting (*vide infra*). For evaluating the influence of the applied serum volume on the detection limit the experiment was repeated with 50, 100, and 250 µl serum containing an absolute amount of 5 pg EPO-Fc.

As an alternative strategy immunoaffinity extraction by ELISA was tested. The method was already described in detail elsewhere.[27] In brief, serum samples spiked with EPO-Fc (0.0/25/50/100/200/400/800 pg in 200 µl) were depleted from high abundant proteins using a high-capacity multi-immunoaffinity column (MARS system). The flow-through fractions were collected, concentrated and washed with 50 mM Tris-HCl buffer (pH 7.4) by ultrafiltration (Amicon Ultra-15 and Ultra-0.5). Retentates (*ca* 30 µl) were mixed with 30 µl of buffer A of the ELISA kit and transferred to the ELISA wells. After overnight incubation in the cold-room bound EPO-Fc and shEPO were eluted with 40 µl LDS sample buffer (95°C, 5 min, 800 rpm; Thermomixer) after washing the wells with PBS. Eluates were analyzed by SDS-PAGE and Western double-blotting with chemiluminescence detection.

SDS- and Sarcosyl-PAGE of EPO-Fc

Three different types of experiments were conducted with both methods, namely (1) comparison of the electrophoretic separation behaviour of EPO-Fc with pharmaceutical formulations of recombinant epoetins, determination of the detection limit (LOD) of the EPO-Fc standard in (2) buffer and (3) human serum after immunoaffinity purification followed by Western double-blotting and chemiluminescence detection. Both, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and sarcosyl polyacrylamide gel electrophoresis (SAR-PAGE) were performed as described earlier.[27,28] For the *determination of the molecular mass-based electrophoretic behaviour* about 1 µg of EPO-Fc and EPO-pharmaceuticals (Erypo, NeoRecormon, NESP, Dynepo, MIRCERA, Retacrit, Biopoin) were heated under reducing conditions (100 mM DTT) in 1.5 ml Eppendorf tubes either in LDS or sarcosyl sample buffer (95°C, 5 min, 800 rpm; Thermomixer). SAR sample buffer was prepared as described earlier.[27] The composition of the four-fold (4x) concentrated buffer was 424 mM Tris hydrochloride, 564 mM Tris, 8% sarcosyl, 40% glycerol, and 2.04 mM EDTA. Phenol red was used as front marker. Running buffers were MOPS-based and were either bought as ready-made stock solution (SDS-PAGE; 20x) or prepared immediately before usage (SAR-PAGE; 50 mM MOPS, 50 mM Tris, 0.1% sarcosyl, 1 mm EDTA). Precast BisTris-gels (10 % T, 1.5 mm, 10 wells) were used for all experiments. The gels were run at constant voltage (200 V; maximum 120 mA and 25 W) and until the dye front reached the foot of the gel (*ca* 55 min). Catholytes were supplemented with 5 mM sodium metabisulfite as antioxidant. After completion of the run gels were stained with Coomassie R-250 (*vide infra*). The *detection limit of the EPO-Fc standard* was determined by preparation of a two-fold serial dilution of EPO-Fc in PBS containing 0.05% BSA. The highest and lowest amounts of EPO-Fc applied on gel were *ca* 0.2 ng and *ca* 0.39 pg, respectively. Western double-blots were performed as described below. Immunoaffinity purified EPO-Fc from spiked serum samples (*vide supra*) was run under identical conditions as describe above (SDS-PAGE, SAR-PAGE; Western double-blots).

IEF-PAGE of EPO-Fc

IEF-PAGE gels with precast wells were prepared as described elsewhere.[29] The acrylamide-bisacrylamide solution (5% T/3% C) contained 7 M urea and 4% (*w/v*) carrier ampholytes. Gels (*ca* 25 × 11.5 cm, 1 mm) were cast in three different pH-ranges (pH 2–6, pH 4–8, pH 3–10) and on GelBond PAG film.[29] Mixtures of Servalytes 2–4/4–6 and Servalytes 4–6/6–8 were used for the pH 2-6 and pH 4-8 gels, respectively. Servalyt 3–10 Iso-Dalt was employed for the pH 3–10 gel. Decane was utilized as contact fluid between the PAG film and the cooling plate (Multiphor II). Different types of catholytes and anolytes were used for the three pH-ranges, namely 2% (*w/v*) Servalyte 6–8 and 0.5 M phosphoric acid (pH 2–6),[30] 1 M NaOH and 0.04 M L-glutamic acid (pH 4–8),[31] and 1 M NaOH and 0.04 M L-aspartic acid (pH 3–10),[31] respectively. EPO-Fc standard (*ca* 0.5 ng) and EPO pharmaceuticals (*ca* 0.2 ng) were applied after prefocusing (250 V constant, 30 min; 10 °C; *ca* 10 cm inter-electrode distance) on the gels and then focused for 2000 Vh (pH 3–10), 3600 Vh (pH 2–6), and 4000 Vh (pH 4–8) at 25 W constant power (10 °C).[31] Typically, all EPO standards were supplemented with 1% Tween-80 before electrophoresis. Standard dilutions were prepared with 0.05% BSA in PBS. Additional experiments were conducted with EPO-Fc containing Tween-20 (1%), no detergent, and after reduction with DTT (20 mM) in DeStreak solution (see section on two-dimensional polyacrylamide gel electrophoresis (2D-PAGE); again with and without addition of detergent). All standards and samples were applied on the cathodic side of the gels. After isoelectric focusing proteins were blotted to polyvinylidene fluoride (PVDF) membranes (Immobilon-P; *vide infra*).

