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Rapid affinity purification of erythropoietin from biological samples using disposable monoliths

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ABSTRACT

Identification of post-translational modifications of proteins in biological samples often requires access to preanalytical purification and concentration methods. In the purification step high or low molecular weight substances can be removed by size exclusion filters, and high abundant proteins can be removed, or low abundant proteins can be enriched, by specific capturing tools. In this paper is described the experience and results obtained with a recently emerged and easy-to-use affinity purification kit for enrichment of the low amounts of EPO found in urine and plasma specimens. The kit can be used as a pre-step in the EPO doping control procedure, as an alternative to the commonly used ultrafiltration, for detecting aberrantly glycosylated isoforms. The commercially available affinity purification kit contains small disposable anti-EPO monolith columns (6 μL volume, Ø7 mm, length 0.15 mm) together with all required buffers. A 24-channel vacuum manifold was used for simultaneous processing of samples. The column concentrated EPO from 20 mL urine down to 55 μL eluate with a concentration factor of 240 times, while roughly 99.7% of non-relevant urine proteins were removed. The recoveries of Neorecormon (epoetin beta), and the EPO analogues Aranesp and Mircera applied to buffer were high, 76%, 67% and 57%, respectively. The recovery of endogenous EPO from human urine was 65%. High recoveries were also obtained when purifying human, mouse and equine EPO from serum, and human EPO from cerebrospinal fluid. Evaluation with the accredited EPO doping control method based on isoelectric focusing (IEF) showed that the affinity purification procedure did not change the isoform distribution for rhEPO, Aranesp, Mircera or endogenous EPO. The kit should be particularly useful for applications in which it is essential to avoid carry-over effects, a problem commonly encountered with conventional particle-based affinity columns. The encouraging results with EPO propose that similar affinity monoliths, with the appropriate antibodies, should constitute useful tools for general applications in sample preparation, not only for doping control of EPO and other hormones such as growth hormone and insulin but also for the study of post-translational modifications of other low abundance proteins in biological and clinical research, and for sample preparation prior to in vitro diagnostics.

1. Introduction

Correct pre-treatment and purification of biological samples in order to isolate low-abundance proteins prior to analytical techniques such as IEF-PAGE, SDS-PAGE, 2D-electrophoresis, capillary electrophoresis and LC–MS/MS, are mandatory in proteomics [1]. Especially when the research is focused on post-translational modifications giving rise to a spectrum of isoforms of a protein; it is essential that the purification regime does not disturb the isoform distribution. In plasma samples, the concentration range of proteins is wide, for albumin at 40 g/L and EPO at 50 ng/L, a range of 10^9 times, making it difficult to prepare a sample free from other proteins for the analyses of low abundance proteins. It has been common in proteomics to remove the most abundant proteins (e.g. 20 proteins constitutes 97–98% of total protein in plasma) to be able to detect the less abundant ones. However, even after removing 98% of the proteins, a concentration range of more than 10^7 times still remains. The advent of specific micro affinity columns

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makes it possible to capture one low-abundance protein, and to concentrate and purify it from other components in the sample.

In order to avoid interference from other proteins, an immunological detection step can be included in the analytical procedure, but insufficient specificity might still result in interference as found in the accredited EPO (erythropoietin) doping control method [2–4]. This control method is based on IEF with a double immunoblotting step, preceded by an ultrafiltration concentration of EPO from the urine sample. However, non-specific interference from abundant urine proteins is claimed to result in bands that are not related to EPO or its analogues [5]. Several different urine proteins, when occurring in sufficiently high concentration, might interfere both outside the actual p*I* range of EPO variants [6,7] and in the actual p*I* range [8]. This interference can be reduced by using EPO affinity purification as sample pre-treatment instead of ultrafiltration. A column with anti-EPO bound to porous beads has been utilized for the purification of serum samples and ultrafiltration retentate from urine samples [6]. For identification of human EPO in equine plasma samples, affinity purification with anti-hEPO bound to magnetic particles has been used, in a lengthy procedure, as a pre-step for LC–MS/MS doping control [9]. The magnetic particles were regenerated for repeated use.

However, it is not acceptable that affinity columns are re-used when analyzing samples to be used for in vitro diagnostics (IVD) or for doping control. Especially for EPO doping control there are so many possible EPO variants and some of them, like EPO dimers or trimers [10], can show unpredictable on- and off-rates in their interaction with the antibodies. This can result in a slow carry-over of doping positive EPO variants from a re-used column to the following samples.

Traditionally affinity columns, using anti-EPO immobilized on porous particles, will be too expensive to be used as disposables. Mouse monoclonal antibodies for EPO are very expensive, and cost in the range of 500–5000 €/mg, and at least one mg antibody will be required per traditional column to achieve a sufficiently high EPO recovery.

