Short communication

Drug Testing and Analysis

Received: 13 July 2011 Revised: 1 February 2012 Accepted: 13 March 2012 Published online in Wiley Online Library: 7 May 2012

(wileyonlinelibrary.com) DOI 10.1002/dta.1361

Easy-to-use IEF compatible immunoaffinity purification of Erythropoietin from urine retentates

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Erythropoietin (EPO) is a peptide hormone responsible for hypoxia-induced promotion of erythrocyte production. The possibility of enhancing oxygen transport through an increase of erythrocytes has led to EPO abuse in sports. Detection of exogenous EPO is most commonly done via isoelectric focusing (IEF) which is a method provided by the Technical Document TD2009EPO of the World Anti-Doping Agency (WADA). Before analysis, collected urine samples need to be concentrated 500- to 1000-fold, leading to high protein abundance in the retentates. Reduction of protein concentration through an immunoaffinity purification using ELISA wells has been successfully used prior to SDS-PAGE. This ELISA kit was used to purify samples using an IEF-compatible elution. The purification showed recovery ratios between 50 and 90% depending on substance and application volume. Application of immunopurified samples to IEF was shown to improve the quality of the gels by reducing streaks and curvatures within the lanes and bands of the gel. The result was an increase of quality for IEF gels.

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

Keywords: erythropoietin; immunoaffinity purification; isoelectric focusing; doping control

Introduction

Erythropoietin (EPO) is a 30.4 kDa glycoprotein which is the humoral regulator of red blood cell production. In the adult EPO is produced mainly in the kidneys.[1,2] It is composed of an invariant amino acid core while three asparagine-linked sugar chains and one serine-linked sugar chain account for a variable carbohydrate component. EPO does not have one defined structure but it is a conglomerate of different EPO isoforms.[3] Successful expression of recombinant human EPO (rhEPO) in different cell lines has led to a variety of pharmaceuticals developed for anaemia treatment. In sports, the use of EPO has been officially forbidden since 1990.[4] Lasne *et al.* have established a direct test using the athlete's urine to detect EPO abuse.[5,6] The test involves isoelectric focusing (IEF) followed by a double-blotting procedure and chemiluminescence detection of EPO isoforms. Human urinary EPO can be distinguished from rhEPO by its differing isoform patterns on IEF. The criteria defining a sample as rhEPO positive are specified in the recent World Anti-Doping Agency (WADA) technical document. IEF data which do not fulfil certain criteria can be rendered useless for analysis: 'spots, smears areas of excessive background or absent signal invalidate a sample lane'.[7] Good quality IEF data are essential for a competent and failsafe analysis of urine samples. Sample overloading due to high protein abundance can lead to streaking and curvature of the detectable bands making densitometric measurements unreliable. Heparin and possibly anionic detergents from gloves that might be used during doping control have been shown to disturb EPO isoform patterns during IEF.[8,9] Cross-reactivity of the anti-EPO antibody with a second urinary protein has also been observed.[10] Immunoaffinity purification of urine samples is a convenient way to improve signal to noise ratio, avoid cross-reactivity, and purge the sample of IEF interfering substances. Urine sample purification has been performed using enzyme-linked immunosorbent assay (ELISA) wells prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) yet this method was not compatible with IEF.[11,12] Immunoaffinity columns and WGA-Sepharose have been used prior to IEF.[13,5,6] This paper now discusses an easy-to-use, IEF-compatible purification using ELISA wells from STEMCELL.

Materials

Rotiphorese Gel 30 Acrylamide/bisacrylamide solution for isoelectric focussing was provided from Roth (Karlsruhe, Germany). Ammonium persulfate (APS), N,N,N´,N´-tetramethylethylenediamine (TEMED), Servalyte carrier ampholytes (2–4, 4–6, 6–8) and sucrose were bought from Serva (Heidelberg, Germany). Glacial acetic acid, phosphoric acid (85%), glycine, Tris (hydroxymethyl) aminomethane, potassium chloride and di-sodium hydrogen phosphate dodecahydrate were obtained from Merck (Darmstadt, Germany). Sodium chloride was used from VWR International (Leuven, Belgium). The protease inhibitor cocktail Complete was bought from Roche Diagnostics (Indianapolis, IN, USA). AppliChem (Darmstadt, Germany) provided dithiothreitol. Urea, bovine serum