2D-PAGE of EPO-Fc

Epo-Fc (10 µg) was solubilized in 125 µl DeStreak rehydration solution supplemented with 15 µl of IPG-buffer pH 3-10 (linear) per 3 ml and containing 20 mM DTT.[32] A pH 3–10 (linear) IPG-strip (7 cm) was overnight rehydrated with the sample and then run at 20 °C on an IPGphor II instrument. Focusing conditions were selected according to the instructions of the manufacturer (1) 300 V/200 Vh (step), (2) 1000 V/300 Vh (gradient), (3) 5000 V/4000 Vh (gradient), (4) 5000 V/2000 Vh (step); 50 µA per strip).[33] After completion of IEF the IPG-strip was equilibrated in reducing buffer (LDS-sample buffer containing 6 M urea and 65 mM DTT; 2 × 7.5 min) followed by equilibration in alkylating

buffer (LDS-sample buffer with 6 M urea and 135 mM iodoacetamide; 2 × 7.5 min). Subsequently, the strip was transferred to the IPG-well of a BisTris ZOOM gel (4–12% T, 1 mm) and sealed with MOPS running buffer containing 0.5% (*w/v*) low melting point agarose (55 °C). After solidification of the sealing solution (*ca* 30 min) the gel was run under SDS-PAGE conditions (200 V constant; maximum 120 mA and 25 W) using MOPS running buffer as electrolyte. The catholyte contained 5 mM sodium metabisulfite as antioxidant. Gels were stained with Coomassie R-250 as soon as the front had reached the bottom of the gel.

Staining of SDS/SAR-PAGE and 2D-PAGE gels with Coomassie R-250

After electrophoretic separation gels were fixed in 100 ml of a solution containing 50% methanol and 10% acetic acid (15 min). Gels were then stained overnight with 0.025% Coomassie R-250 in 40% methanol/10% acetic acid (100 ml). Destaining was performed by washing gels with 20% methanol/10% acetic acid for several hours. The staining solution was prepared immediately before usage.[27]

Western double-blotting of EPO-Fc and EPO standards

After SDS-, SAR-, and IEF-PAGE proteins were transferred via semi-dry blotting to Immobilon-P membranes. Procedures were already described elsewhere.[27,28,34] In brief, Bjerrum buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol)[35] and Towbin buffer (25 mM Tris, 192 mM glycine, no methanol)[34] were used for the transfers of SDS/SAR-PAGE and IEF-PAGE gels, respectively. After buffer equilibration (3 × 5 min SDS/SAR-PAGE, 1 × 2 min IEF-PAGE) proteins were blotted at constant current (1.0 mA/cm^2) for 60 min (SDS/SAR-PAGE) or 30 min (IEF-PAGE). One layer of extra thick blotting paper (SDS/SAR-PAGE) or three layers of thick blotting paper (IEF-PAGE) were used on each side of the Immobilon-P membrane/gel sandwich. For IEF-gels an additional Durapore membrane was used to separate the soft gel from the Immobilon-P membrane.[34] After the transfer Immobilon-P membranes were briefly washed in PBS and then incubated in 5 mM DTT/PBS solution (37 °C, 45 min). Incubation in primary antibody (clone AE7A5; 1:1000 (*v/v*) in 1% non-fat milk (NFM)/PBS) was done overnight (cold room) and after blocking in 5% NFM/PBS for 45 min. Subsequently, membranes were washed (0.5% NFM/PBS; 3 × 7 min) and bound primary antibodies transferred to a second Immobilon-P membrane (0.7% acetic acid; 0.8 mA/cm^2, 10 min). After blocking (5% NFM/PBS; 45 min) transferred antibodies were detected by incubation of the membranes in biotinylated secondary antibody (goat anti-mouse IgG; 1:2000 (*v/v*), 1% NFM/PBS, 60 min) and streptavidin horseradish peroxidase (HRP) complex (1:2000 (*v/v*), 1% NFM/PBS; 60 min). Between incubations washing steps (3 × 7 min) were performed with 0.5% NFM/PBS and PBS, respectively. After reaction with chemiluminescence substrate (West femto) membrane images were acquired with a LAS-4000 CCD-camera. GASepo was used as image analysis software.

Results and discussion

For developing a detection strategy for EPO-Fc, several *enrichment* (protein A/G, ACN precipitation) and *purification* (immunoaffinity) methods had to be tested regarding their ability to capture low pg amounts of the fusion protein from a rather complex protein matrix (human serum). From data of a published pulmonary administration study it was known that typical maximum concentrations of EPO-Fc in serum were in the low ng/ml range.[20] Hence, the detection strategy also required a *detection method* capable of measuring these low amounts without interference of contaminating proteins and shEPO in particular. From routine anti-doping testing it was known that ELISA and electrophoretic (SDS/SAR-PAGE, IEF-PAGE) methods may be good candidates for fulfilling these requirements.

Mass spectrometric characterization of EPO-Fc

After tryptic digestion EPO-Fc peptides were separated by nano-LC and sequenced in an LTQ-Orbitrap mass spectrometer (nano-ESI MS/MS). Spectra identification was automatically performed using SEQUEST search algorithm and UniProt human database. According to the manufacturer (ProSpec), EPO-Fc was produced in a CHO cell-line and is a homodimeric glycoprotein consisting of two EPO molecules linked to the hinge-CH2-CH3 part of human IgG1. No additional sequence information was given. Sequence coverage was about 44% assuming a fully processed fusion protein as declared by the manufacturer (794 amino acids; 2 × human EPO (UniProt P01588, 2 × 165 amino acids), 2 × human IgG1 chain C region (UniProt P01857, hinge-CH2-CH3 fragment, 2 × 232 amino acids). Higher sequence coverage would be obtained if glycosylation sites, possible missed cleavage sites and amino acid modifications (e.g. oxidation of Met, Cys, Trp, Tyr, Phe, His, Ile, Leu, Pro, deamidation of Asn)[36,37] were taken into account. However, no attempt was undertaken to further characterize the protein. Figures 1 and 2 show MS/MS spectra of two tryptic EPO-Fc peptides, which are characteristic for human EPO (EAISPPDAASAAPLR) and the CH3 domain of human IgG (GFYPSDIAVEWESNGQPENNYK).