The use of monoliths for separation [11,12] shows several advantages useful for rapid and efficient affinity chromatography for biological samples. Membranes, one type of monoliths, have rapid mass transfer due to the absence of pore diffusion together with an efficient convective flow [13,14]. A short monolithic column (60 μL) with a length of 3 mm has been used for affinity purification of specific proteins [15]. Such column requires coupling of 1–2 mg of antibody per monolith, which will make repeated use necessary. In our publication is presented a 6 μL column with a length of only 0.15 mm, which can be used for purification and concentration of EPO from biological samples. The column is designed to be used as a disposable to simplify the pre analytical sample treatment and to avoid carry-over effects.

2. Materials and methods

The water used in all experiments was prepared using Milli-Q Academic equipment (Millipore, Billerica, MA, USA). Reagents, unless specified, were of analytical grade and purchased from Sigma (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane, protease free, was obtained from Acros Organics (New Jersey, USA).

2.1. Biological samples

The collection of urine specimens from healthy volunteer donors (men and women between 20 and 60 years old) was approved by the local ethical committee. The serum samples were leftovers from a health control and used after informed consent from the healthy individuals. The specimens were stored in aliquots at −20.

2.2. Erythropoietins and analogues

Eprex®, recombinant epoetin alpha, was purchased from Janssen-Cilag AB (Sollentuna, Sweden); Neorecormon®, recombinant epoetin beta, and MIRCERA®, methoxy polyethylene glycol-epoetin beta, were obtained from Roche GmBH (Mannheim, Germany). Aranesp®, the recombinant EPO analogue darbepoetin alpha, was purchased from Amgen (Thousand Oak, CA, USA). One IU of epoetin corresponds to 8.4 and 8.3 ng recombinant EPO for Eprex and Neorecormon, respectively. Retacrit™, recombinant epoetin zeta, was purchased from Hospira Enterprises B.V. (Hoofddorp, Netherlands) and Dynepo (epoetin delta) was obtained from Shire Pharmaceuticals. Other epoetins were obtained from different countries like Epomax (epoetin omega) from Slovenia, and EPIAO, Jia Lin Hao, Ji Mai Xin and Ning Hong Xin from China. The Second International Reference Preparation of erythropoietin, human, urinary (67/343) was obtained from National Institute for Biological Standards and Control (NIBSC). Recombinant EPO Biological Reference Preparation (BRP) was obtained from European Directorate for the Quality of Medicines & HealthCare, Strasbourg, France.

2.3. Vacuum manifold

Vacuum manifold for processing 1–24 columns, QIAvac 24 Plus, and the connecting system (Qiagen, www.qiagen.com) was used together with a vacuum pump for passing the liquids through the columns.

2.4. Sample preparation before affinity purification

The procedure was carried out as described in the instructions in the EPO Purification Kit (Art. No. 0250, MAIIA Diagnostics, Uppsala, Sweden, www.maiiadiagnostics.com).

The thawed and to room temperature adjusted urine was gently turned end-over-end to distribute precipitates evenly and an aliquot was then transferred to another tube after which urine precipitate dissolvation (UPD) buffer, provided in the EPO Purification Kit, was added, in proportions 10 parts of urine and 1 part of UPD buffer. The urine precipitates were usually instantly dissolved. The UPD–urine mixture was heated to about 82–85 °C, as specified in the instruction, and thereafter rapidly cooled by immersing the tubes in a cold water bath. For affinity purification of EPO from sample volumes of less than 5 mL urine, the heating step was mostly omitted. The urine-UPD mixture was diluted 1:1 by adding dilution buffer (dilution in water from the supplied Detergent aid and Exposure aid to 2%, respectively). The urine mixture was filtered through a HPF Millex-HV filter (Millipore, Bedford, MA, USA).

Samples like serum and cerebrospinal fluid were diluted to 10% in a buffer containing 20 mM Tris, pH 8.0, 0.1 M NaCl, 0.02% NaN_3 supplemented with 0.1% Tween 20 (1% of Detergent aid supplied in the kit). The sample mixture was filtered through a 0.22 μm filter, type GV or GP, 25 mm diameter (Millipore), before applied to the column.

2.5. Purification using EPO Purification Kit

The procedure was in accordance with the instructions in the kit (Art. No. 0250, and the previous kit, Art. No. 0160, MAIIA Diagnostics). The kit includes UPD buffer and buffers for washing, desorption and pH adjustment. The anti-EPO monolith was obtained as a clear plastic column holder containing a 6 μL monolith (7 mm Ø monolith, height 0.15 mm) with about 20–40 μg antiEPO 3F6 immobilized according to the supplier. The pretreated filtered sample mixture, see Section 2.4, was passed through the prewashed (1 mL) anti-EPO column, using the vacuum manifold, at

a flow rate of about 1 mL/min, and finally the column was washed with 1 mL of washing buffer.