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albumin (BSA) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were supplied by Sigma-Aldrich (St Louis, MO, USA) A 10% Tween-80 Solution was acquired from ThermoScientifc (Rockford, IL, USA) by the name Surfact-Amps 80. Devices for sample preparation and western blotting membranes were provided by Millipore (Billerica, MA, USA), namely Steriflip filters (0.22 μm) for microfiltration, for ultrafiltration Amicon Ultra-4 and Amicon Ultra-15 (nominal molecular weight limit for both filter types 30 kDa), for blotting polyvinylidene difluoride membranes (PVDF; Immobilon-P, Durapore). NovaBlot blotting paper, GelBond PAGfilm and Electrode strips were supplied be GE Healthcare (Uppsala, Sweden). Low fat milk (lait écréme) for blocking and antibody incubation buffers was bought from Regilait (Saint-Martin-Belle-Roche, France). The ELISA kit used for immunoaffinity purification and quantification of erythropoietin was purchased from STEMCELL Technologies (Vancouver, Canada). For detection of erythropoietin a monoclonal mouse antibody (clone AE7A5; R&D Systems, Minneapolis, MN, USA), a biotinylated polyclonal antibody (ImmunoPure goat anti-mouse IgG (H + L); Pierce, Rockford, IL, USA) and a streptavidin horseradish peroxidase complex (Biospa, Milano, Italy) were used. The chemiluminescence substrate kit ChemiGlow West was acquired from Biozym (Vienna, Austria). Standards for human urinary erythropoietin (uhEPO; second international reference preparation) were from the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK) and standards for human recombinant erythropoietin (rhEPO; BRP-EPO batch 3) were the European Directorate for the Quality of Medicines (Strasbourg, France). The following recombinant and modified EPO pharmaceuticals were used: Binocrit from Sandoz (Kundl, Austria), Neorecormon from Roche (Indianapolis, IN, USA), Dynepo from Shire (Hampshire, England), Eporatio from Ratiopharm (Ulm, Germany), Mircera from Roche (Indianapolis, IN, USA), Aranesp from Amgen (Thousand Oaks, CA, USA) and Silapo from Stada (Bad Vibel, Germany). NuPAGE BisTris gels (10% T, 1mm thickness), lithium dodecyle sulfate (LDS) sample buffer, antioxidant, 4-morpholinepropanesulfonic acid (MOPS) electrophoresis running buffer were supplied by Invitrogen (Carlsbad, CA, USA). PageSilver Staining Kit was acquired from Fermentas (St Leon-Rot, Germany).

Samples

Blank urine samples used were acquired from three healthy volunteers (30–56 years) who were not taking any medication or suffering from diseases that may influence the EPO production, metabolism, or excretion. Spiked urines were obtained by spiking blank urines with 3.5U/L BRP. Samples from two different excretion studies were used. In these studies urine samples were taken from a single healthy volunteer (59 years, 80kg) before a single subcutaneous application of 50U/kg Silapo or respectively 0.75μg/kg Aranesp and at various time points afterwards. Urine samples were stored at −20°C until sample preparation. All volunteers gave written consent for participation in this study. Furthermore the Silapo and Aranesp excretion studies were performed in agreement with the ethical committee of the German Sports University Cologne. Buffers used were spiked as follows: 40μl of 50mM Tris buffer pH 7.4 containing 0.1% BSA were spiked with one of the following: 20mU Binocrit, 20mU Neorecormon, 20mU Dynepo, 20mU Eporatio, 20mU Silapo, 0.2ng Aranesp, 0.1ng Mircera or 20mU uhEPO standard from the NIBSC, respectively.

Sample preparation

Urine sample preparation was performed for blank urines, rhEPO spiked urines, and excretion study urines according to Lasne *et al.*, with minor changes in centrifugation time, speed, and washing volume.[5,6] A 20-ml urine sample was centrifuged 7 min at 4000 rcf. The supernatant was microfiltrated with Steriflip units. The filtrate was then concentrated in Amicon Ultra 15 units at 4000 rcf for 18 min. The retentate of about 200 μl was washed with 15 mL 50mM Tris (pH 7.4) buffer and centrifuged again for 18 min at 4000 rcf. The retentate was then transferred to an Amicon Ultra 4 unit and centrifuged for 7 min at 4000 rcf to concentrate the sample to about 50 μl and then washed with 4 ml of 50 mM Tris (pH 7.4) buffer. The final centrifugation step was done at 4000 rcf for 25 min leaving a retentate of about 30 to 50 μl. The retentate was then stored at −20°C until analysis.