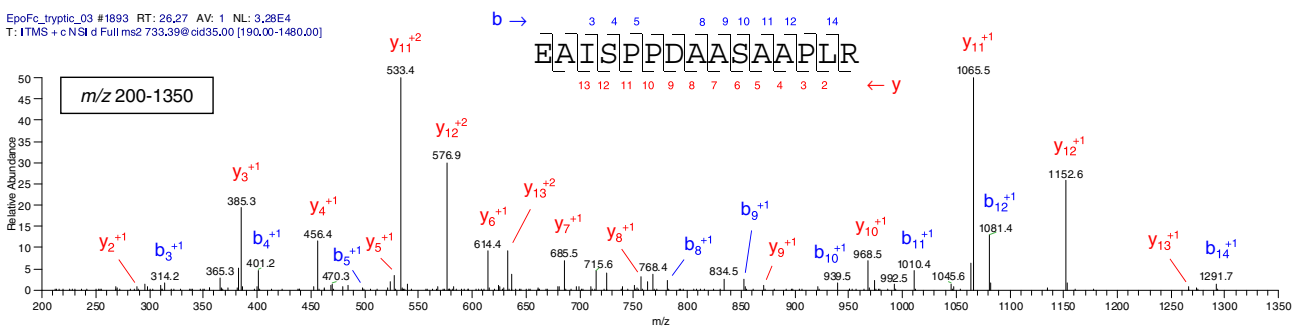

Figure 1. Nano-ESI-MS/MS spectrum of EPO peptide EAISPPDAASAAPLR (*m/z* 733.4, z = 2) obtained after cleavage of EPO-Fc with trypsin. The spectrum was recorded in the linear ion trap of the LTQ-Orbitrap hybrid mass spectrometer, observed b- and y-ions are marked.

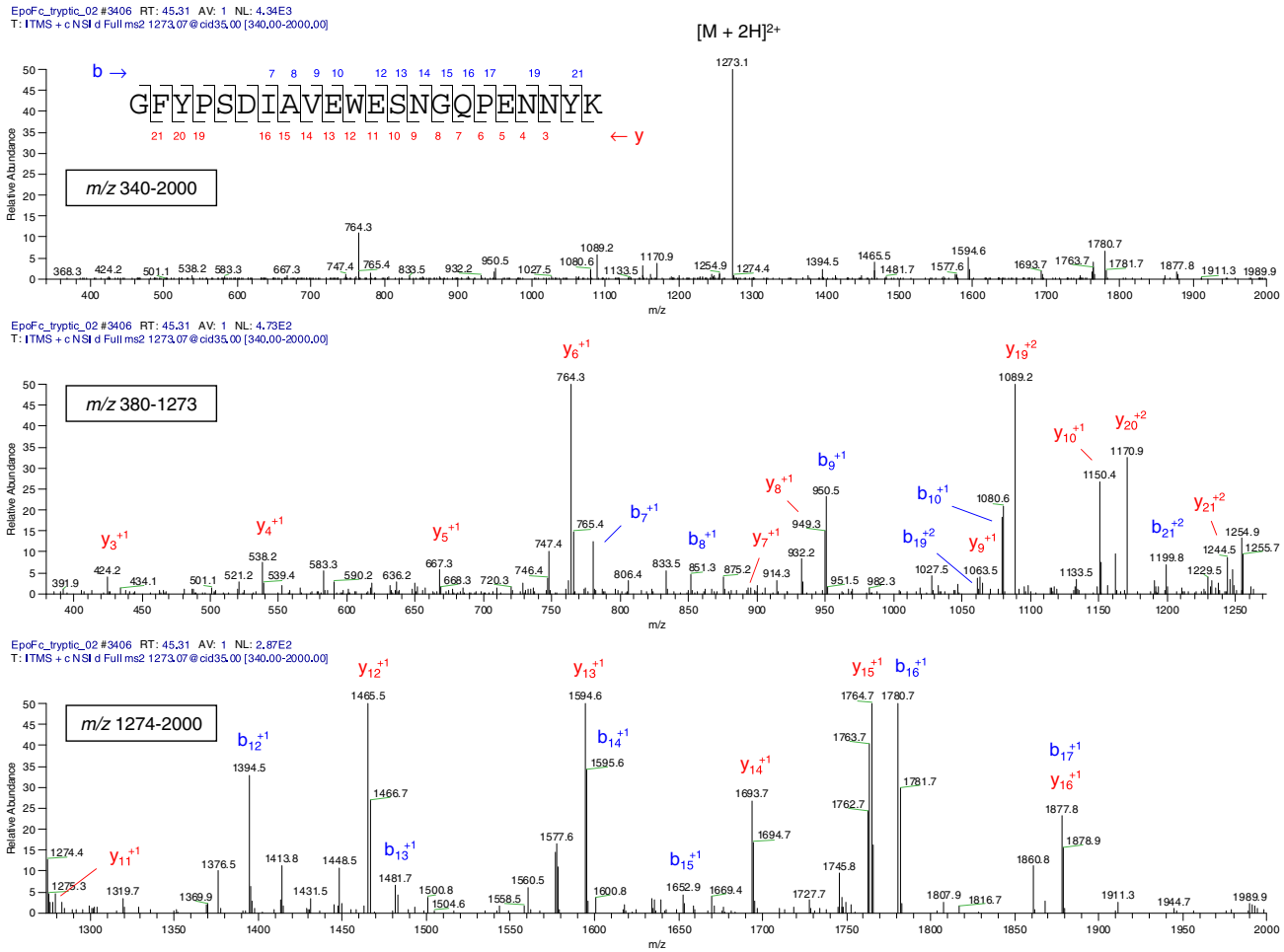

Figure 2. CID spectrum of tryptic peptide GFYPSDIAVEWESNGQPENNYK of EPO-Fc (*m/z* 1272.571, z = 2; accurate monoisotopic mass as recorded in the Orbitrap analyzer). The peptide is characteristic for the CH3 domain within the Fc chain of the EPO-Fc fusion protein. (A) shows the entire mass range of the fragment ion spectrum (*m/z* 340–2000), the other two spectra (B, C) magnify mass regions below and above the precursor ion. Note that due to the long chain length of the peptide (22 amino acids) the precursor $[M + 2H]^{2+}$ was only partly fragmented in the ion trap and hence is still present in the MS/MS spectrum; b- and y- fragment ion series are indicated.