The column was placed in a vial in the microcentrifuge and further dehydrated by spinning for 1 min at 1000 × *g* after which the vial was discarded. EPO was eluted by placing the column in a new vial containing pH-adjustment buffer supplemented with BSA and detergent, adding the desorption buffer to the column and spinning for 1 min at 1000 × *g*. The eluate was kept at −20 °C until analysis.

2.6. Ultrafiltration

Ultrafiltration of urine specimens was performed using membranes with a cut-off at 30 kDa. To 20 mL urine sample, 0.4 mL of Complete protease inhibitor cocktail (Roche, prepared by 1 tablet to 2 mL water) and 2 mL of 3.75 M Tris buffer pH 7.4 were added. The urine mixture was centrifuged for 10 min and the supernatant was filtered through a 0.22 μm Steriflip Filter Unit (Millipore). 10 mL filtrate was applied to a Centricone Plus-20, MWCO 30 kDa (Millipore) and after 5 min centrifugation the remaining filtrate was added and centrifuged for another 15 min. The retentate was washed with 20 mL of 50 mM Tris pH 7.4 buffer supplemented with Complete inhibitor (0.01 tablet/mL); the filter was reverted and the retentate was centrifuged in a new tube. This retentate was applied to a Microcone YM30 (Millipore) where a maximal volume of 0.5 mL sample was centrifuged for 15 min. Then several centrifugations were performed in 5 min intervals, as each urine specimen required different centrifugation time to be concentrated to 20–40 μL. The volume of each final retentate was estimated by weighing.

2.7. EPO lateral flow immunoassay for concentration determination

EPO Quantification Kit (MAIIA Diagnostics) was used for the measurement of EPO. The kit for the EPO test containing the anti-EPO strip, carbon black anti-EPO suspension, and washing solution was kindly provided by MAIIA Diagnostics and used as described by the producer. Typically, 25–200 μL of sample was dispensed into microtiter wells (F8 polysorp, Nunc Roskilde, Denmark). Other wells were prepared by dispensing aliquots of 25 μL carbon black anti-EPO and of 25 μL washing solution. An Epson Expression scanner (Epson, Sollentuna, Sweden) using an optical resolution of 600 ppi and 16 bits sample depth for greyscale was used in reflection mode for detection. The EPO concentration in the urine specimens was estimated after desalting as described elsewhere [16]. EPO in cerebrospinal fluid was measured after ½ dilution, while serum from anaemic mice was diluted more than ten times. For measuring EPO in serum and plasma samples there were some additional reagents added to the EPO Quantification Kit. The human serum samples were diluted 8 times in a 20 mM phosphate buffer pH 7.5, supplemented with 6% BSA, 0.1 M NaCl and 0.02% NaN_3. In each well, 25 μL of diluted sample was mixed with 25 μL 20 mM phosphate buffer pH 7.5 supplemented with 1% BSA, 0.1 M NaCl, 0.1% bovine gammaglobulin, 0.02% monoclonal mouse IgG, 1% Tween 20 and 0.05% NaN_3. To the working solution of anti-EPO CBNS 0.02% monoclonal mouse IgG was added. The equine plasma samples were treated in the same way but without the addition of monoclonal mouse IgG.

2.8. Isoelectric focusing electrophoresis

The accredited doping control IEF procedure was performed as previously described [4] and in accordance with the 2009 WADA technical document [17]. Affinity purified EPO was applied to the gel using the same procedure as for the buffer preparation of EPO or the ultrafiltered sample used for comparison. The samples were focused on an IEF gel (pH range 2–6), EPO and anti-EPO (mouse monoclonal AE7A5, R&D Systems) were blotted in two steps, and the enzyme-generated chemiluminescent signal was finally detected.

2.9. Protein determination

The amount of protein in the affinity purified eluates (the recommended BSA addition was omitted) and the ultrafiltration retentates was measured by the BCA protein determination test (Art. No. 23223 and 23224, Pierce, www.piercenet.com). 20 μL of a dilution series of BSA, 10–500 μg/mL, or sample, was dispensed into microtiter wells; 0.2 mL of BCA reagent was added, and incubation was performed during shaking for 30 min at 37 °C. The obtained absorbance was measured in a spectrophotometer at 560 nm. The amount of protein in the samples was calculated using the BSA dilution series as standard.

2.10. Calculation of recovery

The EPO concentration of the buffer preparations of recombinant EPO and analogues was, in the same assay, measured in the applied sample and in the eluate, and the recovery was calculated. For urine specimens, the most recently obtained value for EPO in urine, analyzed before the purification, was used for calculating the recovery.

3. Results and discussion

3.1. EPO Purification Kit performance

3.1.1. Kit components

The small disposable MAIIA anti-EPO column, see Fig. 1, has a porous monolith with a volume of only 6 μL (Ø7 mm, length 0.15 mm) containing anti-EPO 3F6 mounted in a plastic housing. Such minute monolith volume allows complete release of EPO using a desorption volume of only 50 μL.