ELISA affinity purification

The immunoaffinity purification was performed with an ELISA kit from STEMCELL. Similar procedures have been described for purification with ELISA wells prior to SDS-PAGE analysis.[11,12] Samples were either applied together with 50 μl of assay diluent (buffer B supplied with the ELISA kit), similar to the ELISA purification published for SDS-PAGE[11,12] or the samples were directly applied without addition of assay diluent. With addition of assay diluent total application volume added up to 80 to 100μl. ELISA wells were then sealed with sealing foil and incubated for 15min at room temperature (RT) on a microplate shaker at 650 rpm. This was followed by 19 h of incubation at 4°C without shaking. After incubation, the solution in the wells was discarded. Each well was washed five times with 300 μl PBS and tapped dry. Elution was performed with 25 μl 4.4% CHAPS (in water) at 90°C and 650 rpm for 5 min. The wells were then allowed to cool down for 10 min on a microplate shaker. They were then centrifuged for 1 min at 4000 rcf to merge the condensate on the well sides with the rest of the elution. Samples were then ready for downstream IEF analysis and EPO ELISA quantification.

EPO ELISA quantification

EPO was quantified using the EPO ELISA from STEMCELL. The EPO amount was determined before incubation, after incubation, in the elution and in the washing fractions. The assay was used as depicted in the instructions of the kit. Measurements were performed with a Victor 3 multi-label plate reader (PerkinElmer; Waltham, MA, USA). Calculations were performed with the Masterplex2010 curve-fitting software v.2.0.0.72 (Hitachi Software Engineering; New York, NY, USA)

SDS-PAGE

Sodium dodecyle sulfate polyacrylamide gel electrophoresis was performed with precast BisTris-gels. Prior to gel application samples were heated under reducing conditions in LDS-buffer to 90°C for 5min. Electrophoresis was performed with a reducing electrophoresis running buffer at constant voltage (160V) for 50 min. After this the SDS-gels were silver- stained.

Staining

Silver stains were performed as described in the manual of the silver staining kit.

Isoelectric focusing, double-blotting and detection

IEF was performed using polyacrylamide slab gels with cast-in slots. Tween-80 was added to both samples and spiked buffers to a final concentration of 1% (*v/v*) before application to the gel. Focusing conditions, double-blotting and detection was performed as described by Lasne *et al.*[5,6] Detection of chemiluminescence was performed with a CCD-camera (LAS-4000; Fujifilm, Tokyo, Japan). Densitometric analysis of the immunoblot was performed with GASepo software v.2.1.[14]

Results and discussion

Washing efficiency

The purification has to be efficient in terms of depleting proteins and other substances from the sample. This efficiency of depleting proteins from the sample was tested by eluting retentates either directly without washing or after one to five washing steps. The elutions were subjected to SDS-PAGE and the gel was then silver stained. The purification of urine retentates depleted samples of most unwanted proteins. The stain shown in Figure 1 illustrates that the first two washing steps are extremely efficient in depleting protein abundance. The following washing steps reduced the total protein amount only marginally. As a standard procedure five washing steps have been established as described in the methods.

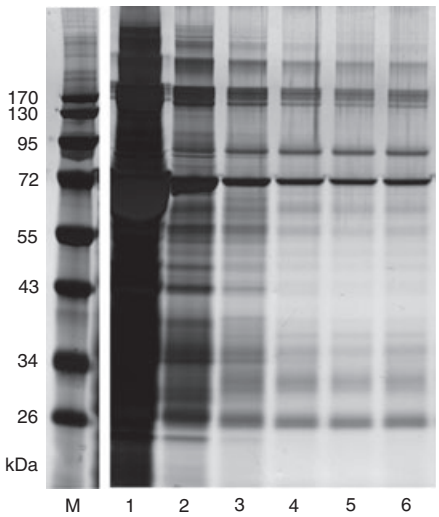

Figure 1. SDS-PAGE with following silver staining of ELISA elutions after no washing step (1) and after one (2), two (3), three (4), four (5) and five (6) washing steps (M; PageRuler molecular weight marker).