Selective enrichment of EPO-Fc using Protein A and G beads

Protein A and G selectively bind to the Fc-region of antibodies with high affinity at neutral and weakly basic pH conditions. The interaction is disrupted at acidic pH-values. Hence, both protein A and G have been frequently used, for example, for the purification of monoclonal antibodies as well as immonoprecipitation and site-directed antibody immobilization experiments. Both proteins exhibit slightly different selectivities for species and IgG subclasses.[38,39] Typically, protein A is recommended for capturing human antibodies of the IgG_1, IgG_2, and IgG_4 subclasses, while protein G can be additionally used for IgG_3 antibodies.[39] Since EPO-Fc was described to contain the hinge, CH2, and CH3 domains but lacks the CH1 domain of the constant region of the human IgG_1 heavy chain, its behavior to proteins A and G had to be evaluated regarding their binding behavior. According to the manufacturer the dynamic binding capacity of POROS A beads (20 μm) is 30 mg/ml and for POROS G beads (20 μm) 15 mg/ml (both for human IgGs and at pH 7.5).[40] Contrary to that POROS MabCapture A beads (45 μm), which were specifically developed for purifying monoclonal antibodies with perfusion chromatography, show an increased binding capacity of > 45 mg/ml (human IgGs, pH 7.5).[41] Reference values for human IgG (total), IgG_1, IgG_2, IgG_3, and IgG_4 in serum are 7.0–16.0, 4.9–11.4, 1.5–6.4, 0.2–1.1, and 0.08–1.4 g/l (adults).[42,43] Hence, 50 μl of, for example, packed POROS G beads (or 100 μl of a 1:1 bead slurry) should be able to capture a maximum of 750 μg of human IgGs, which corresponds to a serum volume of approximately 47–190 μl. Based on these considerations the *selection of the most suitable type of beads* was performed (50 μl serum spiked with 0.0/5.0/12.5/25/50 pg EPO-Fc, 100 μl POROS bead slurry). Figure 3A shows the results obtained for POROS A, G, and MabCapture A beads after elution and EPO ELISA readout. Due to the higher IgG binding capacity POROS MabCapture A beads performed slightly better than the other two bead types. Hence, MabCapture A beads were chosen for the next experiments.

For *determining the optimum bead volume*, 50 μl of the EPO-Fc spiked sera were incubated with 50, 100, and 300 μl beads. Less EPO-Fc was captured by the 50 μl than the 100 μl beads, but no further improvement was observed when 300 μl beads were used (data not shown). The *impact of serum amount* on EPO-Fc capture was studied by incubating 100 μl beads with 50 μl and 200 μl of spiked sera. A significant relative decrease in EPO-Fc recovery was seen for the experiments performed with 200 μl of serum (Figure 3B). Consequently, it was decided to use 100 μl of washed beads for experiments with 50 μl serum (2:1 ratio of bead