Addition of the supplied UPD buffer to the urine samples dissolved both pink and white precipitates frequently found in the samples. For 85% of the samples it was sufficient to add 1 part of UPD to 10 parts of sample while 15% of the samples required an additional 0.5 part of UPD. The precipitates may sometimes contain up to 85% of the total amount of EPO in the sample [16]. It is thus essential to dissolve the precipitates.

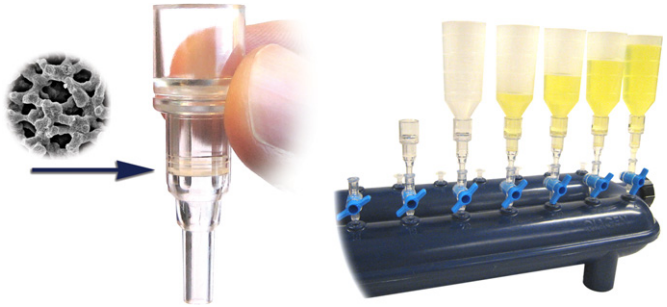

Fig. 1. The evaluated easy-to-use affinity purification set-up is intended to be used as a pre-step for analysis of post-translational modifications of proteins. The thin antibody monolith, inside the plastic housing, has a separation pass-way of only 0.15 mm and a total volume of 6 μL. Both large (>40 mL) and small volumes of samples can be applied to the funnel using a multi-channel vacuum equipment to suck the sample through the monolith. After washing, 50 μL of desorption buffer is applied and the elution is performed by centrifugal forces. This small column, although containing expensive monoclonal antibodies, allows single-use, which is required to avoid carry-over between samples.

3.1.2. EPO recovery after affinity purification

EPO and EPO analogue recoveries were measured after performing the purification process of recombinant proteins added to buffer (20 mM Tris buffer pH 8.0, 0.1 M NaCl, 0.1% Tween 20 and 0.02% NaN_3). The recoveries of Neorecormon (epoetin beta), and the EPO analogues Aranesp and Mircera were as high as, 76, 67 and 57%, respectively, calculated as the average values for 23 different purifications for each protein during three months. The recovery estimation varied with a CV of 15%, 12% and 20%, respectively. Several different epoetins such as Eprex (epoetin alpha), Dynepo (epoetin delta), Epomax (epoetin omega), Retacrit (epoetin zeta), and the Chinese epoetin biosimilairs EPIAO, Jia Lin Hao, Ji Mai Xin and Ning Hong Xin have been purified applied to buffer showing the same recovery as for Neorecormon.

The recoveries of Neorecormon, Aranesp and Mircera added to human plasma, equine plasma and human urine samples were 75%, 61%, 40%; 69%, 47%, 35%; and 67%, 57%, 30%, respectively.

Affinity purification of endogenous EPO from urine specimens from healthy individuals resulted in a recovery of 65 ± 17% (mean ± SD) for 115 tested samples, with no difference if the sample volume applied was 5, 10 or 20 mL. Endogenous EPO from human, equine or mouse serum, or from human cerebrospinal fluid, was affinity purified with a mean recovery of 60–80%.

3.1.3. Problems encountered when measuring recovery

The determination of EPO in urine, and thus the calculated recovery, may have some uncertainties to be noted. In some urine samples, EPO seemed to be degraded very rapidly as reported earlier [16]. The urine specimen used as affinity purification control of EPO recovery, stored frozen at −20 °C in aliquots, showed reduced EPO concentration to 61% of the first measured value, after storage in 15 months. The large amount of heavy urine precipitates in some frozen urine samples can make the distribution into smaller volumes, as required for repeated testing, difficult. It is essential to make sure that the same amount of precipitates is found in all the aliquots, as precipitates bind high amounts of EPO [16]. Volume loss and EPO adsorption in filter and tubes also have to be considered when measuring the recovery.

3.1.4. Volume of desorption buffer

Buffer solutions of Neorecormon and Aranesp, and a urine sample with added Neorecormon, were passed through anti-EPO affinity monoliths. The bound EPO was thereafter desorbed and eluted with different volumes (20–200 μL) of desorption buffer. The relative recovery obtained, as compared to when 200 μL was used, was 96%, 100%, 98%, 86% and 77%, for 100, 50, 40, 30 and 20 μL of desorption buffer, respectively. The lowest suitable elution volume seems to be between 30 and 40 μL, whereas the kit instruction recommends 50 μL as the lowest desorption buffer volume.

3.1.5. Flow rate and recovery

The recovery of endogenous EPO in urine, and Neorecormon, Aranesp and Mircera when purified after addition to three different urine specimens, were compared using flow rates of 0.6, 1 and 2.1 mL/min. The recoveries for all EPO variants were the same when using 0.6 and 1 mL/min, but for 2.1 mL/min the recovery was slightly reduced with 16%, 8%, 26% and 24% for endogenous EPO and Neorecormon, Aranesp and Mircera, respectively. It seems that the recovery of the EPO analogues Aranesp and Mircera is slightly more affected by higher flow rate than EPO. A flow rate up to 1 mL/min can be used with good recovery, which corresponds to a linear flow rate of 4.5 cm/min during the passage of the monolith. The time of passage across the 0.15 mm thin monolith is thus only 0.2 s. This short antigen/antibody ligand exposure time sets heavy demands on the capturing ability of the immobilized antibody.