Isoform distribution

A significantly varying binding affinity of the anti-EPO antibody used in the ELISA wells to EPO isoforms could adulterate downstream analysis. Two sets of experiments were used to investigate if the ELISA purification ensures unchanged isoform distribution before and after purification. In the first approach eight spiked buffers were directly applied to IEF or after immunoaffinity purification. In Figure 2 the immunoblot of the IEF of these samples is shown. Neither the huEPO nor any of the rhEPOs subjected to immunoaffinity purification showed an isoform distribution which differed from the distribution of the directly applied samples. A loss of signal intensity after purification could be seen for all samples tested (Figure 2, 1–8). This was most notable for the epoetins with the greatest structural difference to huEPO, the pegylated epoetin beta (Mircera; Figure 2, 2), and the genetically engineered darbepoeitin alpha (Aranesp; Figure 2, 3). In the second approach urine samples were applied to IEF either purified or unpurified. The most acidic isoforms of an Aranesp excretion study sample showed a decreased recovery compared to other isoforms contained in the sample (Figure 3, B). Analogs to epoetin alpha can show after administration isoform patterns in urine that have resemblance to effort urines or be identical to unstable samples. The purification needs to have a good recovery for these isoforms to prevent false negative results. In the case of Aranesp a decreased recovery as shown in this paper is acceptable since even slight increases in band intensity in the acidic area change the isoform pattern in a way that can only be explained by administration of NESP. Despite this, the sample remained NESP positive after purification. Band and lane shape of the sample were improved, increasing the precision of densitometric analysis. The blank urine tested (Figure 3, A) remained negative after purification, as defined by the technical document, without altering of isoform distribution. Signal intensity was lower as in the unpurified sample. Interestingly, isoform distribution of the purified Silapo excretion study urine seemed to be affected in comparison to the directly applied sample. This is due to high protein abundance in the directly applied sample leading to heavy disturbances in band and lane definition (supplementary fig. 1). Both excretion samples fulfilled the WADA criteria of a positive sample prior to purification and still did afterwards. The decrease of signal in purified samples seen on the immunoblots in Figures 2 and 3 was determined with a quantification of EPO before and after purification.

EPO quantification

Recovery was determined using an EPO-ELISA from STEMCELL. The concentration of spiked buffers was measured before subjection to purification and afterwards. The EPO content in the spiked

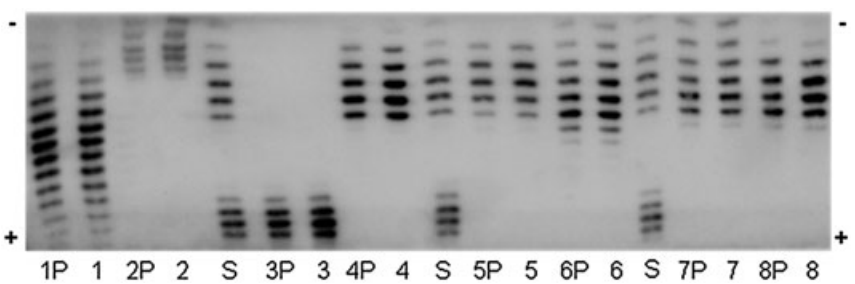

Figure 2. Immunoblot of an IEF-PAGE of 20 mU uhEPO (1), 0.1 ng Mircera (2), 0.2 ng Aranesp (3), 20 mU Binocrit (4), 20 mU Neorecormon (5), 20 mU Dynepo (6), 20 mU Silapo (7) and 20 mU Eporatio (8) directly applied and immunopurified (P). Standard lanes (S) contain a BRP-EPO/NESP mix.

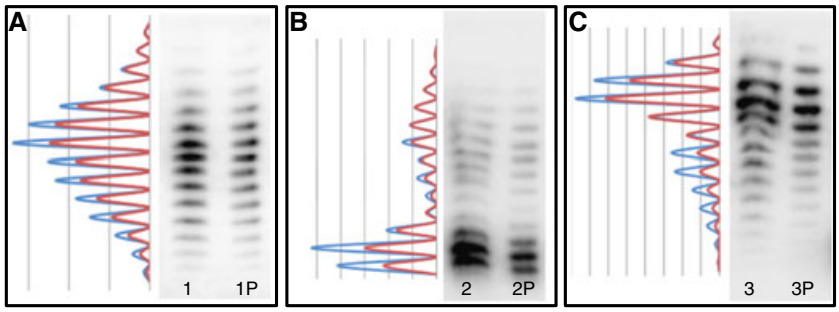

Figure 3. Immunoblots with corresponding histogramms of an IEF-PAGE. A blank urine (1; A), an Aranesp excretion study urine (2; B; 76 h after application of 0.75 μg/kg Aranesp) and a Silapo excretion study urine (3; C; 28 h after application of 50 U/kg Silapo) were either directly applied (1–3) or immunopurified before application to IEF (1P-3P). The histogramms (A-C) illustrate the pixel density for each band of the purified (red) and unpurifed (blue) samples shown in the immunoblots.