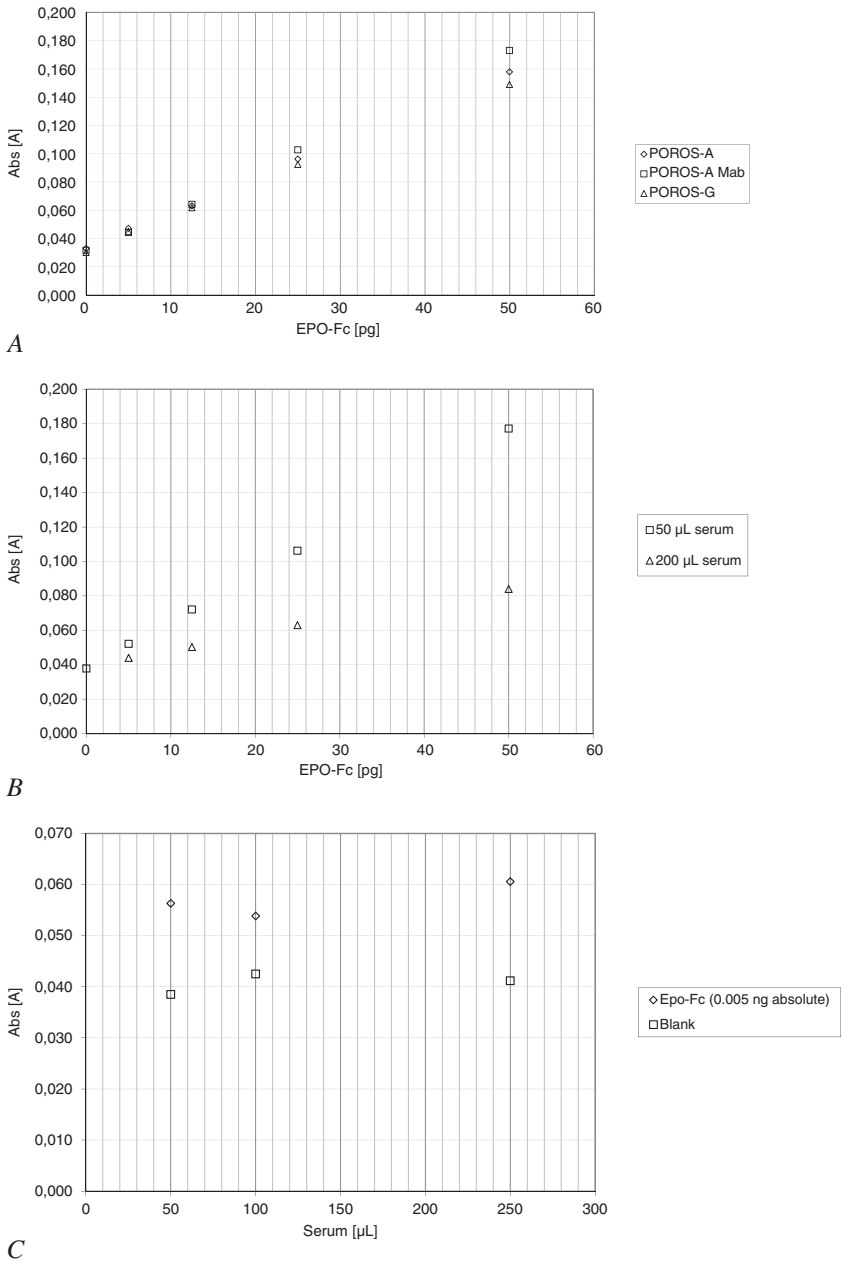

Figure 3. Affinity extraction of EPO-Fc in serum with POROS beads and measurement by EPO ELISA. (A) Performance characteristics of POROS A, G, and MabCapture A beads. Dependence of extraction efficiency on serum volume, EPO-Fc concentration (B), and LOD (5 pg; C) for POROS MabCapture A media.

slurry to serum), which allowed the *detection of at least 5 pg of EPO-Fc* by ELISA. No interference by shEPO was observed due to non-specific capture on POROS beads. For evaluation of the *dependence of the detection limit on the serum EPO-Fc concentration*, 5 pg of EPO-Fc were spiked into increasing amounts of serum (50, 100, 250 µl). Bead volumes were adjusted accordingly. No interference due to the higher IgG and shEPO amounts was observed. In summary, 5 pg of EPO-Fc could be detected by the EPO ELISA kit from R&D Systems and the POROS MabCapture A protocol (Figure 3C), which makes it ideally suited for fast screening purposes.

Selective enrichment of EPO-Fc using ACN precipitation

As shown earlier, the precipitation of high abundant serum and plasma proteins by organic solvents (e.g. ethanol, acetonitrile),[24,44,45] polyethylene glycol-containing solutions (e.g. PEG-6000 in saline),[46] and acidification (e.g. perchloric acid)[47] can selectively enrich EPO and MIRCERA in the supernatant. Hence, it was tested whether a precipitation strategy (acetonitrile) might be also useful for enrichment of EPO-Fc in serum. However, it was expected that higher losses might occur for EPO-Fc due to the immunoglobulin part and its decreased solubility compared to unmodified EPO (e.g. PEG-6000 precipitates antibodies including IgG).[48] Hence, experiments were performed at much higher concentrations of EPO-Fc in serum (0.25–3.0 ng/ml) than for POROS beads and also with higher serum sample volumes (200 µl). The EPO/EPO-Fc content in the supernatant was measured by EPO ELISA and compared with the results obtained for 100 µl of non-precipitated samples. Acetonitrile precipitation led to a complete loss of EPO-Fc and only a rather

constant amount of shEPO was measurable in the supernatant by ELISA (Figure 4). Hence, precipitation strategies were not further investigated.

SDS- and Sarcosyl-PAGE of EPO-Fc

SDS- and SAR-PAGE are currently used together with IEF-PAGE for detecting and confirming doping with various recombinant erythropoietins.[28,30,49] SAR-PAGE was shown to be particularly useful for the detection of MIRCERA doping in blood due to its increased sensitivity compared to SDS-PAGE.[27,50] Hence, SAR-PAGE might also be a good candidate method for confirming the presence of EPO-Fc in human blood. The EPO-Fc standard was separated on SDS- and SAR-PAGE and stained with *Coomassie R-250* (Figures 5A and 5B). Both methods gave similar results. Two bands were observed, with the more intense band showing an apparent molecular mass of *ca* 60 kDa and the less intense band a mass of $>$ 116 kDa (Figure 5A). Since gels were run under reducing conditions, the band with the lower molecular mass can be explained by splitting of the disulphide bridges between the two Fc-chains, which hold the EPO-Fc monomers together. The band with the higher mass might consist of intact dimers, aggregates, or re-associated monomers. Since a preceding reduction and alkylation step did not alter the position of the band and its intensity (data not shown), it is assumed that the band does not correspond to re-associated EPO-Fc monomers.

Based on the data of the Coomassie stained gels, EPO-Fc can be differentiated from all other epoetins including originator formulations of recombinant EPO, NESP, MIRERA, and biosimilars by its characteristics in apparent molecular mass. Next, the *immunological detectability of EPO-Fc* by Western double-blotting and the monoclonal anti-EPO antibody, which has to be mandatorily used for the detection of EPO doping by IEF-PAGE,[21] was studied. Figure 6A shows the results obtained with SAR-PAGE and in comparison with the uhEPO standard (NIBSC) and the other epoetins, which were already tested by Coomassie staining. The same band pattern was observed as on the dye-stained SAR-PAGE gel (one intense band with an apparent molecular mass between the masses of NESP and MIRCERA, followed by a low intensity but higher mass band with a molecular mass

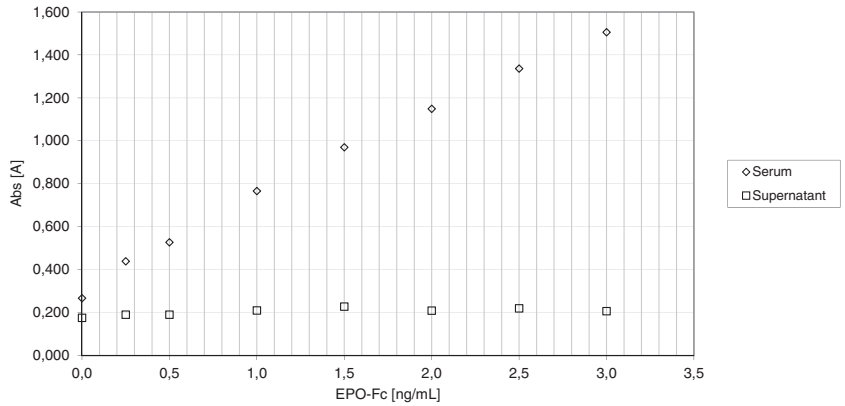

Figure 4. Evaluation of acetonitrile precipitation for enrichment of EPO-Fc in serum. Due to lower solubility compared to shEPO or MIRCERA hardly any EPO-Fc is detectable in the 50% ACN (*v/v*) supernatant.