3.1.6. Loading capacity of the monolith column

Up to 60,000 pg of Neorecormon and Aranesp, and 300,000 pg of Mircera in 20 mL of a buffer containing 20 mM Tris pH 8, 0.1 M NaCl, 0.1% Tween 20 and 0.02% NaN_3 were passed through the columns. The high concentrations of the applied solutions, 3000 ng EPO/L, about 1000 times the normal concentration, and 3000 and 15,000 ng/L of Aranesp and Mircera did not significantly reduce the recovery.

When two different volumes of human plasma were applied on a column, the percentage of recovery of endogenous EPO was 1.2 times higher for 2 mL than for 0.5 mL of plasma. Equine plasma was applied on the column in volumes of 0.5, 1 and 2 mL. In these cases the percentages of recovery were 0.95 and 1.2 times higher for 1 and 2 mL as compared to 0.5 mL.

3.1.7. Heat treatment of urine specimens before affinity purification

Application of large urine volumes, although prefiltered with the recommended 0.45 μm HPF filter, occasionally reduces the flow rate through the monolith due to the presence of certain urine components, e.g. Tamm-Horsfall glycoprotein that can clog the pores. When 17 urine samples with a volume of 20 mL were purified by omitting the recommended heating process, and were only diluted 1:1, filtered through a 0.45 μm filter and applied to the affinity column, this problem seemed to occur. The first 10 mL of urine mixture passed the column during 10–20 min, while it took 57–87 min for the passage of 40 mL for 15 out of 17 samples, when using constant vacuum. Two of the samples did not go through the column even if the vacuum was increased to maximal values, about 400 mbar below normal pressure. This problem was reduced when the urine samples were pretreated by rapid heating to between 82 and 85 °C by immersing the tubes with the urine samples in a bath with simmering water, and then rapidly cool them by immersing them in a bath with cold water. About hundred different urine specimens have been tested with this heating procedure and they all had a flow rate through the column comparable to buffer.

In order to know if a urine volume of 5 mL could be affinity purified without heat treatment, urine specimens from 25 different individuals were collected before and after strenuous exercise. 10 mL of 1:1 diluted urine was applied to the column in accordance with the kit instructions, and for each sample the 4 min heat pretreatment up to 82–85 °C was used, as well as a procedure with this step excluded. The EPO concentrations obtained in the eluates were about the same for the two procedures. After heat treatment of the urine specimen, the flow rate was comparable to the flow rate for buffer, with low variations between the specimens. Without heat treatment, the mean flow rate was reduced to 68%, and for 19% of the specimens the vacuum had to be increased to ensure passage through the column. But, by using a higher initial vacuum 5 mL of non-heated samples can be processed without problems. The urine samples collected before and after exercise showed no differences in flow rate.

The possibility that the heating process has introduced conformational changes in EPO seems unlikely as a corresponding heating step was used for the preparation of the uEPO NIBSC standard, and in the IEF sample preparation procedure [4].

3.1.8. Repeated use of the anti-EPO affinity column

The monoliths are intended for single-use, but it seems possible to reuse them. When purifying one buffer sample containing EPO and two urine specimens, it was found that the monoliths could be used four times with the same recovery, but for the fifth purification the recovery was reduced by 20%.

It is however not recommended to reuse the columns for urine specimens from different individuals. There are so many different types of EPO analogues available, and more to be expected, for

which the elution conditions are unpredictable. Especially some EPO analogues, like dimers and trimers of erythropoietin [10], might not be totally released from the column during the conditions of desorption, and instead slowly leak during desorption of the succeeding samples.

When using affinity purification as a pre-step for doping analysis or in vitro diagnostics, it is absolutely not recommended to use regenerated columns due to the risk for carry-over. Porous bead columns on the contrary, become too expensive for single use as they for acceptable performance require high amounts of immobilized antibodies.

3.1.9. Addition of protease inhibitors

Protease inhibitors seem to be required for the ultrafiltration pretreatment step for the accredited IEF method. As the cost for the inhibitors is quite high several experiments have been performed to see if this precaution is necessary when the affinity monoliths are used. Additions of protease inhibitors (Complete protease inhibitor cocktail and Pepstatin (Roche), and protease inhibitor cocktail P8340 (Sigma)) to the urine specimens and to different buffers were performed before processing them with the affinity monoliths. There were no indications that addition of inhibitors was necessary. The reason for this is most probably that affinity purification is a very fast procedure compared to ultrafiltration, and that proteases are removed during the purification process. To avoid bacterial growth the urine specimens should be collected and stored under proper conditions and preferably be frozen directly after collection.