buffers after 19h of incubation was measured to ascertain the amount of unbound EPO. Also, The EPO content within the washing fractions was measured to determine the loss of EPO during the washing steps. All measurements were performed at least in triplicate. The recovery was then expressed as the amount of EPO recovered from the amount of EPO subjected to purification in percent. The calculated recovery rates for most tested epoetins (Binocrit, Neorecormon, Dynepo, Silapo, and Eporatio) were between 60 and 75%. There was no EPO found in the washing fractions and only about 1.5% remained unbound after 19 h incubation. The recovery for Mircera and Aranesp was lower, 50–60% and 40–50% respectively. Also the amount of unbound EPO in the wells was significantly higher for Mircera 12% and for Aranesp 7%. The high percentage of unbound Mircera and Aranesp are an indication of reduced binding affinity. A reduced binding affinity of Aranesp and Mircera to an anti-EPO antibody has already been published for the immunoaffinity columns from MAIIA.[13,15] Though the antibodies are not identical, the phenomenon is similar. The washing fractions did not contain EPO. This confirmed the results acquired by densitometry which had also shown a decreased recovery for Aranesp in comparison to other epoetins. For all epoetins tested with this purification 30 to 40% of the applied EPO is missing. This EPO is neither eluted, nor unbound, nor is it washed away. Which leads to the question where the remaining EPO is to be found? The ELISA, if used for the purpose it was manufactured for, i.e. the quantification of EPO, is performed by default with volumes of about 100–125 μl. The anti-EPO antibody will be coated to the wells to at least this volume. If these ELISA wells are used for purification and the applied volume of the urine retentate is higher than the volume used for elution, then EPO bound to upper parts of the well could not be reached by the elution buffer. Four different volumes, 25 μl, 50 μl, 75 μl and 100 μl, of a 400U/L Binocrit spiked buffer were incubated for 19 h at 4°C. After incubation EPO was eluted with 25 μl elutionbuffer. As shown in Figure 4, the recovery decreased with rising application volume. If 25 μl were applied, recovery was 85%; at a volume of 100 μl, it was only 55%. This was also tested for different application volumes of urine retentates. The results were comparable when using varying application volumes of urine retentates instead of spiked buffer as can be seen in Figure 4. The best recoveries were achieved with low application volumes. It seems that the EPO bound to the upper parts of the well cannot be eluted with an elution volume of 25 μl. The elution volume should not exceed 25 μl since this is roughly the maximum volume which can be applied to an IEF gel. Optimal recoveries depend on efficient concentration of urine retentates to assure low application volumes to the ELISA wells.

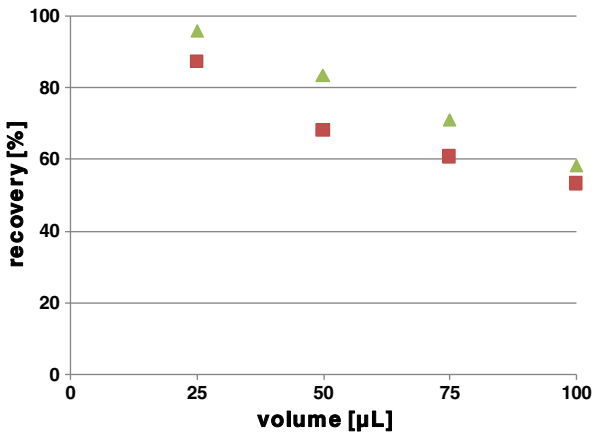

Figure 4. Calculated recoveries after immunoaffinity purification of spiked buffer and urine retentates. Different modes of application were tested to elucidate recovery problems. Recoveries of varying application volumes of a 400U/L Binocrit solution which were eluted with 25μl are shown in squares. Recoveries of varying application volumes of urine retentates with 25μl are shown in triangles.

Conclusion

The tests done so far with this method have shown it to be a valuable tool in improving gel quality. Purified urine retentates have far less disturbances and irregularities in lane and band. The efficacy of the purification in depleting protein abundance was tested with silver staining and was shown to be satisfactory. High recoveries rely on an efficient concentration of urine prior to purification. The higher the application volume the lower the recovery will be. The purification is easy-to-use and not work-time consuming. When using SDS-PAGE as an auxiliary method to IEF the ELISA immunoaffinity purification is a very flexible way to purify samples. By changing the elution buffer samples can be applied to either IEF- or SDS-PAGE. The demonstrated method is a good addition to the WADA-accredited IEF method for EPO detection. It can improve band and lane shape in certain samples without altering isoform distribution and improve densitometric measurements.

Supporting information

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

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