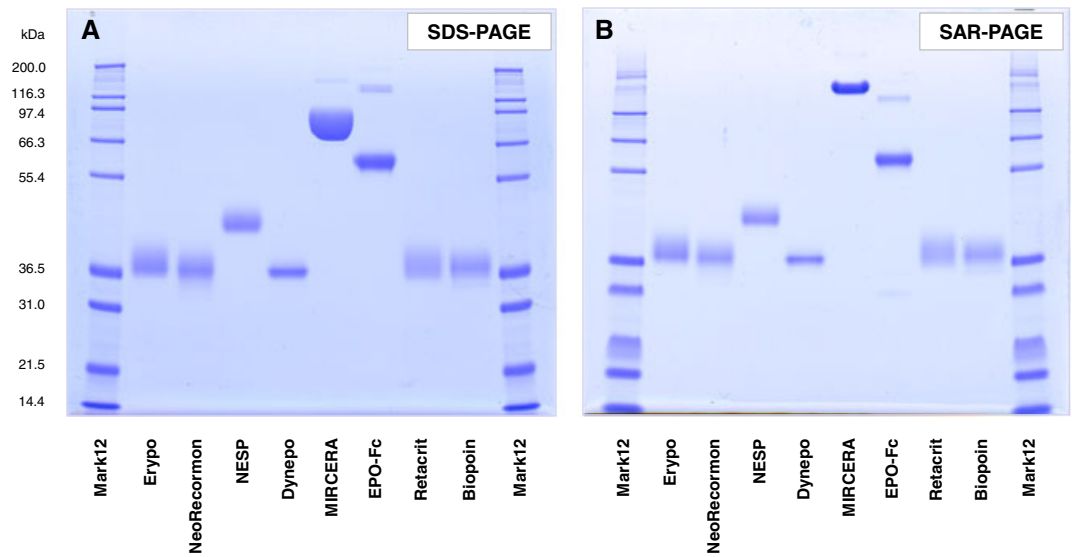

Figure 5. Performance characteristics of EPO-Fc on SDS- and SAR-PAGE. Under reducing conditions the majority of the fusion protein is split into its two monomers, which migrate with an apparent molecular mass of *ca* 60 kDa on SDS-PAGE (A). Identical behavior is seen on SAR-PAGE (B). Note the enhanced distance of EPO-Fc to the MIRCERA band on SAR-PAGE, which is due to the favorable binding properties of sarcosyl for PEGylated EPO.

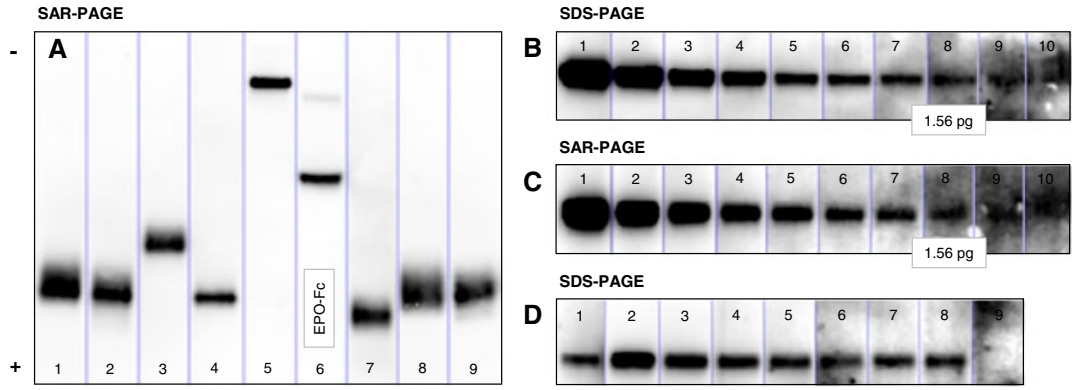

Figure 6. (A) SAR-PAGE of EPO-Fc and various epoetins (lane 1/Erypo, 2/NeoRecormon, 3/NESP, 4/Dynepo, 5/MIRCERA, 6/EPO-Fc, 7/uhEPO (NIBSC), 8/Retacrit, 9/Biopoin). A two-fold serial dilution was used for determination of the detection limit (LOD) of EPO-Fc in 0.05% BSA/PBS on SDS-PAGE (B) and SAR-PAGE (C) (lanes 1–10: 200/100/50/25/12.5/6.25/3.13/1.56/0.78/0.39 pg). Identical LODs were received for both methods (*ca* 1.56 pg (14 amol)). (D) Immunoaffinity purification of EPO-Fc in serum followed by SDS-PAGE (lane 1/Epo-Fc standard (12.5 pg), 2-5/EPO-Fc dilution series in 200 μL serum (200/100/50/20 pg), 6–8/EPO-Fc (5 pg) in 50/100/250 μl serum, 9/blank serum). Western double-blots with chemiluminescence detection.

slightly below MICERA). The applied amount was *ca* 0.2 ng EPO-Fc. Similar results were obtained for EPO-Fc on SDS-PAGE, but due to the different migration behavior of MIRCERA, the MIRCERA band was positioned between the two EPO-Fc bands (data not shown).