3.1.10. Storage of EPO on the monolith

Three samples of Neorecormon added to buffer (20 mM Tris buffer pH 8.0, 0.1 M NaCl, 0.1% Tween 20 and 0.02% NaN_3) were passed through their columns and finally washed. One sample was eluted immediately as usual, while the two other columns were kept in the refrigerator for 1 and 6 days until the bound EPO was eluted. The recoveries were equal for these three procedures. EPO seems thus not to be affected when stored on the monolith. Large volumes of frozen urine specimens are today sent to analytical laboratories to a very high cost and the possibility to send EPO bound to the monolith without refrigeration should be more investigated.

3.2. Ultrafiltration of urine samples

The ultrafiltration procedure, using a 30 kDa filter preceding the accredited IEF doping test, with Neorecormon, Aranesp or Mircera added to buffer, showed 47%, 51% and 50% recovery, respectively. When measuring the retentates from 27 different 20 mL urine samples the recovery of EPO was 51%. The recovery of about 50% is in accordance with earlier estimations [4].

The procedure for concentrating EPO from 20 mL urine to 20–40 μL retentate is very tedious with several hands-on steps. The frequently appearing clogging problems for individual urine samples made it very difficult to schedule the required time for this step, which took at least two to four hours for 20 samples.

3.3. Protein determination of eluate and retentate from affinity purified and ultrafiltered urine

The amount of protein in the eluate obtained after affinity purification ($n = 10$) and in the ultrafiltration retentate ($n = 14$) was 3.4 and 586 μg, respectively, when applying 20 mL of urine. The amount of urine proteins was thus reduced by 99.4% in the eluate compared to the retentate. The amount of protein in urine can roughly be estimated to 3 mg/20 mL, and 20% was left in the retentate after ultrafiltration and only 0.1% in the eluate after affinity purification, thus roughly 80% and 99.9% were removed.

After strenuous exercise, the protein concentration in urine can increase significantly, for albumin 4 times and for α_1-microglublin 22 times [18] which might result in worse interference in the analysis. The affinity purification pre-treatment thus significantly reduces the interference from urine proteins, which always give non-specific binding with the anti-EPO antibodies in the accredited IEF based doping control method.

3.4. IEF results

The accredited doping control method based on IEF (pH 2–6), with double-blotting and immunodetection, reveals charge differences for EPO variants and analogues that react with the selected anti-EPO antibody. The antibody binds to an epitope within the first 26 amino acids at the N-terminal part of hEPO.

The isoform distribution was found to be retained after affinity purification as shown in Fig. 2, when rhEPO BRP (alpha and beta), the EPO analogue Aranesp, one Chinese epoetin analogue, and the uEPO NIBSC standard were analyzed with the IEF method, with and without affinity purification. The same result, with no change of band distribution after affinity purification, was found for Epomax (epoetin omega) and the epoetin biosimilairs ESPO, Repotin, Ning Hong Xin, Erythrostim and Epocrine as reported in a recent validation of the affinity purification kit [19]. Urine samples collected after an injection series with Dynepo (epoetin delta) showed the same band position after ultrafiltration and affinity purification. Affinity purification of Mircera added to urine and to serum has resulted in the typical narrow band distribution pattern in the less acidic part of the IEF gel. A recent study, using the monolith for affinity purification of Mircera and endogenous EPO from 0.2 mL of serum, showed very good results in terms of specificity and selectivity when the distribution was detected with IEF and SDS-PAGE [20].

Several urine samples were also affinity purified and the IEF pattern was compared with the results for the ultrafiltered urine retentate. The results for three urine specimens can be found in Fig. 3. The band intensity (difference between signal and noise), the intensity ratio between bands, and position of the bands, when applying affinity purified EPO instead of ultrafiltered, were much easier to interpret as the background staining was lower and the bands showed no bends and disturbing smears.

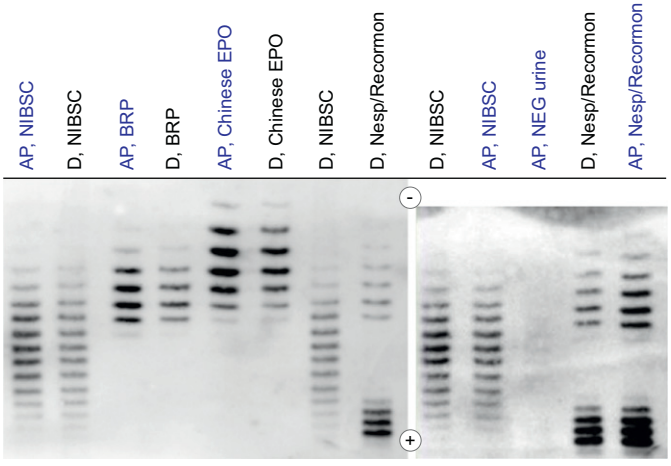

Fig. 2. IEF (pH 2–6) analysis of EPO isoform distribution. The EPO preparations were applied directly to the gel (D), or applied after affinity purification (AP). The urine EPO preparation from NIBSC is partially purified from urine proteins. The BRP rhEPO preparation is a mixture of 50% epoetin alpha and 50% epoetin beta, Recormon is pure epoetin beta and the Chinese EPO represents epoetin biosimilairs. The EPO analogue Aranesp (NESP) shows considerably lower p*I* compared to EPO, due to two additional N-linked carbohydrate structures. Affinity purification did not to change the band distribution for any of the tested EPO variants.