For determining the *detection limit of EPO-Fc* on SDS- and SAR-PAGE a two-fold serial dilution ranging from *ca* 0.2 ng to *ca* 0.39 pg EPO-Fc was used. The lowest detectable amount was *ca 1.56 pg*, which corresponds to *ca 14 amol* assuming a molecular mass of *ca* 112 kDa for EPO-Fc.[11,20] Identical LODs were received for the SDS- and SAR-PAGE gels and the combination with Western double-blotting/chemiluminescence detection (Figures 6B and 6C). However, regardless identical LODs it should be kept in mind, that for comprehensive EPO anti-doping testing SAR-PAGE is the method of choice due to its higher sensitivity for MIRCERA.[27,50]

Next, the *detectability of EPO-Fc in serum* was evaluated. Due to the high total protein content of serum (65-85 μg/μl),[51] EPO-Fc had to be first enriched before serum samples could be applied on SDS- and SAR-PAGE gels. Three strategies were investigated, namely specific enrichment of EPO-Fc by protein A (POROS MabCapture A) beads, enrichment of EPO and EPO-Fc by depletion of high abundant proteins followed by immunoaffinity purification with an EPO ELISA (StemCell Technologies), and direct immunoaffinity purification of EPO-Fc and EPO with a commercial EPO purification kit (MAIIA Diagnostics). Only the latter strategy proved useful in practice. Due to the still high protein content of the protein A bead eluate, which contains EPO-Fc and almost the entire serum IgG fraction (*vide supra*), the eluate could not be directly applied on SDS- and SAR-PAGE gels without obtaining severe gel-overloading effects as demonstrated by Coomassie R-250 staining (data not shown). The ELISA purification strategy on the other hand was also not of practical value due to its poor sensitivity. The lowest detectable amount of EPO-Fc was around 0.2 ng (data not shown). Keeping the detection limit of the proposed screening method (*vide supra*) in mind (POROS MabCapture A beads plus EPO-ELISA (R&D Systems) readout), which was *ca* 5 pg EPO-Fc, the LOD of the ELISA purification strategy was far beyond practical relevance for being a useful confirmation method. Consequently, the only strategy, which was further explored, was direct immunoaffinity purification. A dilution series ranging from 200 to 20 pg EPO-Fc in 200 μl serum easily allowed the detection of 20 pg EPO-Fc (Figure 6D). Hence, the detectability of *5 pg EPO-Fc* (the LOD of the proposed POROS/ELISA screening method) in different volumes of serum (50, 100, 250 μl) was tested. EPO-Fc was detectable in all three samples (Figure 6D). Thus, the combination of direct immunoaffinity purification with SDS- or SAR-PAGE electrophoretic separation and Western double-blotting/chemiluminescence detection can be either used as confirmation method for the results obtained by the POROS/ELISA screening method or as a stand-alone method for screening and confirmation of EPO-Fc in serum.

IEF-PAGE of EPO-Fc

Carrier ampholyte-based gels in three different pH-ranges (pH 2–6, pH 4–8, pH 3–10) were evaluated in combination with Western double-blotting and chemiluminescence detection. Previous studies with a homodimeric EPO-Fc fusion protein found isoforms within the pH-range of 4.6 to 7.0.[11,52,53] Homemade immobilized pH-gadient (IPG) gels were used with a pH-range of pH 4 to 8, and gels were rehydrated in a solution of 6 M urea, 10 mM DTT, 2% 3-3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.4% bromophenol blue and 2% Pharmalyte 3-10. Hence, it was first tested how EPO-Fc performs on a regular pH 2–6 carrier ampholyte gel used in EPO anti-doping control. EPO-Fc was supplemented with Tween-80 (1%) as usually done with EPO standards and samples (BRP-EPO, NESP, NIBSC uhEPO), also after treatment with DTT-containing (20 mM) DeStreak rehydration solution with and without addition of Tween-80. DeStreak solution contains 2-hydroxyethyl disulfide, which improves electrophoretic separation by converting protein SH-groups into stable disulfides and by preventing disulfides from oxidation. This usually leads to less protein streaking in the basic pH range.[32,54,55] DeStreak rehydration solution also contains urea, thiourea, and CHAPS. However, regardless which treatment was used, no satisfying results were obtained for the pH 2–6 carrier ampholyte gels. EPO-Fc (*ca* 0.5 ng) with Tween-80 led to a broad smear towards the anode with no resolved isoforms (Figure 7A, lane 3). The two samples treated with DeStreak showed few isoforms (Figure 7A, lanes 4 and 5), but as in lane 3 EPO-Fc did hardly migrate towards the anode. Also, this basic part of the gel contains the region where zinc-alpha-2-glycoprotein (ZAG) interacts with clone AE7A5 anti-EPO antibody[56] – a gel region which is not required in current EPO anti-doping testing.