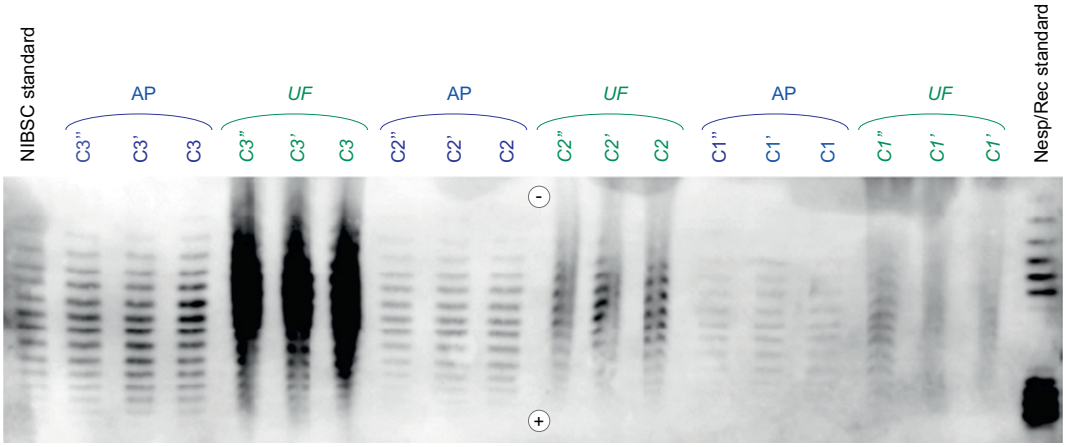

Fig. 3. IEF (pH 2–6) analysis of isoform distribution for endogenous EPO. Endogenous EPO from a 20 mL urine sample was obtained in 15–30 μL retentate after ultrafiltration (UF) and in 55 μL eluate after affinity purification (AP). To the IEF gel was applied 20 μL of retentate and eluate. C1, C2 and C3 represent three different urine samples with an estimated original concentration in urine of 4, 7 and 60 ng EPO/L. Affinity purification of the urine sample removed the background staining and made the interpretation of band intensity, intensity ratio calculation and estimation of position much more reliable.

3.5. Problems encountered when working with Mircera

The EPO analogue Mircera is constituted of epoetin beta linked via amide bonds (position 1 or 45 or 52) to a single methoxy-polyethylene glycol polymer of about 30 kDa, resulting in a molecule with 60 kDa molecular mass. This molecule shows much lower affinity to the EPO receptor than epoetin beta with a mean elimination half-life of 130 h compared to 9 h for epoetin beta after intravenous injection [21]. In IEF the Mircera bands appear as less acidic than epoetin beta with a characteristic distribution of the bands [17]. Mircera reacts about 2.5 times less efficient than EPO in the lateral flow sandwich immunoassay as judged from the calculated amount of epoetin beta in the protein–PEG conjugate. The obtained affinity purification recovery of 57% for Mircera, when added to buffer, compared to 76% for epoetin beta, is quite high considering the presence of the large PEG structure.

The addition of Exposure aid to the urine sample before affinity purification is proposed by the kit supplier to enhance the recovery by exposing Mircera correctly to the antibodies. This was tested by purification of nine urine specimens to which Mircera was added. By omitting the supplied Exposure aid to the specimens the mean Mircera recovery was reduced in the range 20–80% with a mean value of about 57%.

3.6. Further applications

Analysis of other proteins and peptides used for doping, like insulin and growth hormone, should benefit by using a rapid purification and concentrating device before the analysis. For doping analysis of insulin in human and equine samples, affinity purification has been used as a pre-step to LC–MS/MS analysis [22–24]. However, the procedures, using antibodies bound to gel columns or magnetic beads, are tedious and the required re-use of the antibodies might result in carry-over between samples.

In the doping analysis of recombinant growth hormone (rhGH) in human plasma samples [25], where the ratio between two hGH immunoassays is calculated in order to distinguish between pituitary and recombinant hGH, several samples from blood donors were not measurable due to too low pituitary GH concentration. There is also a request for a test that can identify rhGH in urine, which appears in very low concentrations, to avoid invasive sample collection. In both these cases a pre-concentration affinity purification step of the sample should enhance the detection. In addition, by stacking two monoliths, one for binding hGH and the other for hEPO, the same urine volume can be used for capturing both these low-abundant proteins.