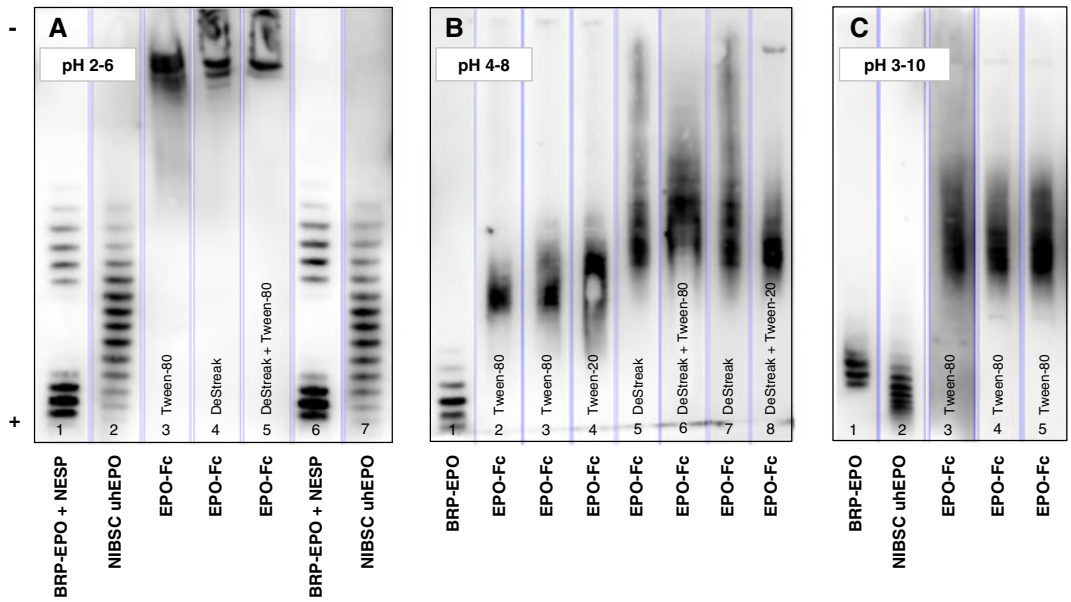

Figure 7. IEF-PAGE of EPO-Fc on carrier ampholyte-based gels. Results obtain on pH 2-6 (A), pH 4-8 (B), and pH 3-10 (C) gels. Under focusing conditions perfectly suited for isoform separation of recombinant (BRP-EPO, NESP) and endogenous (uhEPO) epoetins no satisfying results were obtained for EPO-Fc. Reduction and alkylation (DeStreak reagent) of EPO-Fc led to no significant improvement. Western double-blots with chemiluminescence detection (BRP-EPO/NESP/uhEPO: 0.2 ng, EPO-Fc: 0.5 ng (except (C), lanes 3–5: 0.1/0.5/1.0 ng)).

In conclusion, pH 2–6 carrier ampholyte gels appeared to be not well suited for the detection of EPO-Fc. Hence, pH 4–8 and 3–10 carrier ampholyte gels were tested, too. Figure 7B shows the results obtained with the pH 4-8 gel. Regardless how EPO-Fc was treated (Tween-80, Tween-20, DeStreak without and with Tween-80 or Tween-20; lanes 2-8), no separation of EPO-Fc in discrete isoforms was achieved. Further expansion of the pH-range (pH 3–10 carrier ampholyte gel) did not improve resolution (Figure 7C, lanes 3–5). The main reasons for the poor results obtained with carrier ampholyte-based gels probably were (1) the rather low maximum voltage gradients reached during the run (typically below 150 V/cm) and (2) the rather low total Volt-hours used for focusing of EPO-Fc (usually below 4000 Vh). However, conditions like these work perfect for regular epoetins (e.g. uhEPO, shEPO, rhEPO).

2D-PAGE of EPO-Fc

In order to investigate whether EPO-Fc can be separated into discrete isoforms by using the same chemical treatment as for the carrier ampholyte gels, commercial pH 3–10 IPG-strips (7 cm) were tested with higher maximum voltage gradients (715 V/cm) and total Volt-hours (6500 Vh). Under these conditions, DTT/DeStreak-treated EPO-Fc was easily resolved into fourteen isoforms (Figure 8). Due to the fact that 2D-PAGE is a low-throughput method compared to the POROS/ELISA and SDS/SAR-PAGE protocols, 2D-PAGE was not further taken into account as basis for an EPO-Fc anti-doping test.

Conclusion

Out of the five evaluated strategies for detecting EPO-Fc in human serum (POROS MabCapture A media plus EPO-ELISA, ACN precipitation, immunoafffinity purification plus SDS/SAR-PAGE, IEF-PAGE, 2D-PAGE) only two methods proved useful within the context of anti-doping requirements (the development of an EPO-Fc specific ELISA method was not considered as no EPO-Fc specific antibody was available). The POROS/ELISA strategy first enriches EPO-Fc on high capacity protein A beads (POROS MabCapture A media) and then detects it with high sensitivity (LOD *ca* 5 pg EPO-Fc) by employing a commercial EPO-ELISA kit. The method is

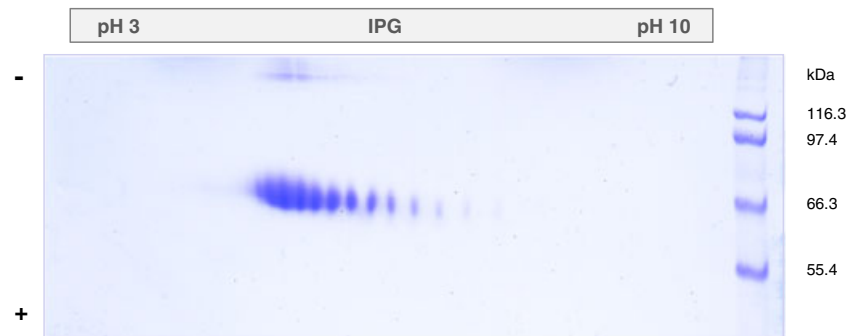

Figure 8. 2D-PAGE of EPO-Fc (10 μg) after isoelectric focusing on an immobilized pH-gradient gel (IPG; pH 3–10, 7 cm) in the first dimension. Fourteen isoforms are detectable on the Coomassie R-250 stained gel.

well suited for *fast screening* purposes. For *screening and/or confirmation* purposes the combination of fast immunoaffinity purification (commercial EPO purification kit) with SDS/SAR-PAGE followed by Western double-blotting/chemiluminescence detection provides the necessary high sensitivity (same LOD of *ca* 5 pg EPO-Fc). Without the purification losses the lowest detectable amount of EPO-Fc on SDS/SAR-PAGE is *around 1.56 pg (14 amol)*. For comprehensive sports drug testing SAR-PAGE is recommended due to its additional higher sensitivity for PEGylated erythropoietin (MIRCERA).

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