Moreover, when frozen urine samples in large volumes have to be transported to the analysis laboratory, like for EPO analysis, the cost is very high. It seems possible to easily send EPO immobilized on the monolith, without requiring expensive refrigerated transports.

4. Conclusions

The EPO Purification Kit seems to fulfil the requirements for use as a pre-step for human and equine EPO doping analysis for both urine and plasma samples.

The risk of appearance of non-specific bands in the IEF, due to interference from urine proteins other than EPO, was significantly reduced by removing 99.4% of non-relevant proteins compared to the ultrafiltration procedure for concentrating EPO. The column, although containing expensive monoclonal antibodies, allows single-use of the columns, which is required to avoid carry-over between samples.

The recoveries of EPO and EPO analogues were higher than those obtained with the ultrafiltration procedure. The quality of the IEF analysis was improved as the reduction of non-specific background signal between the bands made them easier to distinguish, which enabled better ratio calculation between their intensities. The presence of smears and bent bands, which is common for samples with high protein concentration, was no longer found. The purification procedure was simple and possible to implement at every laboratory, and can be scaled up for rapid processing of a several samples simultaneously.

The purification regime, using only 0.15 mm thin disposable monolithic columns with immobilized specific antibodies, can be expected to be useful for preceding analysis of other hormones, like growth hormone and insulin, interesting for the doping control laboratories but also for in vitro diagnostics applications.

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References

[1] M. Azarkan, J. Huet, D. Baeyens-Volant, Y. Looze, G. Vandenbussche, J. Chromatogr. B 849 (2007) 81.
[2] F. Lasne, J. Immunol. Methods 253 (2001) 125.
[3] F. Lasne, J. de Ceaurriz, Nature 405 (2000) 635.
[4] F. Lasne, L. Martin, N. Crepin, J. de Ceaurriz, Anal. Biochem. 311 (2002) 119.
[5] M. Beullens, J.R. Delanghe, M. Bollen, Blood 107 (2006) 4711.
[6] F. Lasne, L. Martin, J.A. Martin, J. de Ceaurriz, Int. J. Biol. Macromol. 41 (2007) 354.
[7] C. Reichel, J. Mass Spectrom. 43 (2008) 916.
[8] A. Khan, J. Grinyer, S.T. Truong, E.J. Breen, N.H. Packer, Clin. Chim. Acta 358 (2005) 119.
[9] F. Guan, C.E. Uboh, L.R. Soma, E. Birks, J. Chen, Y. You, J. Rudy, X. Li, Anal. Chem. 80 (2008) 3811.
[10] A.J. Sytkowski, E.D. Lunn, K.L. Davis, L. Feldman, S. Siekman, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 1184.
[11] D. Josic, J.G. Clifton, J. Chromatogr. A 1144 (2007) 2.
[12] A. Jungbauer, R. Hahn, J. Sep. Sci. 27 (2004) 767.
[13] T.B. Tennikova, F. Svec, J. Chromatogr. 646 (1993) 279.
[14] J. Thömmes, M.-R. Kula, Biotechnol. Progr. 11 (1995) 357.
[15] M. Rucevic, J.G. Clifton, F. Huang, X. Li, H. Callanan, D.C. Hixson, D. Josic, J. Chromatogr. A 1123 (2006) 199.
[16] M. Lonnberg, M. Drevin, J. Carlsson, J. Immunol. Methods 339 (2008) 236.
[17] C. Ayotte, G. Gmeiner, F. Lasne, P.A. Pascual, C. Reichel, M. Saugy, WADA Technical Document – TD2009EPO, 2009.
[18] A. Clerico, C. Giammattei, L. Cecchini, A. Lucchetti, L. Cruschelli, G. Penno, G. Gregori, O. Giampietro, Clin. Chem. 36 (1990) 562.
[19] Y. Dehnes, S. Lamon, M. Lönnberg, J. Pharm. Biomed. Anal. 53 (4) (2010) 1028.
[20] N. Leuenberger, manuscript in preparation.
[21] I.C. Macdougall, Curr. Hematol. Rep. 4 (2005) 436.
[22] E.N. Ho, T.S. Wan, A.S. Wong, K.K. Lam, B.D. Stewart, J. Chromatogr. A 1201 (2008) 183.
[23] T. Kuuranne, A. Thomas, A. Leinonen, P. Delahaut, A. Bosseloir, W. Schanzer, M. Thevis, Rapid Commun. Mass Spectrom. 22 (2008) 355.
[24] M. Thevis, A. Thomas, P. Delahaut, A. Bosseloir, W. Schanzer, Anal. Chem. 78 (2006) 1897.
[25] M. Bidlingmaier, J. Suhr, A. Ernst, Z. Wu, A. Keller, C.J. Strasburger, A. Bergmann, Clin. Chem. 55 (2009) 445.